NEW STRATEGIES TO INHIBIT CELL DEATH IN MYOCARDIAL ISCHEMIA-REPERFUSION INJURY: HOW TO SUCCEED?

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EDITORIAL
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Editorial: New Strategies to Inhibit Cell Death in Myocardial Ischemia-Reperfusion Injury: How to Succeed?

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Editorial on the Research Topic

New Strategies to Inhibit Cell Death in Myocardial Ischemia-Reperfusion Injury: How to Succeed?

INTRODUCTION

Despite an active research activity since more than 30 years, there is no specific treatment nowadays against myocardial ischemia-reperfusion (IR) injury. Development of strategies based on inhibition of cell death appeared as a main issue for the reduction of injury to provide cardioprotection in the myocardial tissue. However, despite numerous putative drugs identified in animal models, no one of potential clinical utility has emerged. What would be the new ways to follow for discovering novel strategies of cardioprotection after a heart attack?

I-MULTIPLE TARGETS THERAPIES

Among cardioprotective strategies under investigation, cell therapy using various types of stem/stromal cells fully responds to the concept of a therapy based on a pleiotropic effect to fight multifaceted cardiac injury including cell death, inflammation and fibrosis. Mesenchymal Stromal/Stem Cell (MSC)-based therapy has been reported to improve the functional recovery of the ischemic myocardium by promoting endogenous cell survival, proliferation and angiogenesis. In particular, Shi et al. reports that neovascularization is the main mechanism of MSCs to improve the status of ischemic hearts. Blood supply being fundamental for the survival and the function of the myocardium, the formation of an efficient vascular network is a prerequisite for restoring durably the blood flow. MSCs activated by the hypoxic environment are able to differentiate into pericytes, endothelial, and smooth muscle cells. They regulate both angiogenesis and vasculogenesis through paracrine factors secreted throughout the neovascularization process (Shi et al.). Secretion of paracrine factors, rather than the differentiation process, is the main mechanism of action also evidenced in endothelial progenitor cells (EPCs) for their cardioprotective effects. Current knowledge on exosomes of EPC as putative therapeutic agents for treating cardiovascular disease as well as their mechanism of action is reviewed by Zeng et al..

For the treatment of multifaceted IR injury, MSC represent a lead candidate cell type during myocardial infarction. Nernpermpisooth et al. report that the cardioprotective effect of bone marrow-derived MSC depends on the presence of PPARβ/δ (*Peroxisome proliferator-activated β/δ*)

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receptors reported to play key roles in metabolism, angiogenesis and cell survival. In addition, they show using genetically modified MSC knockout for PPARβ/δ that the acute cardioprotective effect of MSC injected in the coronary network of *ex vivo* ischemic hearts is not related to their anti-inflammatory properties.

MSC therapeutic effects associated to their ephemeral presence in the IR myocardium suggests that cardioprotection is mediated through the release of paracrine factors such as extracellular vesicles (EV) that contain a variety of bioactive components able to rapidly educate immune cells and to protect cardiac cells. Zhang et al. report for the first time the expression profile of circular RNA involved in the cardioprotective effect mediated by EV derived from Human Umbilical Cord MSC (HuMSC) on cultured cardiac cells subjected to hypoxia-reoxygenation (HR). HuMSC-EVs treatment increases their survival rate due to the high level of expression of 10 circular RNA (circRNA) identified by High throughput RNA sequencing. GO (*Gene ontology*) and KEGG (*Kyoto Encyclopedia of Genes and Genomes*) pathway analyses allowed identifying that these circRNAs were related to important biological functions including cellular response to hypoxia and that the VEGF signaling pathway could be a mediator.

Although several small chemical compounds targeting cell death have been developed as potential therapeutic drugs, alternative medicine using natural extract from herbs, plant, or foods has also been explored and tested in preclinical models. One important issue is that such medicine is currently used in human. One of the major mechanisms of the natural phytochemical compounds is scavenging reactive oxygen radicals (ROS), which can effectively inhibit cellular damage at different levels. Chen et al. performed constructive review of several promising therapeutic candidate phytochemical compounds for myocardial IR injury. Several studies in different study models of myocardial IR injury were performed. Cardioprotection by phytochemical compounds is due to their anti-oxidant, anti-inflammatory, and anti-apoptotic effects. Several compounds were reported to downregulate cell signaling pathways involved in cell death such as ERK, p38, JNK, JAK/STAT, NF-κB, apoptotic regulatory p53, apoptotic regulatory Bcl-2/Bax proteins, and by contrast, to stimulate cell survival kinases including PI3K/Akt/GSK-3β. The original contribution from Givre et al. describes *in vitro* experiments on cardiac myocytes to evaluate the cardioprotective effects of hibernating bear serum against HR injury. The study shows that cell death inhibition was specific to the serum from brown bears, in contrast to horse or rabbit serum. The discovery of serum molecules coming from hibernating animals in non-hibernating animals opens a new therapeutic avenue for identifying cardioprotective molecules with future applications in humans (Givre et al.).

II-PHARMACOLOGICAL THERAPIES

Since the discovery of small non-coding micro RNAs (miRNAs) in 1993, their roles and effect have been studied both in the regulation of normal physiological conditions and in pathogenesis. Wang and Zheng reviewed the protective role of miRNA against cellular apoptosis in myocardial IR injury as well as in post-ischaemic remodeling. Moreover, the article highlighted the relevance of targeting regulatory molecules of miRNA expression as alternative potential therapeutic strategies against myocardial IR injury (Wang and Zheng).

As another pharmacological approach, Parra-Flores et al. demonstrated that stimulation of Toll-Like Receptor (TLR) 4 could attenuate (simulated IR protocol) cardiac fibroblast cell death *in vitro via* activation of Akt and ERK survival kinases, suggesting that TLR4 could possibly be a novel therapeutic target to prevent cardiac cell death.

Proteins and peptides could also act as therapeutic agents to protect the myocardium. Pilose antler polypeptide (PAP-3.2KD) is one of the main active components from the traditional Chinese Medicine Pilose antler, known for its benefits in cardiomyopathy. Xu et al. demonstrated the protective effects of PAP against Adryomycin-induced myocardial injury mainly by inhibiting apoptosis. Recently, Li et al. demonstrated that the herbal extract Sweroside could protect cardiac cell death from IR-induced pyroptosis *via* a novel mechanism based on Kelch-like ECH-associated protein 1 (Keap1) inhibition and induced nuclear factor E2-associated factor 2 (Nrf2) nuclear translocation, which is a pathway involved in inflammation-related cell death.

A review article from Fernandez Rico et al. describes all the peptides that have been developed to treat myocardial IR injury. Therapeutic peptides could target apoptosis, necroptosis and inflammation activated during IR injury and some have been evaluated also in clinical trials. The authors present also their optimization in terms of targeting the ischemic area to limit off targets (Fernandez Rico et al.).

III-NON-PHARMACOLOGICAL THERAPIES

Nowadays, non-pharmacological therapeutic strategies have also been evaluated and implemented to reduce the aggravation of myocardial IR injury. One of the well-known strategies to reduce cellular injury is based on lowering temperature or hypothermia. This technique has been proven to successfully reduce cardiac injury and infarct size, as well as improve cardiac function, not just only in experimental animal but also in clinical trials. Yamada et al. reviewed the implementation of therapeutic hypothermia in several study models. The gap of knowledge, which is related to the efficiency and safety of the technique, was also intensively identified. Another interesting article by Wang et al. introduces the physical cardioprotective strategy by electroacupuncture. An *in vivo* experiment is laboratory animals shows that electroacupuncture could precondition the heart by reducing cardiac cell injury and infarct size. The possible explanation of cardioprotection is based on apoptosis inhibition and survival kinases activation. However, several factors concerning

safety need to be intensively investigated before proceeding to clinical applications.

IV-CONSIDERATION OF COMORBIDITIES AND TREATMENTS

Until now, the translation of cardioprotection from animal experiments to randomized clinical trials has been rather disappointing. Unfortunately, there is a gap to be overcome and for that it is necessary to consider several elements that prevent from success.

First, most preclinical studies are performed in rodents animal models, as published in the collection "*New Strategies to Inhibit Cell Death in Myocardial Ischemia-Reperfusion Injury: How to Succeed*?." A mandatory step toward clinical translation would be to repeat the experiments in a large species, most often the pig, as recommended by the working groups for the development of cardioprotective therapeutics, in order to confirm the results (1).

Second, laboratory animals are very different, in terms of pathophysiological conditions, from patients at risk of infarction. Rodents used in experimental studies are generally healthy young adults who differ from AMI patients with an average age of about 62 years as reported in most clinical studies. Aging is reported to impact on the increased susceptibility of cardiac cells to IR injury and could affect the effectiveness of cardioprotective strategies [see (2) for review].

Comorbidities such as diabetes and medications used in patients with acute myocardial infarction may also blind the beneficial effects of cardioprotective strategies studied in young and healthy animals. To overcome this critical issue, new experiments need to use more clinically relevant animal models.

Dia et al. compared the impact of type 2 diabetes on infarct size between STEMI patients and mice. They found that diabetic mice had larger infarcts than nondiabetic mice. However, they observed no difference between the two groups of patients, highlighting the fact that the diabetic patients were all treated with antidiabetic drugs, mainly Metformin. The authors also showed that Metformin was able to prevent an increase in the rate of cell death associated with the diabetes phenotype cultured cardiomyocytes following a hypoxia-reoxygenation protocol. treatment. Yu et al. also demonstrated that Dapagliflozin, a new type of antidiabetic medication that inhibit the sodium-glucose co-transporter-2, significantly improves ischemia-reperfusion induced cell death in non-diabetic mice by the selective autophagy degradation of the inflammasome component NLRP3.

The hallmarks of myocardial aging may also account for the discrepancy between animal and clinical studies. In a mini-review, Díaz-Vesga et al. provide un update concerning potential new cardioprotective strategies that could be used for the treatment of aging hearts.

CONCLUSION

In order to ensure the successful development of new strategies to treat patients with AMI, it is important to consider drugs with pleiotropic effects acting (i) different pathways or (ii) different cell types, or by (iii) an improved tissue or subcellular targeting. Also, the therapeutical time window should be considered as well as aging, comorbidities and associated medicine treatments. The combination of these strategies should provide advantages for future clinical outcomes.

AUTHOR CONTRIBUTIONS

SB-L wrote the outline, introduction, conclusion, and the paragraph on cell therapy in Chapter I as well as Chapter II on pharmacological therapies concerning peptidic and protein tools. CP wrote Chapter IV on comorbidities and treatment options. SK wrote the paragraph on phytochemicals and traditional Chinese medicine in Chapter II and the paragraph on non-pharmacologic therapies in Chapter II. SB-L and SK reviewed, shortened, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neovascularization: The Main Mechanism of MSCs in Ischemic Heart Disease Therapy

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Mesenchymal stem cell (MSC) transplantation after myocardial infarction (MI) has been shown to effectively limit the infarct area in numerous clinical and preclinical studies. However, the primary mechanism associated with this activity in MSC transplantation therapy remains unclear. Blood supply is fundamental for the survival of myocardial tissue, and the formation of an efficient vascular network is a prerequisite for blood flow. The paracrine function of MSCs, which is throughout the neovascularization process, including MSC mobilization, migration, homing, adhesion and retention, regulates angiogenesis and vasculogenesis through existing endothelial cells (ECs) and endothelial progenitor cells (EPCs). Additionally, MSCs have the ability to differentiate into multiple cell lineages and can be mobilized and migrate to ischemic tissue to differentiate into ECs, pericytes and smooth muscle cells in some degree, which are necessary components of blood vessels. These characteristics of MSCs support the view that these cells improve ischemic myocardium through angiogenesis and vasculogenesis. In this review, the results of recent clinical and preclinical studies are discussed to illustrate the processes and mechanisms of neovascularization in ischemic heart disease.

Keywords: mesenchymal stem cells, ischemic heart disease, neovascularization, angiogenesis, vasculogenesis

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INTRODUCTION

Ischemic heart disease (IHD) is characterized by reduced blood supply to the heart and is the leading cause of death and disability worldwide. Long-term myocardial ischemia and acute massive myocardial infarction often result in decreased left ventricular function. Although the development of new drugs and the use of stent implantations have benefited numerous patients with coronary heart disease, some patients still have no effective treatment due to issues associated with diffuse coronary artery lesion, postoperative restenosis and heart failure after myocardial infarction (MI).

The foundation of IHD treatment is the reconstruction of vessels and the recovery of blood flow. Over the past decades, with the introduction of the concept of therapeutic angiogenesis, more and more studies have demonstrated that neovascularization can effectively improve the blood supply of ischemic myocardium. There are two primary mechanisms by which neovascularization occurs: vasculogenesis and angiogenesis. Vasculogenesis is the *in situ* assembly of endothelial progenitors into capillaries, while angiogenesis is a process through which new blood vessels form from pre-existing vessels through sprouting and intussusception (1). Cytokine-based therapeutic angiogenesis from the bench to clinical trials has been a major focus of medical research, and the efficacy of vascular endothelial growth factor (VEGF) blockers has led to the approval of

anti-angiogenesis drugs for cancer and eye disease. Conversely, the use of angiogenesis factors, such as VEGF and basic fibroblast growth factor (bFGF), has been shown to promote notable increases in collateral vessel and myocardial perfusion in ischemic myocardium, reduced infarct size and improved cardiac function (2), demonstrating the theoretical and experimental promise of this approach in treating ischemic diseases. Unfortunately, despite the exciting results obtained using angiogenesis factors to treat IHD, gene therapy is also limited by its restricted efficacy and resistance (3). For example, VEGF also accelerates angiogenesis in atherosclerotic plaques and promotes plaque growth, which may eventually lead to plaque instability, while it promotes angiogenesis in ischemic tissue, an observation referred to as the famous Janus phenomenon (4). Angiogenesis greatly improves blood flow in myocardial ischemia, but the safety of growth factor-based angiogenesis therapy is an issue that remains to be overcome. Thus, how to avoid the risks associated with angiogenesis therapy is a problem that must be considered.

Stem cell-based therapies provide a promising new method for the formation of new blood vessels. MSCs have become the most promising seed cells for the treatment of IHD, with advantages of rapid self-renewal, multidifferentiation potential, and weak immunogenicity in autologous transplantation. Clinical and preclinical studies have shown that MSCs therapy effectively limits the infarcted area and improves heart function. However, the mechanisms associated with the activities of MSCs in IHD therapy remain controversial. We primarily attribute the cardiac protective effect of MSCs to their ability to promote neovascularization for the following two reasons. First, MSCs secrete soluble paracrine factors that contribute to angiogenesis and vasculogenesis. Second, MSCs are able to differentiate into ECs, pericytes and smooth muscle cells (SMCs), which form the foundation of vessels, processes that both participate in the protective ability of MSCs toward IHD. In this review, we focus on the mechanisms and clinical applications of MSCs in IHD therapy through neovascularization to provide reference for the application of stem cells in IHD.

COMPARISON OF MSCS FROM DIFFERENT SOURCES

MSCs can be isolated from bone marrow, adipose tissue, umbilical cord blood, peripheral blood and almost every tissues in adults. Although MSCs can be harvested from different sources, regardless of their origin, they all have the capability of differentiating into adipocytes, osteoblasts and chondroblasts *in vitro* under specific conditions and can adhere to plastic under culture conditions. Furthermore, the surface of MSCs displays CD73, CD90, and CD105 but lack CD34, CD45, HLA-DR, CD14 or CD11b, CD79a or CD19. The International Society for Cell Therapy proposed the three criteria described above as identification standards for MSCs (5). Although MSCs from different sources share many of the same biological features, there are also some differences between distinct MSC populations. Bone marrow-derived MSCs (BMSCs), adipose-derived MSCs (AMSCs) and umbilical cord-derived MSCs (UCMSCs) are the most popular MSCs in clinical and preclinical experiments and trials, and some of their capabilities are compared below (**Table 1**).

TABLE 1 | Comparison of MSCs from different sources.

	BMSCs	AMSCs	UCMSCs
Differentiation capacity			
Osteogenesis	++	+	+++
Chondrogenesis	++	+	+++
Adipogenesis	+	++	+++
Endothelial cells	+	+	+
Pericytes	+	+	+
Smooth muscle cells	+	+	+
Proliferation capacity	+	++	+++
Migration capacity	+++	+	++
Tube formation	+	+	+

Differentiation Capacity

MSCs have the ability to differentiate into adipocytes, osteoblasts and chondroblasts. The amount of calcium deposits and sulfated proteoglycans stained by Alizarin red and Alcian blue, respectively were both higher in BMSCs than that observed in AMSCs, indicating that BMSCs have a higher capacity toward osteogenic and chondrogenic differentiation than AMSCs. While similar adipogenic differentiation potential was observed between these two types of cells (6), some studies have reported that AMSCs are more prone to adipogenic differentiation than BMSCs (7). Baksh et al. (8) observed that compared to BMSCs, UCMSCs underwent osteogenic differentiation more rapidly, exhibited higher alkaline phosphatase activity, and generated significantly more fat-containing cells when grown under adipogenic conditions by day 21. The differentiation ability of stem cells is affected by donor sex, age, isolation and culture conditions, etc. (9). Thus, which types of MSCs have a greater ability to differentiate into adipocytes, osteoblasts and chondroblasts remains disputed. In addition, MSCs also have the ability to differentiate into ECs, pericytes and SMCs, which are necessary components of blood vessels (10–13). Lu et al. (14) showed that MSCs from adipose tissue may have significantly greater ability to promote angiogenesis both *in vitro* and *in vivo* than UCMSCs and endometrial MSCs.

Proliferation Capacity

MSCs from different tissue sources do not have the same proliferative ability *in vitro*. Choudhery et al. (15) observed that UCMSCs have higher population doublings than AMSCs (33.0 $\pm$ 1.5 vs. 25.8 $\pm$ 0.6), with the doubling time being longer for AMSCs (2.7 $\pm$ 0.03 days) than UCMSCs (2.0 $\pm$ 0.04 days). Moreover, after prolonged passaging (30 times), the proliferative ability of UCMSCs did not change significantly, while BMSCs showed decreased proliferation after 6 passages (8), indicating that UCMSCs have a stronger proliferative ability than BMMSCs and AMSCs. Under human platelet lysate-supplemented culture conditions, AMSCs were observed to have greater proliferative

potential than BMSCs (6). Therefore, the proliferative ability of UCMSCs is the strongest, followed by AMSCs and BMSCs.

Migration Capacity

MSCs play an important role in posttraumatic tissue repair and cell therapy, and their migration ability is a key factor affecting their therapeutic efficacy. The migration capacity of BMSCs and placenta-derived MSCs (PMSCs) was observed to be 5.9- and 3.2-fold higher than that of UCMSCs, respectively. These results were consistent with the observed levels of migration-enhancing proteins in UCMSCs, including cathepsin B, cathepsin D and prohibitin, which were significantly lower than those observed in BMSCs and PMSCs, while the levels of migration-inhibiting proteins such as plasminogen activator inhibitor-1 and manganese superoxide dismutase were higher (16). Vimentin also contributed to the higher migration capability of BMSCs than UCMSCs (17). In contrast, UCMSCs exhibited an enhanced migration capacity toward factors released by hepatocellular carcinoma compared with BMSCs (18).

Capacity of MSCs to Promote Tube Formation of ECs

Tube formation is the last step in the formation of vessels and is necessary to supply blood for ischemia. Pill et al. (19) showed that AMSCs and BMSCs are both promising cell types to induce vascularization with ECs *in vitro* and are promising candidates to support *in vivo* vascularization. Nevertheless, Kim et al. (20) observed that conditioned medium from human AMSCs showed better tube formation-promoting effects than that from BMSCs *in vitro*, and AMSC group showed better recovery of blood flow than BMSC group in hindlimb ischemia model of nude mice. Furthermore, young AMSCs may have a higher tube formation capacity than old ones (21). UCMSCs are also capable of forming tubular networks (22). Du et al. (23) reported tube numbers of 11.65 ± 2.92, 0.91 ± 0.76 and 0.41 ± 0.20 for BMSC, AMSC and UMSC groups, respectively, indicating that BMSCs may have better angio-vasculogenic capacities than UMSCs and AMSCs. In contrast, Panepucci et al. (24) thought that UCMSCs would be more committed to angiogenesis and BMMSCs would be more committed to osteogenesis. Above all, there is still no consensus on which cell type has the greater capacity to promote tube formation.

PARACRINE FUNCTION OF MSCS THROUGHOUT THE NEOVASCULARIZATION PROCESS

The mechanism of MSC therapy is still controversial, because few MSCs can be found in myocardium after injection *in vivo* study. Wang et al. (25) showed that most intravenously injected MSCs remain in the lungs and liver, with only a small portion reaching the myocardial tissue. Similarly, Uemura et al. (26) observed only a few GFP-labeled MSCs in the periinfarct myocardium. Even so, clinical and preclinical studies still indicated the cardiac function of ischemic heart was improved, and infarct size and the number of apoptotic cardiomyocytes were significantly reduced after MSCs intervention. Which suggested the efficacy of MSCs did not benefited from themselves in some degree.

Paracrine hypothesis was firstly advanced by Gnecchi et al. (27). They found genetically modified BMSCs overexpressing the Akt1 released paracrine factors that exert cytoprotective effects on cardiomyocytes exposed to hypoxia and limited infarct size and improved ventricular function (27, 28). Furthermore, high VEGF, bFGF, IGF-1 and SDF-1 expression in hypoxia-preconditioned MSCs medium was examined, the results of which indicated that the paracrine function of MSCs may play more important role than their differentiation ability (26). Recently, it has been reported that MSCs secreted a wide array of cytokines that exerted beneficial angiogenesis in ischemic tissue, including PDGF, thrombopoietin, and angiogenin (29, 30). These factors are all involved in the neovascularization process, including MSC mobilization, migration, homing, adhesion and retention, and the differentiation of ECs. Especially VEGF and bFGF, which both have high affinity toward heparin and participate in angiogenic processes such as migration and amplification of ECs, are also necessary substances to induce the transformation of stem cells into ECs (10, 31). MSCs overexpressing Akt and angiopoietin-1 showed higher Flk1 and Flt1 positivity and promoted intrinsic $Flk1^+$ and $Flt1^+$ cell mobilization into the infarcted heart (32). Huang et al. (33) observed that overexpression of miR-126 promoted the differentiation of MSCs toward ECs through activation of the PI3K/Akt and MAPK/ERK pathways and the release of VEGF and bFGF factors. Therefore, paracrine factors secreted by MSCs may have pivotal functions throughout the neovascularization process. The role of various secretory factors in the neovascularization process will be discussed below.

INVOLVEMENT OF MSCS IN THE NEOVASCULARIZATION PROCESS

The process by which MSCs promote neovascularization involves in a number of steps. First, once ischemia occurs which also follows stress change, MSCs can perceive the associated changes and are mobilized from their niches to migrate and adhere to ischemic tissue to proliferate and differentiate. Notably, MSCs secrete various factors, including chemokines and growth factors, and this paracrine function is carried out throughout the neovascularization process (**Figure 1**). The completion of all biological processes depends on the cooperation of different types of cells, and the neovascularization process requires the collaboration of ECs, endothelial progenitor cells (EPCs) and pericytes. In addition, exosomes derived from MSCs act as a messenger that participate in cell-to-cell communication.

Environmental Perception by MSCs

Perception of Hypoxia by MSCs

Despite the benefits of MSC transplantation in cardiac tissue, detailed *in vivo* observations have shown that MSCs only survive for a brief period after engraftment due to harsh microenvironmental conditions (including ischemia, inflammation and anoikis) in the infarcted myocardium (34).

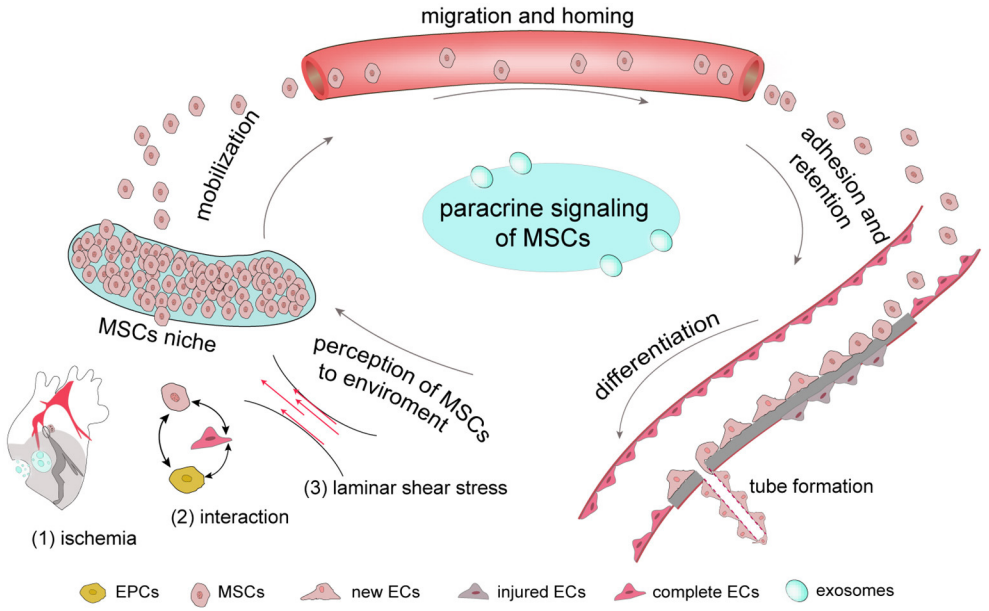

FIGURE 1 | The complete process by which MSCs respond to ischemia In general, MSCs are stored in their niches, which retain adult stem cell in a dormant state. Once tissue is damaged, signals, including those involve in ischemia-associated pathways, cell-cell interaction and stress mobilize stem cells to migrate from the stem-cell niche to damaged tissues, where they adhere, self-renew and differentiate. Once ischemia occurs, MSCs have the ability to secrete a number of growth factors through their paracrine function to promote new tube formation of ECs to provide new blood for ischemic tissue.

However, this environment contributes to the mobilization of MSCs from their niches.

MSCs originated from the bone marrow microenvironmental niche exhibit low oxygen tension. O_2 is a necessary factor in the maintenance of cell life as the final receptor in the intracellular aerobic respiration electron transport chain and is a substrate of some enzymes. Once the supply of O_2 is insufficient, the hypoxia signal will be rapidly transmitted to nucleus and initiate related gene expression to maintain oxygen homeostasis and the balance of energy metabolism between the cells and organism. Hypoxia inducible factor 1 (HIF-1), which has a dimeric complex composed of HIF-1a and HIF-b subunits, is oxygen-sensitive and the most important transcription factor affecting gene regulation under hypoxia (35). Once ischemia occurs, HIF-1 increases the expression of angiogenesis-associated genes, including VEGF, its receptors Flk-1 and Flt-1, bFGF and the fibrinogen system (36, 37). At the same time, HIF-1 improves the expression of proteases, such as membrane type matrix metalloproteinases, which hydrolyzes extracellular protein to promote cell migration, matrix reconstruction and the formation of tubule-like structures (38).

Hypoxia is also a basic aspect of the microenvironment that determines the differentiation of MSCs. Compared with a normoxia group, VEGF expression in embryonic and MSCs under hypoxia was observed to be significantly increased (39–41). Likewise, the *in vivo* administration of hypoxia-inducible VEGF-engineered MSCs was shown to induce ischemia-responsive VEGF production and lead to a significant increase in myocardial neovascularization after myocardial infarction in rats (39).

Cell-Cell Interactions

In 1997, Asahara et al. (42) identified and named a small population of $CD34^+$ cells as "EC progenitors." Indeed, EPCs are involved in a number of processes during angiogenesis, including mobilization, differentiation into ECs, homing, paracrine function and others (43, 44). Coculture of EPCs and MSCs significantly increased the transcription levels of endothelial specific markers, including vWF, CD31, VE-cadherin, Flk-1 and Flt-1 (45) and enhanced tube-like formation (46) through platelet derived growth factor (PDGF), Notch and TACE/TNF alpha signaling (45, 47). Joensuu et al. (48) noted that in cocultures of human MSCs and peripheral blood mononuclear cells, the previously nonadherent cells attached and started to elongate and form tube-like structures within 1 week concomitant with VEGFR1 upregulation, and platelet endothelial cell adhesion molecule 1 (PECAM-1) and endoglin-positive vessel-like structures were observed after 20 days. In addition, MSC-EC interactions were observed to decrease endothelial permeability induced by lipopolysaccharide through hepatocyte growth factor (HGF) by restoring the integrity of endothelial monolayers and remodeling endothelial intercellular junctions (49). VEGF secreted by stem cells from apical papilla is also used by human umbilical vein endothelial cells to increase the number of endothelial tubules, tubule lengths, and branching points (50).

Laminar Shear Stress and Pulsatile Stress

There are many force-sensitive molecules on the cell surface, such as cilia, integrins, ion channels and plaque proteins. Integrins connect the cytoskeleton and extracellular matrix through adhesive plaque and transform the force signals into intracellular biological signals through this plaque (51, 52). Considering the key role of shear force in the differentiation of ECs, researchers reported that such mechanical stimulation in cell culture *in vitro* was equally effective for the differentiation of stem cells into ECs (53). MSCs are highly reactive to mechanical stimuli in the environment, and different types of stress on the same MSC population will lead to different differentiation results (54). After generating canine BMSCs under shear stress provided by a pulsatile bioreactor for 4 days, the expression of endothelial cell markers, such as PECAM-1, VE-cadherin and CD34 was observed to be significantly increased (55). Fisher et al. (56) noted that AMSCs could form cords but failed to take up acetylated low density lipoprotein (acLDL) or express molecular markers after being cultured in endothelial cell growth supplement. Only the subsequent exposure of stem cells to shear stress did the cells exhibit realignment, acLDL uptake and CD31expression, indicating that stem cells differentiation to ECs requires the synergism of biochemical and shear force.

Dynamic Process of MSCs to Repair Ischemic Tissue

Mobilization

MSC mobilization is key for its involvement in tissue repair following their sensing of hypoxia, stress or other signals. An anoxic environment is one of the factors that induces stem cells to migrate out of their niches. Prolyl hydroxylase (PHD) and factor inhibiting HIF-1 (FIH) are key oxygen sensors in MSCs. HIF-1α upregulation by double knockdown of PHD and FIH synergistically increases stem cell mobilization and myocardial angiogenesis and improves cardiac function (57). The high concentration of growth factors outside of stem-cell niches may be another factor causing MSCs to mobilize from their original niches. Stromal cell-derived factor-1 (SDF-1 α)/Cxc chemokine receptor 4 (CXCR-4) are part of the most important chemotactic axis regulating MSC mobilization and migration. VEGF and insulin-like growth factor-1 (IGF-1)-overexpressing MSCs accelerate BMSC mobilization via the activation of SDF-1 α/CXCR4 signaling to promote myocardial repair (58, 59). Wan et al. (60) showed that active transforming growth factor β (TGF-β) also control the mobilization and recruitment of MSCs to participate in vascular repair. In addition, high-intensity exercise may be a potent stimulus that promotes circulating mesenchymal cells mobilization in patients with stable coronary artery disease (61).

Migration and Homing of MSCs

Homing and migration comprise a key step after MSC mobilization. Microenvironmental interactions between hypoxia and MSCs may control the ability of MSCs to migrate and their migration direction. In hypoxic tissue, SDF-1 and CXCR-4 are also important factors for cell migration. Ischemic myocardial and vascular tissues secrete SDF-1 to attract CXCR-4-expressing cells, particularly their therapeutic progenitors. Yu et al. (62) showed that SDF-1/CXCR-4 may mediate the migration of BMSCs toward heart MI through activation of PI3K/Akt signaling. Growth factors play an important role in the process of MSC migration. The stimulation of SDF-1α expression in infarcted hearts by VEGF-overexpressing MSCs was observed to result in the massive mobilization and homing of BMSCs (59). TGF-β1, HGF, IGF-1 and endothelial nitric oxide synthase (eNOS) also promoted the migration and homing BMSCs to the ischemic myocardium (63–65). Schmidt et al. (31) showed that low concentrations of bFGF attracted cells, indicating that bFGF may direct the migration of MSCs. In addition, Yan et al. (66) observed that C1q/tumor necrosis factor-related protein-9 (CTRP9) enhances AMSC proliferation and migration through the ERK1/2-MMP-9 signaling pathway and also promotes anti-apoptotic/cell survival via ERK-Nrf2/anti-oxidative protein expression. MiRNA, like miR-206 also involved in migration of MSCs by targeting Pim-1 (67).

Proliferation and Survival

Although MSCs transplantation is a promising therapeutic approach for IHD, the low viability of MSCs after transplantation needs to be improved. Hypoxic preconditioning may improve the functional survival and therapeutic efficiencies of engrafted BMSCs, at least in part through autophagy regulation (68). Some growth factors, including increased VEGF, TGF-β, IGF-1, SDF-1a and angiogenin were shown to enhance MSC survival and vasculogenesis in an MI model (69). Preconditioning with other factors, such as protein kinase C epsilon (εPKC), CTRP9, dimethyloxalylglycine and connexin-43 improves the retention and survival of transplanted MSCs in rat MI through the SDF-1/CXC and PI3K/AKT pathways (66, 70–72). Qu et al. (73) showed that atorvastatin, a hypolipidemic agent, has a protective effect on cardiomyocytes against apoptotic cell death in infarct and peri-infarct areas and could also increase the survival rate of implanted BMSCs in acute myocardial ischemia.

Adhesion and Retention

MSCs need to stay and adhere to ischemic tissue to play their important role in ischemic tissue repair. Molecular imaging studies have shown that <5% of MSCs engraft in ischemic tissues after being intravenously injected, with most of them dying within few hours after administration (74). This poor engraftment may be attributed to the constant blood flow and the harsh environmental conditions after acute ischemic injury. Since the long-term efficacy of cell therapy is proportional to the number of retained cells, this low retention and viability needs to be improved. Increasing the ability of MSCs to adhere to the ischemic tissues is key to improving their retention and viability. IGF-1 can increase the adhesion of MSCs and prolong their survival under hypoxia *in vitro* through PI3K activation (75). MSC adhesion can also be promoted by increasing the expression of integrin-linked kinase, periostin, and 2, 4-dinitrophenol (76–78). Reactive oxygen species (ROS) inhibit the cellular adhesion of engrafted MSCs, indicating that the elimination of ROS may be a novel strategy for improving the survival of engrafted MSCs

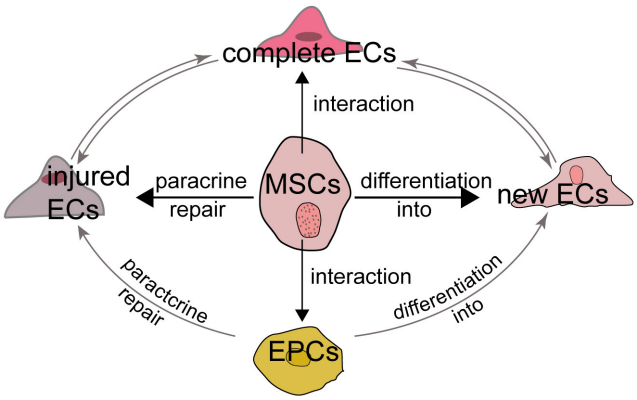

FIGURE 2 | MSCs and endothelial cells The relationship between MSCs and ECs can be summarized as follows. First, MSCs can repair the injured but not dead ECs through their paracrine function to induce the release of growth factors. Second, MSCs have the potential to differentiate into new ECs although it is controversial. Third, the interaction and crosstalk between MSCs and existing ECs promotes the formation of new ECs and the repair of injured ECs. Last, MSCs also interact with EPCs to influence EC formation and function.

(79). Bortolotti et al. (80) demonstrated that cardiotrophin-1 (CTF1) increases the retention and adhesion of BMMSCs to protect BMSCs from apoptosis, identifying it as a new powerful cytokine promoting cell engraftment. The retention and survival of transplanted MSCs was also shown to be improved by the overexpression of εPKC in AMI rats through the SDF-1/CXC and PI3K/AKT pathways (70).

INTERACTIONS AMONG MSCS AND ECS, EPCS AND PERICYTES

Multiple cell types are known to be involved in the processes of angiogenesis and vasculogenesis, including MSCs, ECs, EPCs and pericytes. In particular, ECs are indispensable for angiogenesis and the relationship between MSCs and ECs mainly attribute to the following aspects. First, MSCs secret growth factors which can repair the injured but not dead ECs through their paracrine function. Second, the interaction and crosstalk between MSCs and existing ECs promotes the formation of new ECs and the repair of injured ECs. Third, MSCs have the potential to differentiate into new ECs although it is controversial. Last, MSCs also interact with EPCs and pericytes to influence EC formation and function (**Figure 2**).

The ability of MSCs to limit infarct size may attributed to their pro-angiogenesis activity through existing ECs (**Figure 2**). The stimulated angiogenic activity of ECs is associated with the secretion of various growth factors and cytokines, including VEGF, HGF, IL-6, TGF-β1 and monocyte chemoattractant protein-1 (81). Lu et al. (82) showed that nestin(+) BMSC transplantation improved cardiac function in a mouse AMI model by recruiting resident cardiac ECs to the infarcted border region. BMSCs also rescued injured ECs through modulation of mitophagy or activation of signaling pathways such as PI-3K/AKT/m-TOR/eNOS and p38/MAPK (83, 84). Hypoxia also influences the interactions between the endothelium and MSCs (85).

In addition to the interactions between MSCs and ECs, studies showed MSCs had the ability to differentiate into ECs to promote angiogenesis in some degree although it was still controversial. For example, Otto et al. (86) did not observe MSC transdifferentiation into cardiomyocytes, ECs or SMCs and that the transdifferentiation of MSCs into cardiomyocytes or vascular cells did not significantly contribute to the improvement of cardiac function. Conversely, Silva et al. (87) showed that BMSCs promoted the angiogenesis of dog ischemic myocardium by differentiating into ECs, which accelerated the establishment of collateral circulation. Studies support MSCs own the potential to differentiated into ECs according to the below reasons. First, MSCs are multipotent stem cells derived from the mesoderm. Theoretically, MSCs can be differentiated into all mesoderm derived cells, and since ECs are mesoderm-derived cells, MSCs have the potential to differentiate into ECs. Second, MSCs express molecular markers of early ECs, such as VEGF receptor 2 (VEGFR-2/Flk-1/KDR) and bFGF, indicating that ECs can be derived from mesenchymal colonies and that MSCs arise from precursors with angiogenic potential (31, 88). Last, a series of *in vivo* and *in vitro* experiments proved that MSCs can differentiate into ECs. Oswald et al. (10) successfully used 2% fetal bovine serum supplemented with 50 ng/mL VEGF to induce BMSCs to differentiate into ECs *in vitro*. They observed that differentiated cells increased the expression of endothelial-specific markers, such as KDR and VEGF receptor 1 (VEGFR-1/Flt-1), and formed capillary-like structures. Furthermore, the process of MSC differentiation into ECs may require the synergy of bFGF, IGF, epidermal growth factor (EGF) (89, 90). ERK signaling may also involve in the differentiation of porcine AMSCs into ECs (90).

Furthermore, MSCs also function with EPCs to promote tissue repair. As a precursor of ECs, EPCs also differentiate into ECs and promote ischemia angiogenesis through their paracrine function (91, 92). MSCs could attract and promote the migration and vascularization of EPCs, which may depend on a positive feedback loop between CXCR-2 and CXCR-4 (93, 94). The viability and ability of MSCs to promote nerve regeneration is also improved by EPCs through PDGF-BB/PDGFR-β signaling (95). Rossi et al. (96) found MSCs and EPCs into the hind limbs of ischemia model together accelerated ischemic muscle recovery through an endoglin-dependent mechanism. Consequently, MSCs, ECs and EPCs may have a synergistic effect in ischemic tissue repair (**Figure 2**).

Pericytes, also known as mural cells, wrap around ECs in arterioles, capillaries and venules to regulate the maturation of ECs, stabilize the microvascular wall and promote angiogenesis. Although pericytes are surrounded by a basement membrane, they contact the ECs with through a "peg and socket" mechanism through holes in the basement membrane. Studies have shown that pericytes also communicate with ECs via paracrine signaling to improve tissue repair (97, 98). It is notable that pericytes also have stem cell-like properties and exhibit the morphology, mitotic activity and surface antigens of MSCs (99) and are

seemingly able to differentiate into adipocytes, chondrocytes, osteoblasts, neurons, astrocytes and oligodendrocytes, leading them to be identified as MSCs (100–102). However, it is still debated whether pericytes are MSCs. Guimaraes-Camboa et al. (103) challenged this concept and suggested that mural cells do not intrinsically behave as MSCs during aging and repair in multiple adult organs using a transgenic cell line. Over 2 years, the study showed that Tbx18 lineage-derived cells maintained their perivascular identity in the brain, heart, muscle and fat, indicating that mural cells do not exhibit an overt potential to give rise to other cell types. In contrast, MSCs can serve as a potential source of pericytes and induce vasculogenesis as mentioned previously (13, 104, 105), but similar to the multi-differentiation potential of MSCs, there needs to be standard guidelines for assessing pericyte differentiation in future studies. Furthermore, MSCs secrete various growth factors, including PDGF, which serves as a biomarker and crucial factor controlling the differentiation and recruitment of pericytes (106–108). These findings indicate that MSCs may regulate the recruitment of pericytes to injured tissue to participate in angiogenesis, but the associated mechanisms between MSCs and pericytes need to be further elucidated.

MSCS AND MSC-DERIVED EXOSOMES

Exosomes are a type of extracellular microvesicle secreted by multiple eukaryotes. Compared with cell therapy, MSC-derived exosomes (MSC-exos) have lower immunogenicity and are safer and more efficient, providing a new strategy for tissue regeneration via cell-free therapy (109, 110). MSC-exos are a type of message carrier that harbor a modifiable content of microRNAs, mRNAs and proteins, mediating communication between cells and functioning as key mediators of the paracrine effect of MSCs (111, 112). The pro-angiogenesis function of MSC-exos has been demonstrated in a number of studies. For instance, exosomes from MSCs overexpressing Akt, HIF-1α or CXCR-4 were shown to accelerate EC proliferation, migration and tube-like structure formation *in vitro*, as well as blood vessel formation to improve cardiac function in an MI model (113–115). MSC-exosomes may also have anti-inflammatory activities in MI model (116). Currently, the application of MSC-exos primarily focuses on preclinical experiments. One of the key problems for exosome clinical therapy is how to collect and purify enough exosomes so that they can be used safely. Andriolo et al. (117) developed a GMP-class method for the mass preparation of stem cell-derived exosomes to enable them to be used in future clinical applications. Indeed, as they are secreted by MSCs, MSC-exos have similar biological properties to MSCs to some extent. MSC-exos also have paracrine functions and mediate communication between MSCs and ECs, and they are also influenced by microenvironmental stress conditions, such as hypoxia and irradiation (118, 119). Furthermore, as they harbor a part of and not the entirety of MSC contents, MSC-exos are not an MSC "mini-me" and cannot replace MSCs in some respects, including their multiple differentiation and proliferation abilities.

APPLICATION OF MSCS IN IHD CLINICAL AND PRECLINICAL PRACTICE

The results of numerous clinical and preclinical studies have indicated that MSC transplantation is safe, significantly improves cardiac function and decreases infarct size and fibrosis in ischemic patients, which may be associated with the survival, retention, angiogenesis, paracrine action and the anti-apoptosis activities of MSCs (**Tables 2**, **3**). Although cellular therapies hold great promise for the treatment of human IHD and have good safety, the efficacy of MSCs remains disputable, especially when used in clinical trials. Meta-analyses of randomized clinical trials showed that the transplantation of BMSCs resulted in limited improvement on cardiac function for MI patients (171, 172). As it was showed in **Table 2**, some clinical trials proved MSC transplantation did not improve LVEF although it may limit infarct size. Different from clinical trials, MSC transplantation in animal experiments showed significantly elevated LVEF in most studies (**Table 3**).

Clinical patients are different from animal ischemia models, and the efficacy of MSCs in clinical practice is influenced by many different factors, such as (1) disease etiology and severity of patients, and (2) the type, number, delivery route and time, retention, survival, proliferation and differentiation of MSCs. Another meta-analysis showed that MSCs are more effective in patients with lower baseline left ventricular ejection fraction (LVEF) ($\leq$50%), and the effects of cells that were transferred at 3–7 days post-AMI was superior to those transferred within 24 h or more than 7 days in improving LVEF and decreasing LV end-systolic and diastolic dimensions (173), which suggested transplantation time was a key factor to influence cardiac function. Compared with clinical trials, animal experiments are easier to obtain positive results because of their simplicity, such as the MI model can be established uniformly by ligation of the left anterior descending coronary artery. Compared with MSCs intervention alone, pretreated MSCs with some growth factors together may get more efficacy (**Table 3**).

It is notable except for growth factors, more attention has been paid to natural botanical medicines. EGb761, an extract of *Ginkgo biloba*, was shown to exhibit a biphasic effect on hypoxia/serum deprivation-induced BMSC apoptosis, and its effect was closely associated with the PI3K/Akt and caspase-9 signaling pathways (174). *Salvia miltiorrhiza* is a widely used traditional Chinese medicine in cardiovascular diseases, and its constituent Tanshinone IIA was observed to decrease infarct size by increasing the recruitment of BMSCs to the infarct region by upregulating the SDF-1/CXCR-4 axis in a rat MI model (175). In addition to botanical medicines, chemicals such as statins, as the most commonly used lipid-lowering agents, exert activity toward a wide spectrum of cellular functions in addition to their lipid-lowering effects, including anti-inflammatory, anti-apoptotic, anti-fibrotic and pro-angiogenesis effects (176, 177). The results of multiple studies have suggested that atorvastatin has the ability to increase the survival rate of implanted BMSCs in an MI model, and combined with MSCs, it also ameliorated the cardiac milieu by reducing inflammatory cell infiltration, myeloperoxidase

TABLE 2 | Completed clinical trials using MSCs to treat ischemic cardiovascular diseases registered at clinicaltrials.gov.

NCT	Conditions	Interventions	Phase	Patients No/ follow-up	Endpoint	LVEF improved	Study type	Cell quantity	Method	Data	References
NCT00950274	MI	$CD133^+$ ABMSCs	III	82/6 mon	LVEF, LVEDV, LVESV, scar size, LV mass, NT-proBNP	Yes	R,PA,D	$0.5\text{-}5x10^6$	IM	2009.7–2016.3	(120)
NCT00669227	AMI	ABMCs	II	42/6 mon	LVEF, LVEDV, LVESV, infarct size	No	R,PA,D	381×10^6	ICA	2005.10–2009.1	(121)
				42/36 mon	LVEF, LVEDV, LVESV, infarct size, MO	Yes	R,PA,D	324×10^6			(122)
NCT00893360	MI/LVD	Autologous cardiosphere-derived stem cells	I	25/6 mon	Safety, LVEF, scar mass, systolic wall thickening	No	R,PA,N	$12.5–25 \times 10^6$	ICA	2009.5–2012.2	(123)
				25/13.4 mon	Safety, LVEF, LVEDV, LVESV, scar mass, scar size,	Yes					(124)
NCT01033617	MI/HF	ABMCs($CD133^+$)	–	40/ 6 mon	LVEF, LVEDV,LVESV,	No	R,PA,Q	$0.5–10 \times 10^6$	IM	2009.10–2016.6	(125)
NCT00279175	AMI	ABMCs	III	204/12 mon	LVEF, safety	Yes	R,PA,D	236×10^6	ICA	2004.4–2010.10	(126, 127)
NCT00114452	MI	Allogeneic hMSCs	I	53/6 mon	Safety, LVEF,LV remodeling	Yes	R,PA,D	$0.5, 1.6, 5.0 \times 10^6$/kg	ICV	2005.2–2009.2	(128)
NCT01291329	STEMI	Human umbilical WJ-MSC	II	116/18 mon	Safety, LVEF, perfusion,	Yes	R,PA,Q	6×10^6	ICA	2011.2–2012.7	(129)
NCT00883727	MI	Allogeneic BMSCs	I/II	20/2 year	Safety, LVEF, perfusion, infarct volume	No	R,PA, D	2×10^6/kg	ICV	2009.4–2012.8	(130)
NCT00765453	MI	ABMCs	II	100/12 mon	LVEF, infarct size, NT-proBNP	No	R,PA,T	$59.8\ (1.9CD34^+) \times 10^6$	ICA	2008.3–2018.3	(131)
NCT00264316	STEMI	ABMCs	II	67/4 mon	LVEF, LVEDV, LVESV, infarct size, systolic wall thickening	No	R,SA,D	$304\ (72MNCs) \times 10^6$	ICA	2003.5–2005.12	(132)
NCT00199823	AMI	ABMMCs	II	100/12 mon	Prothrombotic markers, LV function	No	R,PA,S	68×10^6 (0.7×10^6 $CD34^+$)	ICA	2003.9–2006.5	(133, 134)
NCT00313339	STEMI	ABMSC CD34+	I	31/6 mon	Safety, LVEDV, LVESV, LVEF, infarct size, perfusion	No	R,FA,N	$5, 10, 15 \times 10^6 CD34^+$ cells	ICA	2006.3–2013.3	(135)
NCT00684060	IHD /LVD	ABMSC	II	80/6 mon	LVEF, wall motion, LV volumes, infarct size, relationship of Ratio of $CD133^+$,$CD34^+$ cells on LVEF	Yes	R,PA,D	150×10^6	ICA	2008.7–2012.2	(136–138)
NCT00684021	STEMI/ LVD	ABMSC	II	120/6 mon	Safety, LVEF, wall motion, LV function	No	R,PA,D	150×10^6	ICA	2008.7–2012.11	(139)
				95/12 mon	LVEF,LV volumes, infarct size	No					(140)
NCT00355186	STEMI	ABM-MNCs	II	200/4 mon	LVEF, LVEDV, LVESV	No	R,FA,N	$153\ (119) \times 10^6$	ICA	2006.8–2012.11	(141)
				200/ 12 mon	LVEF, LV volumes, scar size, N-BNP	No					(142)
NCT01167751	MI	BM-MNCs; $CD133^+$ cells	II/III	90/6,18 mon	Safety, LVEF, systolic wall thickening	Yes	R,PA,D	MNCs:564.63×10^6; $CD133^+$cells:8.19×10^6	IM	2008.1–2012.7	(143)

(Continued)

TABLE 2 | Continued

NCT	Conditions	Interventions	Phase	Patients No/ follow-up	Endpoint	LVEF improved	Study type	Cell quantity	Method	Data	References
NCT00268307	AMI	ABMCs	I	40/6 mon	LVEF, LVEDV	Yes	R, CA, D	100×10^6	ICA	2005.12–2010.9	(144)
NCT02439398	AMI	Allogeneic hCSCs	I/II	49/12 mon	Safety, infarct size	–	R,PA,D	35×10^6	ICA	2014.6–2016.11	(145)
NCT00395811	CABG	ABMMNC	I/II	60/6 mon	safety, LVEF, LVEDV, LVESV,	Yes	R,PA,D	100×10^6	Graft	2007.1–2009.6	(146)
NCT00418418	Ischemic HF	ABMMC	II	39/12 mon	LVEF, LVEDV, LVESV, infarct size, safety,	No	R,FA,Q	$5–1,000 \times 10^6$	ICA	2006.10–2010.12	(147, 148)
NCT00363324	STEMI	BMSCs	II/III	80/6 mon	LVEF, LVEDV, LVESV	Yes	R,PA,D	360(2.6 CD34+)x10^6	ICA	2005.1-2009.11	(149, 150)
NCT01495364	STEMI	CD34+	II	161/ 12 mon	Safety, LVEF, infarct size,	No	R,PA,D	10x10^6±20%	ICA	2011.12-2016.4	(151)
NCT00289822	IHD	BMCs/CPCs	II	75/ 3 mon	LVEF	Yes	R,CA,N	BMCs: 205×10^6;CPCs:22×10^6	ICA	2002.1–2005.1	(152)
NCT01234181	MI	Hypoxia BMSCs	–	34/1 year	Safety, LVEF, LVEDV, LVESV, wall motion, perfusion	No	R,PA,N	10×10^6	ICA	2010.11–2012.12	(153)
NCT01076920	CMI/LVD	ABMSCs	I/II	10/2 year	Safety, LVEF, myocardial viability and contraction	Yes	SA;N	61.5×10^6	IM	2009.10–2014.9	(154)
NCT01087996	ICM	BMSCs	I/II	30/13 mon	Safety, LVEF, LVEDV, LVESV, immunologic monitoring, quality of life, pulmonary function	–	R,PA,N	20, 100, 200×10^6	TE	2010.4–2011.9	(155)

R, Randomized; PA, Parallel Assignment; FA, Factorial Assignment; CA, Crossover Assignment; SA, Single Group Assignment; DR, Double Randomized Controlled Trial; Q, Quadruple (Participant, Care Provider, Investigator, Outcomes Assessor); T, Triple (Participant, Care Provider, Investigator); D, Double blind; S, Single (Outcomes Assessor); N, None (Open Label); ABMMCs, Autologous Bone Marrow Mononuclear Cells; ABMCs, autologous bone-marrow cells; hCSCs, human cardiac stem cells; LVEDV, Left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; MO, microvascular obstruction; IHD, ischemic heart disease; LVD, left ventricular dysfunction; ICM, ischemic cardiomyopathy.

TABLE 3 | Representative animal studies performed using MSCs in ischemic models.

Disease model	Animal source	cell source/ type	Dose	Method/ No. of sites	Treatment/end time	Preclinical outcome	Mechanisms	Specific treatment	References
MI/IR	Mice	mice/ADSCs	2×10^5	IM/3	0 h/-	↑LVEF, ↓fibrosis	↑ADSCs survival/retention, migration, angiogenesis; ↑cardiomyocyte adhesion, proliferation; ↑MMP-10/13 and/or HGF	N-cadherin	(156)
IR	Adult male SD rats	SD rat within 6–7 d/ ADSCs	2×10^6	Iv/-	0, 24 h/24 h,72 h,28 d	↑LVEF, ↓infarct size	↓Neutrophil number by enhanced M2 macrophage and macrophage efferocytosis	–	(157)
IR	Female Gottingen mini-swine	Male Gottingen mini-swine/Cortical bone stem cells	2×10^7	IM/-	1.5–2 h/3 d, 7 d, 3 mon	↑LVEF, ↓infarct size	↑Macrophage and T-cell populations; ↓cardiomyocyte apoptosis	–	(158, 159)
MI	Male C57 mice	Human umbilical cord blood-MSCs	2×10^5	IM/4	0/-	Protects cardiac function (↓infarct size)	↓Apoptosis and autophagy of myocardial cells; ↑tube formation of ECs	Exo-SDF1	(160)
MI	Male C57/BL6 mice	-/MSCs	2×10^5	IM/5	30 min/1, 28 d	↑LVEF, ↓LVEDV, LVESV, scar size,	↑Autophagic flux through exosome containing mainly miR-125b-5p	Exo- MSCs	(161)
IR MI	Female Göttingen swine	Male Yorkshire swine MSCs/CSCs	200×10^6/1 $\times 10^6$	TE/10	-/3 mon	↑LVEF, perfusion, ↓LVEDV, LVESV, scar size, remodeling	↑Cardiac regeneration	–	(162)
MI	C57/BL6 mice; CTRP9 knock-out mice	EGFP-TG mice with C57BL/6J background/ADSCs	1×10^5	IM/3	1, 3, 7, 14 d/3 d, 4 w	↑LVEF, fibrotic area	↑Proliferation/ migration by ERK1/2-MMP-9, ↓poptotic /oxidant via ERK-Nrf2	CTRP9	(66)
MI	C57/BL6 Mice	male C57/BL6/BM-MSCs, ATMSCs	2×10^5	IM/1	-/IM after 1, 7, 14, and 21 d, LV after 21, 60 d	↑LVEF, LVFS	↑BM-MSC adhesion, ↓apoptosis, ↑focal adhesion kinase	CTF1	(80)
I/R	Female Large White pigs	male Large White pigs/ATMSCs	10×10^6	ICA/-	15 min after reperfusion/2 days, 60 d	↑Myocardial perfusion (vascular density), not LV Function	Pro-angiogenic factors: VEGF,SDF-1a, GM-CSF; Anti-apoptotic, inflammatory and collagen deposition	–	(163)
MI	SD rats	Aged and young male hMSCs	10×10^6	IM/5	30 min/28 d	↑LVEF, ↓fibrosis/scar size	↑Angiogenesis/ survival; against apoptosis	SRT1720	(164)
MI	C57BL/6 mice	Synthaetic hBMSCS	1×10^5	IM/-	0/15 d	Mitigated LV remodeling	↑Angiogenesis	–	(165)
MI	Male SD Rats	SD rats /BMSCs	22×10^6	Tail vein	2 d/IF: weeks 1, 2, 4; LV: 3 d, 1,3,6 w	↑Cardiac function (LVEF, LVDD, LVSD)	↑Angiogenesis by the tropism of MSCs to the MI area through SDF-1	VEGF-encapsulated	(166)
AMI	Female Large White pigs	Pigs /ATMSCs	50×10^6	IM /7-8	0/2, 15, 30 d	Cardiac function not significantly improved (LVEF)	↓Inflammation, ↑angiogenic process	IGF-1 /HGF	(167)

(Continued)

TABLE 3 | Continued

Disease model	Animal source	cell source/ type	Dose	Method/ No. of sites	Treatment/end time	Preclinical outcome	Mechanisms	Specific treatment	References
MI	Male Corriedale sheep	Corriedale male sheep/ BMSCs	20×10^6	IM/-	30 min/30, 60 d	↓Infarct volume; ↑LVEF	↑Angio-/arteriogenesis, ↓apoptosis by HIF1-mediated overexpression of EPO, iNOS, VEGF, and ANG-1	HIF1-a	(168)
CMI	Female pigs	Human umbilical cord-derived MSCs	30×10^6	ICA/ICV/-	4, 5, 6 w/ 4 w	↑LVEF, perfusion; ↓apoptosis, fibrosis	↑Angiogenesis by VEGF and Ang	–	(169)
MI	Male Cynomolgus monkeys	Cynomolgus monkey/BMSCs	10×10^6	IM/5	0/3, 28, 90, 180, 270 d	↑LV function, ↓infarct size	↑Cardiomyocyte proliferation, vascular density, myocardial glucose uptake, engraftment, paracrine activity(EPO, HIF1-α, ANG-1); ↓endogenous cell apoptosis	Hypoxia	(170)
AMI	SD rat	Male SD rat/BMMSCs	1×10^6	IM/5	0/1 day, 4 w	↑LVEF, ↓LVEDD, LVESD), remodeling improved	↑Retentionl:SDF-1/CXC and PI3K/AKT; ↑survival:VEGF, bFGF, TGFβ, cTnI, vWF, SMA and factor VIII	PKCε	(70)

ICA, intra-coronary artery infusion; ICV, intra-coronary vein infusion; IM, intramyocardial; TE, transendocardial; ↑, indicators increased or improved; ↓, indicators decreased or worsened.

activity and cardiac fibrosis (178, 179). Furthermore, activation of the JAK-STAT pathway may play important role in the ability of rosuvastatin to increase the efficacy of transplanted MSCs (178). Other factors, including glucagon-like peptide-1-eluting (GLP-1), lipopolysaccharide and lysophosphatidic acid also exert pro-angiogenesis effect by promoting the enhanced expression of cytokines and growth factors (180–183).

STRATEGIES AND FUTURE DIRECTIONS

MSCs display robust reparative properties through their paracrine and differentiation abilities that can limit apoptosis, enhance neovascularization and direct positive tissue remodeling. However, some problems with MSCs remain and must be solved before they can have widespread use. MSCs are important infiltrating cells that are also drived by blood and vasoconstriction. So the first problem is the low survival and retention of transplanted cells *in vivo* which limits their overall effectiveness in clinical usage. Consequently, identifying strategies to improve cell survival and retention *in vivo* is a priority. However, cell transplantation is affected by many factors, each of which may have an impact on the survival of transplanted cells, and there is still no consistent recommendation for each factor. The microenvironment of transplanted cells directly affects the survival of stem cells. The blood supply in the marginal area of myocardial infarction is well known to directly affect the survival rate and recovery of cardiac function after cell transplantation. One important goal of cell transplantation is to promote angiogenesis in the ischemic area and reduce the generation of myocardial scars. Studies have shown that hypoxia-induced stem cells release a variety of factors to improve the microenvironment through anti-inflammatory and anti-fibrosis effects and by promoting angiogenesis (170). Hypoxia or other growth factors used to precondition stem cells may allow MSC survival and retention to be improved, but additional comparisons and a set of standards are needed to identify the most powerful factors.

Another important factor limiting the clinical application of stem cells is the shortage of effective monitoring methods for stem cells. The successful implementation of cell therapies requires a better understanding of cell fate after transplantation. Currently, there are three primary labeling methods for stem cells, including reporter genes, fluorescent dyes and nanoparticles, which require optical imaging, MRI and radionuclide imaging to trace the transplanted stem cells, respectively or in combination, with each technique having its advantages and disadvantages (74, 184). Thus, there is an urgent need to develop a nontoxic and noninvasive tracer technology that exhibits long term stability and that can also be used to dynamically monitor the survival status of transplanted cells with respect to processes such as migration and differentiation *in vivo*.

AUTHOR CONTRIBUTIONS

WS contributed to the design and manuscript writing. QX, RY, and YY collected and assembled data. WC contributed the review design and financial support. KC was responsible for proofreading and final approval of the review. All authors contributed to the article and approved the submitted version.

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Effects of Therapeutic Hypothermia on Normal and Ischemic Heart

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Therapeutic hypothermia has been used for treating brain injury after out-of-hospital cardiac arrest. Its potential benefit on minimizing myocardial ischemic injury has been explored, but clinical evidence has yet to confirm positive results in preclinical studies. Importantly, therapeutic hypothermia for myocardial infarction is unique in that it can be initiated prior to reperfusion, in contrast to its application for brain injury in resuscitated cardiac arrest patients. Recent advance in cooling technology allows more rapid cooling of the heart than ever and new clinical trials are designed to examine the efficacy of rapid therapeutic hypothermia for myocardial infarction. In this review, we summarize current knowledge regarding the effect of hypothermia on normal and ischemic hearts and discuss issues to be solved in order to realize its clinical application for treating acute myocardial infarction.

Keywords: hypothermia, myocardial infarction, infarct size, endovascular, physiology, cardiac function, clinical trial, preclinical

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INTRODUCTION

The impact of temperature on human biology has been researched extensively and a number of experimental studies have shown that lowering body temperature is capable of protecting tissues from injury (1–7). To take advantage of this protective effect, the concept of therapeutic hypothermia (TH) has been developed and tested in patients with various diseases. Different levels of hypothermia including mild (32–35°C), moderate (28–32°C), severe (20–28°C), and profound (<20°C) were proposed (8). Clinical application of TH for acute diseases is mainly limited to mild and moderate hypothermia due to technical challenges and increased risks of arrhythmias at lower temperature range. Meanwhile, deeper TH has been applied to protect organs during circulatory arrest for cardiac and aortic surgeries (9). Successful demonstration of injury limitation in experimental studies and early clinical trials (10, 11) prompted researchers to use TH also for ST-elevation myocardial infarction (STEMI). However, much of the current clinical evidence of TH in organ protection was derived from studies that focused on neurological injury, while those focusing on the ischemic heart remain limited. Whereas reperfusion has already taken place in the brains of patients after resuscitation for out-of-hospital cardiac arrest (OHCA), the circumstances of STEMI are unique in that hypothermia can be applied prior to the reperfusion of ischemic myocardium. It therefore offers targeting of reperfusion injury in addition to post-reperfusion injury. The emergence of new devices and techniques that allow rapid cooling of the heart opens the door for discussion on whether priority should be placed on the attainment of a target temperature or on more rapid reperfusion. In this review, we summarize our current knowledge related to the impact of TH on the heart and discuss its potential benefit for treating STEMI.

ECG CHANGES AND ARRHYTHMIAS ASSOCIATED WITH TH

A decrease in heart rate is the most consistently reported electrophysiological change associated with TH in both normal and ischemic hearts (12–15). In addition to a reduced sinus rate, atrial and ventricular conduction velocities seem to decrease under hypothermia as represented by prolongation of PR, QRS, and QT intervals (16–18). Whether profound bradycardia during TH for MI would require intervention remains unclear, but bradycardia was apparently a favorable marker for patients undergoing TH after resuscitation following OHCA (14). Although it is unknown if lower heart rate in this study actually contributed to the good outcome or it was just reflecting less myocardial injury, bradycardia may be treated conservatively unless there is an evidence of organ hypo-perfusion.

The J wave is a characteristic ECG change found in some hypothermic patients. Up to 30% of patients after OHCA presented with J waves during TH and its prevalence was found to be higher in patients with STEMI (19). In cases of accidental hypothermia, the J wave was observed more frequently in patients having lower body temperatures (20, 21), suggesting a temperature dependent increase in its appearance. Interestingly, there was also an inverse correlation between temperature and the size of the J wave (21). These ECG changes sometimes mimic those of STEMI. In fact, Rolfast et al. (19) reported ST changes during TH in some OHCA patients who lacked actual coronary occlusion, which was confirmed by coronary angiograms.

Potential increase in incidence of atrial and ventricular arrhythmias has been a concern for applying TH in STEMI patients. In the COOL AMI EU pilot trial, which used an endovascular cooling method, the incidence of atrial fibrillation was more common in the TH group (32%) compared to the control group (8%, $P = 0.07$) (22). Using naïve pigs, Manninger et al. (18) found that TH prolongs the effective atrial refractory period at 33°C, which was accompanied by an increase in pacing-induced atrial fibrillation. However, the serum potassium level was decreased during hypothermia in this study, suggesting a potential influence of the dysregulated electrolyte. In contrast, in the *post hoc* analysis of a Targeted Temperature Management (TTM) trial, TH was not associated with the incidence of atrial fibrillation in patients with new-onset STEMI (23). Combined analysis of the RAPID MI-ICE and the CHILL-MI trials also exhibited no difference in atrial fibrillation incidence (24). Within the temperature range used in STEMI cooling studies, no significant increase in ventricular arrhythmias has been reported (22, 24), a finding consistent with animal studies (25). Taken together, mild TH seems not to significantly increase the occurrence of arrhythmia in general STEMI patients, but there could be a subpopulation of patients more prone to the development of arrhythmias, such as those with electrolyte dysregulation. Because hypothermia can dysregulate electrolyte balance through volume shift and by influencing kidney excretion (26), careful electrolyte monitoring is likely important.

IMPACT OF TH ON CARDIAC FUNCTION

In non-diseased hearts, several *ex vivo* studies reproducibly showed that hypothermia increased cardiac contractility (27–30). Despite increasing contractility, however, myocardial oxygen consumption remained similar and hypothermia was thus believed to improve myocardial energy efficiency (27, 31). The contractility increase was accompanied by prolonged systolic time (27, 32), resulting in a delay in achieving end-systole during ventricular ejection. Because of the prolonged systole, systolic functional parameters that include time component (e.g., maximum dP/dt, tissue Doppler velocity) did not necessary indicate improvement, whereas time-independent contractility parameters such as Emax or slope of end-systolic pressure-volume relationship generally showed an increase (12, 27–30). Some conflicting results exist for *in vivo* studies showing decreases in stroke volume or cardiac output (33, 34), but this was likely associated with complex biological interactions such as neuromodulation (35) and altered vascular resistance (36, 37).

As discussed above, heart rate slows by TH and helps to compensate for reduced diastolic time associated with prolonged systole. But even with lower heart rate, diastolic functional parameters are usually impaired under hypothermic conditions (37). Both active relaxation, as assessed by cardiac relaxation time constant, tau, or minimum dP/dt, and left ventricular stiffness, as assessed by left ventricular end-diastolic pressure or end-diastolic pressure-volume relationships, can be impaired by cooling (12, 38, 39). Myocardial stiffening associated with lower myocardial temperature might be responsible for increase in end-systolic and end-diastolic elastance. Meanwhile, increasing the heart rate by pacing has been shown to impair systolic function and also to worsen diastolic dysfunction (40). These results suggest mechanistic importance of prolonged systole for maintaining systolic function.

There are also limited data on the impact of hypothermia on cardiac function during myocardial ischemia, but available data suggest that functional changes in response to hypothermia are generally similar to those of the normal heart (41, 42). Interestingly, some previous studies before the reperfusion era showed that myocardial function (cardiac output and left ventricular stroke work) was better in hypothermia-treated animals after rewarming compared to the normothermic animals, despite the absence of reperfusion (33, 43). Whether hypothermia and rewarming also improves cardiac function without coronary reperfusion as these authors suggested or it was associated with rewarming induced vasodilation remains unclear. In either case, more data on rewarming after reperfusion is necessary to devise appropriate exit strategies for cardiac TH.

In summary, TH seems to have positive or at least neutral effects on contractility, but negative effects on diastolic function. Yet, studies that examined the impact of TH on *in vivo* heart function are limited and data are not always consistent with *ex vivo* findings. These are likely related to the difference in experimental settings including method and speed of cooling, animal species, anesthesia, and means of functional assessment.

MECHANISMS OF INFARCT REDUCTION

A large body of data on protective mechanisms associated with hypothermia derives from studies in neurons or in the arrested human heart at much lower temperatures. Limited studies have investigated mild hypothermia mediated protection in the myocardial ischemia setting (44–50). Nevertheless, available studies report similar mechanisms in the ischemic myocardium to those found in neuron studies in preventing post-reperfusion injury (8). However, TH can be applied prior to reperfusion in STEMI and it may offer an additive benefit by alleviating ischemia before reperfusion and also by reducing reperfusion injury at the early phase of reperfusion. Potential mechanisms of infarct size reduction by TH are discussed here.

Alleviating Ischemia

As discussed above, cardiac contractility is expected to be preserved during mild TH whereas myocardial energy efficiency is improved. TH also reduces heart rate. These are expected to reduce myocardial oxygen consumption related to the pump function (mechanical work) and alleviate ischemia progression. TH can also affect cardiac metabolism. Whole body metabolism and oxygen consumption decrease substantially as the temperature decreases (51). The relationship between temperature and oxygen consumption is likely non-linear, with a greater reduction of oxygen consumption in the first few degrees from normothermia (52, 53). It is expected that the ischemic myocardium also follows this relationship, and thus oxygen demand as well as tissue metabolism are likely suppressed early after TH induction. In a rabbit heart, initiation of epicardial cooling before MI preserved tissue adenosine triphosphate (ATP) and glycogen in the ischemic myocardium 20 min after MI (44). Reduced metabolism would also alleviate cellular acidosis, which can trigger cell death (54). However, it remains uncertain if and to what extent reductions in metabolism and oxygen consumption would offer benefit in the already ischemic myocardium, since energy stores are likely depleted by the time TH is initiated, unless started immediately after the onset of ischemia. Using a dog isolated heart, Jones et al. (55) reported that despite a 50% reduction in both ATP utilization and anaerobic glycolytic ATP production, energy deprivation could not be prevented and all hearts resulted in contracture-rigor, although with some delay in the hypothermia treated hearts. Thus, it is convincing that mechanisms other than mere reduction in energy consumption play important roles in myocardial protection during ischemia and reperfusion processes. That being said, slowing of energy utilization might offer large benefits to patients who have rich collateral supply to the ischemic myocardium, those with partially recanalized coronary, or those who arrived hospital early after the ischemia onset.

Reducing Reperfusion Injury

Reperfusion injury is estimated to cause around 50% of total myocardial injury in MI (56). Various mechanisms contribute to reperfusion injury and hypothermia seems to inhibit many of these pathological processes at the cellular level (26, 57). For example, TH has been shown to reduce cellular calcium load after reperfusion (58), which causes cell necrosis via the opening of mitochondrial permeability transition pores. Hypothermia-mediated apoptosis inhibition has been shown in many studies of neuron injury (59, 60), but data in myocardial ischemia-reperfusion injury remain scarce. *In vitro* studies using cardiomyocyte cell lines indicate that apoptosis of these cells at reperfusion following oxygen/energy deprivation is suppressed by TH (61, 62). Using an isolated rabbit heart, Ning et al. (63) reported reduced apoptosis in hearts maintained at 30°C during ischemia compared to those maintained at 34°C, but unfortunately, this study did not include settings at higher temperatures. Upon ischemia-reperfusion, rapid increases in oxygen radicals induce tissue oxidative stress, which has been shown to be inhibited by TH in both the heart (64) and neurons (65). Additionally, maintenance of cellular membrane integrity by hypothermia might prevent cellular edema. This is supported by a study that reported reduced myocardial edema after TH which was detected by magnetic resonance imaging in a pig model of ischemia-reperfusion (66). Hypothermia has also been reported to suppress post-ischemic inflammation via reduction of pro-inflammatory cytokine release (67) and local immune cell activation (66).

At the molecular level, Yang et al. (68) showed that hypothermia (35°C) increases extracellular signal-regulated kinase (ERK) activity in isolated rabbit hearts, the inhibition of which abolished the beneficial effects on infarct size. Using rat isolated hearts, Mochizuki et al. (46) reported that nitric oxide (NO) and phosphatidylinositol 3'-kinase (PI3K) are the key molecules in hypothermia (34°C) mediated infarct size reduction. Other studies also reported that increased AKT phosphorylation (47), reduced p53 expression, and increased heme-oxygenase 1 (50) play major roles in hypothermia-mediated reduction of reperfusion injury.

Interestingly, several reports indicate that hypothermia initiated after reperfusion does not reduce infarct size, whereas its initiation before reperfusion does so even if it is delayed from onset of the ischemia (69, 70). These results suggest that hypothermia may precondition the heart to alleviate injury associated with the very acute phase of reperfusion, which is expected to be the major portion of total reperfusion injury as shown in **Figure 1**. This might be the unique feature of hypothermia that allows alteration of ischemic myocardial wall property i.e., temperature, through endocardial transmission in the absence of coronary recanalization, which is not possible by pharmacological approaches. It remains uncertain, however, what mechanisms underlie in this protective preconditioning effect, since most of the previous reports studied myocardial molecular changes after the reperfusion.

IMPACT OF TH ON CORONARY FLOW – MICROVASCULAR OBSTRUCTION

In addition to the reduction of infarct size, TH has been shown to offer beneficial effects on post-reperfusion coronary flow. In a series of experiments, Hale et al. (71, 72) reported that direct ice

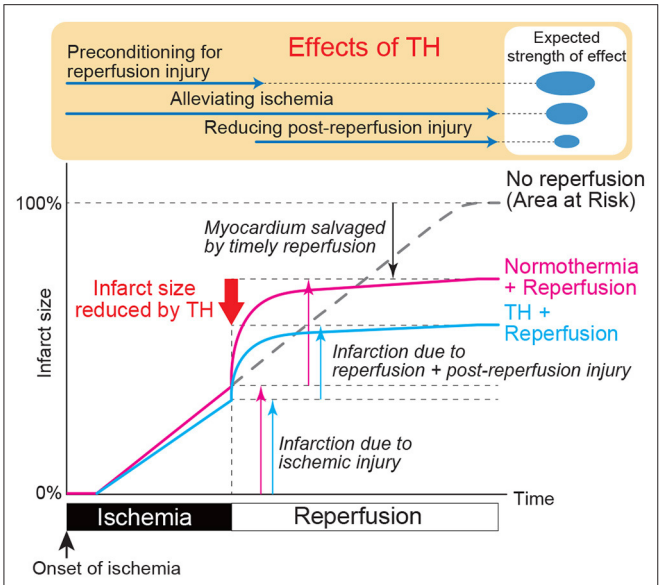

FIGURE 1 | Expected impact of therapeutic hypothermia on myocardial ischemia and reperfusion injury. The pink line shows the time course of myocardial injury associated with ischemia and reperfusion. X axis indicates time after ischemia onset and Y axis indicates infarcted myocardium relative to ischemia area-at-risk. Largest injury is expected just after the reperfusion. Blue line shows the expected time course of myocardial injury with TH when applied at the same time as the ischemia. Depending on when TH is started, the line may diverge at that time point. Preclinical data suggest that TH started at the time of reperfusion does not reduce myocardial injury. Therefore, TH during ischemia likely has preconditioning effect that prepares the myocardium for reperfusion. Expected strength of this preconditioning effect, ischemia alleviation, and attenuation of reperfusion injury are shown in the top of the Figure.

bag cooling of the rabbit heart initiated at the peri-reperfusion period reduced the no-reflow phenomenon without a change in acute infarct size. This result is supported by a study in pigs by Gotberg et al. (73), which demonstrated significant reduction of microvascular obstruction in the ischemic area as assessed by single photon emission computed tomography. However, clinical trials have yet to confirm these findings in humans and one randomized trial failed to find differences in the size of microvascular obstruction areas between patients treated with hypothermia and controls (74). More recently, Testori et al. (75) also reported that microvascular obstruction area assessed by cardiac magnetic resonance imaging was not different between the patients treated with and without TH 4 days after the onset of STEMI.

TH FOR CARDIOGENIC SHOCK ASSOCIATED WITH STEMI

As discussed above, mild TH increases cardiac contractility. Vasoconstriction of the peripheral vasculature increases systemic vascular resistance (36, 37) and raises arterial pressure. In addition, systemic hypothermia reduces metabolic demand of the whole body (76), which improves supply/demand of the non-cardiac organs. Therefore, theoretically, hypothermia would be an appropriate therapy for cardiogenic shock. Clinical studies in cardiogenic shock patients after cardiac surgery reported an increase in venous oxygen saturation upon hypothermia, indicating improved whole body oxygen supply/demand (12, 77, 78). Meanwhile, *post-hoc* analysis of TTM trial found that patients that required high dose of vasopressors were more common in the 33°C group than 36°C group (79). Whether this would be the same for patients in cardiogenic shock remains unclear. In a recent randomized trial involving 40 patients with cardiogenic shock, hypothermia failed to improve cardiac power as well as clinical outcome (80). Because only around half of the patients were STEMI-associated cardiogenic shock, more studies are needed to determine if hypothermia is safe and efficacious in treating STEMI-associated cardiogenic shock.

ANIMAL STUDIES OF TH FOR REDUCING MYOCARDIAL INFARCT SIZE

Infarct size reduction by TH has been studied in various animal species with variety of cooling methods, ischemia duration, cooling duration, and timing of cooling initiation. While small animal studies are useful in studying the mechanisms, large differences in body size, morphology, and heart size that allow much faster cooling may not fully represent cooling conditions in a clinical setting. Large animal models offer a simulation of hypothermia in clinically relevant conditions and also allow endovascular or intracoronary cooling approaches, which are not feasible in small animals. A summary of representative preclinical experiments in large animals that examined the impact of hypothermia on infarct size is provided in **Supplementary Table 1** (33, 42, 64, 66, 69–73, 81–97). In general, studies that initiated cooling prior to reperfusion have shown reduction of infarct size, whereas studies that initiated cooling just prior or after reperfusion tended to show no benefit, regardless of the cooling method. The majority of these studies only looked at the acute impact of TH and there are very few data on the impact of rewarming on infarct size. Accordingly, whether acute benefits on the infarct size can be maintained throughout the chronic phase remain unclear.

CLINICAL TRIALS OF TH FOR STEMI

Similar to preclinical studies, various approaches and devices have been employed for the controlled and efficient cooling of patients in clinical trials targeting STEMI (**Table 1**) (22, 74, 75, 98–109). The ideal cooling method for STEMI application would be one that offers rapid cooling with the ability to control body temperature throughout the temperature management period, from initiation through the rewarming phase. The ideal method would also be minimally invasive and implemented easily, in an ambulance if necessary, and without significant side effects. Shivering in response to cooling in awake patients is

another factor that needs attention because it can significantly increase body oxygen demand and slow cooling speed. Counter-heating of the skin during TH seems to be effective in reducing the shivering (110, 111), which is obviously not available for surface cooling methods and necessitate anti-shivering drug administrations. There is currently no single method that meets all above ideal features, leaving each with its own advantages and disadvantages (**Table 2** and **Supplementary Table 2**).

Ly et al. (101) used surface cooling and reported that target temperature of 34.5°C could be achieved in an average of 79 min. Although the trial did not find safety issues, no follow-up studies have been conducted, likely due to advances in cooling technology that offered much faster cooling. Peritoneal cooling was tested in 54 STEMI patients who were randomized to hypothermia ($n = 28$) and control ($n = 26$) (103). The study demonstrated that peritoneal cooling offers rapid cool-down of patients. However, there was no reduction of infarct size,

TABLE 1 | Summary list of clinical therapeutic hypothermia studies targeting myocardial infarction.

Author	References	Hypothermia method	Patient no.	Target temp (°C)	TH infarct size (%LV)	Control infarct size (%LV)	Significant
Dixon et al.	(93)	Endovascular cooling	42	33	2%	8%	No
O'Neill et al.	(101)	Endovascular cooling	392	33	14.1%	13.8%	No
Kandzari et al.	(97)	Endovascular cooling	18	33.5	4.0% (Day 30)	No control	NA
Ly et al.	(96)	Surface (Arctic Sun)	11	32–34	23%	No control	NA
Knafelj et al.	(105)	Surface + IV saline	72	32–34	NA	NA	NA
Wolfrum et al.	(103)	Surface + IV saline	33	32–34	NA	NA	NA
Schefold et al.	(104)	Surface + IV saline	62	33	NA	NA	NA
Koreny et al.	(100)	Surface (TheraKool)	111	32–34	NA	NA	NA
Götberg et al.	(95)	Endovascular cooling	20	33	13.7%	20.5%	No
Testori et al.	(99)	Surface + endovascular + IV saline	19	35	NA	No control	NA
Erlinge et al.	(102)	Endovascular + IV saline	120	33	40.5%	46.6%	No
Nichol et al.	(98)	Peritonial cooling	54	32.5	16.1%	17.2%	No
Otterspoor et al.	(94)	Intracoronary	10	−6°C Body temp	NA	No control	NA
Noc et al.	(16)	Endovascular cooling	50	32	16.7%	23.8%	No
Testori et al.	(75)	Surface + endovascular + IV saline	101	34	22% (Day4)	22% (Day4)	No

*More detailed information of the studies are provided in **Supplementary Table 2**. CMR, cardiac magnetic resonance; IV, intravenous; LAD, left anterior descending artery; LV, left ventricle; MACE, major advanced cardiac events; MI, myocardial infarction; NA, not available; OHCA, out of hospital cardiac arrest; SPECT, single-photon emission computerized tomography; STEMI, ST elevation myocardial infarction; TH, therapeutic hypothermia.*

TABLE 2 | Approaches to cool down the heart.

	Speed	Access	Earliest starting	Temperature control	Technical feasibility	Other
Systemic						
IV cold fluid	++	Periferal vein	Ambulance	Low	High	Potential lung congestion Evidence suggests negative impact on clinical outcome
Surface cooling	+	Percutaneous	Ambulance	Low	High	Speed depends on the cooling device
Endovascular	+++	Central vein	ER	High	Medium	
Peritoneal	+++	Peritoneal	ER	Medium	Low	Increased adverse events
Inhalation	++	Intubation	ER	Low	Medium	Limited experience
ECMO	++++	Central artery and vein	ER	High	Low	Increased risk of bleeding
Local						
Intracoronary	++++	Artery	Cathlab	Low	Low	Myocardial temperature may be different from coronary temperature
Direct cooling	++++	Open chest	Surgical room	Low	Low	

ECMO, extracorporeal membrane oxygenation; ER, emergency room; IV, intravenous.

whereas some concerns regarding increased safety issues were noted. Intracoronary hypothermia is another invasive approach that has been shown to be effective in rapidly and locally lowering the myocardial temperature (112). In a recent trial conducted in Europe, 10 patients were treated with intracoronary hypothermia by injecting room temperature saline through the coronary balloon catheter wire lumen, which was followed by 4°C saline injection after reperfusion (99). The authors reported arrhythmic events in patients with inferior MI, but not in the anterior MI, concluding that it was safe and feasible in patients with anterior MI. Follow up randomized studies are currently recruiting patients in Europe with an expected enrollment of 200 patients [Clinicaltrials.gov identifier NCT03447834 (113) accessed on Jan 15th, 2021]. Endovascular cooling has been the most popular method in the past STEMI trials likely owing to its relatively fast cooling, feasible application, and without requiring substantial amount of fluid loading. Some of these trials examined feasibility and safety (100, 102, 104), which were successfully confirmed, but none of the subsequent efficacy trials were able to meet the primary efficacy endpoints (22, 74, 75, 98, 106). More recently, Dae et al. (90) combined the data of 6 previous randomized clinical trials that used endovascular cooling method and analyzed the infarct size at 1 month after MI on a patient basis. Overall, 629 patients were included in the analysis and the study identified that anterior MI patients who were cooled to below 35°C at the time or reperfusion did show reduced infarct size over the control group, whereas other patient populations failed to show infarct size reduction. These results strongly suggest that rapid cooling to below 35°C is necessary, and patients with larger MI benefit most from TH. As we illustrated in **Figure 1**, we expect that the inhibition of reperfusion injury offers major benefit in TH. If 35°C is the threshold temperature to inhibit reperfusion injury, some patients may require additional time to reach this point before reperfusion. Existing data is insufficient to determine if delaying reperfusion for a short period to achieve target temperature below 35°C offers more benefit than immediate reperfusion. Currently, a clinical trial that aims to test the safety of new powerful endovascular cooling device is planned in the US [Clinicaltrials.gov identifier NCT03361995 (114) accessed on Jan 15th, 2021] and might provide more information on the temperature threshold at the time of reperfusion.

POTENTIAL REASONS FOR LACK OF EFFICACY IN CLINICAL TRIALS

Although blood temperature is expected to correlate with cardiac temperature, direct monitoring of ischemic myocardial temperature in STEMI setting is challenging. Moreover, cooling speed of the heart and other organs varies depending on the employed method (115). Therefore, inconclusive results in above discussed studies might be associated with insufficient lowering of the ischemic myocardium in contrast to the reported measured temperature. Unlike animal experiments, infarct size measurement in humans relies on imaging modalities and these indirect measures of infarct size assessment could be the source of measurement errors that can obscure the results (75). Additionally, some of the patients presenting STEMI might already have reperfusion at the time of the first coronary angiogram (116). Based on preclinical studies and the recent report by Dae et al. (90), these patients may not benefit from TH since reperfusion has already taken place. The efficacy of some drugs is known to be impaired at low temperature and these drug interactions need careful attention (26). There is a possibility that TH might have synergistic effects when combined with other therapies directed at reducing myocardial infarction (117), and this area remains largely unexplored yet.

CONCLUSIONS AND FUTURE PERSPECTIVES

Available evidence suggests that TH has the potential to reduce myocardial ischemic injury in humans. However, randomized clinical trials have yet to prove promising results in preclinical studies. Compared to the large number of studies focusing on TH for post-resuscitation brain injury or myocardial protection for surgery, that of alleviating myocardial reperfusion injury remains much less. Accordingly, there remain many questions that are only vaguely answered. These include: (1) Optimal target temperature for STEMI application; (2) Optimal TH method; (3) Whether target temperature needs to be achieved prior to reperfusion; (4) Optimal duration of hypothermia; (5) Mechanisms of myocardial protection; (6) Optimal target patient population; and (7) Optimal protocol for rewarming. Emergence of new devices that allow faster cooling may help to better define some of these questions and lead to positive results in forthcoming clinical trials.

AUTHOR CONTRIBUTIONS

KI drafted the manuscript. All other authors critically edited the manuscript and provided key information on this review article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Circular RNA Involvement in the Protective Effect of Human Umbilical Cord Mesenchymal Stromal Cell-Derived Extracellular Vesicles Against Hypoxia/Reoxygenation Injury in Cardiac Cells

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Human umbilical cord mesenchymal stromal cell-derived extracellular vesicles (HuMSC-EVs) can repair damaged tissues. The expression profile of circular RNAs (circRNAs) provides valuable insights into the regulation of the repair process and the exploration of the repair mechanism. AC16 cardiomyocytes were exposed to hypoxia/reoxygenation (H/R) injury and subsequently cultured with or without HuMSC-EVs (Group T and Group C, respectively). High-throughput RNA sequencing was implemented for the two groups. On the basis of the transcriptome data, gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and network analyses were carried out to determine the differential gene expression profiles between the two groups. After screening the circRNA database, the results were proved by quantitative real-time polymerase chain reaction. The survival rate of cardiomyocytes exposed to H/R was increased by treatment with HuMSC-EVs. RNA-seq analysis showed that 66 circRNAs were differentially expressed in cardiomyocytes in the co-cultured group. The cellular responses to hypoxia and to decreased oxygen levels were at the top of the GO upregulated list for the two groups, while the vascular endothelial growth factor signaling pathway, long-term potentiation, and the glucagon signaling pathway were at the top of the KEGG pathway upregulated list for the two groups. In the same samples, the 10 most aberrantly upregulated circRNAs were chosen for further verification of their RNA sequences. Seven of the 10 most aberrant circRNAs were significantly upregulated in the co-cultured group and in the HuMSC-EVs. Our results revealed that upregulated circRNAs were abundant during the repair of damaged cardiomyocytes by HuMSC-EVs, which provides a new perspective for the repair of H/R by HuMSC-EVs.

Keywords: circular RNA, AC16 human cardiomyocytes, human umbilical cord mesenchymal stromal cell-derived extracellular vesicles, hypoxia/reoxygenation injury, repair

INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury is one of the most common cardiac abnormalities following cardiac surgery. To date, there is no effective treatment for I/R injury (1). So, it is urgent need for finding novel therapeutic strategies. Mesenchymal stromal cell (MSC)-based therapy is a promising approach following I/R injury (2, 3). Different mechanisms are associated with this therapy, such as anti-apoptosis, anti-inflammation, and proangiogenesis (4). There is evidence that transplanted MSCs exhibit low survival in the host (5). Hence, the positive effects of MSCs are mainly due to their paracrine effects, especially through extracellular vesicles (EVs) (6).

EVs are nanosized membrane vesicles secreted in an evolutionally conserved manner by all types of cells including MSCs (7). EVs are key mediators of intercellular communication and take part in the transmission of biological information between cells, especially the transport of proteins and various nucleic acids, mainly mRNA and non-coding RNAs (ncRNA) (8). Previous studies have shown that MSC-derived EVs can mimic the biological characteristics of MSCs, such as immune modulation and tissue damage repair (9, 10). Our previous study also showed that human umbilical cord mesenchymal stromal cell-derived EVs (HuMSC-EVs) can protect cardiac cells against hypoxia/reoxygenation (H/R) injury *via* activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway (11). However, the mechanism has not been fully elucidated.

Circular RNA (circRNA) is formed by covalently closed transcripts without a 5' hat structure and 3' poly A tail (12). Because of their closed structure, circRNAs are resistant to exonuclease degradation and have higher stability than linear RNAs (13). Many studies have shown that circRNAs possess various biological functions, including sequestering microRNAs, binding to RNA-binding proteins (RBPs) (14, 15), and even encoding proteins (16). Recently, circRNAs have been implicated in cardiovascular diseases, including myocardial infarction and heart failure (17). However, whether circRNAs play a part in HuMSC-EV-mediated cardiac repair remains unclear.

In the present study, we aimed to investigate the function of circRNAs in the protective effect of HuMSC-EV in H/R injured cardiomyocytes. High-throughput RNA sequencing (RNA-seq) was used to find out the differentially expressed circRNA profiles in damaged cardiomyocytes co-cultured with or without HuMSC-EVs.

MATERIALS AND METHODS

Cell Culture and Extraction of HuMSC-EVs

HuMSCs were isolated as described previously (18). The Institutional Review Board of Shantou University Medical College (Shantou, China) consent the experiment protocol. HuMSCs were obtained from five patients who signed written informed consent forms. The HuMSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium containing 10% exosome-depleted fetal bovine serum (FBS) and incubated at 37°C in an incubator filled with 5% CO_2. We use the third passage of the HuMSCs for EV extraction. AC16 human cardiomyocytes were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), cultured in DMEM added 10% FBS, 100 U/mL streptomycin, and 100 U/mL penicillin, and incubated at 37°C in an incubator filled with 5% CO_2. ExoQuick-TC (System Biosciences, CA, USA) was used for HuMSC-EVs isolated as described previously (11).

Internalization of HuMSC-EVs Into AC16 Human Cardiomyocytes

HuMSC-EVs were labeled with PKH26 (Ca#MIDI26, Sigma-Aldrich, China) for 15 min in the dark at room temperature, washed thrice with PBS, centrifuged at 100,000 × g, and incubated at 4°C for 2 h. The purified EVs were added to the culture medium and incubated with cardiomyocytes for 12 h. Cell nuclei were dyed using 4969-diamidino-2-phenylindole (DAPI, Ca#C1005, Beyotime Biotechnology, Shanghai, China) and the internalization of HuMSC-EVs by the cardiomyocytes was analyzed using the Olympus BX51 confocal microscope (Olympus, Tokyo, Japan).

In vitro H/R Model

To induce hypoxia, the initial culture medium was replaced with buffer (pH 6.2, 137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2 \times 6H_2O$, 0.9 mM $CaCl_2$, 4 mM HEPES, 20 mM Na lactate) (19) and the AC16 human cardiomyocytes were cultured in pure N_2 gas at 37°C for 3, 6, 12, or 24 h. Afterward, cardiomyocytes were reoxygenated in fresh oxygenated culture medium for 1, 3, 6, and 12 h, respectively (H_3/R_1, H_6/R_3, H_{12}/R_6, H_{24}/R_{12}), in an incubator with 5% CO_2. Three experimental groups were designated as follows: normal group, in which AC16 human cardiomyocytes were cultured under normal conditions (Group N); H/R group (Group C); and H/R+HuMSC-EVs group (Group T), the cardiomyocytes were cultured with HuMSC-EVs (8 μg/mL) for 12 hours prior to H/R. Experiments were carried out in triplicate.

MTT Assay

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used for evaluated the viability of the cardiomyocytes. MTT was added to the culture medium, incubated for 4 h at 37°C, and subsequently solubilized with 150 μL DMSO. The optical density (OD) values were measured at 490 nm using a microplate reader. The viability of the cardiomyocytes was calculated as follows: viability (%) = (OD of assay—OD of blank) / (OD of control—OD of blank) × 100%.

Western Blot

Cardiomyocytes or HuMSC-EVs were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer prior to whole-protein purification. The protein concentration was measured by a bicinchoninic acid assay (BCA) kit (Beyotime

Abbreviations: HuMSC, human umbilical cord mesenchymal stromal cell; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; MSCs, mesenchymal stem cells; EVs, extracellular vesicles; circRNA, Circular RNA; ceRNA, competitive endogenous RNA; FBS, fetal bovine serum; ATCC, American Type Culture Collection; OD, optical density; PVDF, polyvinylidene difluoride; DECs:differentially expressed circRNAs; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SD, standard deviation; ANOVA, analysis of variance; VEGF, vascular endothelial growth factor.

Institute of Biotechnology, Haimen, China). Equal amounts of protein underwent 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with the primary antibody overnight at 4°C and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000, Ca#BE0101, EASYBIO, Beijing, China) for 1 h at room temperature. Specific protein bands were observed with an ECL Plus chemiluminescence kit (EMD Millipore) and quantitatively analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). Experiments were carried out in triplicate. The primary antibodies used in this study were as follows: Bax (1:5000, Ca #ab32503, Abcam, Cambridge, MA, USA), Bcl-2 (1:2000, Ca #ab196495, Abcam); cleaved-caspase 3 (1:1000, Ca#184787, Abcam), and β-actin (1:5000, Ca#0035, Abways, Shanghai, China).

CircRNA Identification by RNA-Seq

Total RNA was isolated from the cardiomyocytes using Takara RNAiso Plus (Total RNA Extraction Reagent, Takara, Japan) in the light of the manufacturer's protocol. The Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and Agilent 2100 Bioanalyzer (Applied Biosystems, Carlsbad, CA, USA) were used to evaluate the concentration and integrity of the RNA, respectively. The RNA-seq library was prepared with ∼1 μg of total RNA using a KAPA RNA HyperPrep Kit with RiboErase (H/M/R) for Illumina® (Kapa Biosystems, Inc., Woburn, MA, USA). After the depletion of ribosomal RNA, the ribominus RNAs were fragmented and the first strand and directional second strand were synthesized. Afterward, A-tailing and adapter ligation were performed with purified cDNA. After that, the purified, adapter-ligated DNA was amplified. The DNA library quality and concentration were evaluated with a DNA 1000 chip using an Agilent 2100 Bioanalyzer (Applied Biosystems, Carlsbad, CA, USA). Accurate quantification for sequencing applications was performed using the qPCR-based KAPA Biosystems Library Quantification kit (Kapa Biosystems, Inc., Woburn, MA). Each library was diluted to a final concentration of 10 nM and pooled in equimolar amounts before clustering. Sequencing was carried out in a 150-bp paired-end run (PE150) using the Novaseq 6000 system (Illumina, San Diego, CA, USA).

Bioinformatics Analysis

It has been proven that circRNAs can sponge miRNAs and indirectly regulate the translation of mRNAs. To further illuminate the role of circRNAs in the protective effect of H/R injury in cardiomyocytes, the differentially expressed circRNAs (DECs) were selected to predicted the target miRNA by using miRanda software. The top 10 upregulated circRNAs with the largest fold changes (FCs) were then picked out to create a circRNA-miRNA network diagram. The functions of the parental genes consistent with the upregulated circRNAs were predicted by gene ontology (GO). GO terms with a $p < 0.05$ were chosen and integrated using Venn analysis. The top 18 enriched GO terms among the groups were ranked by fold enrichment, and the enrichment scores were presented. The pathway clusters were analyzed on the basis of the Kyoto Encyclopedia of Genes and Genomes (KEGG). According to the threshold of $P < 0.05$ and overlap gene count ≥ 1, significant correlations between the parental genes of upregulated circRNAs and their potential pathways were determined. The -log10 (P-value) indicates the significance among differentially expressed RNAs.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from cardiomyocytes and HuMSC-EVs were reverse-transcribed to cDNA using the Geneseed® II First Strand cDNA synthesis kit (Geneseed, Guangzhou, China). CircRNA primer sequences were designed *via* Primer Premier 5.0 and synthesized by Geneseed Biotech Co., Ltd. (Guangzhou, China). qRT-PCR was then performed with the SYBR Green qPCR Master Mix (Geneseed). The fluorescence signal emitted was detected and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control to normalize the circRNA expression data. The $2-\Delta\Delta Ct$ method was used to analyzed the relative expression levels. All experiments were performed in triplicate. The primer sequences of the circRNAs for qRT-PCR are listed in **Table 1**.

Statistical Analysis

Data were analyzed in SPSS version 20.0 software (SPSS Inc., Chicago, USA). Quantitative data are presented as the mean ± standard deviation (SD). Statistical differences between groups were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. A p-value <0.05 was considered statistically significant.

RESULTS

Internalization of HuMSC-EVs Into AC16 Human Cardiomyocytes

To demonstrate the internalization of HuMSC-EVs by AC16 cardiomyocytes, the HuMSC-EVs were labeled with PKH-26 and incubated them with the cardiomyocytes. We observed that red fluorescence in the cytoplasm is nearly all cardiomyocytes (as shown in **Figure 1A**), manifesting that abundant HuMSC-EVs were internalized into the cardiomyocytes.

HuMSC-EVs Alleviate H/R Injury

The MTT assay showed that the viability of the cardiomyocytes gradually declined in a time-dependent manner after H/R (**Figure 1B**), indicative of a successful H/R injury. Hypoxia for 12 h and reoxygenation for 6 h (H_{12}/R_6) resulted in a moderate injury, and this condition was selected for our subsequent experiments. As shown in **Figure 1C**, cell viability reduced significantly suffer to H/R injury (57% compared with group N) and HuMSC-EVs ameliorated cell viability (74% compared with group N) in cardiomyocytes subjected to H/R ($^{\#\#}p < 0.01$, vs. Group N, $^{**}p < 0.01$, vs. Group C). Compared to Group C, HuMSC-EV treatment upregulated Bcl-2 while downregulating the protein expression of Bax and cleaved-caspase 3 ($^{\#}p < 0.05$ vs. Group N; $^{*}p < 0.05$ vs. Group C) (as shown in **Figures 1D–F**).

TABLE 1 | The characteristics and primers of the ten circRNAs.

Number	circbaseID	Gene	Sequence (5′->3′) (Forward/Reverse)	Product length
1	hsa_circ_0008199	ATXN10	CATCTCCAATGTGGCCAATG	103
			CTGGGTGCTGTTTCTCTTGT	
2	hsa_circ_0007928	DCUN1D4	N/A	
3	hsa_circ_0110664	PRDM2	AAATCTAGAGAGCGGAGTGG	136
			CTGGGATTTTTTCTTCCCTG	
4	hsa_circ_0023919	PICALM	GTAGCAAGTACATGGGGAGG	105
			CTGCTTGCAGCTGTAGAATC	
5	hsa_circ_0002190	KLHDC10	TCTACCAGAAGAGAGTTGTG	131
			GAGCAAAGCCCATCTCTTAT	
6	hsa_circ_0001988	ATXN7	GGGCTCTGTCGGGAAGATC	112
			TTCTTTGGAGGGTAGGCCAA	
7	hsa_circ_0003203	SBDS	GACCATACACCGTGATCCTT	100
			CATCGAGGTCTTTTTCCCTG	
8	hsa_circ_0001971	FAM126A	CTGTGTCAAATTTGTTCAAGACA	115
			TGTGGCTCCTGGATAACTTT	
9	hsa_circ_0039400	FTO	GAGAATGGCATGCCAGATGA	143
			GCCAACTGACAGCGTTGTAA	
10	hsa_circ_0001746	MKLN1	GAAAAGGCTGTAAATGGAAC	114
			CAGGCCTTTCGAGCTTTAGA	

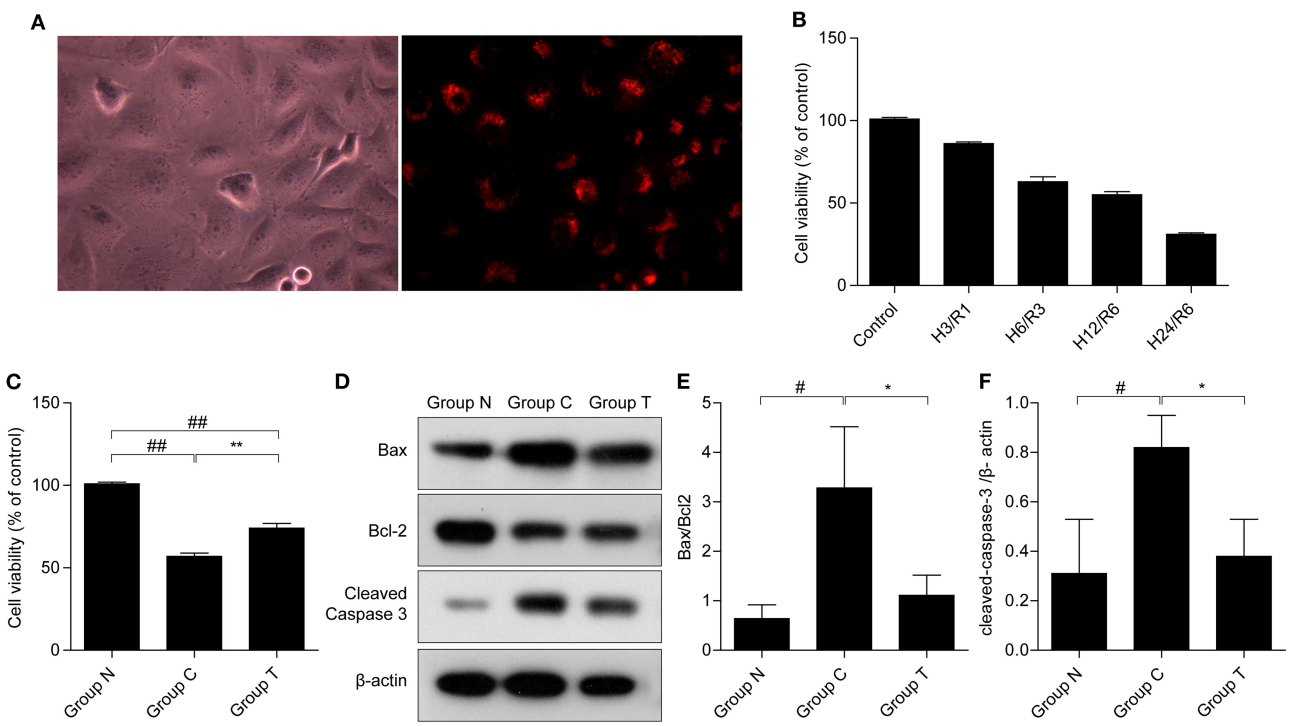

FIGURE 1 | Internalization of HuMSC-EVs by AC16 human cardiomyocytes and its effects on cell survival after H/R injury. **(A)** Internalization of HuMSC-EVs by cardiomyocytes, as detected by fluorescence microscopy. **(B)** MTT was used to measure cardiomyocytes viability after exposure to H/R. **(C)** HuMSC-EVs promote survival after H/R. **(D–F)** The protein expression of cleaved-caspase 3, and Bax/bcl2 in cardiomyocytes. Values are means ± SD. $^{\#}P < 0.05$ vs. Group N;$^{\#\#}P < 0.01$ vs. Group N; $^{*}P < 0.05$ vs. Group C; $^{**}P < 0.01$ vs. Group C. β-actin served as an internal control. H/R: hypoxia/reoxygenation. Group N: cardiomyocytes cultured under normal conditions; Group C:H/R group; Group T:H/R+HuMSC-EVs group.

The above data suggested that HuMSC-EVs could ameliorate cell survival and mitigate apoptosis caused by H/R injury in cardiomyocytes.

Analysis of circRNA Expression Profiles

In total, 15,880 circRNAs were detected in RNA-sequence analysis. About 89.49% of circRNAs were from protein-coding exons (e-circRNAs), 1.28% of circRNAs were from intronic circRNAs (i-circRNAs), and the rest were exon-intron circRNAs (e/i-circRNAs). First, we analyzed the chromosomal distribution of DECs in Groups C and T. The results indicated that these circRNAs were located at all chromosomes (**Figure 2A**). Sequence length analysis showed that the circRNA transcripts were mainly 400–800 bp in length (**Figure 2B**). A heat map and a volcano plot were used to show the DEC profiles between the two groups (**Figures 2C,D**). Sixty-six circRNAs were significantly differentially expressed (fold change > 1.5 or < -1.5, $p < 0.05$). Compared to Group C, 33 circRNAs were upregulated (**Supplementary Table 1**) and 33 circRNAs (**Supplementary Table 2**) were downregulated in Group T. Then, we narrowed the scope of our analysis to the 10 most aberrantly upregulated circRNAs in Group T. These 10 upregulated

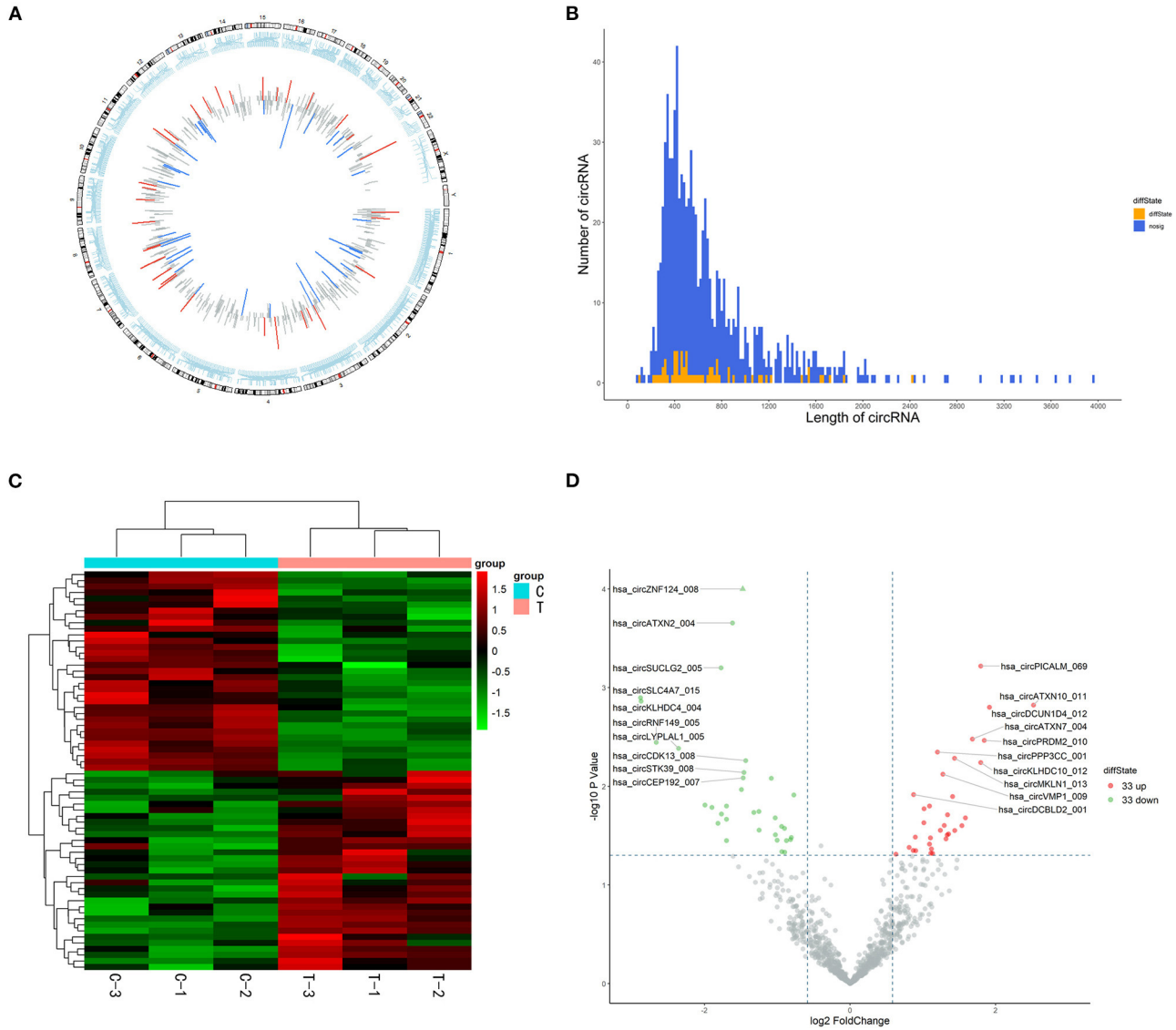

FIGURE 2 | High-throughput sequencing analysis of circRNAs in cardiomyocytes**. (A)** chromosomal distribution of the of the DECs between the Group C and Group T. **(B)** Length distribution analysis of DECs, the up and downregulation circRNAs have been marked in red and green bars. **(C)** Hierarchical clustering of circRNAs. Each group contains three individuals. circRNAs are represented by single rows and samples by single columns. **(D)** Volcano plot of circRNAs. The values on the X-axes present normalized fold changes and Y-axes present P values. The color scale indicates relative expression, upregulation (red) and downregulation (green). CircRNAs with fold change > 1.5 or < -1.5, $P < 0.05$ were regarded as differentially expressed. CircRNAs: circular RNAs; DECs: differentially expressed circRNAs; Group C: H/R group; Group T: H/R+HuMSC-EVs group.

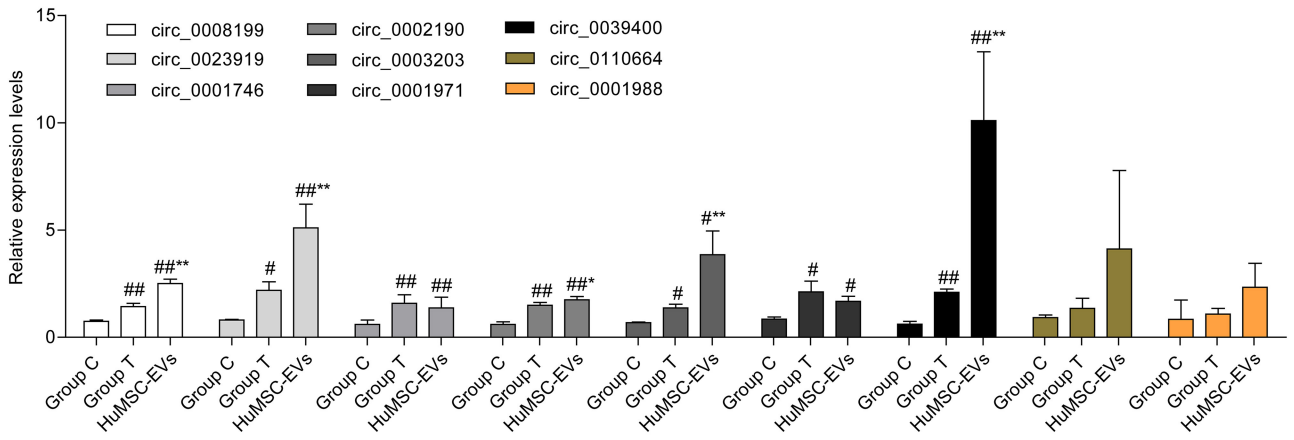

FIGURE 3 | The expression of selected circRNAs (hsa_circ_0008199,hsa_circ_0110664,hsa_circ_0023919,hsa_circ_0002190,hsa_circ_0001988,hsa_circ_0003203,hsa_circ_0001971,hsa_circ_0039400,hsa_circ_0001746) in Group C, Group T, and HuMSC-EVs according to qRT-PCR. Data are shown as mean ± standard deviation (SD). #$p < 0.05$, ##$p < 0.01$ vs. Group C. *$P < 0.05$, **$P < 0.01$ vs. Group T.

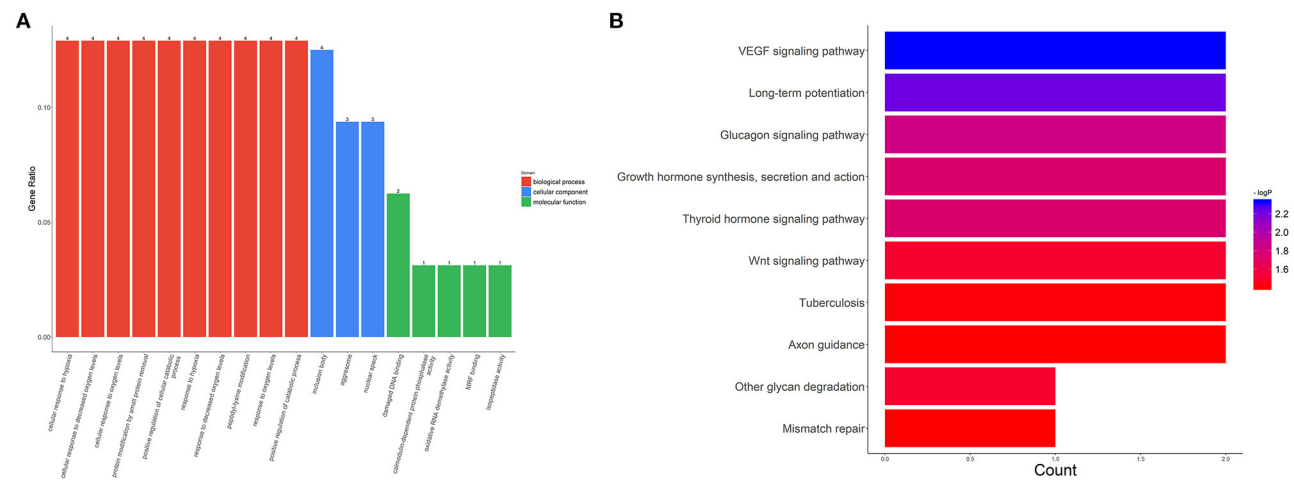

FIGURE 4 | GO and KEGG analysis for circRNA-targeted mRNAs. GO enrichment for the target mRNAs **(A)** and KEGG pathway analysis **(B)**. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

circRNAs, which had the largest FCs in the RNA-seq, are shown in **Table 1**. Furthermore, we confirmed the expression levels of the 10 circRNAs using qRT-PCR with the same samples analyzed using RNA-seq. Seven of the 10 circRNAs were confirmed to be significantly upregulated in Group T ($p < 0.05$), namely hsa_circ_0008199, hsa_circ_0023919, hsa_circ_0002190, hsa_circ_0003203, hsa_circ_0001971, hsa_circ_0039400, and hsa_circ_0001746 (**Figure 3**). The expression levels of these seven circRNAs were in accord with the RNA-seq analysis. Nevertheless, primers for hsa_circ_0007928 could not be designed. Additionally, the expression levels of hsa_circ_0110664 and hsa_circ_0001988 showed no differences between the two groups. The expression of the seven circRNAs was assessed in the HuMSC-EVs and was found to be similar to that in Group T (**Figure 3**).

We also evaluated the functions of the upregulated circRNAs. GO analysis showed that the gene functions were mainly focused on three terms: biological processes (BP), cellular components (CC), and molecular functions (MF). Under BP, cellular response to hypoxia, cellular response to decreased oxygen levels, and cellular response to oxygen levels were the three most enriched items. CC analysis indicated that the upregulated circRNAs were significantly enriched in the inclusion body, aggresome, and nuclear speck. Under MF, damaged DNA binding, calmodulin-dependent protein phosphatase activity, and oxidative RNA demethylase activity were the three most enriched items (**Figure 4A**). KEGG analysis suggested that the upregulated circRNAs could target genes associated with several biological pathways, including the vascular endothelial growth factor (VEGF) signaling pathway,

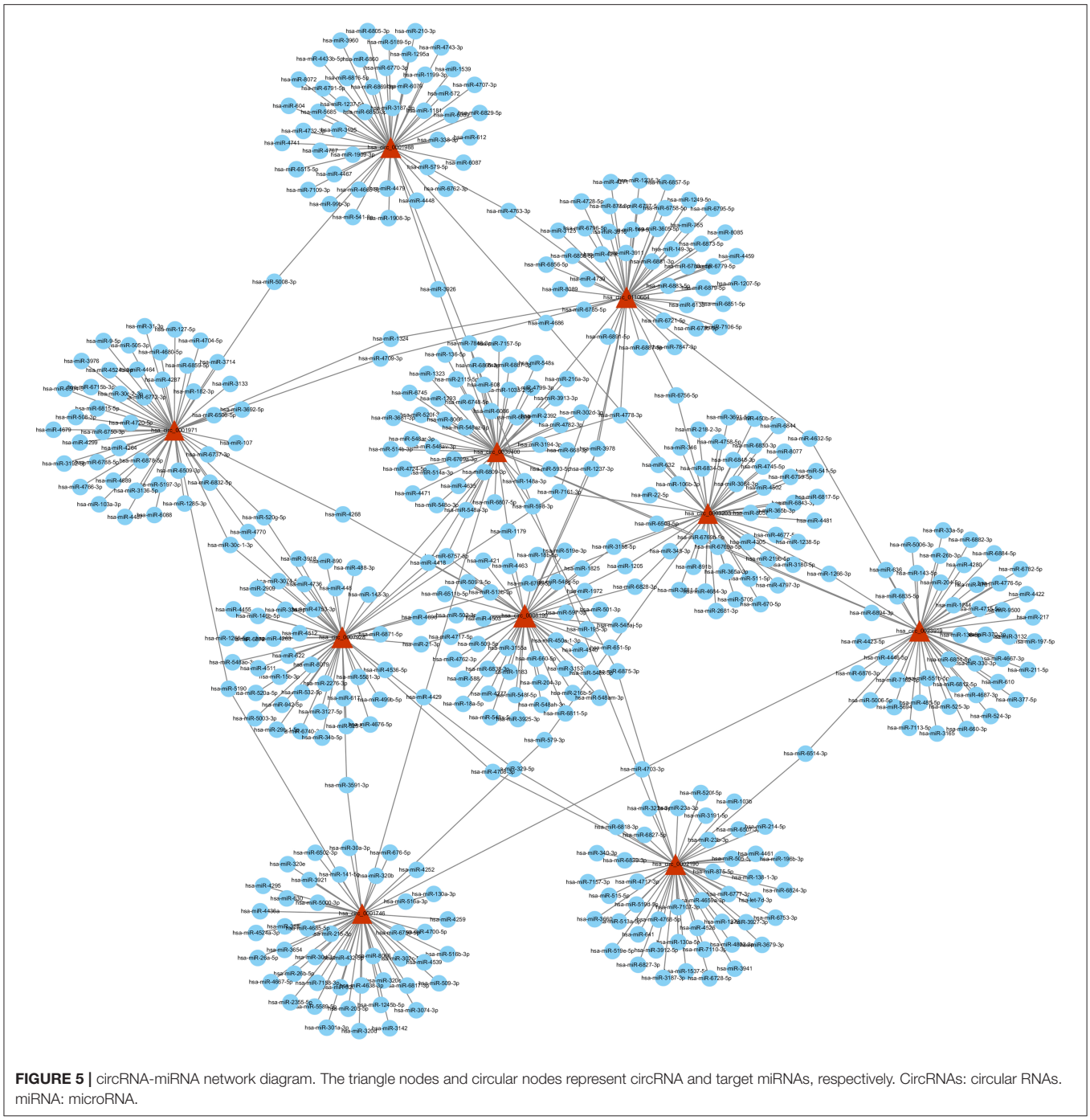

FIGURE 5 | circRNA-miRNA network diagram. The triangle nodes and circular nodes represent circRNA and target miRNAs, respectively. CircRNAs: circular RNAs. miRNA: microRNA.

long-term potentiation, and the glucagon signaling pathway (**Figure 4B**).

Prediction of circRNA-miRNA-mRNA

Based on the competitive endogenous RNA (ceRNA) theory, the first 10 most aberrantly upregulated circRNAs were chosen to construct a circRNA-miRNA network diagram. As shown in **Figure 5**, the network diagram contained 10 circRNAs and 2070 miRNAs. To investigate whether the VEGF signaling pathway is regulated by ncRNAs, we predicted the targeted miRNAs in the VEGF signal pathway using multiMiR and identified 14 miRNAs that targeted two genes involved in the VEGF signal pathway, namely PIK3CD and PTGS2 (**Figure 6**).

DISCUSSION

Myocardial ischemia/reperfusion injury is characterized by cardiac damage as a result of restriction and subsequent

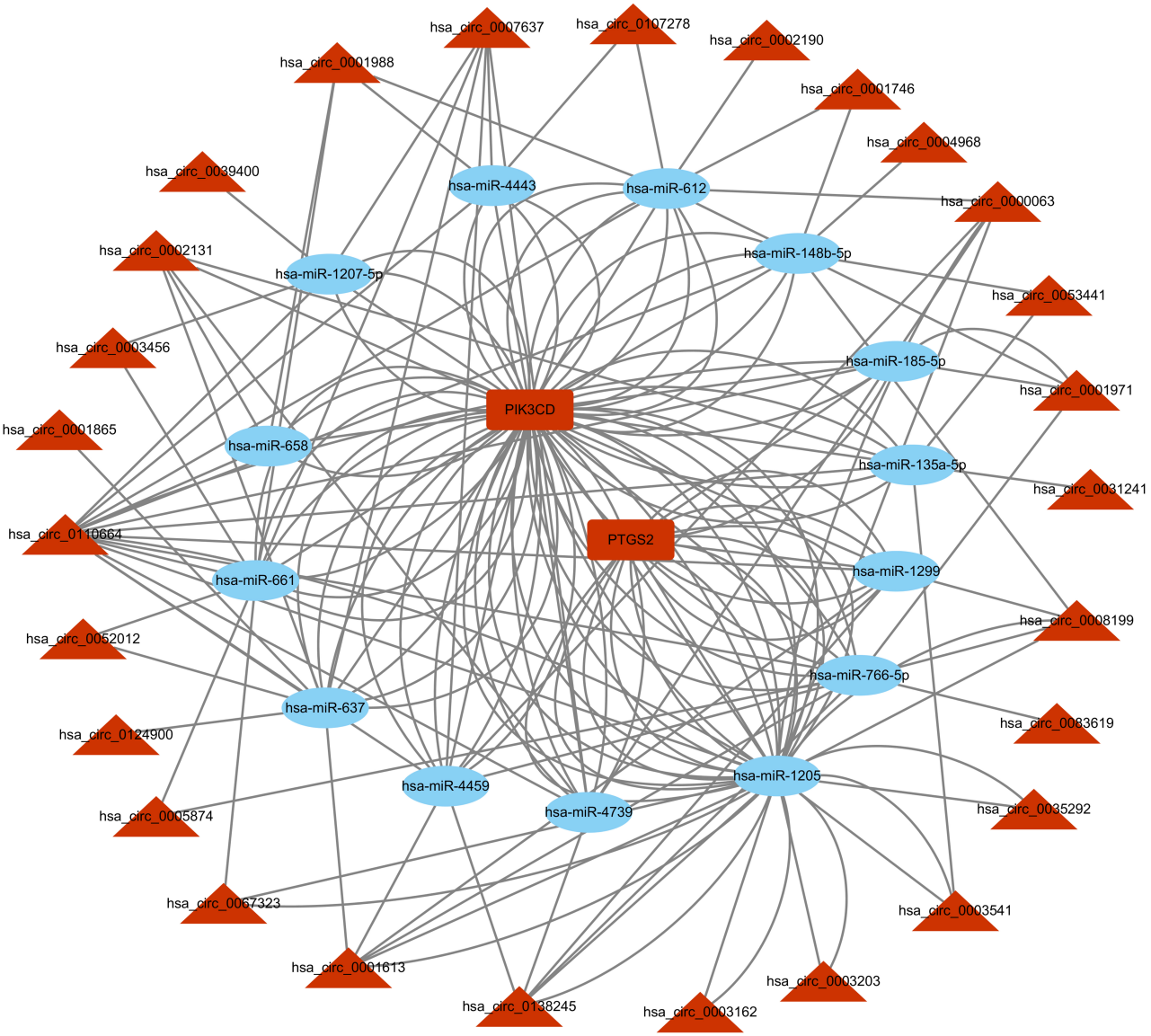

FIGURE 6 | circRNA-miRNA-VEGF signal pathway network diagram. CircRNAs: circular RNAs. miRNA: microRNA.

restoration of blood supply to myocardium. Accumulation of reactive oxygen species (ROS) production and endoplasmic reticulum (ER) stress prominently contribute to the cell death in myocardial ischemia/reperfusion injury (20).Our previous research indicated that HuMSC-EVs could protect cardiac cells against H/R injury. MSC-derived EVs are considered to promote the repair of damaged tissues by forming abundant bioactive compounds such as microRNA and lncRNA (21). In this study, circRNAs are found abundantly expressed and upregulated during the repair of cardiomyocytes by co-cultured HuMSC-EVs following H/R injury. This result suggests that circRNAs may play a vital role in the therapeutic effect of HuMSC-EVs on cardiomyocytes after H/R injury.

Previous studies have suggested that circRNAs are related to tissue repair. Sun et al. (22) used microarray analysis to identify nine key circRNAs that were significantly increased during the Wharton's jelly-derived mesenchymal stem cell repair of damaged human endometrial stromal cells. Wu et al. (23) also reported 29 upregulated and 34 downregulated circRNAs in heart tissue following heart failure induced by myocardial infarction. In another study, Li et al. (24) used RNA-seq analysis to identify six significantly altered circRNAs during the repair of liver injury. Collectively, these data show that circRNAs act as key regulators of the biological and pathogenic process of damaged tissue repair. However, whether circRNAs play a part in the repair of cardiomyocytes that suffer H/R injury remains unclear.

High-throughput RNA sequencing was employed to screen for DECs following culture with or without HuMSC-EVs. Sixty-six significant DECs were detected, 33 of which were upregulated, and 33 of which were downregulated. Then we paid attention to the 10 upregulated circRNAs in Group T with the largest FCs in expression. Seven of these 10 circRNAs were abundantly expressed in the HuMSC-EVs. We also found that PKH26-labeled HuMSC-EVs were internalized by cardiomyocytes. Therefore, it would be logical to conclude that the circRNAs were delivered from the HuMSC-EVs to the cardiomyocytes, leading to the elevated expression of circRNAs in the cardiomyocytes.

GO and KEGG pathway analyses were carried out to investigate the biological function and relevant pathways of the upregulated circRNAs. In this study, important biological functions, such as cellular response to hypoxia, cellular response to decreased oxygen levels, and cellular response to oxygen levels determined through GO analysis, as well as significant pathways such as the VEGF signaling pathway and glucagon signaling pathway estimated from KEGG pathway analysis, were implicated in several physiological and pathophysiological activities related to anoxia. For example, VEGF is an angiogenic growth factor that can stimulate the proliferation, differentiation, and survival of vascular endothelial cells. Several experiments have indicated that VEGF plays a cardio-protective role in myocardial I/R injury (25, 26). Further, most of the host genes of the seven validated circRNAs, such as ATXN10, FAM126A, SBDS, PICALM, and FTO, are closely associated with cell survival, cell proliferation, and cell differentiation (27–31). These results indicated that the upregulated circRNAs were derived from cell development genes related to cell proliferation, which may play an important role in the cardio-protection of HuMSC-EVs.

CircRNAs may regulate the function of miRNAs by acting as ceRNAs (32). We found that the VEGF signaling pathway was involved in the cardio-protection of HuMSC-EVs. Therefore, we constructed a circRNA-miRNA-VEGF signaling pathway regulatory network for upregulated circRNAs based on the ceRNA theory (**Figure 6**). The results showed that two upregulated circRNA-targeted mRNAs were differentially expressed. PTGS-2, one of the circRNA-targeted mRNAs, also called cyclooxygenase (COX)-2, was increased significantly in response to a variety of stimuli, including IR injury (33).Further investigation of the gene function revealed that PIK3CD, another circRNA-targeted mRNAs, has been reported to play a protective role in renal ischemia-reperfusion injury *via* activating the PI3K/Akt signaling pathway (34). Combining our previous study results, we propose that the upregulated circRNAs may act as miRNA sponges and release PIK3CD before activating the PI3K/Akt signaling pathway. However, confirmation of the circRNA/miRNA/PIK3CD association and the function and molecular mechanism of circRNAs in the repair of H/R injury by HuMSC-EVs remain indistinct and require further research.

CONCLUSIONS

The present study was the first to determine the expression profile of circRNAs in cardiomyocyte repair of H/R injury mediated by HuMSC-EVs. Several circRNAs may take part in biological pathways for the protection of cardiomyocytes through diverse regulatory mechanisms. Interactions of circRNA-miRNA-PIK3CD are involved in the protection of cardiomyocytes. Our present study provide a clearer understanding of the expression profile of circRNA during HuMSC-EV-mediated protection of cardiomyocytes and supply a new perspective for the treatment of H/R injury with HuMSC-EVs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Shantou University Medical College (Shantou, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CZ, JL, and LM designed the study, supervised the data collection, and analyzed the data. CZ and HW interpreted the data and prepared the manuscript for publication. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.626878/full#supplementary-material

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REVIEW
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Myocardial Infarction: The Protective Role of MiRNAs in Myocardium Pathology

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Cardiovascular diseases have been regarded as the leading cause of death around the world, with myocardial infarction (MI) being the most severe form. MI leads to myocardial apoptosis, cardiomyocyte fibrosis, and cardiomyocyte hypertrophy, ultimately leading to heart failure, and death. Micro RNAs (miRNAs) participate in the genesis and progression of myocardial pathology after MI by playing an important regulatory role. This review aims to summarize all available knowledge on the role of miRNAs in the myocardial pathological process after MI to uncover potential major target pathways. In addition, the main therapeutic methods and their latest progress are also reviewed. miRNAs can regulate the main signaling pathways as well as pathological processes. Thus, they have the potential to induce therapeutic effects. Hence, the combination of miRNAs with recently developed exosome nanocomplexes may represent the future direction of therapeutics.

Keywords: MI, apoptosis, hypertrophy, fibrosis, miRNA

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INTRODUCTION

Myocardial infarction (MI) is defined as the death of myocardial cells due to prolonged ischemia and is the most serious manifestation of coronary artery disease (1). However, MI also results in cardiac remodeling, including myocardial fibrosis and cardiac hypertrophy (2). The pathological changes induced by MI can lead to heart failure, cardiac rupture, sudden death, and other adverse events (3). Antithrombotic agents, percutaneous coronary intervention, and bypass surgery are usually applied to treat patients after MI (4, 5). Nonetheless, these approaches only reduce the severity of the coronary artery disease rather than saving the ischemic myocardium and preventing the development of adverse tissue remodeling (6, 7). Therefore, novel therapeutic strategies to reduce myocardial cell death, inhibit adverse remodeling, and/or stimulate heart regeneration are highly needed.

Micro RNAs (miRNAs) are also involved in differential gene expression in the pathophysiology of MI (5, 8). miRNAs originating from DNA sequences are transcribed by RNA polymerase II in the nucleus to form primary products: primary miRNA (pri-miRNA). Pri-miRNA is generally larger than 1000 bPs and is a double-stranded RNA, similar to a long hairpin, consisting of multiple nucleotide fragments. In the nuclear region, endonuclease Drosha (RNAse III) and cofactor Dgcr8 constitute a unique structure-microprocessor. This complex could precisely cut pri-miRNA and degrade it into a 65 bPs secondary product: miRNA precursor (pre-miRNA) (9). Subsequently, these new pre-miRNAs are transported into the cytoplasm through transport complexes that are made of export protein 5 (EXP5), RAN, GTP, and pre-miRNAs (10, 11). Once the complex passes

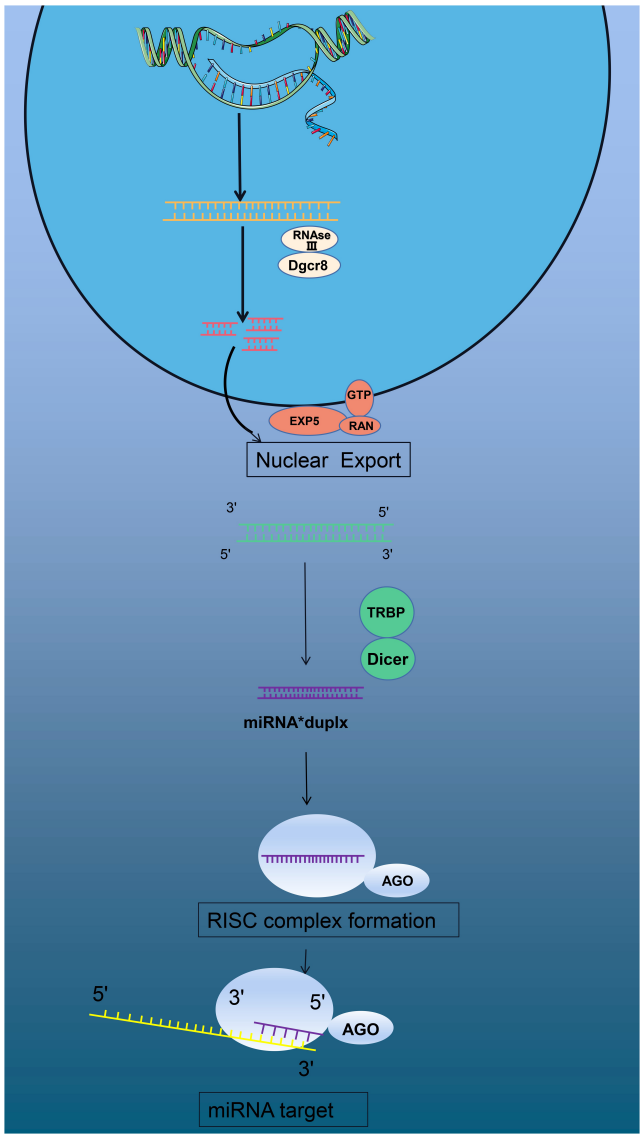

FIGURE 1 | miRNAs form and function.

through the nuclear membrane, the RNAse protein (Dicer) clefts the pre-miRNA into about 19-25 bPs miRNAs, and TAR RNA-binding protein (TRBP or PACT) changes the product into double-stranded miRNAs (12, 13). New double-stranded miRNAs are loaded into a specific AGO protein to form a pre-RNA-induced silencing complex (pre-RISC). As one strand of the double helix in the pre-RISC degrades, it is immediately modified to become a mature RISC (14). The complex binds to the 3'UTR region of the target mRNA, resulting in degradation or inhibition of the target mRNA (15) (**Figure 1**).

In the last century, the use of stem cells was thought to be a promising strategy for the treatment of MI (16). As research continues, the exact mechanism of cardiac repair by transplanted cells remains controversial and has yielded inconsistent results. Two main hypotheses exist: (a) direct cardiogenesis/angiogenic differentiation, and (b) indirect stimulation of the regenerative process through paracrine (17, 18). Leda et al. successfully reprogrammed fibroblasts directly into functional cardiomyocytes (19). But low conversion rates and a complex and expensive process have stalled the technology (20). The therapeutic role of exosomes has long been thought to be useful in the treatment of heart injury (21). In combination with nanomaterials, cell transformation is greatly improved (22).

Small non-coding micro RNAs (miRNAs) participate in the pathogenesis and development of myocardial pathology after MI and play an important regulatory role. This study provides a comprehensive overview of miRNAs affecting the pathology after MI and acting on potential targets and access mechanisms. Furthermore, the present therapeutic methods of saving infarct myocardium and latest research progress are summarized. In particular, the challenges and clinical prospects of using miRNA targets for myocardial regenerative therapy are discussed (**Table 1** and **Figure 2**).

CARDIOMYOCYTE APOPTOSIS

Apoptosis is a type of programmed cell death promoted by extrinsic and intrinsic pathways through the activation of death receptors and mitochondria, respectively (23–25). The transduction of apoptosis signals is mediated by several pro- and anti-apoptotic factors, including the caspase family, the B cell lymphoma 2 (Bcl-2) family, cytochrome c, and inhibitor of apoptosis proteins (IAP) (26). miRNAs play an important role in myocardial cell apoptosis and heart protection after MI (27–30). Two studies indicated that upregulation of miR-195 and miR-15 in ischemic cardiomyocytes of rats promotes ischemic apoptosis by targeting Bcl-2 (31, 32). In turn, miR-17 can support apoptosis via apoptotic protease activating factor 1 (Apaf-1) which facilitates the formation of apoptosomes containing cytochrome c and deoxyadenosine triphosphate (dATP) (33). However, a previous study suggested that miR-327 inhibits cardiomyocyte apoptosis *in vitro* and *in vivo* in rats by targeting the apoptosis repressor with the caspase recruitment domain (ARC) (34). In addition, miR-378 was reported to inhibit caspase-3 expression and attenuate ischemic injury in cardiomyocytes (35), whereas miR-155 did not (36). Overexpression of miRNA-488-3p markedly downregulated the levels of caspase-3 in MI (37). Furthermore, a study revealed that autophagy-related 3 (ATG13) interacts with the fas-associated protein with the death domain to promote the activation of caspase-8 and cell apoptosis (38). ATG13 is also a target of miR-206 and activates the apoptotic factor forkhead box protein 1 (FOXP1) (39, 40). Upregulation of miR-133 can inhibit cardiomyocyte apoptosis, inflammation, and oxidative stress through a mechanism that may be related to the regulation of the SIRT3/AMPK pathway (41). miR-133 is a heart- and muscle-enriched miRNA (42). Sirtuin 1 (Sirt1) also has anti-apoptotic effects that are associated with a reduction in the levels of reactive oxygen species (ROS) (43). In turn, silencing of Sirt1 abolishes the protective effects of miR-22 on hypoxia/reoxygenation-induced mitochondrial dysfunction and

TABLE 1 | Micro RNAs targets and functions in cardiomyocyte apoptosis.

	Functions	miRNA	Up/down	Targets	Reference
Cardiomyocyte apoptosis	Pro-apoptosis	195	Up	SIRT1	PMID:21622680
		195	Up	Bcl2	PMID:27489501
		22	Up	SIRT1	PMID:27174562
		15	Up	Bcl2	PMID:28814571
		155	Up	Capase3	PMID:31191799
		665	Up	AKT/Cnr2	PMID:31026731
		206	Down	ATG3	PMID:29880830
		206	Down	ATG3	PMID:30551524
		17	Down	Apaf-1	PMID:26265044
		762	Down	ND2	PMID:31235686
		340	Down	Act1	PMID:30989715
		124	Down	CircHipk3	PMID:31799682
		498	Down	PAWR	PMID:32767028
	Anti-apoptosis	133	Up	SIRT3	PMID:32575874
		378	Up	Capase3	PMID:22119805
		488	Up	Capase3	PMID:31210328
		206	Up	FoXP1	PMID:26333362
		21	Up	PDCD4	PMID:29674977
		325	Up	RIPK3	PMID:31248365
		24	Up	–	PMID:25352422
		210	Up	AIFM3	PMID:32513270
		410-3p	Up	TRAF5	PMID:31696495
		182	Up	Nogo-C	PMID:27763637
		24-3p	Up	Nrf2	PMID:30622671
		486	Up	PI3k/AKT	PMID:30844685
		7a-5p	Up	BTG2	PMID:32945347
		323-3p	Up	TGF-β2	PMID:32633390
		125-b	Up	p53/BAK1	PMID:30613290
		146a	Up	EGR	PMID:30362610
		let-7d	Up	HMGA	PMID:30934671
		23a/92a	Down	–	PMID:28662151
		145	Down	Dusp6	PMID:30883744
		489	Down	IGF1	PMID:32880387
		7a-5p	Down	–	PMID:33029099
		363	Down	Notch	PMID:28402919
		429	Down	Notch	PMID:27082497
		200-c	Down	GATA-4	PMID:28440427
		327	Down	ARC	PMID:31587299

miRNAs are divided into pro-apoptosis and anti-apoptosis according to functions. The pro-apoptosis of main targets include SIRT, Bcl, caspase, and ATG3 and the anti-apoptosis of main targets include SIRT, caspase, and AKT.

cell injury in cardiomyocytes (44). miRNAs also directly suppress the expression of the programmed cell death (PDCD) family, active IAPs, and accelerates cell transcription to inhibit cell apoptosis after MI (45–47). Recently, receptor interacting protein kinase 3 (RIPK3), apoptosis-inducing factor 3 (AIFM3), and tumor necrosis factor receptor-associated factor 5 (TRAF5) were confirmed to be suppressed by miRNAs (48, 49).

Myocardial cells suffer hypoxic damage when MI occurs (50). Two studies confirmed that the activation of protein kinase B (AKT) (51), as well as the ectopic expression of Notch1 (52), inhibited hypoxia-induced apoptosis in culturing human cardiomyocytes under hypoxic conditions (53). Nogo-C is an endoplasmic reticulum protein ubiquitously expressed in tissues including in the heart, which is upregulated in mouse hearts after MI and in cardiomyocytes upon hypoxic treatments (54). Furthermore, knock-down of endogenous NADH dehydrogenase 2 (ND2) significantly decreases intracellular ATP levels and mitochondrial complex-I enzyme activity, whereas it increases ROS levels and apoptotic cell death in cardiomyocytes (55). A study used a H9C2 cardiomyocyte

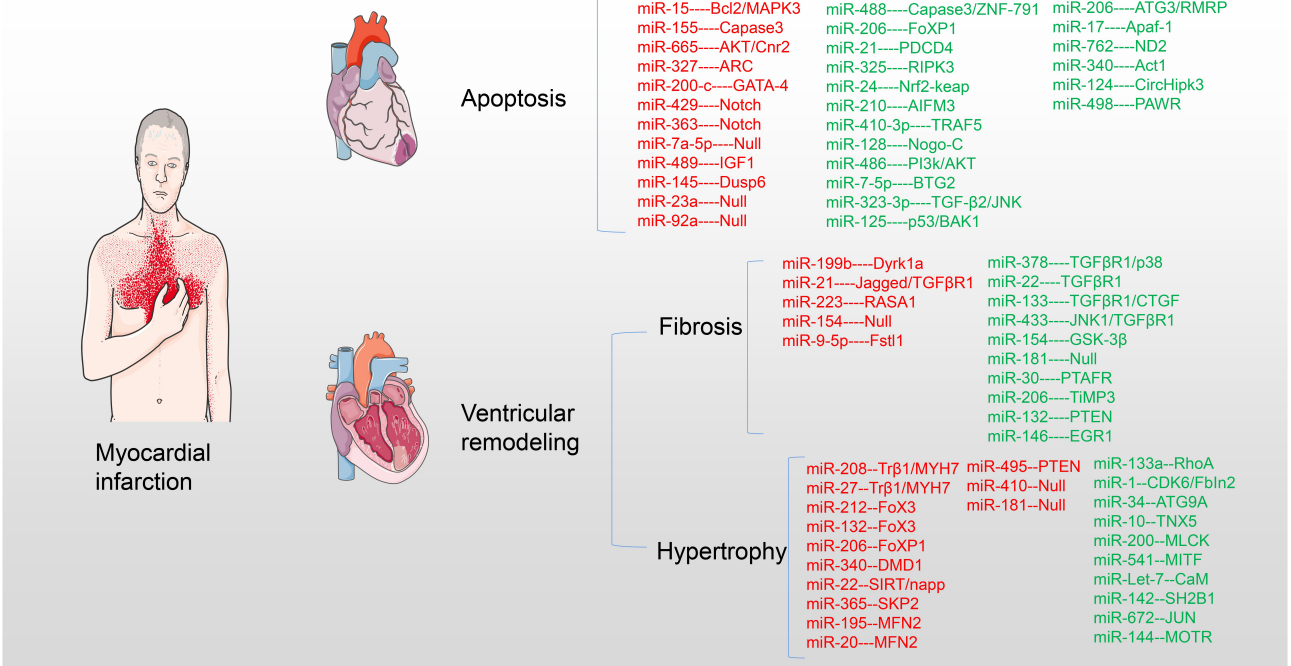

FIGURE 2 | miRNAs target myocardium in pathological process after MI. miRNAs are involved in myocardial cell apoptosis, myocardial fibrosis, and myocardial hypertrophy acting on myocardial targets after MI. Upregulation of red miRNAs promoted the development of pathology, while upregulation of green miRNAs inhibited or even reversed the pathological process.

cell line to perform *in vitro* stimulated ischemia/reperfusion (SI/R) and found a novel function of miR-24-3p in protecting cardiomyocytes from oxidative injury by the activation of the Nrf2/Keap1 pathway (56). Moreover, overexpression of miR-323-3p was also found to reduce oxidative stress and apoptosis of cardiomyocytes via the regulation of the TGF-β2/JNK pathway (57). Additionally, there are still conflicting results regarding miR-7a-5p's protective role on cardiomyocytes upon hypoxic injury (50, 58).

Moreover, upregulation of miR-340-5p suppresses apoptosis and oxidative stress induced by hypoxia/reoxygenation in H9C2 cells by inhibiting the NF-κB activator 1 (Act1) (59). Lastly, a study suggested that bone marrow mesenchymal stem cell (BM-MSC)-derived vascular endothelial growth factor attenuates cardiac apoptosis via regulation of cardiac miRNA-23a and miRNA-92a in a rat model of multiple sclerosis (60). miRNAs from BM-MSCs can interact with myocardial cells through exosomes (61). Interestingly, exosomes originating from adipose-derived stem cells can also attenuate myocardial damage triggered by acute MI via downregulation of early growth response factor 1 (Egr1) (62).

In summary, to date, more miRNAs with anti-apoptotic activity have been reported than those with pro-apoptotic effects, most of which act on classical pathways such as Bcl-2, caspase, AKT, SIRT, and apoptotic factors.

MYOCARDIAL FIBROSIS

Myocardial fibrosis is an important feature of most cardiac pathological conditions (63), characterized by alteration of the extracellular matrix (64). Currently, five types of collagen are known to be expressed in the myocardium, among which fibrillar collagen type I (85%) and type III (11%) are commonly expressed in the cardiac extracellular matrix. And, the basement membrane of myocytes and the pericellular space are rarely composed of collagen type IV and V (65). Additionally, fibrillar collagen type VI is related to the adhesion of cellular fibers (66). An MI model was established in SD rats using the LAD ligation method and the study found transforming growth factor-β 1 (TGF-β1) induces the upregulation of miR-21 and downregulation of Jagged1 in cardiac fibroblasts (CFs), which are activated by MI, thereby inducing myofibroblast transformation (67). Additionally, decreased levels of antizyme inhibitor (AZIN1) activate TGF-β1. Furthermore, downregulation of c-Jun N-terminal kinase 1 (JNK1) results in the activation of the extracellular signal-regulated kinase and p38 kinase, leading to Smad3 activation and ultimately cardiac fibrosis (68). miR-133a expression in the infarct border zone of myocardial tissue was found to be significantly decreased after MI. And, the upregulation of miRNA-133a in the myocardial tissue of rats with MI remarkably improved cardiac function and reduced collagen volume fraction (69). Furthermore, the mRNA and protein levels of TGF-β1, connective tissue growth factor, collagen I and

TABLE 2 | Micro RNAs targets and functions in myocardial fibrosis.

	Functions	miRNA	Up/down	Targets	Reference
Myocardial fibrosis	Pro-fibrosis	199b	Up	Dyrk1a	PMID:21102440
		154	Up	–	PMID:26928825
		21	Up	TGFβR1	PMID:29808534
		223	Up	RASA1	PMID:29689569
		181	Down	–	PMID:32538237
		30	Down	–	PMID:32418505
		30	Down	PTAFR	PMID:32329883
		154	Down	GSK-3β	PMID:29687862
		433	Down	TGFβR1	PMID:27698941
		378	Down	TGFβR1	PMID:25104350
		378	Down	MKK6/p38	PMID:29721099
		22	Down	TGFβR1	PMID:27997889
	Anti-fibrosis	206	Up	TiMP3	PMID:21731608
		132	Up	PTEN	PMID:30216493
		146	Up	EGR1	PMID:30362610
		133	Up	TGFβR1	PMID:31646592
		9-5p	Down	Fstl1	PMID:30101604

miRNAs are divided into pro-fibrosis and anti-fibrosis according to functions. The pro-fibrosis of main targets include TGFβR1 and the anti-fibrosis of main targets include TGFβR1 and PTEN.

III, and α-smooth muscle actin (α-SMA) in myocardial tissue were obviously decreased after miRNA-133a upregulation (70). A study also suggested that miR-223 mimics could enhance cell proliferation and migration, collagen I and III, and α-SMA expression in CFs, which could be mediated via mitogen-activated protein kinase kinase (MEK) 1/2, ERK1/2, and AKT phosphorylation (71). miR-154 has similar functions via glycogen synthase kinase 3 beta (GSK-3β) including reducing the heart and cardiomyocyte size, cardiac fibrosis, lowering the expression of atrial (ANP) and B-type natriuretic peptides (BNP), and of profibrotic markers (72), whereas it increases the expression of p15 (a miR-154 target and cell cycle inhibitor) (73). Furthermore, miR-378 and miR-181a are secreted by cardiomyocytes to act as inhibitors of excessive cardiac fibrosis through a paracrine mechanism (74, 75). Upregulation of miR-132 or phosphatase and tensin homolog (PTEN) silencing activate the PI3K/Akt pathway, thereby repressing cardiomyocyte apoptosis and cardiac fibrosis (76). An earlier study showed that an injection of high mobility group box 1 (HMGB1) into the heart of mice, immediately after MI, had the potential to improve cardiac regeneration and prevent remodeling (77). Recently, a study on CFs isolated from mice hearts upon angiotensin II (Ang II)-induced cardiac fibrosis post-MI revealed that miR-30b-5p and miR-22-3p were downregulated, whereas the platelet activating factor receptor (PTAFR) was upregulated [X. S. (78)]. In addition, miRNAs can directly inhibit myocardial fibrosis and even reverse ventricular remodeling (79, 80). Cardiac CITED4 (CBP/p300-interacting transactivators with E [glutamic acid]/D [aspartic acid]-rich-carboxylterminal domain 4) is sufficient to cause physiological hypertrophy and mitigate adverse ventricular remodeling after MI (81). Although few studies specifically investigated myocardial fibrosis, TGF-β1 is clearly a direct or indirect target underlying this process. Upstream targets of PTAFR and CITED4 have recently been found to be worthy of further exploration (**Table 2**).

CARDIOMYOCYTE HYPERTROPHY

Cardiac hypertrophy is an adaptive response when the heart faces various pathological stimuli, such as energy metabolism disorders, increased load, changes in humoral factors, and neuroendocrine activation (82, 83). With myocardial contractility decreasing after MI, ventricular remodeling always occurs with compensatory hypertrophy of the myocardium (84). Although this mechanism has an important role for cardiac function in the early phase of MI, these changes will eventually develop into heart failure and even death (85). Myocardial contractility depends mainly on the expression of two myocardial myosin heavy chain (MHC) genes α and β, called Myh6 and Myh7, respectively (86). Thyroid hormone T3 signaling controls the expression of these two MHC genes by stimulating the expression of Myh6 and inhibiting the expression of Myh7 after birth (87). miR-208 is a heart- and muscle-enriched miRNA (42). Transgenic overexpression of miR-208a in the heart, which is encoded within an intron of Myh7 and regulates the thyroid hormone-associated protein 1 (TRβ1), was shown to be sufficient to induce hypertrophic growth of the heart in mice (88). Another study showed that infarcted hearts have a higher abundance of extracellular vesicular miRNA-27a compared with normal hearts, and that miRNA-27a inhibited PDZ and LIM domain 5 (PDLIM5) translation, leading to cardiomyocyte hypertrophic gene expression (89). Probably, Myh7 is also regulated by the T-box transcription factor 5 (Tbx5) (90). Mice injected with an adeno-associated virus expressing miR-1

TABLE 3 | Micro RNAs targets and functions in cardiomyocyte hypertrophy.

	Functions	miRNA	Up/down	Targets	Reference
Cardiomyocyte hypertrophy	Pro-hypertrophy	208	Up	Trβ1/MYH7	PMID:19726871
		27	Up	Trβ1/MYH7	PMID:21149577
		27	Up	PDLIM5	PMID:32370947
		212/132	Up	Ang II	PMID:23011132
		22	Up	SIRT	PMID:23524588
		22	Up	PTEN	PMID:21618527
		206	Up	FoXP1	PMID:26333362
		340	Up	DMD1	PMID:26084457
		365	Up	SKP2	PMID:28130111
		195	Up	MFN2	PMID:31341888
		20	Up	MFN2	PMID:31295012
		10	Down	TNX5	PMID:28100873
		200	Down	MLCK	PMID:30680929
		34	Down	Ang II	PMID:24728149
		1	Down	CDK6/NFAT	PMID:26699910
		133a	Down	RhoA	PMID:17468766
	Anti-hypertrophy	1	Up	Fbln2	PMID:23612897
		541	Up	MITF	PMID:24722296
		let-7	Up	CaM	PMID:28123343
		142	Up	SH2B1	PMID:30372837
		672	Up	JUN	PMID:29339068
		144	Up	MOTR	PMID:30084039
		181	Down	–	PMID:32538237
		410/495	Down	–	PMID:26999812
		495	Down	PTEN	PMID:29566365

miRNAs are divided into pro-hypertrophy and anti-hypertrophy according to functions. The pro-hypertrophy of main targets include Trβ1/MYH7, MFN2, and Ang II and the anti-hypertrophy of main targets include MOTR, PTEN, and CaM.

showed reduced, and even reversed, myocardial hypertrophy (91). miR-1 is a heart- and muscle-enriched miRNA (42). miR-1 inhibits the expression of cell division protein kinase 6 (CDK6) to inhibit phenylephrine-induced neonatal rat ventricular cardiomyocytes hypertrophy, thereby attenuating the inhibition of the expression of β-MHC and phosphorylated the retinoblastoma protein (92). miR-340 is a pro-eccentric hypertrophy miRNA that targets the cardiomyocyte structure protein dystrophin (93). miR-22 and miR-495 have the opposite effect, with their upregulation significantly increasing cell size and markedly decreasing the expression of Myh6 (94). Moreover, they negatively regulate the PTEN levels in cardiomyocytes (95). Additionally, overexpression of let-7a was found to repress the expression of ANP, BNP, and Myh7, as well as of CaM levels (96). A dual-luciferase reporter assay also showed that let-7a could bind to the 3'–UTR of CAM1 and let-7a possesses a prominent anti-hypertrophic property by targeting CAM genes (97).

There is a potential link between cardiac hypertrophy and cardiac cell death (98, 99). A study suggested that intravenous miR-144 has a potent effects on cardiac remodeling of rats with MI, which was associated with significant changes in autophagy signaling (100). Cy3-labeled miR-144 was localized to the infarct and border zones and was taken up by cardiomyocytes and macrophages (101). Similarly, knock-down of the autophagy-related protein 9 (ATG9A), which is a direct target of miR-34, downregulated the autophagic activity and cardiomyocyte hypertrophy (102). Furthermore, overexpression of the S-phase kinase-associated protein 2 (Skp2) promoted autophagy and rescued cardiac hypertrophy induced by Ang II. And, Skp2 knock-down further inhibited autophagy and cardiac hypertrophy in mice with MI (103). In contrast, increased miR-206 expression induced cardiac hypertrophy and inhibited cell death in cultured cardiomyocytes. The Yes-associated protein can promote cardiomyocyte growth and survival in postnatal hearts, and increases the abundance of miR-206, which in turn plays an essential role in mediating hypertrophy and survival by silencing FOXP1 in cardiomyocytes (39). miR-133, 541, 200, 624, and 181 can in turn inhibit hypertrophy and improve cardiac function through different mechanisms (104, 105). Recently, a study confirmed that upregulated miR-142-3p could inhibit hypertrophy and mitochondrial SH2B1, a key factor regulating energy metabolism (106). Moreover, miR-195-5p and miRNA-20a-5p can promote cardiac hypertrophy via targeting mitofusin-2 (MFN2), which is a mitochondrial outer membrane fusion protein (107, 108). TRβ1/Myh7, Ang II, and PTEN have been the main targets of research, and MFN2 may be a new major target (**Table 3**).

THERAPEUTIC TARGETS

The majority of patients who survive MI experience a loss of functional cardiomyocytes as a result of the ischemic injury, which leads to ventricular failure with significant alteration of the quality of life and increased risk of mortality (109). Since the proliferation and self-healing capacity of cardiomyocytes in adults is limited, regeneration therapy has emerged as an attractive concept for cardiac repair (110). Compared with traditional interventional stent reperfusion, regenerative therapy can save the myocardium or even regenerate it by promoting angiogenesis, and inhibit, or even avoid adverse cardiac remodeling (111). The main directions of regenerative therapy include stem cell therapy, cardiac fibroblast reprogramming, and exosome therapy.

Scholars have focused on the development of induced pluripotent stem cells, but such treatments have failed to achieve significant benefits in clinical trials (112). This approach has demonstrated limited therapeutic effect mainly due to the risk of immune rejection, genetic instability, tumorigenic potential, low induction efficiency (in the case of induced pluripotent stem cells), and ethical issues (in the case of embryonic stem cells use) (113–115). Leda et al. successfully reprogramed mouse heart and skin fibroblasts into functional induced cardiomyocytes (iCMs) *in vitro* (19). However, the cardiac fibroblast reprogramming efficiency was extremely low and its requirements are too draconian (116). Furthermore, the iCMs carry other risks such as arrhythmias (117). However, the emergence of exosomes provided an additional tool for myocardial regeneration. Exosomes started to attract attention in 2007, when it was discovered that they have the unique property of transferring miRNAs between cells *in vivo*, acting as miRNA nanocarriers (118). Recently, mounting evidence has demonstrated the potential of stem cell-derived exosomes, as well as other exosome types, in repairing damage after MI (62, 119). A study confirmed that mesenchymal stem cell (MSC)-derived exosomes electroporated with miR-132 mimics could markedly enhanced the neovascularization in the peri-infarct zone and preserve heart functions (120). Additionally, an injection of exosomes over-expressing miR-21 directly into the infarct zone was found to markedly inhibit cell apoptosis and significantly improve cardiac function in mice (121). MSC-derived exosomes were also found to protect the heart in a porcine model of MI when administered systemically by intravenous injection (122). However, major hurdles remain for the use of exosomes, primarily due to low yields from cell cultures coupled with complicated purification processes (123). Nevertheless, a study reported the self-assembly of a stem cell membrane-camouflaged exosome-mimicking nanocomplex that recapitulated exosome functions, achieving efficient miRNAs delivery and miRNA-mediated myocardial repair (22). Furthermore, a group constructed a functionalized single-walled carbon nanotube bound to siRNA from caspase 3 (F-CNT-siCas3) that demonstrated good water solubility and biocompatibility, but also had a high transfection efficiency of up to 82%, significantly downregulating the expression of the caspase 3 gene and protein *in vivo* (124). A low molecular weight heparin-encapsulated exosome nanocomplex demonstrated that it could overcome a microvascular obstruction in the infarct, and this structure not only makes myocardial cells uptake miRNAs, which will promote cardiac repair, but will also prevent myocardiocyte apoptosis and attenuate myocardial fibrosis (125). Although the exosome nanocomplex technology is expensive and holds uncertain side effects, it greatly improves the cell conversion rate compared with the previous two regeneration methods, while showing good *in vivo* results. Thus, an exosome nanocomplex is conducive to further clinical research.

CONCLUSION

In particular, miRNAs play an important role in the pathology of myocardial apoptosis, fibrosis, and hypertrophy after MI. Targets of miRNAs have significant therapeutic potential, although there are still some conflicting data. The majority of miRNAs and their targets have consistent actions. In particular, SIRT, Bcl-2, Bax, caspase, TGF-β1, TRβ1/Myh7, and MFN2 are believed to play a more significantly prominent role than other targets. In addition, with the development of exosome therapy in combination with nanomaterials, some of the limitations of stem cell therapy (such as low conversion rates and poor cardiac absorption) can be overcome. Exosome nanocomplexes cannot only carry myocardium-friendly miRNAs, but can also directly deliver analogs of important targets into the myocardium in the future. Whether exosome nanocomplexes can treat infarcted myocardium by acting as vectors for the main targets of miRNAs, similar to cocktail therapy, may be the next major direction of exploration. Exosome nanocomplexes with miRNAs are more likely to be successfully taken forward into clinical evaluation than other experimental strategies; however, they also have several limitations. First, the up- and down-stream relationship with the target needs further verification and improvement. There are still conflicting effects of miRNAs (such as miR-Let-7 and miR-154) and more experimental studies are needed. Second, miRNAs act on multiple targets and are involved in several mechanisms; thus, it is necessary to weigh the advantages and disadvantages of their activities. Lastly, treatment with miRNAs is complicated and expensive, and more clinical studies are needed to confirm their therapeutic potential. With the perfectly targeted mechanism and the continuous improvement of exosome therapeutic materials, we believe that mature technologies and drugs based on miRNAs used to save the infarcted myocardium will soon be available to all.

AUTHOR CONTRIBUTIONS

HZ supervised the writing of the manuscript. WW and HZ prepared the manuscript and wrote the draft together. WW prepared the figures. All authors have read and agreed to the published version of the manuscript.

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Sweroside Protects Against Myocardial Ischemia–Reperfusion Injury by Inhibiting Oxidative Stress and Pyroptosis Partially via Modulation of the Keap1/Nrf2 Axis

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Aims: Sweroside, a secoiridoid glucoside extracted from *Swertia pseudochinensis* Hara, is reported to possess antioxidant and anti-inflammatory activities. However, whether sweroside has a protective effect on myocardial ischemia–reperfusion (IR) injury is yet to be elucidated. The present study aimed to confirm the cardioprotective effect of sweroside and to identify its underlying mechanism.

Methods and Results: H9c2 cells were pretreated with sweroside and then underwent hypoxia–reoxygenation. Cell Counting Kit-8, creatine kinase-myocardial band (CK-MB) and lactate dehydrogenase (LDH) assays were conducted to detect cell viability and myocardial injury, respectively. The Langendorff method was used to induce myocardial IR injury *ex vivo*. Triphenyltetrazolium chloride staining was performed to detect myocardial infarct size, while protein expression was analyzed using western blotting. Overall, the results indicated that sweroside pretreatment dose-dependently led to a significant enhancement in cell viability, a decrease in release of CK-MB and LDH, a reduction in infarct size, and an improvement in cardiac function. Additionally, sweroside pretreatment caused a marked suppression of oxidative stress, as evidenced by the fact that sweroside decreased the accumulation of reactive oxygen species and malondialdehyde, while enhancing the activities of superoxide dismutase and glutathione peroxidase. Moreover, sweroside was found to notably repress pyroptosis, as sweroside blocked pore formation in the cell membrane, inhibited caspase-1 and interleukin (IL)-1β activity, and decreased the expression levels of NLR family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD, cleaved caspase-1, and IL-1β. Mechanistically, it was found that sweroside inhibited Kelch-like ECH-associated protein 1 (Keap1) and induced nuclear factor E2-associated factor 2 (Nrf2) nuclear translocation. Furthermore, the inhibition of oxidative stress and pyroptosis by sweroside could be abrogated via the inhibition of Nrf2 expression, which suggested that the protective effect induced by sweroside was Nrf2-dependent.

Conclusions: The present study demonstrated that sweroside pretreatment could protect against myocardial IR injury by inhibiting of oxidative stress and NLRP3 inflammasome-mediated pyroptosis partially via modulation of the Keap1/Nrf2 axis.

Keywords: myocardial ischemia reperfusion injury, sweroside, oxidative stress, pyroptosis, nuclear factor E2-associated factor 2

INTRODUCTION

Ischemic heart disease (IHD), a major cause of mortality and disability worldwide, is estimated to account for ∼7.4 million deaths globally per year (1). Although myocardial reperfusion therapy, typically represented by percutaneous coronary intervention and coronary artery bypass grafting, has been the mainstream approach for IHD treatment, ischemia–reperfusion (IR) injury remains an unsolved problem that mainly impacts the effectiveness of reperfusion therapy (2). Therefore, how to effectively prevent myocardial IR injury has gained increased interest from researchers.

While the mechanism of myocardial IR injury has not been fully elucidated, oxidative stress (3) and inflammation (4) have proved to be responsible for myocardial IR injury. Nuclear factor E2-associated factor 2 (Nrf2) is a key transcription factor that serves an important role in the regulation of oxidative stress (5) and inflammation (6). In a physiological state, Nrf2 mainly resides in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1), which is a physiological inhibitor of Nrf2. However, under an oxidative stress condition, Nrf2 translocates into the nucleus by separating from Keap1 and subsequently activates the transcription of antioxidant genes, such as heme oxygenase (HO)-1, which protects cells against oxidative stress- and inflammation-induced injury (7, 8). For example, geniposide-induced preconditioning alleviates myocardial IR injury by activating the Nrf2/HO-1 signaling pathway to inhibit oxidative stress (9). Moreover, activation of Nrf2 can repress NLR family pyrin domain containing 3 (NLRP3) inflammasome-mediated pyroptosis via modulation of reactive oxygen species (ROS) in cerebral IR injury (10, 11). Therefore, Nrf2 is considered as a therapeutic target for myocardial IR injury (12).

Accumulating evidence has revealed that some active constituents of traditional Chinese medicine, such as ginsenoside (13, 14) and baicalin (15, 16), could protect against myocardial IR injury via their antioxidant and anti-inflammatory activities. Thus, traditional Chinese medicine provides a novel strategy for the treatment of myocardial IR injury (17). It has been reported that sweroside, a secoiridoid glucoside extracted from the plant *Swertia pseudochinensis* Hara (**Figure 1A**), possesses powerful antioxidant (18) and anti-inflammatory activities (19, 20). For instance, Ma et al. suggest that sweroside pretreatment inhibited aconitine-trigged oxidative stress and intercellular ROS production in cardiomyocytes (18). It also has been found that sweroside could prevent against lipopolysaccharide (LPS) or interleukin (IL)-1β-induced inflammation via suppression of NF-kappaB (NF-κB) signaling pathway (19, 20). Notably, a recent study has demonstrated that sweroside could lead to suppression of NLRP3 inflammasome activation (21). However, the detailed mechanism has not yet been elucidated. Moreover, as predicted by Molecular Operating Environment (MOE) software, sweroside may interact with Keap1. Hence, we hypothesized that sweroside may exert a protective effect on myocardial IR injury via its antioxidant and anti-inflammatory activities via the Keap1/Nrf2 axis.

In the present study, we tested this hypothesis in *in vitro* and *ex vivo* models. The results indicated that sweroside pretreatment alleviated the extent of myocardial IR injury *in vitro* and *ex vivo*, which involved the repression of oxidative stress and NLRP3 inflammasome-mediated pyroptosis partially via modulation of the Keap1/Nrf2 axis.

MATERIALS AND METHODS

Cell Culture

Rat myocardial cells (H9c2 cell line) were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China) and were routinely cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under the conditions of 37°C and 5% CO_2.

Hypoxia/Reoxygenation Model

The hypoxia/reoxygenation (HR) model was established as described previously (22). Specifically, when the cell density reached about 70%, cells were relocated to a tri-gas incubator with an atmosphere of 94% N_2, 5% CO_2, and 1% O_2 and subsequently cultured with Earle's medium without glucose and FBS for 6 h to establish hypoxia. At the end of the incubation, the medium was replaced with DMEM supplemented with 10% FBS, and cells were transferred to CO_2 incubator with an atmosphere of 5% CO_2 for 3-h reoxygenation.

Cell Treatment

Sweroside (CAS no. 14215-86-2; purity ≥98%) was purchased from Meilun Biochemical Co., Ltd. (Dalin, China). Sweroside was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck KGaA) and further diluted with culture medium until the DMSO concentration was <0.1%. Cells were pretreated with various concentrations of sweroside (0–100 μM) for 24 h and then underwent HR.

Cell Transfection

Small interfering (si) RNA targeting Nrf2 and its negative control were designed and synthesized by Guangzhou RiboBio Co., Ltd.

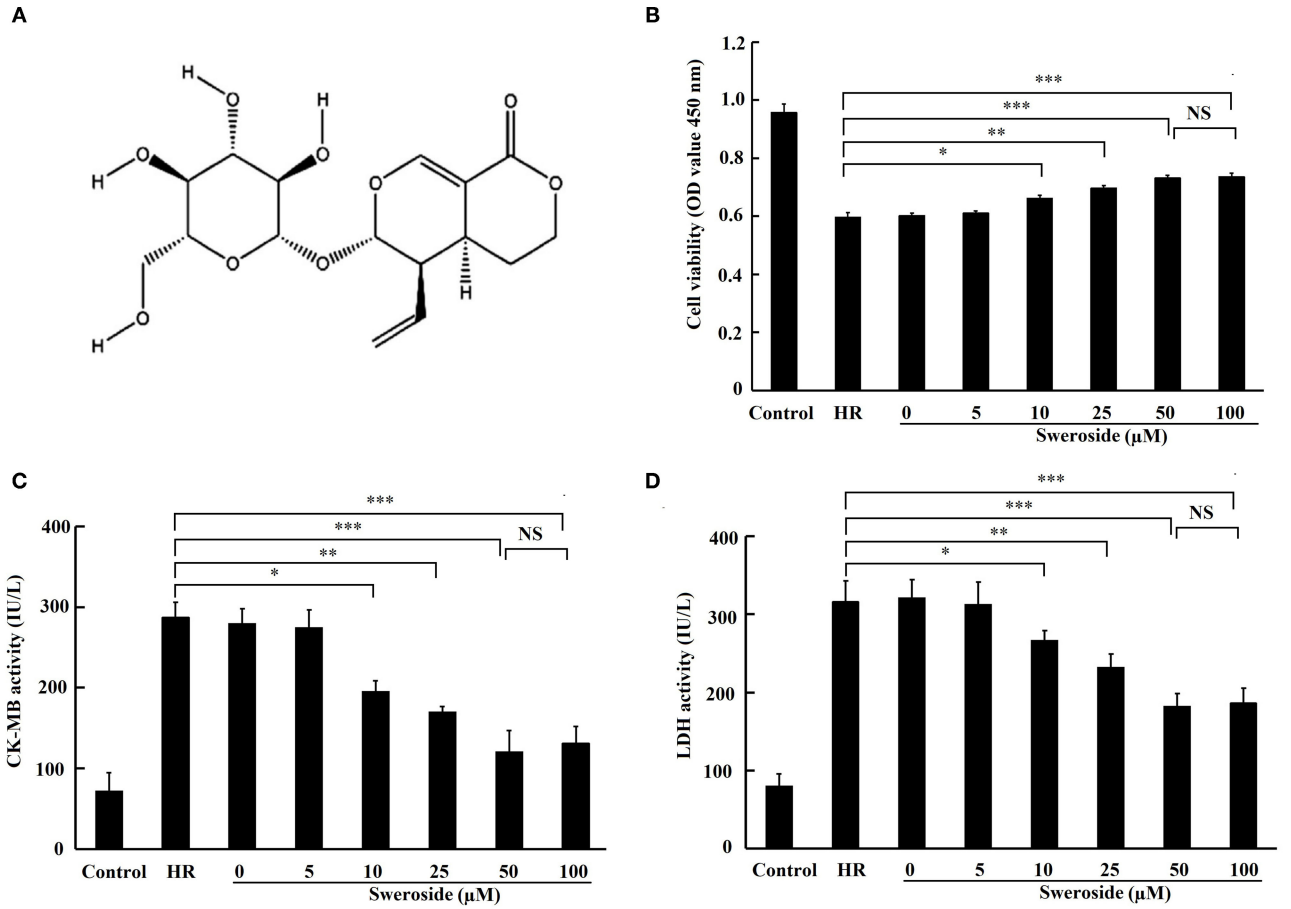

FIGURE 1 | The protective effect of sweroside on hypoxia/reoxygenation (HR)-induced myocardial injury *in vitro*. H9c2 cells were pretreated with a various concentrations of sweroside for 24 h and then underwent HR. **(A)** The chemical structure of sweroside. **(B)** Cell viability was assessed using Cell Counting Kit-8 assay. **(C,D)** Detection of creatine kinase isoenzyme-myocardial band (CK-MB) and lactate dehydrogenase (LDH) levels in culture medium. Each cell experiment was repeated at least three times. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant.

(Guangzhou, China) and were then transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Following transfection for 24 h, cells were further processed for sweroside treatment, followed by HR.

Cell Viability Assay

Cells were seeded into 96-well plates at a concentration of 5,000 cells/well, treated with sweroside, and subsequently underwent HR. A Cell Counting Kit (CCK)-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was performed to evaluate cell viability, following the manufacturer's instructions. The absorbance was measured at 450 nm on a microplate reader.

Myocardial Enzyme Tests

Lactate dehydrogenase (LDH) and creatine kinase-myocardial band (CK-MB) are known as myocardial injury markers (23). Hence, the levels of LDH and CK-MB in the culture medium were measured to evaluate the extent of myocardial injury using LDH and CK-MB assay kits, respectively (provided by Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's instructions.

Measurement of Reactive Oxygen Species Content

The cells were cultured on a small glass sheet and fixed with 4% paraformaldehyde at room temperature for 15 min. After being washed three times with phosphate-buffered saline (PBS), cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which acts as a sensitive ROS probe (Jiancheng Bioengineering Institute, Nanjing, China), at 37°C for 1 h. DCFH-DA-stained cardiomyocytes were observed and imaged under a fluorescence microscope.

Determination of Malondialdehyde, Superoxide Dismutase, and Glutathione Peroxidase Enzymes

After being digested by trypsin, cells were collected and subsequently resuspended in 0.5 ml of PBS prior to being

comminuted with an ultrasonic disintegrator. Cell homogenates were centrifugated at 12,000 × g for 10 min, followed by collection of the supernatant. The protein concentration of the supernatant was determined using an enhanced bicinchoninic acid (BCA) Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's guidelines. The malondialdehyde (MDA) content, and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured using MDA, SOD, and GSH-Px detection kits (all from Jiancheng Bioengineering Institute, Nanjing, China), respectively, following the manufacturer's instructions.

Detection of Cell Membrane Integrity

Pyroptosis is characterized by pore formation in the cell membrane (24). Thus, propidium iodide (PI) may permeate

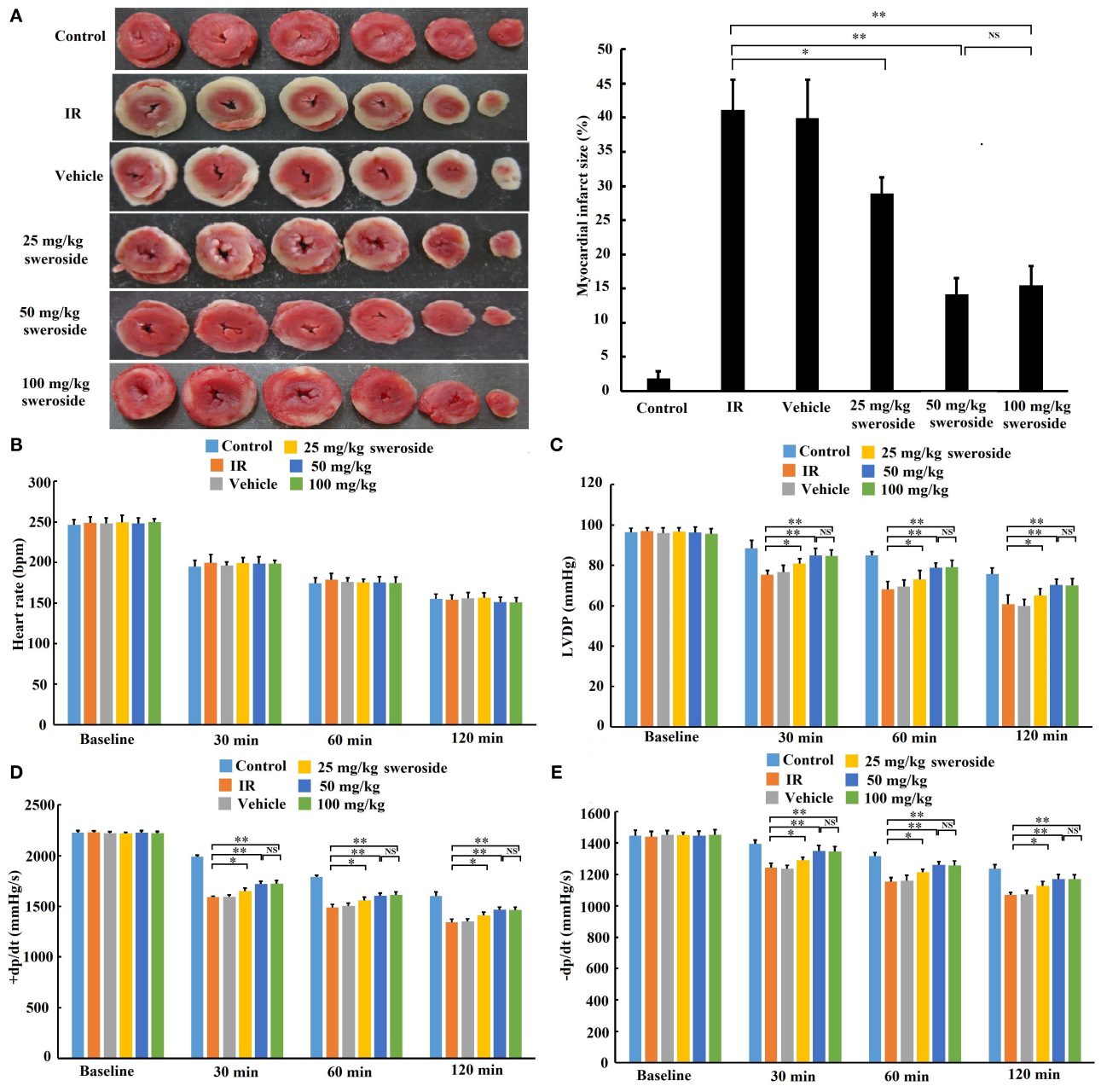

FIGURE 2 | The protective effect of sweroside on ischemia–reperfusion (IR)-induced myocardial injury *ex vivo*. After pretreatment with various doses of sweroside, the rat heart was isolated and then subjected to IR. **(A)** Determination of myocardial infarct size using TTC staining. **(B–E)** Comparison of the difference in heart rate, left ventricular developed pressure (LVDP), positive first-order derivative of ventricular pressure (+dp/dt), and negative first-order derivative of ventricular pressure (–dp/dt) between groups. Data are presented as the mean ± standard deviation, $n = 6$. $^{*}P < 0.05$; $^{**}P < 0.01$; NS, not significant.

into pyroptotic cells and stain nucleus due to the loss of cell membrane integrity. For this experiment, cells were incubated with PI (10 μM) for 30 min at room temperature, followed by re-staining with 4′,6-diamino-2-phenyl indole (DAPI). The results of the staining were observed under a fluorescence microscope.

Measurement of Caspase-1 Activity

After treatment, cells were digested with pancreatic enzymes and collected. The activity of caspase-1 in the collected cells was detected using a caspase-1 activity assay kit (Beyotime Institute of Biotechnology) following the manufacturer's instructions.

Measurement of Interleukin-1β Concentration

The concentration of IL-1β in the culture medium was measured using an enzyme-linked immunosorbent assay (ELISA) with the Rat IL-1β ELISA kit (MULTY SCIENCE) according to the manufacturer's instruction.

Molecular Docking Simulation

The binding between sweroside and Keap1 was stimulated using MOE software. Information regarding the Keap1 protein was downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), and an sdf format of sweroside was obtained from ChemDraw version 19.0. Sweroside and the molecular structure of Keap1 were protonated in 3D at 300°C, pH = 7 and with minimized energy using MOE software. Sweroside was docked to active sites of Keap1 using the Triangle Matcher placement and Induced Fit refinement methods.

Immunofluorescence Staining

Cells were placed on glass slides, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 30 min, and then blocked with 5% goat serum for 15 min at room temperature. Subsequently, cells were incubated with diluted primary antibody against Nrf2 (1:200; cat. no. ab89443; Abcam, Hong Kong) at 4°C overnight, followed by incubation with Cy3-labeled goat antimouse IgG (1:2,000; cat. no. 33208ES60; Yeason, Shanghai, China) for 60 min at room temperature. Nuclei were re-stained with DAPI, and the results of the staining process were observed under a fluorescence microscope.

Animals

The animals used in the present study were healthy male Wistar rats (age, 8 weeks; weight, 240–260 g), which were purchased from the Animal Laboratory Center of China Medical University (Shenyang, China). The rats were kept at 22–24°C under 12-h daylight:12-h dark conditions and had free access to clean water. The body weight of the rats was monitored twice a week. The use of animals in the present study was authorized by the Institutional Animal Care and Use Committee of China Medical University, and the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH), USA] was followed closely.

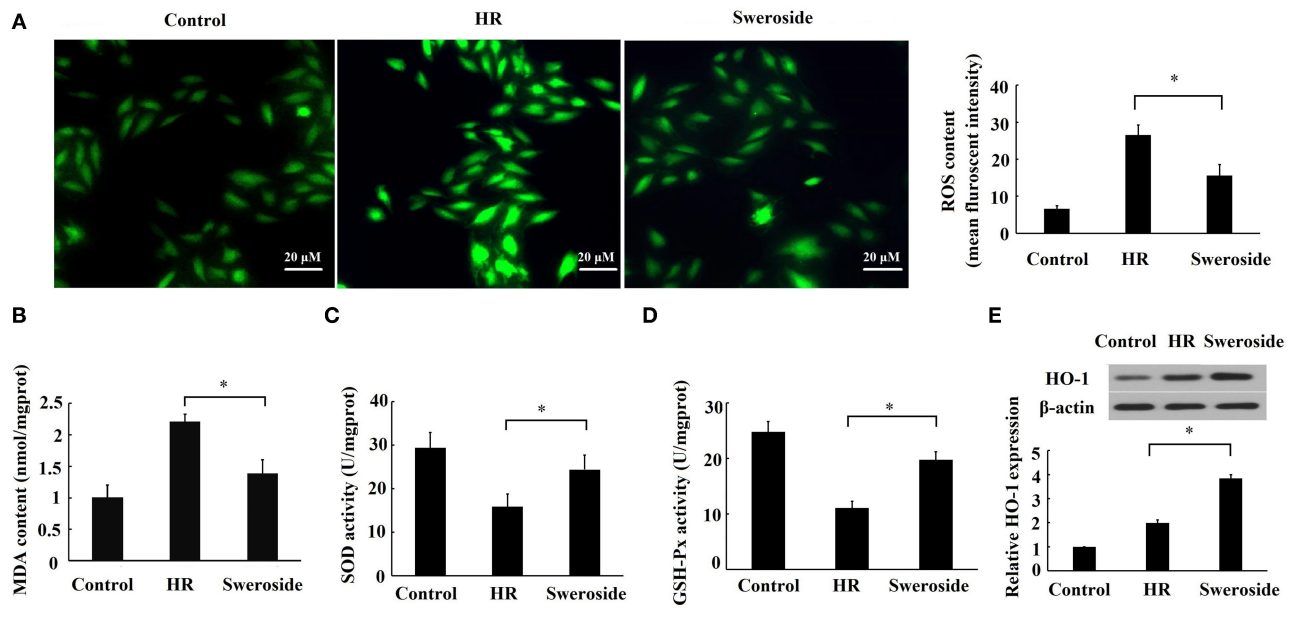

FIGURE 3 | Sweroside inhibited hypoxia/reoxygenation (HR)-induced oxidative stress. H9c2 cells were pretreated with 50 μM of sweroside for 24 h and then underwent HR. **(A)** Reactive oxygen species (ROS) in cells was detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). **(B)** Measurement of malondialdehyde (MDA) content. **(C)** Measurement of superoxide dismutase (SOD) activity. **(D)** Measurement of glutathione peroxidase (GSH-Px) activity. **(E)** HO-1 level detected via western blotting. Each cell experiment was repeated at least three times. *$P < 0.05$.

Establishment of the Isolated Rat Heart Ischemia–Reperfusion Model

Animal anesthesia was induced via an intraperitoneal injection of sodium pentobarbital (30 mg/kg) (22). The rat heart was isolated from the thoracic cavity, hung on a Langendorff perfusion device from the root of the aorta, and perfused with O_2-saturated Krebs–Henseleit (K-H) solution (127 mmol/L of NaCl, 17.7 mmol/L of $NaHCO_3$, 5.1 mmol/L of KCl, 1.5 mmol/L of $CaCl_2$, 1.26 mmol/L of $MgCl_2$, and 11 mmol/L of D-glucose, pH = 7.4) at a constant pressure of 75 mmHg and in a constant temperature (37°C), as described previously (22). Myocardial IR was induced by stopping K-H solution perfusion for a period of 30 min, followed by reperfusion with K-H solution for 90 min. The fluid-filled latex balloon, which was connected to a pressure sensor, was inserted into the left ventricle to dynamically monitor the alteration of cardiac function using a homodynamic system (MP150; BIOPAC Systems, Inc.; Goleta, CA, USA).

Animal Treatment and Experimental Groups

Sweroside at the dose of 25, 50, and 100 mg/kg was intraperitoneally injected into rats once a day for 5 consecutive days before myocardial IR injury was induced. The dose of sweroside used in this study was based on a previous study (18). A total of 48 rats were separated equally into six groups ($n = 8$), as follows: (i) the control group, the isolated heart underwent 120 min of perfusion without interruption; (ii) the IR group, the isolated heart was subjected to interruption of perfusion for 30 min, followed by reperfusion for 120 min; (iii) the vehicle group, the rats received an intraperitoneal injection of 1 ml of saline once a day for 5 consecutive days prior to heart operation,

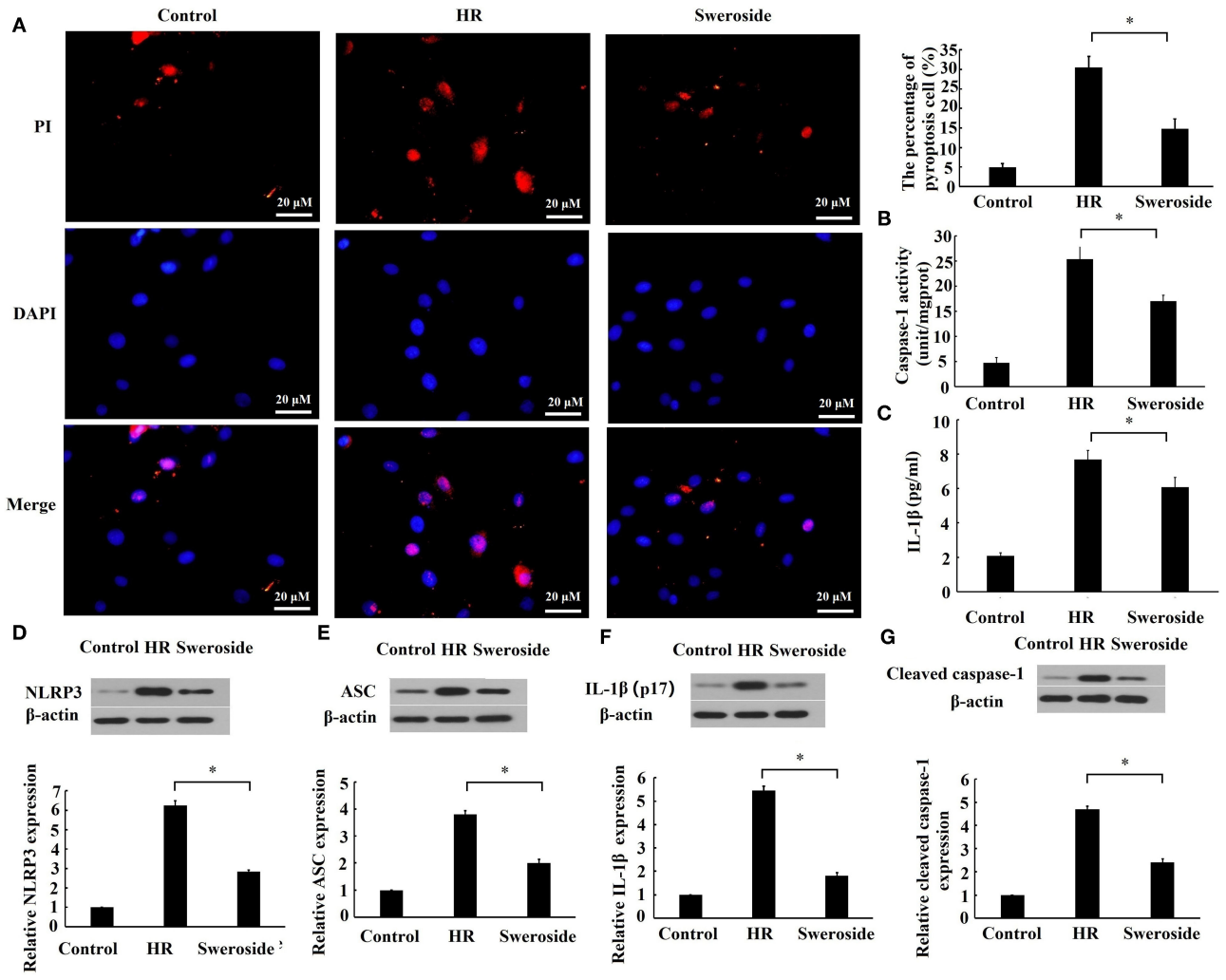

FIGURE 4 | Sweroside inhibited hypoxia/reoxygenation (HR)-induced pyroptosis. H9c2 cells were pretreated with 50 μM of sweroside for 24 h and then underwent HR. **(A)** Pyroptosis cell was characterized by pore formation in the cell membrane. The cell nucleus stained by propidium iodide (PI) indicated a pyroptotic cell. **(B)** The activity of caspase-1 and **(C)** the level of IL-1β in culture medium were detected using enzyme-linked immunosorbent assay (ELISA). Western blotting was performed to detect the expression levels of NLRP3 **(D)**, ASC **(E)**, IL-1β **(F)**, and cleaved caspase-1 **(G)**. Each cell experiment was repeated at least three times. *$P < 0.05$.

followed by IR, as described for the IR group; (iv) the 25 mg/kg sweroside treatment group, the rats received an intraperitoneal injection of sweroside (25 mg/kg) once a day for 5 consecutive days before heart isolation, followed by IR, as described for the IR group; (v) the 50 mg/kg sweroside treatment group, the rats received 50 mg/kg of sweroside treatment and underwent IR, as described for the 25 mg/kg sweroside group; and (vi) the 100 mg/kg sweroside treatment group, the rats received 50 mg/kg of sweroside treatment and underwent IR as described for the 25 mg/kg sweroside group.

Measurement of Infarct Size

At the end of the reperfusion, the hearts were detached from the device, refrigerated at −20°C for 1 h, and then cut into 1-mm-thick sections. These sections were stained with triphenyltetrazolium chloride solution, as described previously (22).

Western Blotting

Total proteins were obtained from the collected cells using radioimmunoprecipitation assay (RIPA) buffer. Nuclear and cytoplasmic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Shanghai, China), and the protein concentration was determined using an enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology), in accordance with the manufacturer's instructions. Protein samples were thermally denatured, separated by 8 or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, transferred to polyvinylidene difluoride (PVDF), membranes and blocked with 1% bovine serum albumin for 1 h at room temperature. Then, membranes were incubated with diluted primary antibodies, including anti-Nrf2 (cat. no. WL02135), anti-HO-1 (cat. no. WL02400), anti-Keap1 (cat. no. WL03285), anti-NLRP3 (cat. no. WL02635), anti-associated speck-like protein containing a CARD (ASC; cat. no. WL02462), anti-cleaved-IL-1β (cat. no. WL00891), and anti-cleaved caspase-1 (cat. no. WL03450) (all antibodies diluted 1:1,000 and purchased from Wanleibio Co., Ltd, Shenyang, China) at 4°C overnight. After being washed three times with PBS, the membranes were incubated with horseradish peroxidase-labeled IgG (1:5,000; Zhongshan Jinqiao Biotechnology, Beijing, China) at room

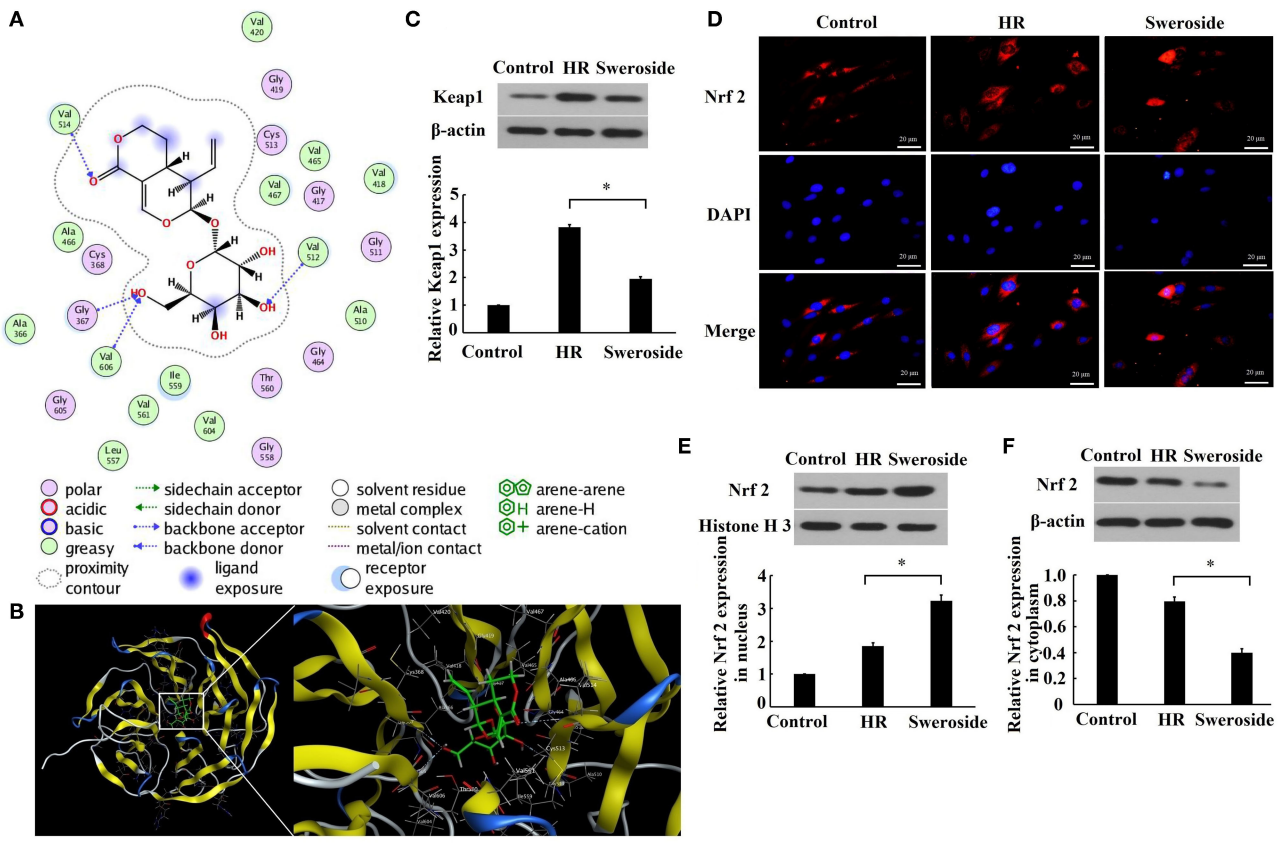

FIGURE 5 | Sweroside repressed Keap1 and promoted nuclear translocation of Nrf2. **(A)** 2D diagrams for the complex between sweroside and Keap1 was simulated using Molecular Operating Environment (MOE). The chemical structure of sweroside is shown in the center, surrounded with the key interacting amino acids. **(B)** 3D models of the docking conformation between sweroside and Keap1. H9c2 cells were pretreated with 50 μM of sweroside for 24 h and then underwent hypoxia/reoxygenation (HR). **(C)** Western blot analysis of Keap1 expression. **(D)** Nrf2 nuclear translocation was observed via immunofluorescence staining. Scale bar = 20 μm. **(E,F)** Western blot analysis of the expression level of Nrf2 in the cytoplasm and nuclei. Each cell experiment was repeated at least three times. *$P < 0.05$.

temperature for 30 min. The membranes were then developed using a BeyoECL Plus kit (Beyotime Institute of Biotechnology) following the operation guideline. Image J2x analysis software (NIH, Bethesda, MD, USA) was used to analyze relative densitometry values.

Statistical Analysis

Data are presented as the mean ± standard deviation. The statistical significance between groups was determined using one-way analysis of variance, followed by the least significant difference *post-hoc* test. SPSS version 17.0 software (SPSS, Inc.,

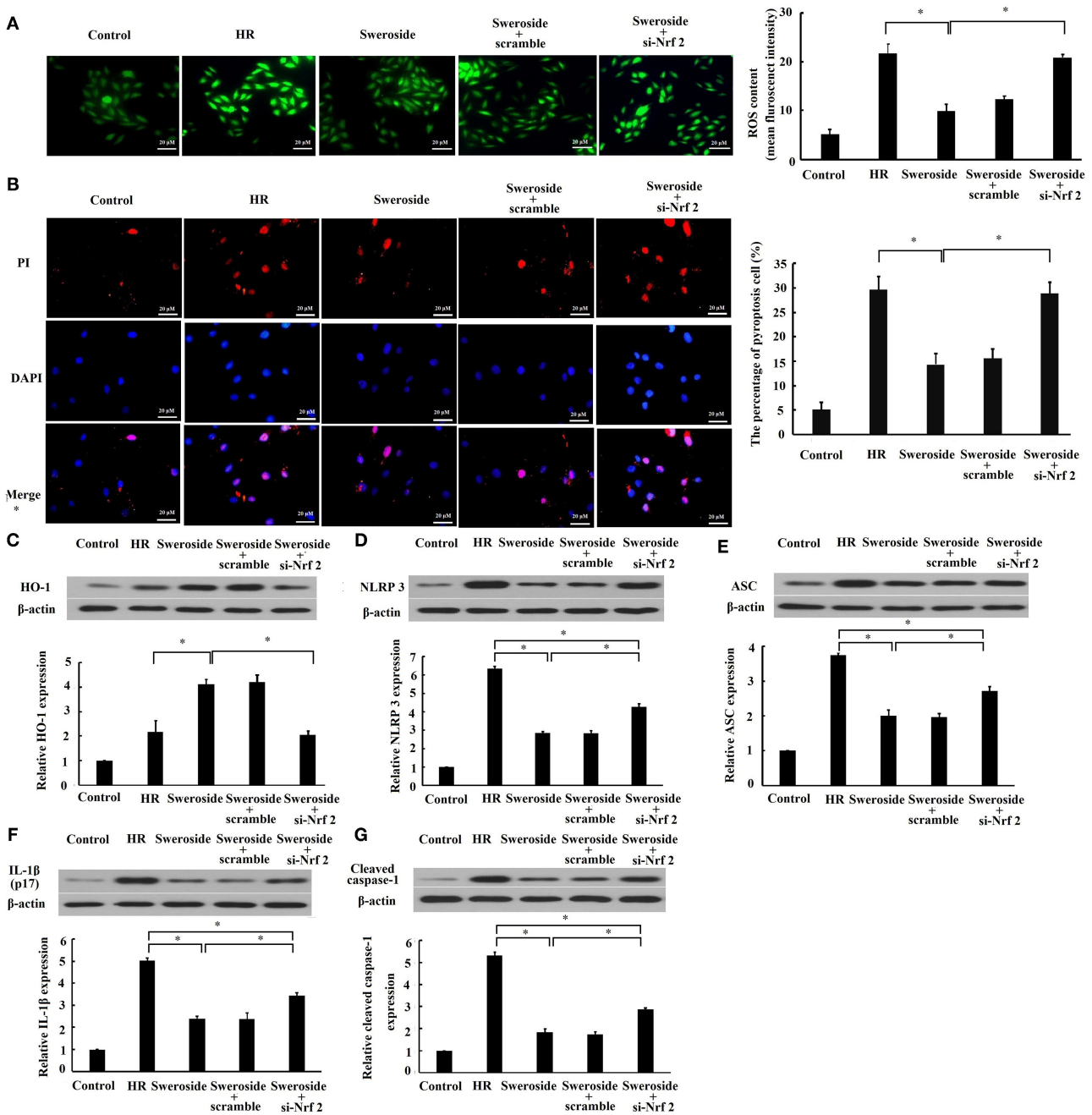

FIGURE 6 | Inhibition of Nrf2 abrogated the effect of sweroside on oxidative stress and pyroptosis. H9c2 cells were transfected with small interfering RNA targeting Nrf2 (si-Nrf2) and its negative control (scrambled siRNA). Following transfection for 24 h, cells were further processed for sweroside treatment and then subjected to hypoxia/reoxygenation (HR). **(A)** Reactive oxygen species (ROS) in cells were detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). **(B)** A pyroptotic cell was characterized by pore formation in the cell membrane. The cell nucleus stained by propidium iodide (PI) indicated a pyroptotic cell. Western blot analysis of the expression levels of HO-1 **(C)**, NLRP3 **(D)**, ASC **(E)**, IL-1β **(F)**, and cleaved caspase-1 **(G)**. Each cell experiment was repeated at least three times. *$P < 0.05$.

Chicago, IL, USA) was employed to conduct all statistical analyses, and $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Sweroside Pretreatment Alleviated Hypoxia/Reoxygenation-Induced Myocardial Injury *in vitro*

The results demonstrated that cell viability was remarkably enhanced (**Figure 1B**; 10, 25, 50, and 100 μM of sweroside vs. HR: 0.652 ± 0.009, 0.697 ± 0.007, 0.732 ± 0.009, 0.735 ± 0.011 vs. 0.597 ± 0.016), and the release of CK-MB and LDH (**Figures 1C,D**) was significantly decreased by 10, 25, 50, and 100 μM of sweroside pretreatment. Moreover, the 25 μM sweroside treatment group displayed higher cardioprotection than the 10 μM sweroside treatment group, while the 50 μM sweroside treatment group had an improved cardioprotection than the 25 μM sweroside treatment group. However, no additive protective effect was found in the 100 μM sweroside treatment group compared with the 50 μM sweroside treatment group. Therefore, these results suggested that sweroside pretreatment dose-dependently alleviated HR-induced myocardial injury *in vitro*, and 50 μM was used for subsequent experiments.

Sweroside Pretreatment Ameliorated Ischemia–Reperfusion-Induced Myocardial Injury *ex vivo*

Myocardial infarct size in the sweroside treatment group (25, 50, and 100 mg/kg) was significantly decreased compared with that in the IR group (25, 50, and 100 mg/kg of sweroside vs. IR: 28.90 ± 2.35%, 14.14 ± 2.38%, 15.51 ± 2.78% vs. 41.14 ± 4.37%). Moreover, the infarct size in the 50 mg/kg sweroside treatment group was small compared with that in the 25 mg/kg sweroside treatment group. However, there was no significant difference in the infarct size between the 50 mg/kg sweroside treatment group and the 100 mg/kg sweroside treatment group (**Figure 2A**). In addition, the left ventricular developed pressure (LVDP) (**Figure 2C**) and positive/negative first-order derivative of ventricular pressure (±dp/dt) (**Figures 2D,E**) values in the sweroside treatment group (25, 50, and 100 mg/kg) were significantly increased compared with those in the IR group, although there was no significant difference in the heart rate (**Figure 2B**). Similar to the result of the infarct size, the LVDP and ±dp/dt values in the 50 mg/kg sweroside treatment group were higher compared with those in the 25 mg/kg sweroside treatment group, but these showed no significant difference as compared with the 100 mg/kg sweroside treatment group. Taken together, these results suggested that sweroside decreased myocardial infarct size and ameliorated cardiac function in a dose-dependent manner and that 50 mg/kg of sweroside treatment induced the best cardioprotection *ex vivo*.

Sweroside Inhibited Hypoxia/Reoxygenation-Induced Oxidative Stress

The level of ROS, MDA, SOD, and GSH-Px can reflect cellular oxidative stress (25). Sweroside pretreatment significantly attenuated the levels of ROS and MDA content (**Figures 3A,B**) that were induced by HR, while it enhanced the activities of SOD and GSH-Px (**Figures 3C,D**) that were repressed by HR. HO-1 is a major antioxidant molecule, and the level of HO-1 can also reflect the status of oxidative stress (26). It was found that sweroside pretreatment led a nearly two-fold increase in the expression of HO-1 (**Figure 3E**). Collectively, these results suggested that sweroside could inhibit HR-induced oxidative stress.

Sweroside Inhibited Hypoxia/Reoxygenation-Induced Pyroptosis

To evaluate the effect of sweroside on pyroptosis, the cell membrane integrity, the activities of caspase-1 and IL-1β, and pyroptosis-related proteins were detected. As shown in **Figure 4A**, sweroside pretreatment markedly prevented the loss of cell membrane integrity induced by HR, and the percent of pyroptotic cells was reduced by sweroside pretreatment (sweroside vs. HR: 14.76 ± 2.58% vs. 30.44 ± 2.82%). Moreover, the activities of caspase-1 and IL-1β in culture medium were significantly decreased by sweroside pretreatment (**Figures 4B,C**). The expression levels of NLRP3, ASC, IL-1β, and cleaved caspase-1 were also decreased by 55, 48, 65, and 49% by sweroside pretreatment, respectively (**Figures 4D–G**). Altogether, these results indicated that sweroside inhibited HR-induced pyroptosis.

Sweroside Repressed Keap1 and Promoted Nuclear Translocation of Nrf2

As predicted by the molecular docking model, it was found that sweroside may interact with Keap1 (**Figures 5A,B**). Furthermore, it was identified that the expression level of Keap1 was significantly decreased by 51% after sweroside treatment (**Figure 5C**). The results demonstrated that the expression level of Nrf2 in the nucleus was nearly enhanced two-fold, while the level of Nrf2 in cytoplasm was reduced by 50% (**Figures 5D–F**), which supported the hypothesis that sweroside repressed Keap1 and promoted nuclear translocation of Nrf2.

The Protective Effect of Sweroside on Oxidative Stress and Pyroptosis Was Nrf2-Dependent

To determine the regulation of Nrf2 in the inhibition of sweroside on oxidative stress and pyroptosis, we examined whether inhibition of Nrf2 by transfection of si-Nrf2 could abrogate the effect of sweroside on oxidative stress and pyroptosis. It was demonstrated that the suppression of ROS and the preservation of cell membrane integrity by sweroside were rescued by inhibition of Nrf2 (**Figures 6A,B**). Additionally, the enhancement of HO-1 (**Figure 6C**) was completely rescued by

inhibition of Nrf2. However, the repression of NLRP3, ASC, IL-1β, and cleaved caspase-1 expression levels (**Figures 6D–G**) by sweroside was only rescued 49, 41, 38, and 39% by inhibition of Nrf2, respectively. Taken together, these results suggested that the effect of sweroside on oxidative stress was Nrf2-dependent and its anti-pyroptotic effect was partially Nrf2-dependent.

DISCUSSION

Swertia pseudochinensis Hara, based on its antioxidant and anti-inflammatory activities, has been used to treat hepatitis in China for a long time (27, 28). Sweroside, a main active component of *S. pseudochinensis* Hara, has also been reported to exert multiple pharmacotherapeutic actions. Yang et al. revealed that sweroside alleviated non-alcoholic fatty liver disease in obese mice via the modulation of lipid metabolism and inflammation (29). In addition, Yang et al. indicated that sweroside protected against non-alcoholic steatohepatitis by inhibiting NLRP3 inflammasome-mediated pyroptosis (21). More interestingly, sweroside has been found to prevent myocardial cells against aconitine-induced cardiac toxicity (18). However, the protective effect of sweroside on myocardial IR injury is yet to be determined. To the best of our knowledge, the present study is the first to demonstrate that sweroside could protect against myocardial IR injury, which indicates a potential use for sweroside.

Although a previous study has reported that sweroside could suppress ROS and MDA and enhance SOD activity (18), the specific mechanism for its antioxidant effect is not fully understood. In the present study, we found that sweroside repressed Keap1 expression and subsequently promoted nuclear translocation of Nrf2. Accumulating evidence has revealed that some small molecules could lead to Nrf2 activation by interrupting the Keap1–Nrf2 protein–protein interaction (30, 31). Considering that the interaction between Keap1 and sweroside was predicted in the molecular docking model, we inferred that Nrf2 was activated by interrupting the Keap1–Nrf2 protein–protein interaction by sweroside. Additionally, we confirmed that the effect of sweroside on HO-1 and ROS was rescued by inhibition of Nrf2, which further demonstrated that the antioxidant effect of sweroside was Nrf2-dependent.

Pyroptosis, a new form of programmed cell death, commonly starts with the formation of an inflammasome complex containing NLRP3, and the adaptor proteins ASC and pro-caspase-1 (32). Pro-caspase-1 is cleaved into its active form by the inflammasome complex, and then, on the one hand, active caspase-1 cleaves GSDMD to facilitate the formation of pore at the cell membrane (24), eventually leading to cell lysis (33). On the other hand, the precursors of IL-1β and IL-18 are cleaved by active caspase-1 to produce active IL-1β and IL-18. The inflammatory response is induced when active IL-1β and IL-18 are released out of cells (4). In the present study, we found that sweroside inhibited the expression levels of NLRP3, ASC, IL-1β, and cleaved caspase-1, which suggested that sweroside could repress NLRP3 inflammasome-mediated pyroptosis. This was consistent with findings by Yang et al. (21). In the process of IR, ROS largely accumulate in the tissue and induce NLRP3 inflammasome activation (34). Additionally, Nrf2 activation could inhibit NLRP3 inflammasome activity by repressing ROS (35). In the present study, we also found that the inhibition of NLRP3 inflammasome-mediated pyroptosis by sweroside could be partially reversed by inhibition of Nrf2. This finding suggested that the inhibition of pyroptosis by sweroside may be partially regulated by Nrf2/ROS/NLRP3 axis. Admittedly, this reversion was partial rather than complete, which suggested that other potential pathways might be involved in the anti-pyroptotic effect of sweroside. For instance, NF-κB signaling pathway also plays a key role in mediating the activation of NLRP3 inflammasome-mediated pyroptosis (36, 37). Sweroside is reported to suppress NF-κB signaling pathway (19, 20). Thus, sweroside may inhibit the activation of NLRP3 inflammasome-mediated pyroptosis mediated by NF-κB signaling pathway. However, due to lack of direct experimental evidence for this speculation, additional experiments need to be done to define the strongest/the most potential pathways of sweroside on its anti-pyroptotic effect in the future study.

The limitation of *ex vivo* model established by the Langendorff system is that it cannot completely mimic the pathos-physiological changes during IR due to lack of neurohumoral regulation. However, the isolated heart model has an advantage in control of lots of the variables influencing IR as well as real-time analysis of the functional changes on the heart (38). Although the elimination of systemic influence should be accounted for analyzing our data, we still gain some invaluable results in understanding the cardioprotection induced by sweroside pretreatment.

In conclusion, our study demonstrated that sweroside pretreatment could protect against myocardial IR injury by inhibiting of oxidative stress and NLRP3 inflammasome-mediated pyroptosis at least partially via modulation of the Keap1/Nrf2 axis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University.

AUTHOR CONTRIBUTIONS

JL, NW, and CM: conceptualization, methodology, software, and validation. JL, CZ, QZ, YW, GL, XL, YL, and NW: formal analysis, investigation, and visualization. CZ, QZ, YW, GL, XL, and YL: resources. JL, CZ, QZ, YW, GL, XL, and YL: data curation. JL and NW: writing—original draft preparation. NW and CM: writing—review and editing, supervision, project administration, and funding acquisition.

All authors contributed to the article and approved the submitted version.

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Myocardial Ischemia-Reperfusion and Diabetes: Lessons Learned From Bedside to Bench

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In front of the failure to translate from bench to bedside cardioprotective drugs against myocardial ischemia-reperfusion, research scientists are currently revising their animal models. Owing to its growing incidence nowadays, type 2 diabetes (T2D) represents one of the main risk factors of co-morbidities in myocardial infarction. However, discrepancies exist between reported animal and human studies. Our aim was here to compare the impact of diabetes on cell death after cardiac ischemia-reperfusion in a human cohort of ST-elevation myocardial infarction (STEMI) patients with a diet-induced mouse model of T2D, using a high-fat high-sucrose diet for 16 weeks (HFHSD). Interestingly, a small fraction (<14%) of patients undergoing a myocardial infarct were diabetic, but treated, and did not show a bigger infarct size when compared to non-diabetic patients. On the contrary, HFHSD mice displayed an increased infarct size after an *in vivo* cardiac ischemia-reperfusion, together with an increased cell death after an *in vitro* hypoxia-reoxygenation on isolated cardiomyocytes. To mimic the diabetic patients' medication profile, 6 weeks of oral gavage with Metformin was performed in the HFHSD mouse group. Metformin treatment of the HFHSD mice led to a similar extent of lower cell death after hypoxia-reoxygenation as in the standard diet group, compared to the HFHSD cardiomyocytes. Altogether, our data highlight that due to their potential protective effect, anti-diabetic medications should be included in pre-clinical study of cardioprotective approaches. Moreover, since diabetic patients represent only a minor fraction of the STEMI patients, diabetic animal models may not be the most suitable translatable model to humans, unlike aging that appears as a common feature of all infarcted patients.

Keywords: diabetes mellitus, myocardia infarction, human, animal model, medication

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INTRODUCTION

In front of the failure of cardioprotective approaches against myocardial infarction (MI) in several clinical studies (1–3), reconsidering animal models by taking into account all the patients' confounding factors may become inevitable for researchers. Aging, gender, co-morbidities, accompanying diseases and medications are underestimated factors that disrupt the translation of basic research into humans (4). Among them, diabetes appears as one of the most relevant due to its growing rise in prevalence and incidence nowadays.

The World Health Organization reports 422 million people living currently with diabetes worldwide. Diabetes increases the risk of mortality compared with non-diabetic patients and mainly cardiovascular diseases such as stroke and acute coronary syndromes (5, 6). Indeed, diabetes slightly increases the risk of mortality in all type of acute coronary syndromes (7). However, patients with type 2 diabetes (T2D) are often followed and treated with medications to regulate their metabolic dysfunction and these treatments may have an impact on the response to other injury such as an ischemic stress. While a higher risk to develop MI for a diabetic patient is recognized, the effect of diabetes on post-MI infarct size is still not clear. In fact, some studies, focusing on ST-segment elevation myocardial infarction (STEMI), have showed that diabetic patients may develop a larger infarct size, as demonstrated in both clinical trials CORE and EMERALD (8, 9). On the contrary, De Luca et al. have indicated no changes in infarct size between non-diabetic and diabetic patients after primary angioplasty (10). Similar infarct size was also observed in a clinical study comparing diabetic patients with and without insulin treatment (11). Interestingly, these discrepancies among clinical trials mirror the results in diabetic animal studies (12). For example, using the high-fat insulin resistant rat, bigger infarct sizes were reported after *in vivo* cardiac ischemia-reperfusion (13), while the recent study using the T2D Zucker rats showed no difference in infarct size between lean and fatty animals (14).

In the diabetic animal models, the heterogeneity in findings may be related to the diabetic inducers (genetic, treatment, diet…) and/or to the timing (early diabetic cardiomyopathy vs. late heart failure stage); while in patients, the intra- and inter-variabilities between humans, as well as their companion medications schedule could have an impact. It should be noted that *in vivo* cardiac ischemia-reperfusion animal protocols rather represent STEMI patients. In this context, infarct size remains an important determinant of the post-MI outcome and is used as an endpoint in both animal and clinical studies of cardioprotective strategies. Therefore, we ought to investigate further the effect of T2D on myocardial infarct size by confronting the results of STEMI patients to a mouse model of early diabetic cardiomyopathy (15) in order to question the relevance of diabetic animal models in studies of cardioprotection against MI.

METHODS

Human Cohort and Consent Information

The study was approved by our institution Review Board and Ethics Committee and is registered with the ClinicalTrials.gov identifier NCT03070496. Patients have given their written consent. From the previously described cohort composed of 250 consecutive patients admitted to the Louis Pradel Hospital for a suspected ST-elevation myocardial infarction (STEMI) from 2016 to 2020 (16), all patients underwent coronary angiography at admission with subsequent reperfusion by primary percutaneous intervention (PCI); but only 177 patients underwent contrast enhanced Cardiac Magnetic Resonance (CMR) at one month after MI for infarct size and LV function measurements.

Type 2 Diabetes Animal Model and *in vivo* Ischemia-Reperfusion Protocol

All animal procedures performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the institutional animal research committee from Université Claude Bernard Lyon 1 and the French ministry (#15,627–2018062118508398 and BH2012-65). Male C57BL/6JOlaHsd (Envigo, France) mice were from the same cohort as characterized previously (15): they were subjected to either a standard diet (SD: LASQC diet Rod16-A, Genobios: 16.9% proteins, 4.3% lipids) or a high-fat high-sucrose diet (HFHSD: 260HF U8978 version 19, from SAFE: 20% proteins, 36% lipids, and 35% carbohydrates) for 16 weeks (**Figure 1B**). For the last 6 weeks of the feeding protocol, metformin gavage (200 mg/kg) was performed daily for half of the HFHSD mice, with the other control group being given the vehicle (0.5% methylcellulose) (17).

For the *in vivo* ischemia-reperfusion (**Figure 1C**), mice were anesthetized with ketamine (100 mg/kg body weight, intraperitoneal injection) and xylazine (5 mg/kg body weight, intraperitoneal injection) and were orally intubated and ventilated via a rodent ventilator (Physiosuite® system from Kent Scientific). Body temperature was monitored by a rectal thermometer and maintained within the normal range by using a heating pad. A left thoracotomy was performed and the pericardium was opened to expose the heart. An 8-0 polypropylene suture was passed around the left anterior descending artery, under an Euromex microscope, for further creating ischemia. Ischemia was confirmed by ST segment shift on the ECG and appearance of epicardial pallor. After 45 min of coronary artery occlusion, the snare was loosened, and reperfusion was confirmed by visual inspection and reduction of ST segment shift on the ECG. The chest wall was closed with a 5-0 vicryl suture and the endotracheal tube was removed once spontaneous breathing had resumed. The mice were then allowed to recover from the anesthesia in a temperature-controlled area enriched with O_2. At the end of the 24-h reperfusion, the mice were deeply anesthetized to allow reocclusion of the artery (the suture material was still in place from the previous surgery) and Unisperse blue pigment (0.5 mg/kg i.v.; Ciba-Geigy) was injected in the inferior vena cava. With this technique, the non-ischemic myocardium appears blue, whereas the ischemic myocardium [area at risk (AR)] remains unstained. The heart was then excised and the atrial and right ventricular tissues were removed. The left ventricle was then cut into four 1 mm thick transverse slices. The basal surface of each slice was photographed for later measurement of the area at risk. Slices were then incubated for 15 min in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 34°C. With this technique, the viable myocardial tissues appear brick red, whereas the infarcted tissues remain pale. The slices were then photographed for later measurement of the area of necrosis (AN). The extent of the area at risk and the infarcted

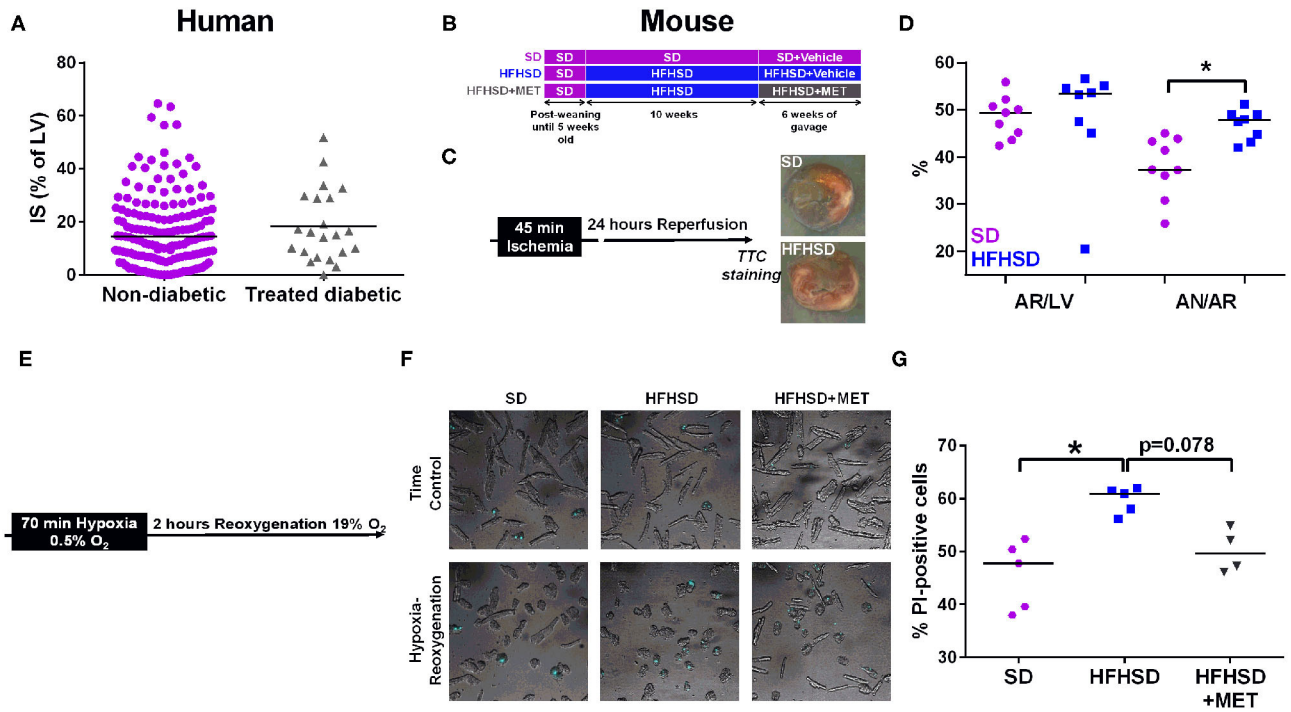

FIGURE 1 | Effect of diabetes on infarct size and cardiomyocyte cell death in humans and mice. **(A)** Measurement of infarct size (IS) as a percentage of the left ventricle (LV), assessed by MRI one month post-MI in patients (155 non-diabetics vs. 22 diabetics). p = ns. **(B)** Study design of the diet groups SD and HFHSD together with the oral gavage of Metformin (MET) or Vehicle for the last six weeks of feeding. **(C)** *in vivo* model of myocardial infarction in mice consists in 45 min of ischemia induced by ligation of the left descending coronary artery followed by 24 h of reperfusion before assessment of infarct size. Representative images of infarct size are depicted for each group. **(D)** Measurement of the area at risk (AR/LV), expressed as a percentage of area at risk (AR) over left ventricle (LV), and of the infarct size (AN/AR), calculated as a percentage of area of necrosis over (AN) area at risk. n = 9 SD and 8 HFHSD, $^*p < 0.05$. **(E)** Timeline of the hypoxia-reoxygenation protocol: hypoxia is induced for 70 min at 0.5% O_2 followed by reoxygenation at 19% O_2 for 2 h before assessment of cell death. **(F)** Representative images of combined white light and propidium-iodide (in blue) cardiomyocytes from SD, HFHSD, and HFHSD+MET mice, in normoxic condition (TC) and after hypoxia-reoxygenation (HR). **(G)** Assessment of cell death by propidium iodide (PI) staining after hypoxia-reoxygenation in cardiomyocytes from SD, HFHSD, and HFHSD+MET mice (n = 4-5/group). $^*p < 0.05$.

area was quantified by computerized planimetry and corrected for the weight of the tissue slices.

Adult Cardiomyocyte Isolation

Mice were heparinized and euthanized by cervical dislocation. Cardiomyocytes were then isolated using O'Connell's protocol (18) and plated for 2 h on glass bottom 35 mm dishes (MatTek) with complete plating medium (MEM Eagle's w/HBSS, FBS 10%, BDM 10 mM, penicillin 100 U/ml, Glutamine 2 mM, ATP 2 mM) prior to the sequence of hypoxia-reoxygenation.

In vitro Hypoxia-Reoxygenation Protocol

Cardiomyocytes were washed twice to remove serum and nutrients with 1 mL Hypoxia Buffer at 37°C (HRB: 0.14 M NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM $CaCl_2$, pH 7.4). Hypoxia was performed in 1 mL of HRB solution for 70 min at 0.5% O_2 / 37°C (including a stabilization period of 25 min to reach the desired level of O_2 in an hypoxic incubator New Brunswick, Eppendorf). Reoxygenation at 19% O_2/120 min/37°C was next achieved by quickly but gently replacing the hypoxic medium with 1.5 mL plating medium. At the end of the 2 h of reoxygenation, counting of propidium iodide-positive cells (PI at 1 μg/ml) and of morphologically dead cardiomyocytes (loss of rod-shape) was performed by confocal microscopy (Nikon) and subsequent analysis was done on ImageJ software.

Statistics

All data were subjected to normality test. Mann-Whitney was applied for the ones which failed the normality test and data were presented as median [Interquartile range]. However, parametric tests were applied for normally distributed data, presented as mean ± SD. For the three groups comparison, a Kruskal-Wallis test followed by a Dunn's multiple comparison test was performed. A p-value < 0.05 was considered statistically significant. Analysis was performed on GraphPad Prism.

RESULTS

Treated Diabetic Patients Exhibit Similar Infarct Size as the Non-diabetics

Patients were included with a median age of 59 ± 12 years. Among the 250 patients, 37 patients suffered from type 2 diabetes, giving a proportion of 14.8% diabetic patients suffering from MI

TABLE 1 | Characteristics of the study population.

	Non-diabetic patients ($n = 213$)	Diabetic patients ($n = 37$)	*p* value
Age, years	57 ± 11	63 ± 10	0.02
Male sex, nb (%)	168 (78.9)	30 (81.1)	0.83
Body Mass Index (BMI), kg/m²	26.3 [23.9-29.4]	25.9 [23.7-29.6]	0.69
Dyslipidemia, nb (%)	50 (23.5)	23 (62.1)	<0.0001
Hypertension, nb (%)	52 (24.4)	22 (59.5)	<0.0001
Current smoker, nb (%)	150 (70.4)	21 (56.8)	0.12
Time from symptoms to PCI, min	205 [145-334.0]	200.0[120.0-251.3]	0.46
Anterior MI, nb (%)	113 (53.3)	19 (51.4)	0.86
Killip status = 1, nb (%)	193 (90.5)	31 (79.5)	0.05
TIMI flow grade at admission = 0-1, nb (%)	140 (72.5)	28 (75.7)	0.84
Post-PCI TIMI flow grade >2 (%)	207 (98.1)	34 (91.9)	0.07
LVEF at baseline (%)	55.0 [46.0-61.3]	50.0 [44.0-62.0]	0.2957
Peak troponin I, ng/L	43907 [16642-107843]	46466 [14353-144943]	0.55
Peak creatine kinase, mUI/L	1529 [684.3-3542.0]	1815 [641.0-4076.0]	0.68
Peak CRP, mg/L	16.1 [7.1-40.5]	42.6 [11.7-74.3]	0.02
Aspirin, nb (%)	24 (11.3)	10 (27.0)	0.02
Betablockers, nb (%)	17 (8.0)	9 (24.3)	<0.0001
ACEi / ARB, nb (%)	27 (12.7)	17 (45.9)	0.006
Statins, nb (%)	21 (9.9)	13 (35.1)	0.0002

Values are expressed as Mean ± SD, Median [interquartile range] or number with percentage (%). PCI, percutaneous coronary intervention; MI, myocardial infarction; TIMI, thrombolysis in myocardial infarction; LVEF, left ventricular ejection fraction; CRP, C-reactive protein. ACEi, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

in this cohort. The characteristics of the study population are presented in **Table 1**. Type 2 diabetic patients were significantly older than non-diabetic patients with a median age of 62 ± 11 vs. 57 ± 12 years ($p < 0.05$), and were more prone to hypertension (**Table 1**). Diabetic patients had a higher percentage of medications taken, including aspirin, statins, betablockers, and angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers (ACEi/ARB) (**Table 1**). All diabetic patients were treated with antidiabetics, mainly Metformin and 9 were under insulin, therefore being considered as treated diabetic. Based on the analysis of the Killip Status, we observed a higher rate of patients with sign of heart failure development in the treated-diabetic patients group compared to the non-diabetic patients (**Table 1**). No differences were observed between the two population regarding the coronary flux evaluated with the TIMI flow grade. Treated-diabetic patients displayed a higher level of C-reactive protein compared to non-diabetic patients (42.6 mg/L interquartile range (IQR): [11.7-74.3] compare to 16.1 mg/L IQR [7.1-40.5], $p = 0.02$). In our cohort population, dyslipidemia affected more patients with treated diabetes than non-diabetic patients (23.5% compare to 62.1%, $p < 0.001$).

No differences were observed neither on the infarct size measurement between treated-diabetic and non-diabetic patients (**Figure 1A**) (respectively, 14.5% [6.8-24.2] of the left ventricle mass compared to 15.6% [IQR: 8.1-29.1]), nor on the left ventricular ejection fraction assessed at one month (treated-diabetic, 53% [IQR: 46.0-58.5] compared to non-diabetic, 50.5 [IQR: 41.5-57.0]).

Increased Cell Death After Hypoxia-Reoxygenation in Diabetic Cardiomyocytes Is Prevented by Metformin Treatment

The mice used in this study were from the same cohort in which we previously characterized the diet-induced T2D mouse model recapitulating the early stage of diabetic cardiomyopathy in human, notably glucose, and insulin intolerance, hyperglycemia, and hyperlipidemia (15). We thus investigated the effect of T2D on infarct size after an *in vivo* ischemia-reperfusion sequence in the 16 weeks diet-fed mice, at the age of 21 weeks (**Figure 1C**). While the areas at risk were comparable between SD and HFHSD mice, HFHSD displayed a significant bigger infarct size compared to the SD mice (**Figure 1D**: HFHSD, 47.8 [43.5, 49.0] vs. SD, 37.3 [33.5, 43.6] % AR/AN, $n = 8$-9 mice/group, $p < 0.05$). Similarly, cardiomyocytes freshly isolated from HFHSD diabetic mice displayed a significant increased cell death upon simulated ischemia-reperfusion, namely hypoxia-reoxygenation, compared to the SD cardiac cells (**Figures 1E–G**, HFHSD, 60.9 [57.1, 61.8] vs. SD, 47.8 [38.8, 51.4] % of PI-positive cells, $n = 5$/group, $p < 0.05$).

One could wonder whether the difference in diabetes-induced cell death after an ischemic event between the patients and the mouse model could rely on the antidiabetic medication regimen taken by the diabetic patients of the cohort, notably Metformin. To this end, the HFHSD mice received an oral gavage with Metformin for the last 6 weeks of the feeding. As previously

described, Metformin did not decrease the body weight of the HFHSD mice but partially rescued the sensitivity to insulin and glucose (17) (data not shown). Interestingly, Metformin treatment of the HFHSD mice led to a reduction of cell death after hypoxia-reoxygenation compared to the HFHSD cardiomyocytes (HFHSD+MET, 49.7 [46.4, 54.2] % of PI-positive cells, $n = 4$-5/group, $p = 0.078$ vs. HFHSD), reaching a similar extent as in the standard diet group ($p =$ ns vs. SD) (**Figures 1F,G**).

DISCUSSION

The goal of our study was to compare the impact of diabetes on cell death after cardiac ischemia-reperfusion in a human cohort of STEMI patients with a diet-induced mouse model of T2D. In our human study population, no differences have been observed regarding the infarct size between treated-diabetic and non-diabetic patients. The baseline characteristics of our study populations are in line with the literature. Indeed, dyslipidemia is a common feature of diabetes (19) and diabetic patients displayed a higher level of C-reactive protein (20). Our population cohort displayed a small proportion of diabetic patients (14.8 %), which may be explained by the selection of only STEMI patients. Indeed, the proportion of diabetic patients in non-STEMI population is more important than in STEMI, as previously reported in the FAST-MI study (21) (16.5% of STEMI patients are diabetic while 27% of non-STEMI patients are diabetic) and in a larger database study (7) (28.8% of non-STEMI patients were diabetic compared to 18.2% in the STEMI group). Interestingly, a temporal study between 1995 and 2003 also highlighted that the diabetic patients are now more prompt to non-STEMI events (5).

As to our results in the HFHSD mice, they revealed an increased infarct size in an *in vivo* model of myocardial infarction as well as an increased cell death following a simulated ischemia-reperfusion, which was prevented by a Metformin daily treatment, as usually performed in diabetic patients. Importantly, Metformin has been shown to exert a cardioprotection through activating the AMPK pathway and upregulating PGC-1α, which improves mitochondrial organization and function (22, 23). Metformin is used as a first line antidiabetic drug, not only due to its glucose lowering potential, but also to its cardiovascular safety and protective contribution. Altogether, our data suggest that the similar infarct size seen between all STEMI patients regardless of their diabetic history may be mainly due to the protective effect afforded by their antidiabetic medication, such as Metformin, insulin, and sulfonylurea drugs, as previously described (13, 24). Therefore, evaluation of the protective effect of new therapeutic drugs in diabetic animal models is effectively of interest but should be combined with the routinely used antidiabetic medications, such as Metformin, to rule out any confounding action between the pre-existing medications and the potential protective therapy. However, while similar results were observed after *in vivo* and *in vitro* ischemia-reperfusion, i.e., increased infarct size/cell death in the HFHSD group vs. SD group, thus validating the relevance of the *in vitro* experiments, one limitation of our study relies on the absence of *in vivo* measurement of infarct size in the HFHSD+MET group. Moreover, besides their antidiabetic treatments, diabetic patients were significantly receiving more treatments than non-diabetic patients, i.e., aspirin, betablockers, and ACEi, which may also have cardioprotective effects not directly on infarct size but on the major adverse cardiovascular events (25). Therefore, taking into consideration also the current medications used routinely by the patients and at the time of reperfusion in the clinical settings would be an invaluable asset in assessing the relevance of cardioprotective drugs in *in vivo* animal studies.

However, as shown by our human cohort of STEMI patients and by others, diabetic patients only represent a minor fraction (around 15%) of the STEMI patients while closer to 30% in the non-STEMI population. Since the current animal models of *in vivo* ischemia-reperfusion usually rely on a coronary artery occlusion, thus representing the STEMI population, diabetes may not be the best co-morbidities to be taken into account to evaluate new cardioprotective strategies. While aging and hypertension were significantly more present in the diabetic patients and could also explain a form of cardioprotection by favoring coronary collateral circulation (26), one main common factor of all patients is aging (4). Although this factor is more complicated to pursue in animal models, future studies are required to assess the relevance of aging in our animal models to study cardioprotection against MI.

Finally, it has been recently shown that infarct size is not the only factor to take into consideration regarding the patient post-MI clinical outcome (25), therefore highlighting the importance to evaluate the effect of cardioprotective strategies not only on infarct size but on the contractile function and the survival notably. Indeed, the higher rate of mortality observed in diabetic patients after MI may not be due to the infarct size but to several confounding factors observed in diabetic patients such as the diabetic cardiomyopathy and dyslipidemia.

All these discrepancies between studies, whether among animal models or clinical testing, have raised a lot of questioning about scientists' attempt to translate their fundamental research into clinics. Here, we provide some advices for translational research in the field of cardioprotective strategies against MI by: (1) considering comorbidities, such as diabetes, together with their daily medication, into the animal models; (2) evaluating the relevance of each comorbidity in the protective approaches, notably between diabetes and aging; and (3) extending our experimental endpoints beyond infarct size, i.e., contractile function and survival study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP of the Hospices Civils de Lyon. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed

and approved by Université Claude Bernard Lyon 1 and the French ministry.

AUTHOR CONTRIBUTIONS

MP, MD, AP, CC, and TB: conceptualization. MD, CL, BP, and MP: experimental investigation. MD, AP, TB, and MP: data collection and analysis. MD and AP: manuscript writing. MP, TB, and CC: manuscript reviewing. MP and MO: funding acquisition. All authors contributed to the article and approved the submitted version.

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Pilose Antler Peptide-3.2KD Ameliorates Adriamycin-Induced Myocardial Injury Through TGF-β/SMAD Signaling Pathway

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Adriamycin (ADR)-based combination chemotherapy is the standard treatment for some patients with tumors in clinical, however, long-term application can cause dose-dependent cardiotoxicity. Pilose Antler, as a traditional Chinese medicine, first appeared in the Han Dynasty and has been used to treat heart disease for nearly a thousand years. Previous data revealed pilose antler polypeptide (PAP, 3.2KD) was one of its main active components with multiple biological activities for cardiomyopathy. PAP-3.2KD exerts protective effects againt myocardial fibrosis. The present study demonstrated the protective mechanism of PAP-3.2KD against Adriamycin (ADR)-induced myocardial injury through using animal model with ADR-induced myocardial injury. PAP-3.2KD markedly improved the weight increase and decreased the HW/BW index, heart rate, and ST height in ADR-induced groups. Additionally, PAP-3.2KD reversed histopathological changes (such as disordered muscle bundles, myocardial fibrosis and diffuse myocardial cellular edema) and scores of the heart tissue, ameliorated the myocardial fibrosis and collagen volume fraction through pathological examination, significantly increased the protein level of Bcl-2, and decreased the expression levels of Bax and caspase-3 in myocardial tissue by ELISA, compared to those in ADR-induced group. Furthermore, ADR stimulation induced the increased protein levels of TGF-β1 and SMAD2/3/4, the increased phosphorylation levels of SMAD2/3 and the reduced protein levels of SMAD7. The expression levels of protein above in ADR-induced group were remarkably reversed in PAP-3.2KD-treated groups. PAP-3.2KD ameliorated ADR-induced myocardial injury by regulating the TGF-β/SMAD signaling pathway. Thus, these results provide a strong rationale for the protective effects of PAP against ADR-induced myocardial injury, when ADR is used to treat cancer.

Keywords: pilose antler peptide, adriamycin, myocardial injury, protective effect, TGF-β/SMADs

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INTRODUCTION

Adriamycin (ADR) is a broad-spectrum anthracycline antibiotic derived from *Streptomyces peucetius*. Clinically, ADR was used to treat acute leukemia, various malignant tumors, and other diseases (1). At therapeutic doses, it could cause a series of severe toxic reactions, including bone marrow suppression, nausea, vomiting, nephrotoxicity and cardiac toxicity (2). Such side effects

were shown to limit its use and increase the incidence of cardiovascular disease and associated mortality in cancer survivors significantly (3, 4).

Pilose antler (Deer antler, *Cornu Cervi Pantotrichum* from *Cervusnippon Temminck*), a traditional Chinese medicine preparation, was mainly produced in Jilin, China. Pilose antler polypeptide (PAP), one main component of Pilose antler, has multiple biological activities, including the amelioration of inflammation, oxidative stress, organ injury, and fibrosis (5, 6). PAP exerted protective effects against myocardial fibrosis. However, its mechanism of action in myocardial injury was unclear (7). The transforming growth factor-β1 (TGF-β1)/Drosophila mothers against decapentaplegic proteins (SMADs) pathway had been considered to play an important role in the pathogeneses of myocardial infarction, cardiomyopathy and heart failure (8). When myocardial injury occurred, the overexpression of TGF-β1 induced the activation of SMADs and exacerbated disease progression (9). Since ADR toxicity in the heart of patients with cancer is observed while ADR is used to treat cancer, the protective roles and mechanisms of PAP against ADR-induced myocardial injury are investigated.

MATERIALS AND METHODS

Reagents

ADR was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Hematoxylin and eosin (H&E) staining kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for cardiac troponin T (cTnT) and cardiac troponin I (cTnI) were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). ELISA kits for B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), and caspase-3 were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). All antibodies were obtained from ProteinTech Group, Inc. (Wuhan, China).

Extraction of Pilose Antler peptide

Pilose Antler (Deer antler, *Cornu Cervi Pantotrichum,* No. 20180325) obtained from Zhenyuan Deer Industry Co., Ltd., Jilin, China was confirmed. The voucher specimen was prepared and deposited at Department of Pharmacy in Changchun University of Chinese Medicine. Pilose antler polypeptide (PAP), one of its main active components, was isolated.

Fresh deer antler (1 kg) was chopped into 1 cm^3 pieces at 4°C, the blood was quickly washed off with distilled water at 4°C, and then 1,000 ml of acetic acid solution (pH 4.0) was added, followed by colloid grinding, repeated homogenization, and centrifugation at 6,500 × g for 20 min at 4°C. The super solution was collected, 90% ethanol was added to a final concentration of 65%, and the mixture was stored at 4°C with stirring every 20 min for 6 h, followed by standing for 12 h. Subsequently, the mixture was centrifuged at 6,500 × g for 20 min at 4°C. The supernatant was collected and freeze-dried to obtain the crude extract of PAP, which was stored at −20°C. PAP was further separated by SuperdexG-75 gel chromatography column, and the components are collected according to the 280 nm ultraviolet absorption peak and then freeze-dried. Furthermore, Molecular weight (MW) of PAP was measured using western Blotting. MW of PAP are 3.2KD and 10KD separately. In the present study, 3.2 KD PAP (PAP-3.2KD, with a purity of 91%) was used for ADR-induced myocardial injury (**Supplementary Figure 1**) (10). The yield rate of PAP-3.2KD from fresh deer antler is 7.28%.

Animals

Forty 6-week-old Wistar rats (male to female ratio = 1:1, weighing 200–240 g) were obtained from Changchun Yisi Experimental Animal Technology Co., Ltd. (Changchun, China). They were maintained under standard conditions (23°C, constant relative humidity as 26% and 12-h dark/light cycle). All experimental procedures for the care and use of laboratory animals and animal handling followed the guidelines of the National Animal Welfare Law of China. The protocols (No. 20180003) for the animal experiments were approved by the Ethics Committee of Changchun University of Chinese Medicine.

Experimental Groups and Treatments

The rats were randomly divided into the following four groups: control, ADR and ADR+PAP-3.2KD 100 and 200 mg/kg, 10 rats in each group. Except the control group, Rats from the other three groups were intraperitoneally injected with ADR (2.5 mg/kg) every 2 days for six times at a cumulative dose of 15 mg/kg (11–13). The rats in control group were intraperitoneally injected with an equal volume of normal saline (ADR solution medium was saline, PAP-3.2KD or ADR solution medium had no effect on healthy rats, data not shown). The rats from two PAP-3.2KD groups were separately administrated orally with PAP-3.2KD 100 or 200 mg/kg for 21 days after six-time ADR stimulation. The rats from ADR group were given orally with an equal volume of water. After 21-days PAP-3.2KD or water treatment, the rats were starved for 12 h, and were anesthetized by an intraperitoneal injection of 3% sodium pentobarbital (35 mg/kg). Blood samples (6–10 mL) were collected from the aorta abdominalis and centrifuged at 1,000 × g for 10 min at 4°C. The serum samples were then stored at −80°C for future analysis. The cardiac tissues of rats were harvested.

Body Weight, Heart Weight, and HW/BW Index Assay

The rats in each group were weighed every 3 days. After the rats were euthanized, the hearts were isolated and weighed to calculate the HW/BW index.

Heart Rate and ST Height Measurement

According to the animal experimental ethics inspection form of Changchun university of Chinese medicine, all rats were anesthetized with isoflurane and fixed in a supine position 30 min following the final drug administration. The Powerlab biological signal acquisition and processing system (AD Instruments, Sydney, Australia) were connected to an electrocardiogram to record heart rate and ST height within 2 min.

Myocardial Histopathology

Heart tissue specimens were fixed in normal 4% paraformaldehyde for 48 h and dehydrated using a graded series of alcohol concentrations. After the specimens had been embedded and sliced, they were stained with H&E (magnification, ×200; Olympus, Tokyo, Japan). The severity of pathological changes was evaluated and graded by two independent observers according to the evaluation criteria shown in **Supplementary Table 1**.

Masson's Trichrome Stain

Myocardial fibrosis was detected by Masson's trichrome staining. Frozen tissue sections were fixed in Bouin's solution for 1 h at 56°C, followed by staining according to the manufacturer's protocol from Trichrome Stain (Masson) kit (Solable Technology, Beijing, China). Under light microscopy, collagen fibers were stained for blue, while cardiomyocytes were stained for red. The collagen area in each field was measured using Image-Pro Plus 6.0 image analysis software, and the collagen volume fraction (CVF) was calculated as follows: CVF = (collagen area/myocardial area) × 100%.

TUNEL

The heart tissues of rats were cut into 5 μm paraffin sections. Myocardial apoptosis was detected by TUNEL staining, according to the commercial kit protocols (Beyotime Biotechnology Co. Ltd., Shanghai, China). TUNEL mix contained 50 μL enzymesolution and 450 μL label solution. Heart sections were incubated with 50 μL TUNEL mix at 37°C for 1 h. The sections were washed in PBS and stained with DAB for 30 min. Methyl green complex staining was carried out. After PBS wash, the sections were mounted and observed. Under light microscopy, the normal myocardial cell nucleus was blue-green, and the apoptotic cells were dark brown in different shades. Each slice was randomly selected with 5 high-power fields (×400). The percentage of myocardial apoptosis area to myocardial area was referred to as the apoptosis index (AI).

ELISA

Serum cTnT and cTnI concentrations were determined to evaluate myocardial injury, according to the commercial kit protocols (Jiancheng Institute of Biotechnology, Nanjing, China). The serum samples were added to the wells pre-coated with antibody in one plate, and were incubated with HRP labeled first antibody. Plates were analyzed with a spectrophotometer after incubation with substrate (450 nm).

Myocardial tissues were homogenized at 4°C with lysis buffer (PBS containing 0.05% sodium azide, 0.5% Triton X-100, and a protease inhibitor cocktail, pH 7.2) (Jiancheng Institute of Biotechnology, Nanjing, China). The expression levels of Bcl-2, Bax, and caspase-3 were determined according to the ELISA kit protocols (Boster Biological Technology, Co. Ltd.).

Western Blotting

Total protein in the heart tissues was extracted according to the instructions of the protein extraction kits (Solaibao Technology, Co. Ltd., Beijing, China), and quantified using bicinchoninic acid (BCA) protein assay kits (SolarBio, Beijing, China). Equal amounts of protein were loaded and separated using 8–12% sodium sulfate dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes, which were blocked in 5% skimmed milk at 25°C for 1 h on a shaking table. Membranes were incubated with the appropriate concentrations of the following antibodies (Proteintech Group, Inc., Wuhan, China) at 4°C overnight: anti-Bcl-2 (1:2,000, Cat. No. 12789), anti-Bax (1:4,000, Cat. No. 50599), anti-Caspase-3 (1:2,000, Cat. No. 19677), anti-TGF-β (1:1,000, Cat. No. 21898, anti-SMAD2 (1:6,000, Cat. No. 12570), anti-SMAD3 (1:1,000, Cat. No. 25494), anti-SMAD4 (1:1,000, Cat. No. 10231), anti-SMAD7 (1:2,000, Cat. No. 25840), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:40,000, Cat. No. 10494). After three rounds of washing with Tris-buffered saline plus Tween (TBST), the membranes were incubated with a secondary anti-rabbit antibody (1:10,000, cat. no. 21991) at room temperature (RT) for 1 h. Finally, the immunoreactive bands were visualized using an enhanced chemiluminescence kit with a gel imaging system (Proteintech Group, Inc.).

Statistical Analysis

The results were presented as the means ± standard error of mean (SEM). Differences between groups were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. A $P < 0.05$ was considered significant, and all data analyses were conducted using the statistical package for the social sciences (SPSS) 21.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

PAP-3.2KD Reversed HW/BW Index in ADR-Induced Rats

After 6 days of PAP-3.2KD treatment, three experimental groups including ADR and PAP-3.2KD (100 and 200 mg/kg) treatment displayed significantly lower BW than those in the control group. BW levels in two PAP-3.2KD groups (100 and 200 mg/kg) groups were significantly increased at 12 days after PAP-3.2KD administration, compared to those in the ADR group ($P < 0.05$ and <0.01), even though BW levels in two PAP-3.2KD groups were still significantly lower than those in the control group (**Figure 1A**).

The heart weights of rats in the ADR group were significantly reduced compared with those in the control group ($P < 0.05$). After treatment with PAP-3.2KD, the heart weights were increased, even though there was no significant difference, compared to those from ADR group ($P > 0.05$; **Figure 1B**). In addition, The HW/BW index was calculated, since it is a basic indicator of cardiac edema and hyperplasia, which reflects the degree of myocardial injury. HW/BW index of rats in the ADR group was increased compared to that in the control rats ($P < 0.01$). The HW/BW index of rats in two PAP-3.2KD groups (100 and 200 mg/kg) were markedly decreased, compared to those in ADR groups separately ($P < 0.05$ and $P < 0.01$; **Figure 1C**).

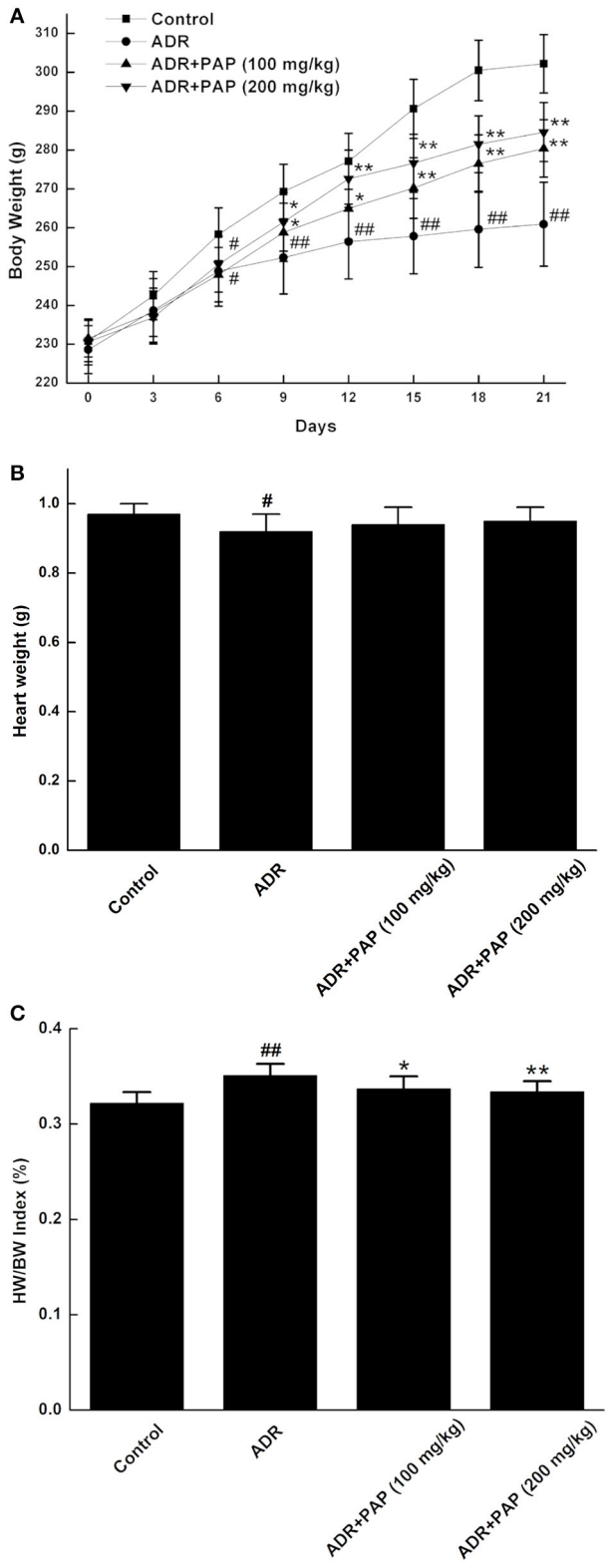

FIGURE 1 | PAP-3.2KD reversed HW/BW index of ADR-induced rats. **(A)** Every 3 days, body weight (BW) was measured in four groups, includingcontrol, ADR, ADR+ PAP-3.2KD (100 mg/kg), ADR+ PAP-3.2KD (200 mg/kg). **(B,C)** Both Heart weight (HW) and HW/BW index were detected in four groups after the rats were euthanized. Data were presented as Mean ± standard error of mean ($n = 10$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the ADR group, ADR, Adriamycin; PAP-3.2KD, 3.2 KD pilose antler polypeptide.

PAP-3.2KD Recovered Heart Rate and ST Height in ADR-Induced Rats

To study the effects of PAP-3.2KD and/or ADR on cardiac function, heart rates and ST heights of ADR-induced rats were measured. Both heart rates and ST heights were remarkably increased by ADR induction compared to those of the control group ($P < 0.01$). However, in the two PAP-3.2KD treatment groups, there were the obvious decrease in these parameters compared to those in the ADR group separately ($P < 0.05$ and <0.01), close to those in control group (**Figure 2**).

Protective Roles of PAP-3.2KD on Serum cTnT and cTnI

The levels of serum cardiac troponin T (cTnT) and cardiac troponin I (cTnI) in ADR group were increased significantly compared to those of the control group. After PAP-3.2KD (100 and 200 mg/kg) treatment, the levels of cTnT and cTnI were markedly decreased relative to those of the ADR group (**Figure 3**).

PAP-3.2KD Attenuated the Histopathological Damages of ADR-Induced Myocardial Tissue

In the control group, the pathological changes of heart tissues were rarely observed, and well-organized muscle bundles and no broken muscle fibers were found (**Figure 4A**). For the heart tissues of the ADR group, the typical characteristics of myocardial injury was found, such as disordered muscle bundles, myocardial fibrosis and diffuse myocardial cellular edema (**Figure 4B**). In the PAP-3.2KD groups, relatively well-organized muscle bundles, lower levels of myocardial fibrosis and diffuse myocardial cellular edema were revealed compared to those in the ADR-stimulated group (**Figures 4C,D**). To evaluate the damage of myocardia tissues quantitatively, the pathological scores were measured based on the ratios of pathological change areas (including inflammation, myocardial fibrosis and diffuse myocardial cellular edema) to the areas of the viewed entire field, as described in **Supplementary Table 1**. The pathological scores from the ADR group were significantly higher than those from the control group, whereas the scores in the PAP-3.2KD (100 and 200 mg/kg) groups were significantly attenuated, compared to those in ADR group separately ($P < 0.01$, both; **Figure 4E**). Those results indicated that PAP-3.2KD alleviated ADR-induced pathological injury of the myocardial tissues.

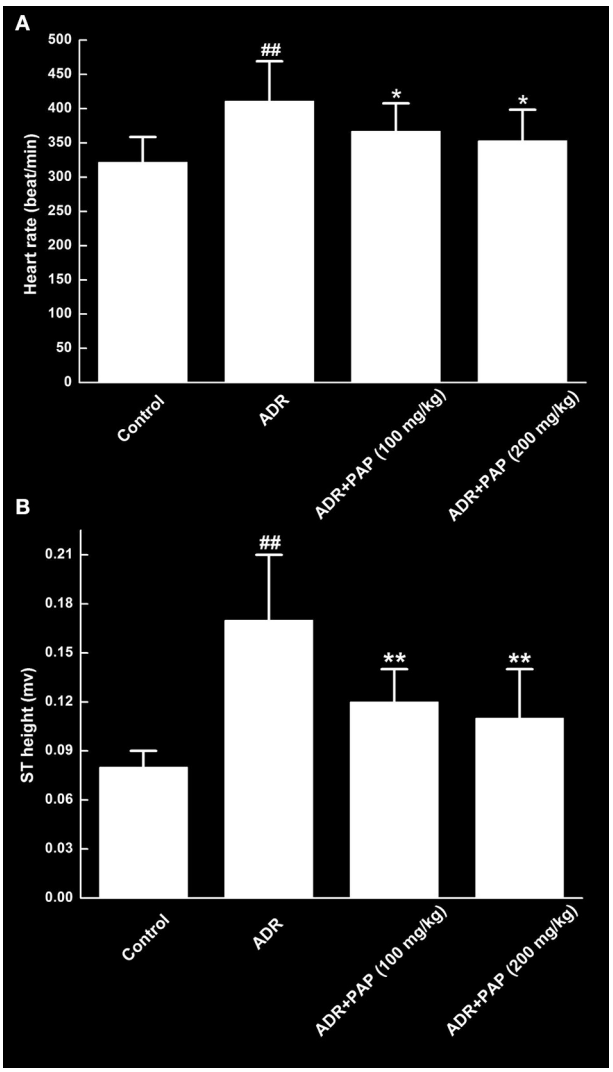

FIGURE 2 | PAP-3.2KD recovered heart rates and ST heights of ADR-induced rats. Both heart rate **(A)** and ST height **(B)** were determined by an eight-channel biological signal acquisition and processing system. Data were presented as Mean ± standard error of mean ($n = 10$). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the ADR group.

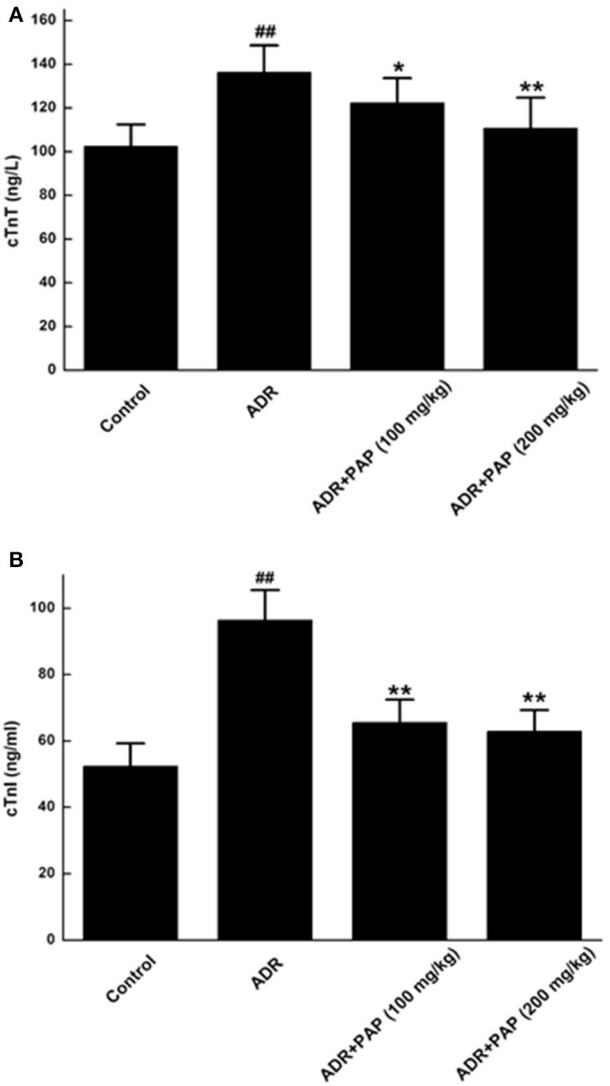

FIGURE 3 | PAP-3.2KD restored the levels of cTnT and cTnI in ADR-induced rats. Serum concentrations of both cTnT **(A)** and cTnI **(B)** were determined by ELISA. Data were presented as Mean ± standard error of mean ($n = 10$). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the ADR group, Cardiac troponin T (cTnT); Cardiac troponin I (cTnI).

PAP-3.2KD Ameliorated ADR-Induced Myocardial Fibrosis

The fibrotic areas were stained blue, after Masson's trichrome staining. There was no obvious fibrosis in the myocardium of the control group (**Figure 5A**). Compared with the control group, fibrosis in myocardial tissue from the ADR group was increased significantly (**Figure 5B**). Histological quantification results were demonstrated with the collagen volume fraction (CVF). After PAP-3.2KD administration, the CVF was decreased with the reduction of fibrosis significantly (**Figures 5C–E**). Thus, PAP-3.2KD reduced ADR-induced cardiac fibrosis.

PAP-3.2KD Reduced the Apoptosis of ADR-Induced Myocardial Tissue

TUNEL staining results demonstrated that there was no obvious apoptosis in the myocardium of the control group. The nucleus of normal cardio myocytes was blue-green, the apoptotic cells were rarely found for dark brown in different shades, the muscle bundles were neatly arranged and the cell gap was uniform (**Figure 6A**). In ADR group, myocardial tissue apoptosis was obvious. The dark brown areas with different sizes appeared and the arrangement of myocardial muscle bundles was relatively disordered (**Figure 6B**). The muscle bundles in both PAP-3.2KD group were relatively neatly arranged, and dark brown areas were reduced, especially in the PAP-3.2KD (200 mg/kg) group

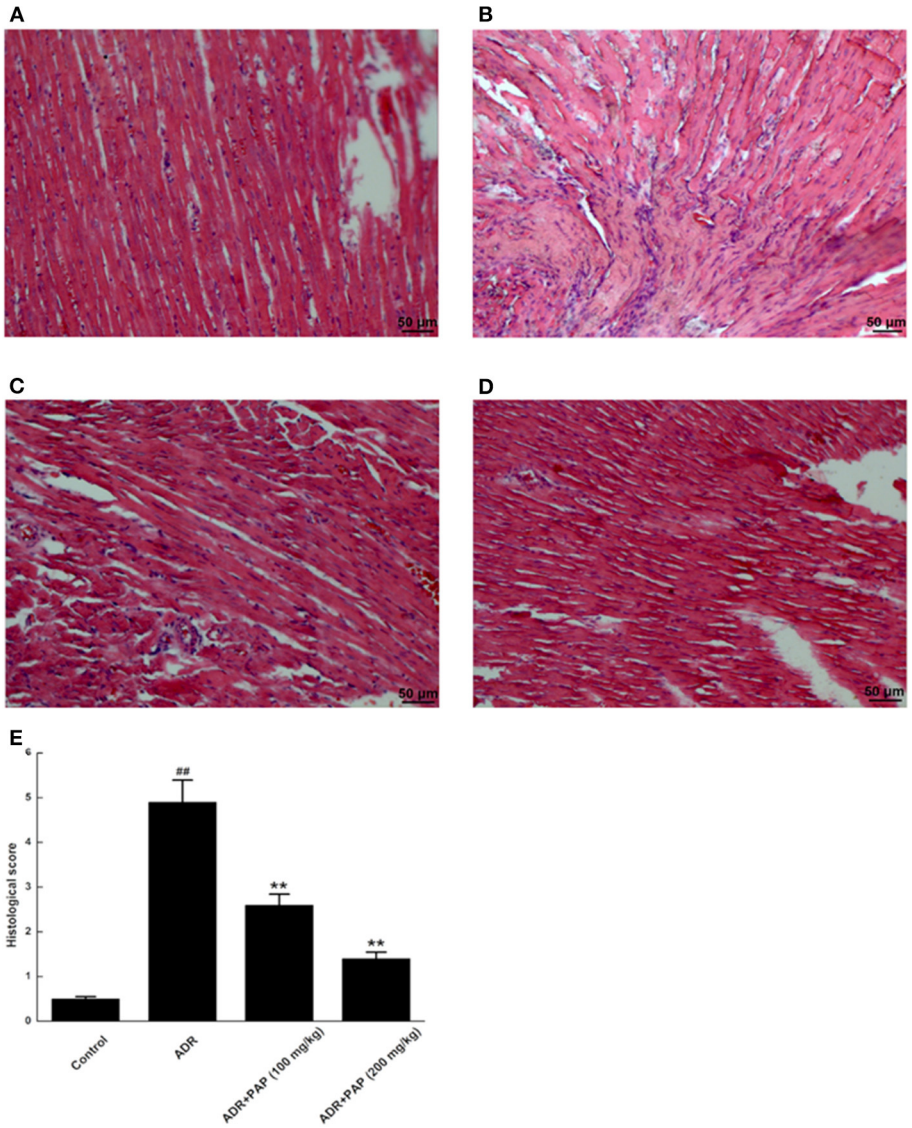

FIGURE 4 | PAP-3.2KD ameliorated the histopathological damages of ADR-induced myocardial tissue. Histopathological damages of myocardial tissues were detected by HE staining. **(A)** Control group, **(B)** ADR group, **(C)** PAP-3.2KD group (100 mg/kg), **(D)** PAP-3.2KD group (200 mg/kg). (Magnification, ×200). **(E)** Mean histopathological scores in each group. Data were presented as Mean ± standard error of mean ($n = 6$). $^{\#\#}P < 0.01$ vs. the control group; $^{**}P < 0.01$ vs. the ADR group.

(**Figures 6C,D**). The apoptosis areas in myocardial tissue were decreased significantly after PAP-3.2KD treatment ($P < 0.01$; **Figure 6E**).

The functional pathways of PAP-3.2KD on ADR-induced apoptosis of cardiac myocytes were examined further. The expression levels of apoptotic protein, Bcl-2, Bax, and caspase-3 in cardiac tissue were analyzed with ELISA and Western blotting. Bcl-2 expression level in myocardial tissue was decreased with the increased expression of Bax and caspase-3 in the ADR group significantly, compared to the expression levels of those protein in the control group (All, $P < 0.01$). However, after PAP-3.2KD treatment, the expression levels of those proteins in the ADR group were reversed. Compared with expression levels of those proteins in the ADR group separately, Bcl-2 expression levels in both PAP-3.2KD (100 and 200 mg/kg) group were significantly increased with the distinctly reduced expression levels of Bax and caspase-3 ($P < 0.05$ and <0.01; **Figure 7**).

PAP-3.2KD Attenuated ADR-Induced Myocardial Injury Through TGF-β_1/SMAD Pathway

The treatment mechanism of PAP-3.2KD on ADR-induced myocardial injury was investigated further. The expression levels of TGF-β1, a major profibrotic cytokine in hearts, were first measured using Western blot analysis. The result

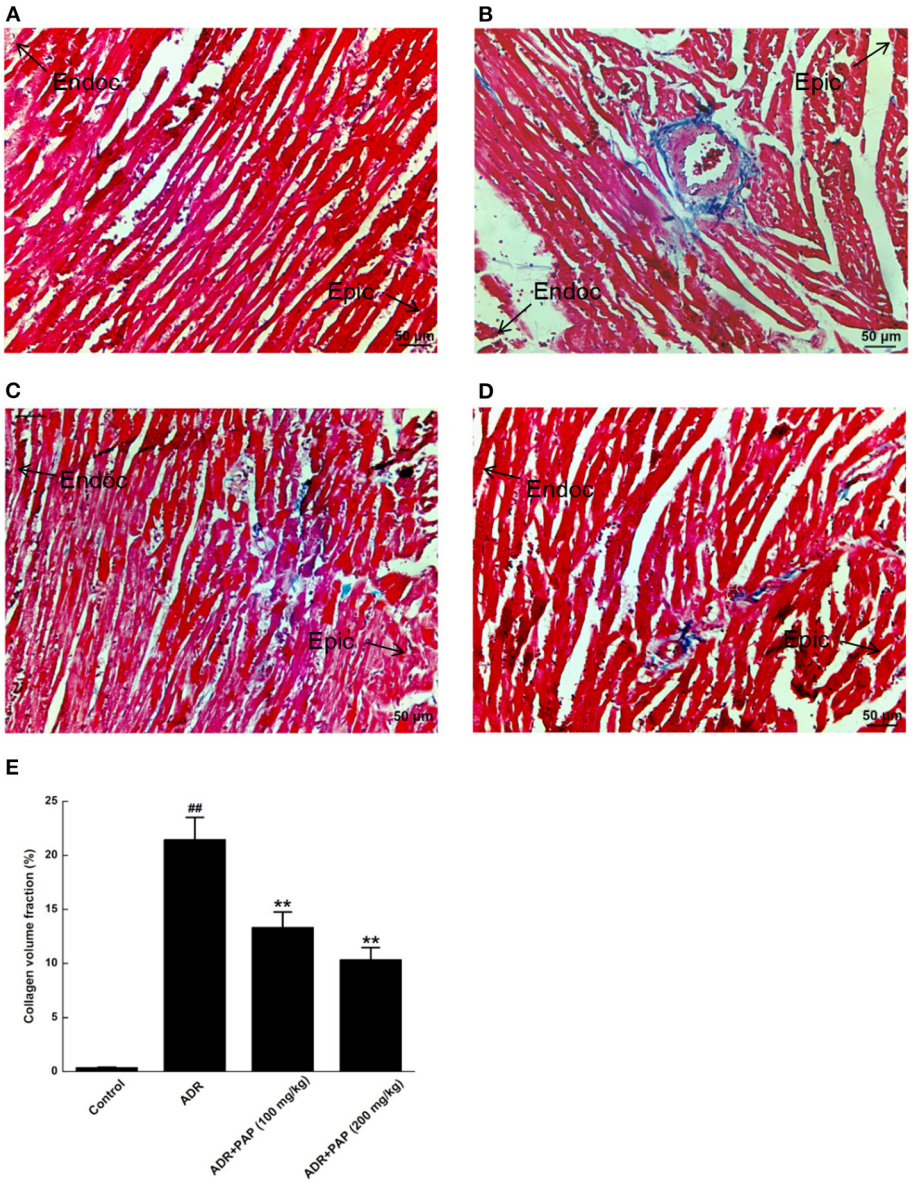

FIGURE 5 | PAP-3.2KD inhibited ADR-induced myocardial fibrosis. Myocardial Fibrosis in each group were detected by Masson's trichrome staining. **(A)** Control group, **(B)** ADR group, **(C)** PAP-3.2KD group (100 mg/kg), **(D)** PAP-3.2KD group (200 mg/kg). (Magnification, ×200). **(E)** Collagen volume fraction (CVF) was calculated in each group. All the myocardial section were made by Left ventricular apex. The zone of endocardium (Endoc)-epicardium (Epic) was indicated. Data were presented as Mean ± standard error of mean ($n = 6$). $^{\#\#}P < 0.01$ vs. the control group; $^{**}P < 0.01$ vs. the ADR group.

demonstrated that PAP-3.2KD treatment inhibited ADR-induced TGF-β1 production. In addition, the increased expression of TGF-β-related SMAD4 and reduced expression of SMAD7 in ADR-induced groups were also efficiently reversed after PAP-3.2KD treatment (100 and 200 mg/kg) (All, $P < 0.01$; **Figures 8A,B**). Compared with control group, there were no significant changes in total protein levels of SMAD2/SMAD3 from ADR group, whereas, there were significantly increased phosphorylation levels of them. PAP-3.2KD (100 and 200 mg/kg) treatment obviously reversed the phosphorylation levels of SAMD2/SMAD3, which were induced by ADR (All, $P < 0.01$; **Figures 8C,D**). These results indicate that PAP-3.2KD treatment may attenuate ADR-induced myocardial injury through the TGF-β_1/SMAD pathway.

DISCUSSION

In the present study, there were two key novel findings, the first was that PAP-3.2KD reversed ADR-induced pathological changes such as abnormal HW/BW index/heart rate/ST height, damage of the heart tissue, myocardial fibrosis and collagen

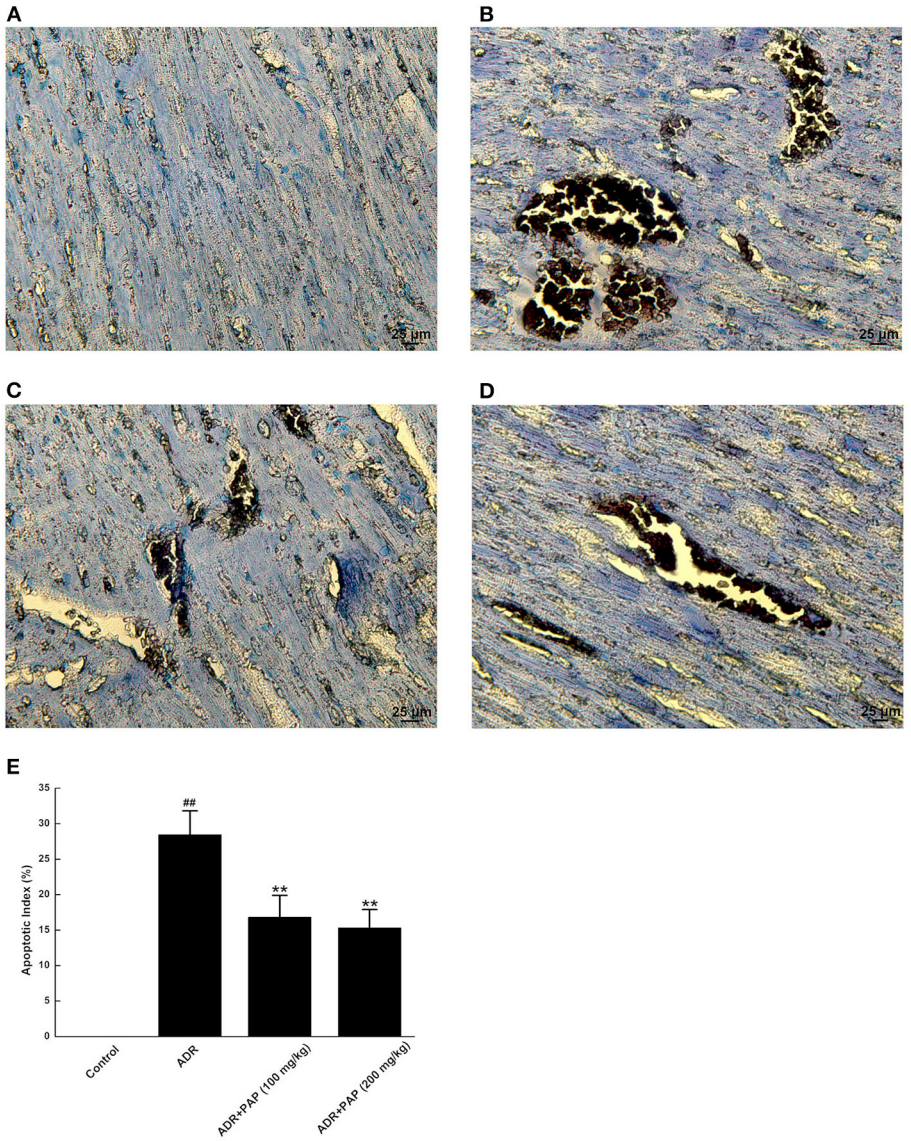

FIGURE 6 | PAP-3.2KD blocked the apoptosis of ADR-induced myocardial tissue. Apoptosis of myocardial tissues was detected by TUNEL staining. TUNEL-positive cells exhibited dark buffy nuclei staining. **(A)** Control group, **(B)** ADR group, **(C)** PAP-3.2KD group (100 mg/kg), **(D)** PAP-3.2KD group (200 mg/kg). (Magnification, ×400). **(E)** Statistical analysis of apoptotic index in each group. The independent experiments ($n = 6$) were performed in each group. $^{\#\#}P < 0.01$ vs. the control group; $^{**}P < 0.01$ vs. the ADR group.

volume fraction. PAP-3.2KD also increased the protein level of Bcl-2, and decreased the expression levels of Bax and caspase-3 in myocardial tissue, compared to those in ADR-induced group. The second was that PAP-3.2KD treatment blocked ADR-induced apoptosis and inflammatory response. ADR was effective when combined with chemotherapy for the treatment of various tumors; however, its toxic side effects caused multiple organ damage (14). ADR has specific toxicity toward the heart due to the high binding affinity of ADR to the anionic phospholipid cardiolipin within the inner mitochondrial membrane of the myocardial cells (15). The clinical application of ADR was restricted due to its side effect on cardiac tissues. Therefore, many studies have been focused on the protective effects of antioxidants and natural products against ADR-induced myocardial injury. In the present study, the HW/BW index was first checked as the most rational indication of changes in heart tissue pathology and under normal conditions, their levels were relatively constant (16). ADR induction significantly increased the HW/BW index, since BW levels were reduced more than those of HW (**Figure 1**), and impaired cardiac function through increasing the levels of heart rate and ST (**Figure 2**) and protein levels of cTnT/cTnI (**Figure 3**). Those results above were consistent with those from previous reports about ADR-induced abnormalities of cardiac function (17, 18). In the present study, PAP-3.2KD treatment

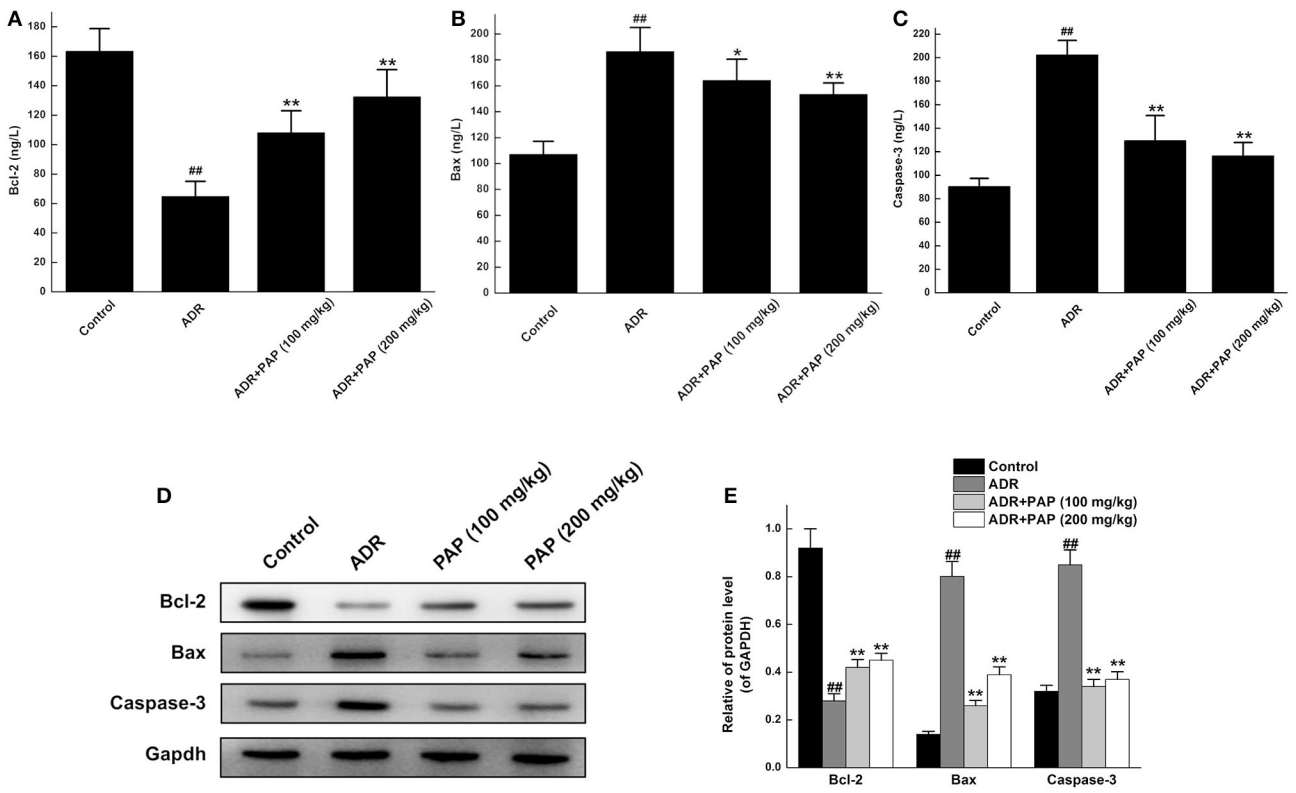

FIGURE 7 | PAP-3.2KD regulated the expression levels of Bcl-2, Bax, and caspase-3 in ADR-induced rats. The concentrations of Bcl-2 **(A)**, Bax **(B)** and Caspase-3 **(C)** in cardiac tissue were determined by ELISA. Data were presented as Mean ± standard error of mean ($n = 10$). **(D)** The protein expression levels of Bcl-2, Bax and Caspase-3 were determined by western blotting. **(E)** Statistical analysis for expression levels of relative protein in **(D)**, GAPDH protein levels as control. Data were presented as Mean ± standard error of mean ($n = 3$). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the ADR group, ADR, Adriamycin; PAP-3.2KD, 3.2 KD pilose antler polypeptide; Bcl-2, B-cell lymphoma-2; Bax, BcL-2-Associated X.

significantly ameliorated the increased HW/BW index and the increased levels of heart rate/ST and cTnT/cTnI in ADR-treated rats, confirming its cardio-protection property (**Figures 1**–**3**). Even though the results showed no different between male and female rats, sex different effect still need to be studied further.

The two major mechanisms underlying these effects may be involved. First, the abnormal myocardial functions may be caused by notable pathological changes in the myocardial cells, such as disordered muscle bundles and myocardial fibrosis, which were found to be induced by ADR. Cardiac fibrosis is a process of pathological extracellular matrix (ECM) remodeling, leading to excessive and continuous ECM deposition in heart, and impairing heart muscle function above. PAP-3.2KD enhanced cardiac function through reducing the fibrosis and inflammation which ADR stimulated in myocardial tissues significantly (**Figures 4**, **5**) (PAP-3.2KD was revealed to have no toxic effects in myocardial tissues from healthy rats, data not shown). Cardiomyocyte apoptosis was another mechanism by which ADR induced cardiomyopathy (**Figure 6**). ADR induced cardiac oxidative stress to disturb mitochondrial membrane permeability, resulting in the released cytochrome c into the cytosol. The cytochrome c bound to another protein to activate caspase cascade and induce cell death (19). Caspase-3 played a key role in apoptosis, especially in the core of the apoptosis cascade. Caspase-3 effectively hydrolyzed cellular structural and functional proteins, inducing cell apoptosis in different pathological status, such as myocardial tissues during myocardial injury (20, 21) (**Figure 7**). Bcl-2 and Bax were a pair of genes that regulated cell apoptosis to activate the next level caspases enzyme system to cause cell apoptosis (22). ADR induced cardiomyocyte apoptosis through the decreased expression level of Bcl-2 and the increased expression of Bax and caspase-3 (**Figure 7**). ADR-treated rats exhibited more myocardial injuries than those from control group. The phenomenon was also confirmed by the electrocardiogram experiment. The reduction in myocardial fibrosis and cardiomyocyte apoptosis may be an effective strategy to reduce the incidence of ADR-associated myocardial injuries. Compared with the levels of Bcl-2, caspase-3, and Bax in the ADR group, PAP-3.2KD significantly reversed their levels, inhibiting the occurrence of myocardial apoptosis (**Figures 6**, **7**). Therefore, PAP-3.2KD administration reversed these symptoms to reduce ADR-induced myocardial injuries.

The expression levels of TGF-β1 protein were increased in cardiac tissue after ADR induction. After PAP-3.2KD administration on ADR-induced rats, TGF-β1 expression was effectively inhibited, protecting the myocardium from damage,

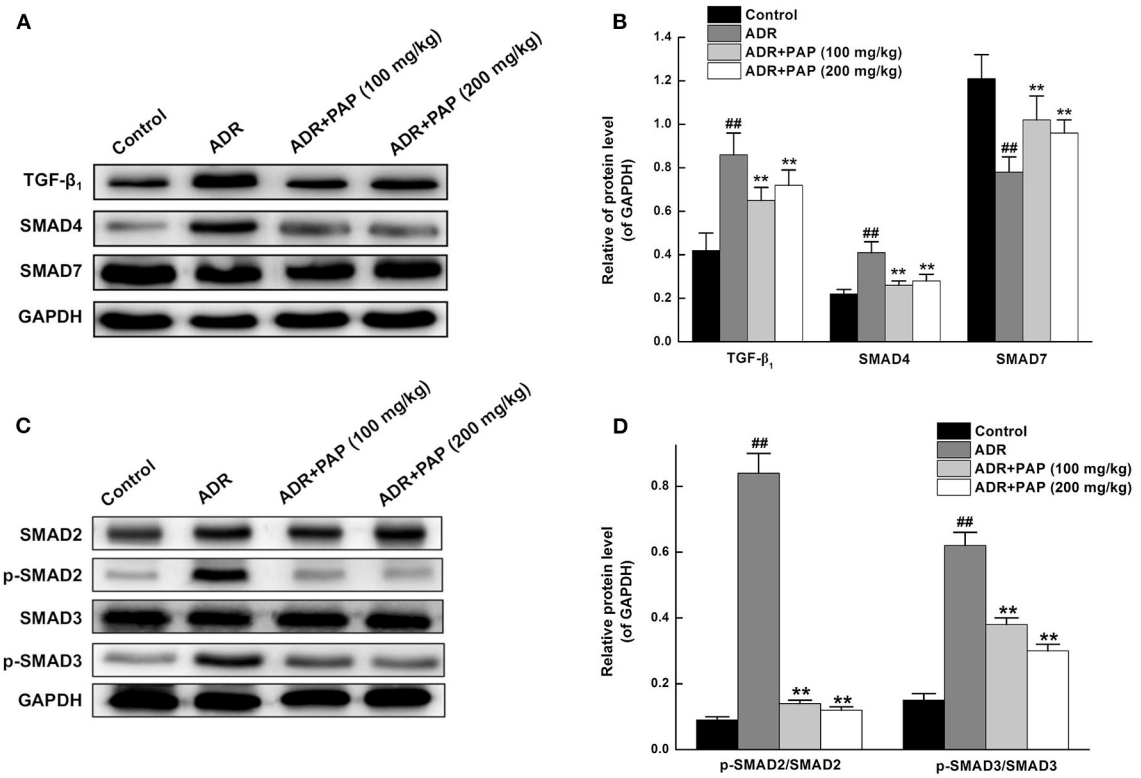

FIGURE 8 | PAP-3.2KD attenuated ACR-induced myocardial injury through TGF-β/SMAD pathway. **(A)** The protein expression levels of TGF-β_1, SMAD4, and SMAD7 in heart tissues were determined by western blotting. **(B)** Statistical analysis of relative levels of target protein in **(A)**, compared to GAPDH protein levels. The representation **(C)** and quantitative analysis **(D)** of western analysis results for SMAD2/SMAD3 and their phosphorylation. Coenzyme Q10 (CoQ10) as positive control group (CoQ10 has been proved to have protective roles for cardiomyocyte after myocardial injury through TGF-β pathway, Data not shown). Thus, splicing occurred between ADR group (Lane 2) and PAP-3.2KD (100 mg/kg) (Lane 3) group in all Western Blotting results. Data were expressed as mean ± standard error of mean ($n = 3$). $^{\#\#}P < 0.01$ vs. the control group; $^{**}P < 0.01$ vs. the ADR group. TGF-β_1, transforming growth factor-β_1; SMAD, drosophila mothers against the decapentaplegic protein.

suggesting that both ADR induced myocardial injury and PAP-3.2KD protected ADR-induced injury through TGF-β1 signaling (**Figure 8**). This was consistent with previous data that ADR promoted fibrosis and apoptosis by upregulation of TGF-β1 (23, 24). TGF-β1, as one multifunctional cytokine, regulated a number of biological responses including fibro-genesis and cell apoptosis, especially on various pathophysiological functions of the cardiovascular system (25–27).

The primary TGF-β signal transduction pathway is the highly conserved TGF-β/SMAD pathway. SMAD proteins, as the main intracellular signal transduction, mediated the transport of TGF-β signals from the cell membrane receptors to the nucleus. SMADs are the target proteins of TGF-β1 signal transduction, which transmits signals in the cell mainly through phosphorylation, such as SMAD2/3, which in turn bind to the common SMAD4 (28). SMAD7 binds to activated type I receptors to regulate signal transduction by the TGF-β family (29). The SMAD complex translocate to the nucleus, where they bind to DNA elements to promote the transcription of various genes that regulate fibrosis. ADR upregulated TGF-β1 expression to promote fibrosis which was a reparative response by which the myocardium compensated for cell loss after myocardial injury (30). PAP-3.2KD treatment inhibited the production of TGF-β_1 and phosphorylation of SMAD2/3 induced by ADR. Therefore, PAP-3.2KD reduced myocardial fibrosis through TGF-β/SMAD signaling. Once the myocardium was damaged, TGF-β1 was activated and secreted, inducing increased apoptosis in cardiac cells (31, 32). ADR-induced myocardial injury down-mediated the levels of TGF-β1 to upregulate the protein expression of p-SMAD2/3, ultimately promoting ADR-induced apoptosis of myocardial cells, and PAP-3.2KD treatment on ADR-induced rats inhibited myocardial apoptosis through the inhibition of TGF-β/SMAD pathway (**Figure 8**). Even though the protective mechanism of PAP-3.2KD against ADR-induced myocardial injury may be clarified, other mechanisms, by which ADR-induced myocardial apoptosis need be investigated further.

CONCLUSION

PAP-3.2KD treatment protects ADR-induced pathological changes, and inhibits ADR-induced myocardial fibrosis and myocardial apoptosis through the inhibition of TGF-β/SMAD pathway, suggesting that PAP-3.2KD may be one potential protective drug during cancer treatment of ADR.

DATA AVAILABILITY STATEMENT

All datasets generated in present study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Department of Pharmacy Changchun University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

XQ conceived and designed the work. XQ and XH coordinated technical support and funding. YX and PQ wrote the manuscript. JZ, GL, and YL performed the experiments and collected the samples. YX and DH acquired, analyzed, and interpreted the data. JL and YC participated in data collection and analysis. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.659643/full#supplementary-material

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frontiers in Cardiovascular Medicine

ORIGINAL RESEARCH
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Toll-Like Receptor 4 Activation Prevents Rat Cardiac Fibroblast Death Induced by Simulated Ischemia/Reperfusion

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Death of cardiac fibroblasts (CFs) by ischemia/reperfusion (I/R) has major implications for cardiac wound healing. In *in vivo* models of myocardial infarction, toll-like receptor 4 (TLR4) activation has been reported as a cardioprotector; however, it remains unknown whether TLR4 activation can prevent CF death triggered by simulated I/R (sI/R). In this study, we analyzed TLR4 activation in neonate CFs exposed to an *in vitro* model of sI/R and explored the participation of the pro-survival kinases Akt and ERK1/2. Simulated ischemia was performed in a free oxygen chamber in an ischemic medium, whereas reperfusion was carried out in normal culture conditions. Cell viability was analyzed by trypan blue exclusion and the MTT assay. Necrotic and apoptotic cell populations were evaluated by flow cytometry. Protein levels of phosphorylated forms of Akt and ERK1/2 were analyzed by Western blot. We showed that sI/R triggers CF death by necrosis and apoptosis. In CFs exposed only to simulated ischemia or only to sI/R, blockade of the TLR4 with TAK-242 further reduced cell viability and the activation of Akt and ERK1/2. Preconditioning with lipopolysaccharide (LPS) or treatment with LPS in ischemia or reperfusion was not protective. However, LPS incubation during both ischemia and reperfusion periods prevented CF viability loss induced by sI/R. Furthermore, LPS treatment reduced the sub-G1 population, but not necrosis of CFs exposed to sI/R. On the other hand, the protective effects exhibited by LPS were abolished when TLR4 was blocked and Akt and ERK1/2 were inhibited. In conclusion, our results suggest that TLR4 activation protects CFs from apoptosis induced by sI/R through the activation of Akt and ERK1/2 signaling pathways.

Keywords: cardiac fibroblasts, ischemia/reperfusion, TLR4, LPS, apoptosis

INTRODUCTION

Cardiovascular diseases (CVDs) have remained as the main cause of death worldwide during the last decades (1). Reperfusion therapy is the primary clinical management in patients with myocardial infarction to restore the normal coronary circulation in the cardiac ischemic tissue and limit the extent of necrosis (2). However, the intracellular biochemical changes during ischemia, followed by the abrupt reperfusion, induce additional cell death associated with the generation of reactive oxygen species (ROS), which produce oxidative modification of macromolecules (proteins, lipids, carbohydrates, and nucleic acids) and trigger apoptotic pathways (3, 4). Currently, many successful cardioprotective strategies against myocardial ischemia/reperfusion (MIR) have been reported in experimental animal models, but translation to clinical practice has proven difficult due to the complexity of MIR (5). Combinations of therapies with synergistic effects, as well as protection of all cardiac cell types—and not just cardiomyocytes—are believed to be essential to achieve cardioprotection in the clinical arena (5, 6).

Cardiac fibroblasts (CFs) are involved in the homeostasis of extracellular matrix (ECM) proteins in normal cardiac tissue. In addition, when an injury occurs in the heart, CFs initiate an inflammation response by secreting many cytokines and growth factors and differentiate into cardiac myofibroblasts (CMFs) to produce collagen, leading to wound healing and scar formation (6, 7). It is well-known that simulated ischemia/reperfusion (sI/R) can produce deleterious effects on CF viability; however, strategies to prevent sI/R injury have been poorly evaluated. This is an important topic since CFs participates in wound healing processes; therefore, viability protection is of utmost importance for their protection (8–11).

Toll-like receptors (TLRs) are a family of receptors present in immune system cells, which recognize and react to highly conserved motifs known as damage-associated molecular patterns (DAMPs; "alarmins") and pathogen-associated molecular patterns [PAMPs, like lipopolysaccharide (LPS)], and mediate the immune innate response and inflammatory process after tissue injury, remodeling, stress, infection, and other situations. In addition, TLRs are highly expressed in vascular cells and cardiac tissue (12, 13), and TLR4 is one of the most studied isoforms. Reports in rat CFs have indicated that TLR4 activation by LPS activates Akt, ERK1/2, and NF-kB signaling pathways. This activation promotes a strong proinflammatory response characterized by the release of cytokines (IL1-β and TNF-α) and chemokines (MCP-1), increased expression of the cellular adhesion molecules ICAM-1 and VCAM-1 and the B1 bradykinin receptor, and prevention of CF-to-CMF differentiation (14–17). Furthermore, other studies in mice have reported that activation of TLR4 by LPS produces a cardioprotective response through the PI3K/Akt pathway against MIR (18, 19).

At the cardiac cellular level, it is well-known that sI/R triggers CF necrosis and apoptosis (10), and we recently showed that the use of antioxidants prevents CF death (11). Therefore, the search of alternative therapies to decrease CF death and, consequently, to improve the wound healing process has become relevant; however, until now, it is unknown whether TLR4 activation can produce a cytoprotective effect in CFs exposed to sI/R conditions. Thus, the aim of this study was to determine whether TLR4 activation (by LPS as a specific agonist) protects CFs from apoptotic death triggered by sI/R and to elucidate the participation of the Akt and ERK1/2 signaling pathways, which are recognized as part of reperfusion injury salvage kinase (RISK). The RISK pathway corresponds to the activation of two parallel cascades: PI3K-Akt and MEK1-ERK1/2, a group of pro-survival protein kinases which that cardioprotection when activated specifically at the time of reperfusion (20).

METHODS AND MATERIALS

Reagents

Dulbecco's modified Eagle's medium F12 (DMEM-F12) and Collagenase Type II (powder) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS), trypsin ethylenediaminetetraacetic acid (EDTA; 0.5%), EDTA 0.2% (10× solution), penicillin–streptomycin–amphotericin B solution, and trypan blue (0.5%) solutions were obtained from Biological Industries (Cromwell, CT, USA). The MEK1/2-ERK1/2 inhibitor PD98059, pancreatin from porcine pancreas, RNAse A, propidium iodide (PI), Bradford reagent, radioimmunoprecipitation assay (RIPA) lysis and extraction buffers, Halt Protease Inhibitor Cocktail (100×), Halt Phosphatase Inhibitor Cocktail, and enhanced chemiluminescence (ECL) western blotting detection reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MI, USA). The MTT Assay Kit and Prestained Protein Ladder-Broad molecular weight (10–245 kDa) were obtained from Abcam (Cambridge, MA, USA). Nitrogen gas (N_2) cylinders were obtained from Linde Group (Santiago, Chile). Primary antibodies for phospho-ERK1/2 (p-ERK1/2), phospho-Akt (p-Akt), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the secondary antibodies anti-rabbit IgG and anti-mouse IgG conjugated with horseradish peroxidase (HRP) were obtained from Cell Signaling Technology (Danvers, MA, USA). All plastic materials were obtained from Corning Incorporated (Corning, NY, USA). The PI3K-Akt inhibitor LY294002 was obtained from Cayman Chemicals (Ann Arbor, MI, USA). LPS-EB, LPS-EB ultrapure, and TAK-242 (inhibitor of TLR4 signaling) were obtained from InvivoGen (San Diego, CA, USA). All inorganic salt products and methanol were obtained from Merck (Darmstadt, Germany).

Isolation and Culture of Cardiac Fibroblasts

Sprague–Dawley neonate rats (1- to 3-day-old) were obtained from the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences at University of Chile. All studies followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and experimental protocols were approved by our Institutional Ethics Review Committee. CFs were isolated as previously described (10). In a sterile zone, rats were swiftly decapitated, and their hearts were removed.

In brief, ventricles were minced and digested in a solution that contained collagenase (0.05%) and pancreatin (0.05%). The digestion homogenized product was pre-cultured on 100-mm-diameter plastic plates, with culture medium containing FBS (10%) and penicillin–streptomycin–amphotericin B, and kept in an incubator with O_2 (95%) and CO_2 (5%) at 37°C. The CFs adhered differentially to plastic, allowing their isolation from cardiomyocytes. After 2 h, the culture medium was replaced with DMEM-F12 containing 10% FBS medium, and CFs were left to proliferate to confluence (3–5 days) in the same environment conditions. The medium was then replaced with DMEM-F12 containing FBS (3%) and penicillin–streptomycin–amphotericin B. Cells underwent up to a maximum of two passages, and detachment was performed using trypsin EDTA (0.5%) and EDTA 0.2% (1×), followed by protease inhibition with DMEM-F12 containing FBS (10%). CFs were then collected and seeded on plastic plates in DMEM-F12 medium without FBS for 24 h before the experiments.

Protocol of *in vitro*-Simulated Ischemia/Reperfusion and Stimulation With Lipopolysaccharide/Inhibitors

After 24 h without serum, CFs were washed with phosphate-buffered saline (PBS) before the ischemic protocol. The cells were exposed to a balanced salt solution (ischemic medium) with pH 6.2: NaCl 139 mM, KCl 12 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 0.9 mM, HEPES 5 mM, lactic acid 20 mM, and 2-deoxy-D-glucose 10 mM. Hypoxia was achieved in a customized chamber with N_2 environment at 37°C for 8 h. A reperfusion protocol was developed by replacing the ischemic medium with DMEM-F12 in a 95% air/5% CO_2 incubator (37°C) for 16 h. Control cells were incubated under normoxic conditions in DMEM-F12 medium and kept in the incubator with O_2 (95%) and CO_2 (5%) at 37°C for the exact duration of simulated ischemia and sI/R experiments. CFs were stimulated with LPS (1 μg/ml) under the following conditions: (i) 24 and 16 h before ischemia; (ii) at the onset of ischemia or reperfusion; and (iii) at the onset of ischemia and consecutive reperfusion, depending on the experimental conditions and with the respective controls. TAK-242 (1 μM), LY294002 (10 μM), and PD98059 (25 μM) were added in the presence/absence of LPS, depending on the experimental conditions and with the respective controls. **Figure 1** shows graphically the different experimental protocols used in the biological assays.

Cell Viability by Trypan Blue Exclusion

CFs were plated at a density of 156 cells/mm^2 on 35-mm plastic dishes and stimulated under the conditions indicated for each experiment. After 16 h of simulated reperfusion, cells were washed two times with PBS and treated with trypsin EDTA (0.5%) and EDTA 0.2% (1×) to detach cells, followed by administration of FBS (10%) to induce inactivation. After detachment, aliquots of 20 μl of sample plus 20 μl of trypan blue (0.5%) reagent were homogenized, and then 8 μl was transferred to a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) to count viable cells (unstained) using optic microscopy. Experiments were performed in duplicate and repeated six times. At least 1,000 cells/ml was counted in each sample.

Cell Viability by the MTT Assay

CFs at a density of 156 cells/mm^2 were plated on 35-mm plastic dishes in DMEM-F12 medium and stimulated under the conditions indicated for each experiment. At the end of reperfusion, cells were washed with PBS and then kept in DMEM-F12 medium. An aliquot of filtered MTT solution (5 mg/ml) was added in a 1:10 ratio, and the cells were incubated for 2.5 h in a 95% air/5% CO_2 incubator (37°C). The cells were then washed with PBS, and an aliquot of isopropanol (1:10 ratio) was added to solubilize the MTT crystals. The solubilized extracts were seeded on 96-well plates with transparent bottom, and the absorbance at 570 nm was registered (Epoch UV-Vis Spectrophotometer, BioTek).

Western Blot Analysis

For protein content analysis, CFs were seeded at a density of 106 cells/mm^2 on 60-mm plastic dishes in DMEM-F12 medium and were stimulated under the conditions indicated for each experiment. At the end of reperfusion, cells were washed with cold PBS and then treated with RIPA lysis buffer containing protease and phosphatase inhibitors. Samples were centrifuged at 252 ×g for 10 min at 4°C, and supernatants were collected. The total protein concentration of samples was determined by spectrophotometry using the Bradford assay reagent, by reading the absorbance at 595 nm (Epoch UV-Vis Spectrophotometer, BioTek). Twenty-five micrograms of total protein extracts (with charge buffer) was separated by 15% acrylamide/bis-acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at constant 90 V for 1.5 h. Proteins were then electro-transferred to a nitrocellulose membrane for 1.5 h, at constant 0.4 A. The membranes were blocked with non-fat milk 5% (w/v) for 1 h and incubated overnight at 4°C with primary antibodies against p-ERK1/2, p-Akt (dilution 1:1,000 for both) or GAPDH (dilution 1:5,000). The membranes were then washed and incubated for 1.5 h at room temperature with anti-rabbit IgG or anti-mouse IgG conjugated with HRP (dilution 1:5,000) secondary antibodies. After being washed, membranes were exposed to the ECL reagent and revealed by two methods: (i) C-DiGit Chemiluminescent Western Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA), wherein images and blots were analyzed and quantified using the Image Studio™ software (LI-COR Biosciences, Lincoln, NE, USA), or (ii) BioMax film in a dark room, wherein films were scanned and blots were quantified with the ImageJ software (LI-COR Biosciences, Lincoln, NE, USA).

Necrosis Determination by Flow Cytometry

CFs were seeded with a density of 106 cells/mm^2 on 60-mm plastic dishes and were stimulated under the conditions indicated for each experiment. At the end of reperfusion, dead cells were collected as a pellet from the medium after centrifuging at 252 ×g for 5 min and kept them on ice. Live cells on plates were detached with trypsin EDTA (0.5%) and EDTA 0.2% (1×) and mixed with

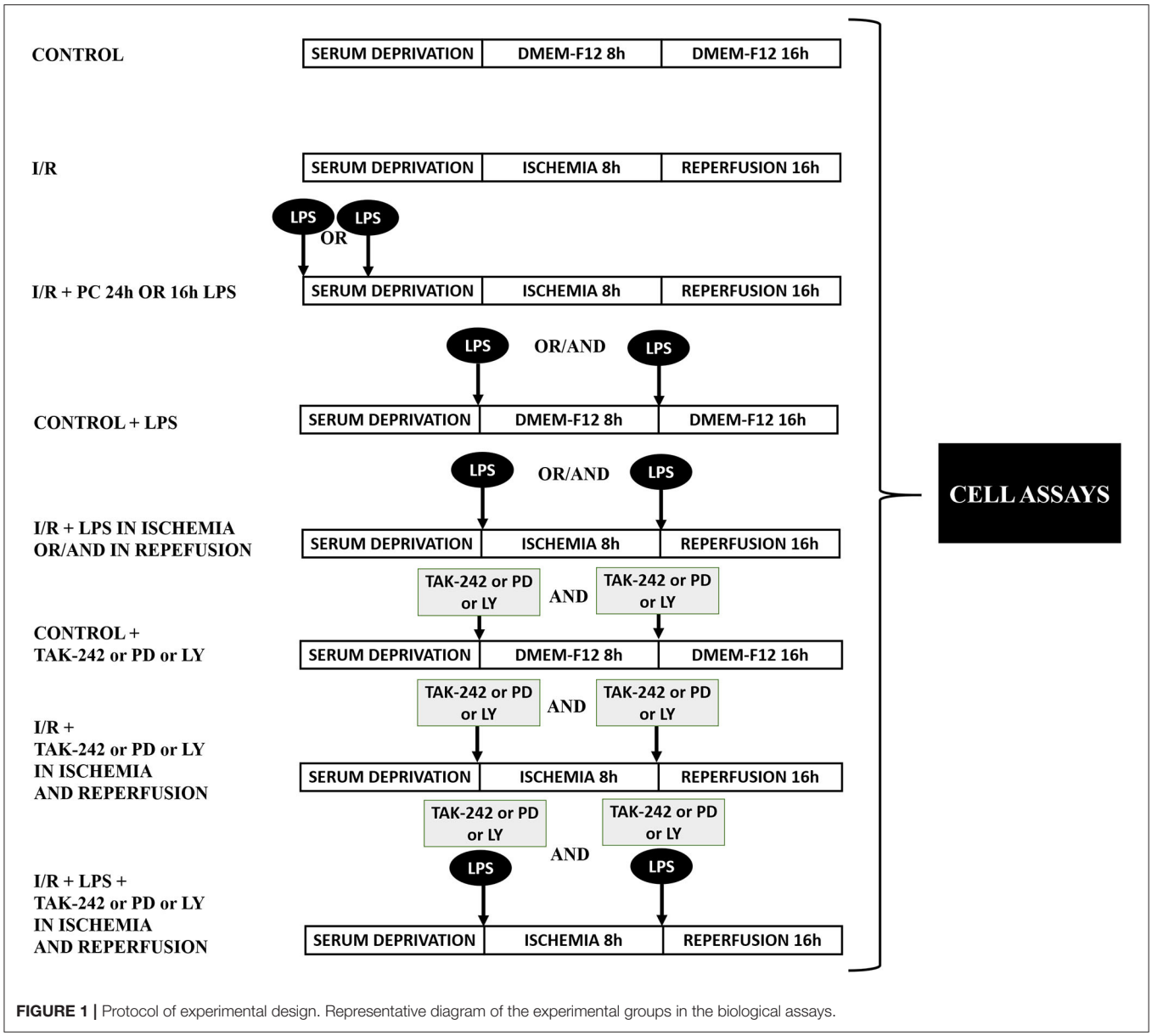

FIGURE 1 | Protocol of experimental design. Representative diagram of the experimental groups in the biological assays.

the dead cell pellet. Subsequently, PI (1 mg/ml) was added to the cell mixture. Cell necrosis was assessed by flow cytometry in a BD FACSCantoA (Becton Dickinson & Company, Franklin Lakes, NJ, USA). A total of 5,000 cells/sample were analyzed.

Determination of the Sub-G1 Population by Flow Cytometry

CFs were seeded at a density of 106 cells/mm^2 on 60-mm plastic dishes and were stimulated under the conditions indicated for each experiment. At the end of reperfusion, dead cells of the medium and living cells on the plates were collected according to the same protocol described in the Necrosis Determination by Flow Cytometry section. Cold methanol was added to the mixture of live and dead cells to permeabilize their cell membranes, overnight at −20°C. Later, RNAse (0.1 mg/ml) was added to the samples for 1 h at room temperature. Finally, PI (1 mg/ml) was added to the cells, and apoptosis was determined using a BD FACSCanto (Becton Dickinson & Company, Franklin Lakes, NJ, USA) flow cytometer. PI marks condensed chromatin and/or fragmented DNA in apoptotic bodies, giving a low-intensity signal (sub-G1 population), under the prominent G1 signal of living cells with integral DNA. A total of 5,000 cells/sample were analyzed.

Statistical Analysis

All data are presented as mean ± SEM of at least four to six independent experiments and were analyzed using the version 5.01 of the GraphPad Prism software (GraphPad, San Diego, CA, USA). The differences between experimental groups were evaluated by one-way ANOVA, followed by

the Tukey post-test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Lipopolysaccharide Treatment Administered at the Beginning of Ischemia and Reperfusion Increased Cell Viability and Reduced Apoptosis of Cardiac Fibroblasts

In order to study the cytoprotective effects of LPS, we first evaluated different LPS treatments times in our model by subjecting neonatal rat CFs to 8 h of simulated ischemia, followed by 16 h of simulated reperfusion; and then we measured cell viability using both the trypan blue and MTT assays. Regarding the sensitivity of cardiac fibroblasts to hypoxia, the ischemia time of 8 h was chosen from previous studies carried out in our laboratory (10) in which it was shown that 8 h of ischemia is the minimum time of ischemia in which a statistically significant decrease in cell viability is not observed, while in longer times of ischemia, the loss of cell viability varies between 40% at 12 h and 85% at 24 h. The results show that cell viability did not increase with LPS treatment (1 μg/ml) in a preconditioning manner during 24 and 16 h, before performing sI/R (**Figures 2A,B**); at the beginning of simulated ischemia (**Figures 2C,D**); or at the beginning of simulated reperfusion (**Figures 2E,F**). However, when we administered LPS (1 μg/ml) at the beginning of both ischemia and reperfusion, cell viability increased when compared with untreated conditions ($p < 0.05$; **Figures 2G,H**). We then evaluated the effects of LPS treatment on cell death types (necrosis and apoptosis) induced by sI/R, using flow cytometry analysis with PI staining. The results indicate that CFs exposed to sI/R increase the percentage of cells in necrosis and apoptosis compared with control, the last one in a higher level than necrotic population. Second, the CFs exposed to sI/R and treated with LPS (1 μg/ml) exhibited a non-significant trend of lower values of necrotic cells (**Figures 3A,C**). On the other hand, treatment with LPS induced a significant decrease in the sub-G1 population of CFs exposed to sI/R, compared with untreated conditions ($p < 0.01$; **Figures 3B,C**).

Protective Role of TLR4 Against Cell Death Induced by Simulated Ischemia and Simulated Ischemia/Reperfusion in Cardiac Fibroblasts

Next, we evaluated the effects of TLR4 blockade on cell viability and intracellular survival pathways when CFs were exposed only to simulated ischemia and to sI/R. First, cell viability (measured by MTT assay) significantly decreased in CFs treated with the specific TLR4 inhibitor TAK-242 after ischemia only or sI/R, compared with the respective untreated groups (**Figure 4**). Furthermore, treatment of CFs with TAK-242 significantly decreased p-Akt and p-ERK1/2 protein levels (determined by western blot) in simulated ischemia only (**Figures 5A,B**) or sI/R (**Figures 5C,D**), compared with the respective untreated groups.

The Cytoprotective Effects of Lipopolysaccharide Against Simulated Ischemia/Reperfusion-Induced Death of Cardiac Fibroblasts Are Mediated by the TLR4, Akt, and ERK1/2

After verifying the participation of the TLR4 in CF survival during sI/R, we continued with the study of the signaling pathways involved in the cytoprotective effects of LPS. To this end, we treated CFs subjected to sI/R with LPS in the presence/absence of TAK-242, LY294002 (Akt-specific inhibitor) and PD98059 (ERK1/2-specific inhibitor) and measured cell viability using the MTT assay. We found that treatments with TAK-242 (**Figure 6A**), LY294002 (**Figure 6B**), and PD98059 (**Figure 6C**) prevented the cytoprotective effects of LPS in CFs exposed to sI/R, compared with the respective groups without inhibitors.

DISCUSSION

In the present study, our main findings were as follows: (a) preconditioning or administration of LPS only in ischemia or only in reperfusion was not cytoprotective for CFs, while in CFs exposed to sI/R, LPS treatment administered during both ischemia and reperfusion increased cell viability and reduced the sub-G1 population, without reducing the number of necrotic cells; (b) in CFs subjected only to simulated ischemia or to sI/R, blockade of the TLR4 reduced cell viability and activity of pro-survival kinases Akt and ERK1/2; and (c) in CFs subjected to sI/R, blockade of the TLR4, or inhibition of the Akt or ERK1/2 signaling pathways abolished the cytoprotective effects of LPS on CF viability.

Lipopolysaccharide Prevents Cardiac Fibroblasts Apoptosis Induced by Simulated Ischemia/Reperfusion

Our results showed that in CFs, LPS preconditioning for 24 and 16 h did not protect against the deleterious effects induced by sI/R. In contrast, several studies have demonstrated that LPS preconditioning during 24 h or longer periods produces cardioprotective effects in animals before the development of MIR (18–21). We consider that the differences with our results could be due to several factors, such as *in vivo* vs. *in vitro* models, the use of different cells types, and LPS concentration and/or exposure/administration time. In this regard, Ha et al. (18) showed in *in vivo* models of cardiac I/R that in order to be effective, TLR4 agonists must be administered at least 8–24 h prior to the induction of I/R. This has been referred to as myocardial preconditioning or tolerance, and this preconditioning effect is reached at lower TLR4 concentrations (in the order of ng/ml). Previous studies have shown that pretreatment of animals with a small dose of LPS for 24 h results in significant inhibition of NF-kB activation and protection of the myocardium against I/R injury (22–24). The authors suggest that LPS pretreatment induces a feedback regulation mechanism of IkBa expression in the myocardium and, consequently, reduces

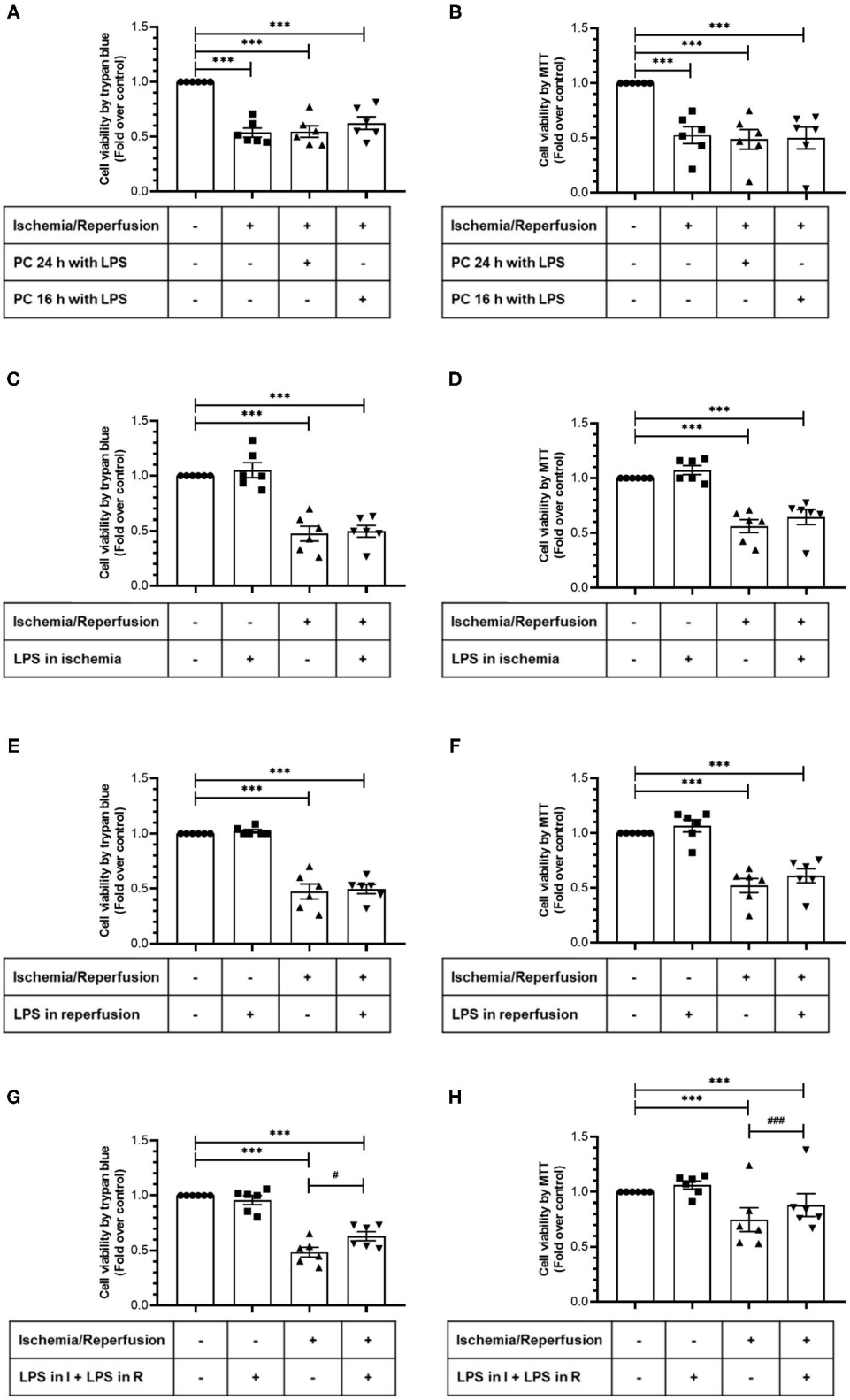

FIGURE 2 | Lipopolysaccharide (LPS) increases the viability of cardiac fibroblasts exposed to simulated ischemia/reperfusion (sI/R). Cardiac fibroblasts were subjected to 8 h of ischemia and 16 h of reperfusion (sI/R). Cell viability was determined using the trypan blue exclusion test **(A,C,E,G)** or the MTT assay **(B,D,F,H)**.

(Continued)

FIGURE 2 | (A,B) Effects of preconditioning (PC) with LPS. Cardiac fibroblasts were pretreated with LPS (1 μg/ml) for 24 and 16 h before performing sI/R. **(C,D)** Effect of LPS only during ischemia. Cardiac fibroblasts were treated with LPS (1 μg/ml) during the 8 h of ischemia and then were perfused (16 h). **(E,F)** Effect of LPS only during reperfusion. Cardiac fibroblasts were treated with LPS (1 μg/ml) only during the 16 h of reperfusion. **(G,H)** Effect exerted by LPS during ischemia and reperfusion. Cardiac fibroblasts were treated with LPS (1 μg/ml) during the 8 h of ischemia and the 16 h of reperfusion. Results are expressed as mean ± SEM of six independent experiments. ***$p < 0.001$ vs. control; #$p < 0.05$ and ###$p < 0.001$ vs. sI/R.

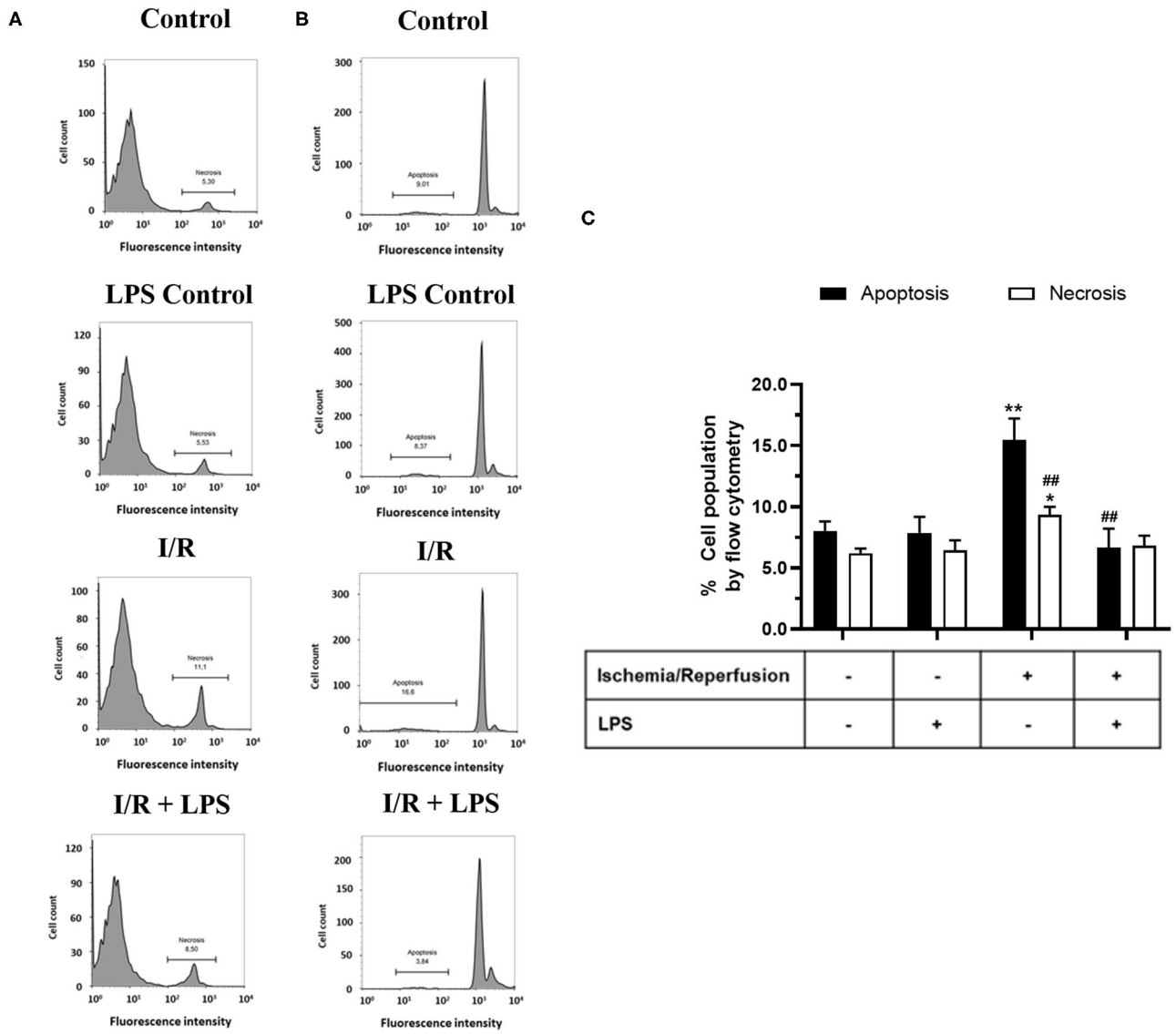

FIGURE 3 | Lipopolysaccharide (LPS) reduces apoptosis induced by simulated ischemia/reperfusion (sI/R) in cardiac fibroblasts. Cardiac fibroblasts were exposed to 8 h of ischemia, followed by 16 h of reperfusion (sI/R). Cells were treated with LPS (1 μg/ml) at the onset of ischemia and at the start of reperfusion. **(A)** The percentage (%) of necrosis was quantified by flow cytometry using propidium iodide (left), with representative histograms of each experimental group and the graphical analysis on the right. **(B)** The percentage (%) of the sub-G1 population (apoptosis) was quantified by flow cytometry using propidium iodide (middle), with representative histograms of each experimental group and the **(C)** graphical analysis on the right panel. The results are expressed as mean ± SEM of five independent experiments. *$p < 0.01$ and **$p < 0.01$ vs. each population control, ##$p < 0.01$ vs. apoptosis population in sI/R.

NF-KB activation. It is well-known that LPS administration increases the expression of inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) in the host through the TLR4-mediated NF-kB activation pathway. Consequently, administration of a large dose of LPS rapidly induces NF-kB activation and leads to the release of inflammatory cytokines, which play a key role in cardiac dysfunction and injury (25, 26). In this sense, we have previously observed that LPS triggers an increase in cytokine and chemokine

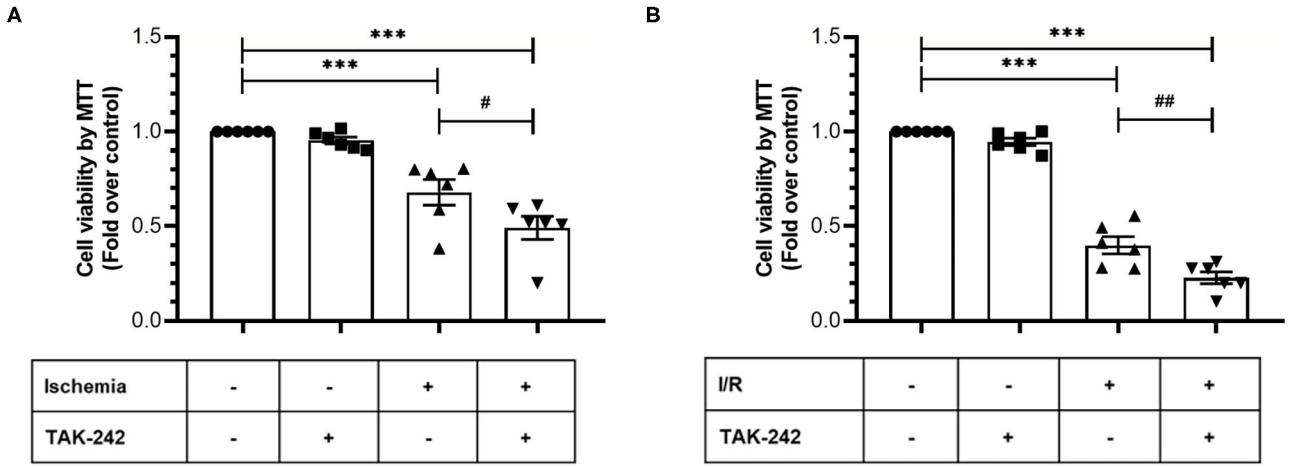

FIGURE 4 | Blockade of TLR4 increases the loss of cardiac fibroblasts viability induced by ischemia or ischemia/reperfusion (I/R). Cardiac fibroblasts were treated with the specific TLR4 inhibitor TAK-242 (4 μM) during the 8 h of ischemia **(A)** or during the 8 h of ischemia and the 16 h of reperfusion **(B)**. Cell viability was determined using the MTT assay. Results are expressed as mean ± SEM of six independent experiments. ***$p < 0.001$ vs. control; #$p < 0.05$ vs. ischemia; ##$p < 0.01$ vs. sI/R.

levels in CFs (15); however, our results indicated that LPS did not produce additional death of CFs at higher LPS concentrations. In addition, an interesting topic to discuss about our results is that LPS did not prevent CF viability loss triggered by I/R only during ischemia or only during reperfusion; however, when LPS was administered during both ischemia and reperfusion, CF viability increased, compared with groups without treatment. These results are similar to those observed by Chao et al. (23) in cardiomyocytes subjected to hypoxia/serum deprivation, where LPS treatment (500 ng/ml) increased cell viability in both conditions and reduced DNA fragmentation, apoptosis, and caspase-3 activation, corroborating that LPS administration during sI/R can confer cytoprotection. The lack of effect of LPS in conditions of ischemia only or reperfusion only suggests that survival kinases such as the RISK must be continuously active.

Lipopolysaccharide Activates AKT and ERK1/2 to Reduce Cardiac Fibroblast Apoptosis Induced by Simulated Ischemia/Reperfusion

The RISK pathway corresponds to the activation of two parallel cascades: PI3K-Akt and MEK1-ERK1/2, a group of pro-survival protein kinases that confer cardioprotection when activated specifically at the time of reperfusion (20). We have previously reported in CFs that TLR4 stimulation with LPS induces activation of Akt, ERK1/2, and NF-kB signaling pathways (17). In this regard, our results showed that in CFs subjected to sI/R conditions, LPS triggers an early activation of Akt and ERK1/2 pathways, probably as a survival cell response. Moreover, our results show now that TLR4 blockade with TAK-246 greatly reduces cell viability and phosphorylation of Akt and ERK1/2 during the treatments of simulated ischemia only and sI/R only, suggesting that TLR4 activation exerts a protective role in CFs during these adverse conditions.

The cytoprotective effects of LPS treatment on CF viability subjected to sI/R were prevented when Akt or ERK1/2 were inhibited, confirming that RISK intracellular signaling is an indispensable survival pathway as a consequence of TLR4 activation by LPS. In this sense, Liu et al. (19) reported that treatment with LPS, through the PI3K/Akt pathway, conferred cardioprotection in an *in vivo* rat model of I/R performed by left coronary artery occlusion, followed by restoration of blood flow. Similarly, Ha et al. (18) reported in an *ex vivo* rat model of MIR that a 24-h pretreatment with LPS decreased the number of apoptotic cardiomyocytes and cardiac injury through an AKT-dependent signaling pathway (18). In addition, other studies have indicated that clenbuterol (potent and selective beta-2 adrenergic agonist) increases the phosphorylation of ERK1/2, thus inhibiting myocardial apoptosis, as indicated by the reduction of Bax/Bcl-2 mRNA and caspase-3 protein levels (27) and that ERK1/2 inhibition exaggerates reperfusion injury in isolated rat hearts (28). Activation of the ERK1/2 and Akt pathways in CFs under sI/R (8, 10) and other related studies (18, 28) have demonstrated the relevance of preventing apoptosis to improve healing process. In our *in vitro* model, LPS treatment showed anti-apoptotic effects with the decrease of the sub-G1 population. Anti-apoptotic effects of LPS have been reported in cardiomyocytes exposed to hypoxia/serum deprivation, in which DNA fragmentation is decreased and pro-caspase 3 cleavage is inhibited (23). With regard to signaling pathways, Chao et al. (23) showed that PI3K/Akt, ERK1/2, and IkappaB kinase beta contribute to the anti-apoptotic effect of LPS since the specific inhibitors wortmannin and PD98059, and dominant negative IKKbeta transgene expression reversed the LPS effect. Our results are reinforced by our previous findings, where we showed that TGF-β1 reduces cell viability loss triggered by I/R through a mechanism involving PI3k/AKT, ERK1/2, and SMAD protein (10). In the same line, we showed that a mixture of ascorbic acid, *N*-acetylcysteine,

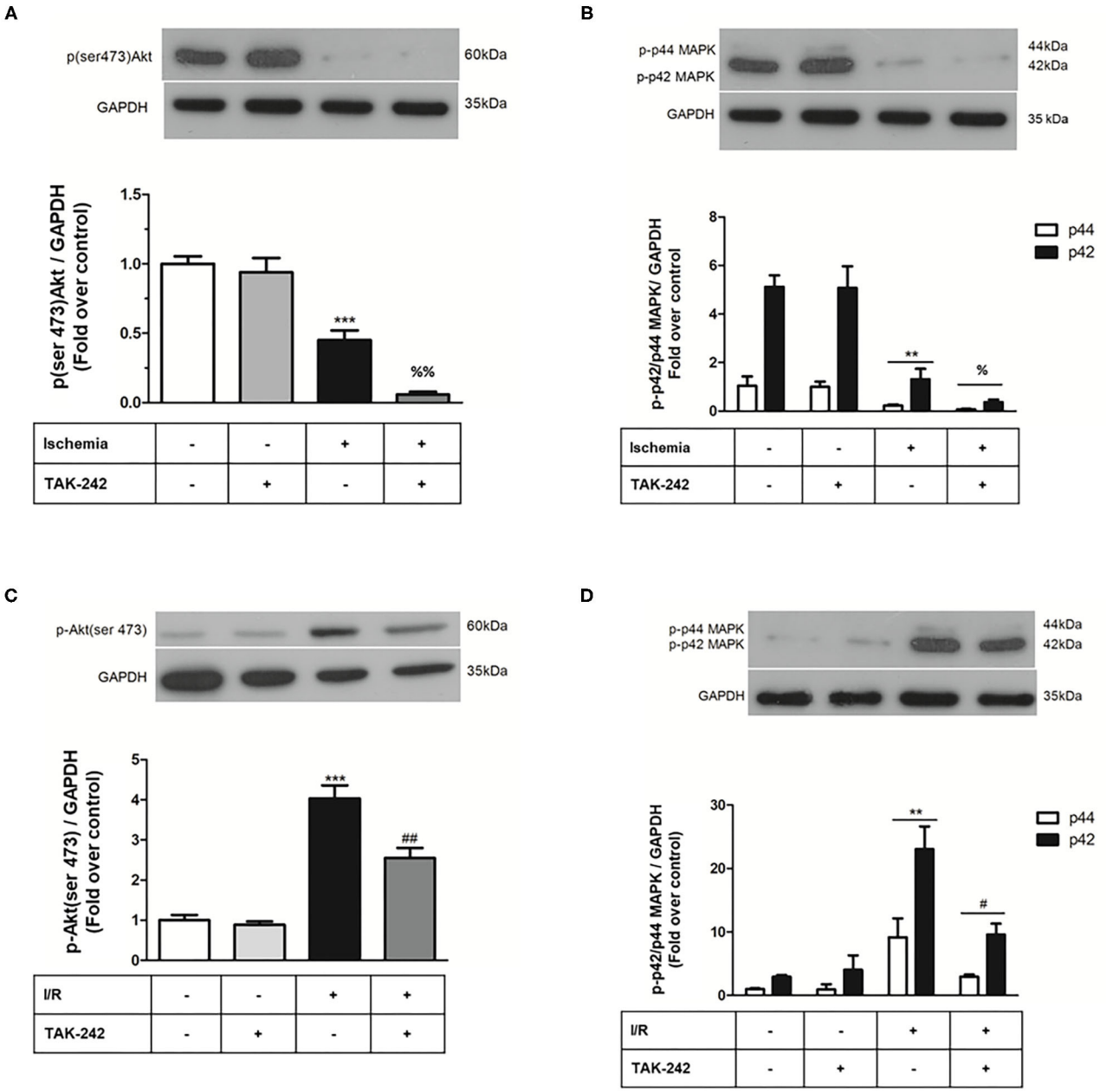

FIGURE 5 | Effect of TLR4 blockade on p-ERK1/2 and p-AKT levels after treatment of cardiac fibroblasts with lipopolysaccharide (LPS) during ischemia and during ischemia and reperfusion. Cardiac fibroblasts were treated with LPS (1 μg/ml) in the presence of the TAK-242 (4 μM) inhibitor during the 8 h of ischemia **(A,B)** and during the 8 h of ischemia and the 16 h of reperfusion (sI/R) **(C,D)**. The expression levels of p-ERK1/2 and p-AKT were determined by Western blot. GAPDH was used as a loading control (we use the same blot for **A,B**). Results are expressed as mean ± SEM of four independent experiments. $^{***}p < 0.001$ vs. control; $^{**}p < 0.01$ vs. control; $^{\%}p < 0.05$ and $^{\%\%}p < 0.01$ vs. ischemia. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. sI/R.

and deferoxamine (A/N/D) prevents CF viability loss though an AKT and ERK1/2 mechanism (11). In addition, clenbuterol—which increases ERK1/2 activation—also reduces caspase-3 expression, and AKT activation also reduces caspase-3 activation in cardiomyocytes exposed to I/R (27). Altogether, our results confirm that PI3K/Akt and ERK1/2 are pro-survival kinase pathways triggered by LPS/TLR4, suggesting TLR4 activation as a potential pharmacological target to confer CF protection in the I/R context.

In contrast, other studies have shown that TLR4 activation triggers cell death under I/R conditions. A recent report indicated that TLR4 deficiency contributes to less myocardial injury and inflammatory symptoms after the induction of I/R (29). The inflammatory reaction is a key hallmark of

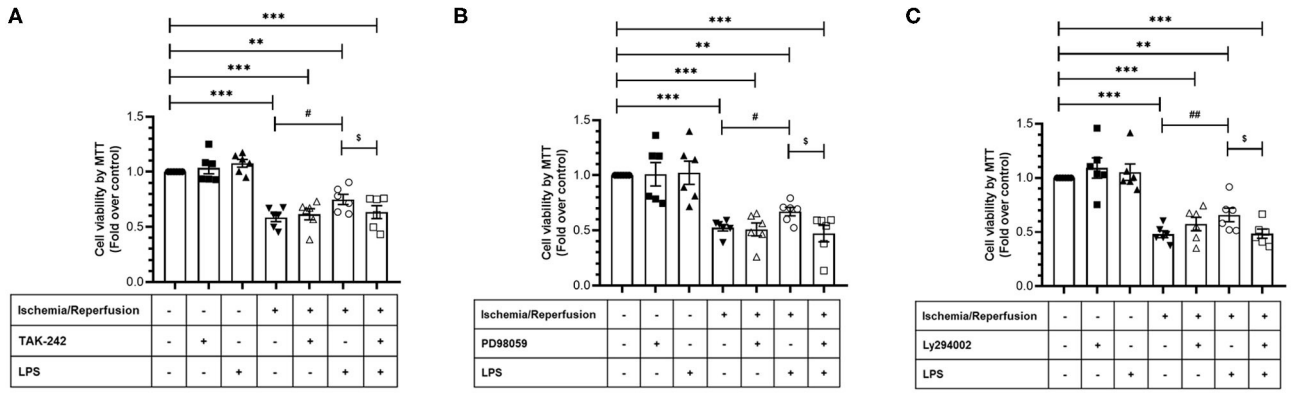

FIGURE 6 | Effect of TLR4 blockade, ERK1/2, and AKT inhibition on the loss of viability induced by ischemia/reperfusion (I/R) in cardiac fibroblasts treated with lipopolysaccharide (LPS). Cardiac fibroblasts were treated with LPS (1 μg/ml) in the presence of **(A)** TAK-242 (4 μM), **(B)** PD98059 (25 μM), or **(C)** LY294002 (10 μM). Cell viability was determined by the MTT assay. Results are expressed as mean ± SEM of six independent experiments. $^{***}p < 0.001$ and $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. sI/R; $^{\$}p < 0.05$ vs. sI/R + LPS.

I/R, and its modulation has been shown to be beneficial in previous investigations (30, 31). Specifically, the TLR4/NF-κB signaling pathway plays a pivotal role in I/R injury, promoting a marked expression of proinflammatory cytokines in cardiomyocytes under I/R conditions (32). It is well-known that LPS administration increases the expression of inflammatory cytokines (such as IL-1β, IL-6, and TNF-α) in the host through the TLR4/NFkB pathway but can also induce the PI3K/Akt pathway, which has been reported as an endogenous negative feedback regulator and/or compensatory mechanism of TLR4/NFkB-mediated proinflammatory responses, thus preventing an excessive innate immune response (25, 26, 33, 34). In this sense, in our previous findings, we showed that I/R triggers the activation of p38 and JNK signaling pathways (11), which correspond to proapoptotic signals. In addition, a recent study showed that miR-20a has a protective action on cardiomyocytes subjected to sI/R by inactivating the p38 MAPK/JNK pathway and promoting cell viability (35). However, in our present study, we did not evaluate whether LPS treatment can reduce p38 and JNK activation, so this topic remains to be elucidated. Finally, several studies have shown that LPS preconditioning protects against I/R damage, although the mechanisms are quite different and depend on the animal model, tissue, or cell type involved. In this sense, He et al. (36) demonstrated that preconditioning with LPS led to the accumulation of HIF-2α in kidneys and mouse endothelial cells, as a result of the activation of NF-κB, which subsequently improved kidney microvascular perfusion and reduced ischemic tubular damage. By contrast, Yao et al. (37) showed that preconditioning with LPS at low doses resulted in significantly higher levels of HSP70 in the myocardium, which could dramatically inhibit the translocation of NF-κB and that NF-κB inhibition, in turn, attenuated the release of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and reduced myocardial apoptosis and infarction area after injury by I/R. In addition, LPS preconditioning protects hepatocytes from I/R injury by inhibiting the ATF4-CHOP pathway, which may be critical in reducing apoptosis-related molecules and modulating innate inflammation (38). Along the same lines, Sano et al. (39) demonstrated that liver preconditioning with LPS caused the positive regulation of specific negative regulators in the TLR4 signaling pathway and that the pre-ischemic induction of these regulators plays an important role as an immunological preparation for subsequent I/R and produces resistance to liver injury. Finally, Merry et al. (40) demonstrated that low doses of LPS protect against subsequent pulmonary I/R injury. LPS preconditioning reduced lung injury and the production of inflammatory mediators after post-I/R exposure. These results as a whole demonstrate that the inhibition of NF-KB, rather than its activation, is an important factor in cytoprotection against I/R injury.

CONCLUSIONS

In conclusion, our findings indicate that LPS treatment, administered in both ischemia and reperfusion, prevents loss of viability and apoptosis in CFs exposed to sI/R, through a mechanism mediated by the activation of the TLR4 and the Akt and ERK1/2 pro-survival kinases, suggesting TLR4 activation as a potential pharmacological target to prevent cell death by sI/R. We recently showed that CF death induced by antioxidant treatment maintains certain cellular functions, such as collagen secretion, CF-to-CMF differentiation, and migration, which are necessary to achieve an affective wound repair process. Therefore, our results suggest that TLR4 activation in CFs could be another effective strategy to prevent excessive cell death and improve the mechanisms associated with wound repair after myocardial infarction.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by our Institutional Ethics Committee (Protocol code CBE2017-13-CYQ-UCH approved on March 14, 2018).

AUTHOR CONTRIBUTIONS

PP-F: experimental analyses, analysis of data, and writing of manuscript. JE-C, CE-P, and CQ: experimental analyses. FB and PA: experimental analyses and analysis of data. AS-H and VP-J: analysis of data. GD-A: conception of the research, analysis of data, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Electroacupuncture Pretreatment Mitigates Myocardial Ischemia/Reperfusion Injury via XBP1/GRP78/Akt Pathway

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Myocardial ischemia/reperfusion injury is a common clinical problem and can result in severe cardiac dysfunction. Previous studies have demonstrated the protection of electroacupuncture against myocardial ischemia/reperfusion injury. However, the role of X-box binding protein I (XBP1) signaling pathway in the protection of electroacupuncture was still elusive. Thus, we designed this study and demonstrated that electroacupuncture significantly improved cardiac function during myocardial ischemia/reperfusion injury and reduced cardiac infarct size. Electroacupuncture treatment further inhibited cardiac injury manifested by the decrease of the activities of serum lactate dehydrogenase and creatine kinase-MB. The results also revealed that electroacupuncture elevated the expressions of XBP1, glucose-regulated protein 78 (GRP78), Akt, and Bcl-2 and decreased the Bax and cleaved Caspase 3 expressions. By using the inhibitor of XBP1 *in vitro*, the results revealed that suppression of XBP1 expression could markedly increase the activities of lactate dehydrogenase and creatine kinase-MB and cell apoptosis, thus exacerbating stimulated ischemia/reperfusion-induced H9c2 cell injury. Compared with stimulated ischemia/reperfusion group, inhibition of XBP1 inhibited the downstream GRP78 and Akt expressions during stimulated ischemia/reperfusion injury. Collectively, our data demonstrated that electroacupuncture treatment activated XBP1/GRP78/Akt signaling to protect hearts from myocardial ischemia/reperfusion injury. These findings revealed the underlying mechanisms of electroacupuncture protection against myocardial ischemia/reperfusion injury and may provide novel therapeutic targets for the clinical treatment of myocardial ischemia/reperfusion injury.

Keywords: electroacupuncture, myocardial ischemia/reperfusion injury, XBP1s, apoptosis, Akt

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INTRODUCTION

Ischemic heart disease is a common cardiovascular problem with high morbidity and mortality (1). Although it is important to timely restore the blood flow of an ischemic myocardium, cardiovascular outcomes can be further aggravated by the so-called myocardial ischemia/reperfusion (MI/R) injury. Therefore, exploring a safe and effective treatment is urgently needed to mitigate MI/R-induced injury.

The endoplasmic reticulum (ER) is an organelle where the target proteins are processed so as to prompt its post-translational modifications, proper folding, and protein transport (2). However, under cellular stress, the imbalance of ER protein processing and accumulation of the unfolded proteins and/or misfolded proteins results in ER stress, which further induces the unfolded protein response (UPR) as an adaptive response to restore ER homeostasis (3, 4). The UPR is initiated by three classical ER transmembrane sensors: protein kinase R-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) (3). As a key player of ER stress, X-box binding protein I (XBP1) expression was induced by ATF6 and then was spliced by IRE1 (5). The resulting spliced form of XBP1 can further activate UPR to cope with ER stress (5). Previous studies further revealed that ER stress participated in the pathogenesis of numerous cardiac diseases (6). In transverse aortic constriction (TAC)-induced hypertrophic and falling hearts, sustained ER stress resulted in cardiomyocyte apoptosis and contributed to the progression from cardiac hypertrophy to heart failure through the canonical and non-canonical pathways (7, 8). Surprisingly, hypoxia induced ATF6 and glucose-regulated protein 78 (GRP78) expressions and protected cardiomyocytes from ischemic injury (9). ATF6 transgenic mice alleviated MI/R damage via decreasing oxidative stress, enhancing catalase expression, and reducing cell necrosis and apoptosis (10, 11). Notably, GRP78 as an XBP1 target was shown to stimulate Akt pathway to protect hearts from I/R injury (12). Furthermore, XBP1 can protect β-cells from lipotoxicity via activation of Akt pathway (13). However, the role of XBP1–Akt pathway in the MI/R injury was not fully investigated.

Based on the Chinese traditional medical theory, acupuncture is used to treat diseases for more than 2000 years (14, 15). Especially, electroacupuncture (EA) pretreatment at specific acupoints has been demonstrated as an effective approach to improve cardiac function in diverse pathological conditions. In spontaneously hypertensive rats, long-term EA reduced the wall thickness of left ventricle via regulating the NOS pathway (16). It was revealed that EA at PC6 (Neiguan) and PC5 (Jianshi) acupoints reduced myocardial malondialdehyde (MDA) level, norepinephrine concentration, and cell apoptosis to attenuate MI/R injury in animal models (17, 18). Furthermore, in our previous clinical study, EA pretreatment significantly attenuated MI/R injury in patients with heart valve replacement surgery (19). However, whether EA pretreatment regulates ER stress signaling pathways to protect hearts against MI/R injury has not been explored previously.

Thus, the present study was designed to investigate the protection of EA pretreatment against MI/R injury and the potential role of XBP1/GRP78 signaling in this process. These findings may provide the theoretical basis for the clinical use of EA pretreatment against MI/R injury.

MATERIALS AND METHODS

Animal Model of Myocardial Ischemia/Reperfusion Injury

The animal experimental protocol in this study was approved by the Animal Care Committee of Air Force Medical University. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Research from US National Institutes of Health (National Institutes of Health Publication No. 8523, revised 1996). Male C57BL/6 mice aged 10∼12 weeks and weighing 22∼26 g were obtained from the Laboratory Animal Center of Air Force Medical University. All mice were housed at 20–25°C under a 12-h light/dark cycle and received a standard diet and water *ad libitum*.

MI/R injury mouse model was established according to the previous study (20). Briefly, mice were anesthetized with 1–2% isoflurane via an isoflurane vaporizer (Matrx, Orchard Park, NY, USA). A skin scar was cut in the left chest, and a tiny hole was made at the fourth intercostal space. Afterwards, the heart was smoothly squeezed out of the thoracic cavity. The left anterior descending (LAD) coronary artery was ligated by a 6-0 silk suture for 30 min, and then the slipknot was released. The reperfusion phase lasted for 2–4 h, and the heart samples were collected for protein expressions analysis. The cardiac function, myocardial infarct size, cell apoptosis, and lactate dehydrogenase (LDH) as well as creatine kinase-MB (CK-MB) were assessed following a 24-h reperfusion. The same procedure except ligation of LAD was performed in the mice of sham group.

Electroacupuncture Pretreatment

EA pretreatment was conducted by using the Hwato Electronic Acupuncture Treatment Instrument (Suzhou Medical Appliances, Suzhou, China). Briefly, mice were anesthetized and maintained by inhalation of 1–2% isoflurane via an isoflurane vaporizer. The needles connected to the electrodes were inserted into 2- to 3-mm depth of muscle layers at the Neiguan acupoint (PC6) of both forelimbs, which are located between the palmar tendon and flexor carpi ulnaris (21). Mice were stimulated at the density of 1 mA with a frequency of 2/15 Hz for 30 min once a day for 3 days. The MI/R surgery or sham surgery was performed within the 30 min after the last EA treatment. Mice from Sham and Sham+EA groups were anesthetized for 30 min to avoid the effects of isoflurane between different groups.

The mice were divided into the following four groups with 12–15 mice for each: sham group (Sham), sham group with EA pretreatment (Sham+EA), MI/R injury group (MI/R), and MI/R group with EA pretreatment (MI/R+EA). Mice in the Sham+EA or MI/R+EA groups received EA preconditioning for 3 consecutive days followed by sham or MI/R surgery, while mice in the Sham or MI/R group underwent the sham or MI/R surgery, respectively.

Echocardiography

Mice were anesthetized and maintained by inhalation of 1–2% isoflurane after 24-h reperfusion. Cardiac function was evaluated by Doppler echocardiography with a 15-MHz linear transducer (Visual Sonic Vevo 2100, Toronto, ON, Canada). Mice were placed on a heating pad to maintain the body temperature during the whole procedure. M-mode echocardiography was recorded and used to assess cardiac function. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were obtained by using Vevo LAB 3.1.0 software.

Activities Measurements of Lactate Dehydrogenase and Creatine Kinase-MB

The serum was obtained by centrifugation of mouse blood at 3,000 rpm for 10 min after 24-h reperfusion and used for LDH and CK-MB determination. The assay was conducted according to the manufacturer's instruction (Jiancheng Bioengineering, Nanjing, China). The activities of LDH and CK-MB were calculated based on the methods described in the manufacturer's instruction.

Triphenyltetrazolium Chloride/Evans Blue Double Staining

The mice were anesthetized with 1–2% isoflurane, and the LAD was occluded at the same position following 24-h reperfusion. The 3% Evans blue solution was injected into the hearts via the aorta to stain the non-ischemic area of the heart. The whole hearts were then collected and frozen on dry ice for 10 min and cut into four slices transversally from the bottom of the hearts. The slices were stained in 1.5% triphenyltetrazolium chloride (TTC) in phosphate solution (pH 7.4) and incubated at 37°C for 20 min. Then the slices were fixed in 4% paraformaldehyde for 12 h, and the images were obtained by using a digital camera. The infarct size was calculated by the ratio of white area to white and red areas. The size was determined by using Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Terminal Deoxynucleotidyl Transferase-Mediated Dutp Nick-End Labeling Staining

Myocardial apoptosis and cell apoptosis were determined by an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (22). In brief, at the end of the experiment, the myocardial tissues and H9c2 cells were fixed in 4% paraformaldehyde for at least 24 h. After the paraffin-imbedded sections were prepared, the manufacturer's instruction for TUNEL staining was followed. The apoptotic myocardial cells and H9c2 cells were stained with TUNEL staining solution, and nuclei were visualized by DAPI staining. Then the images were obtained with an Olympus FV10i microscope (Olympus, Tokyo, Japan); and an apoptotic rate was presented as the count of TUNEL-positive cardiomyocytes to the total number of cells.

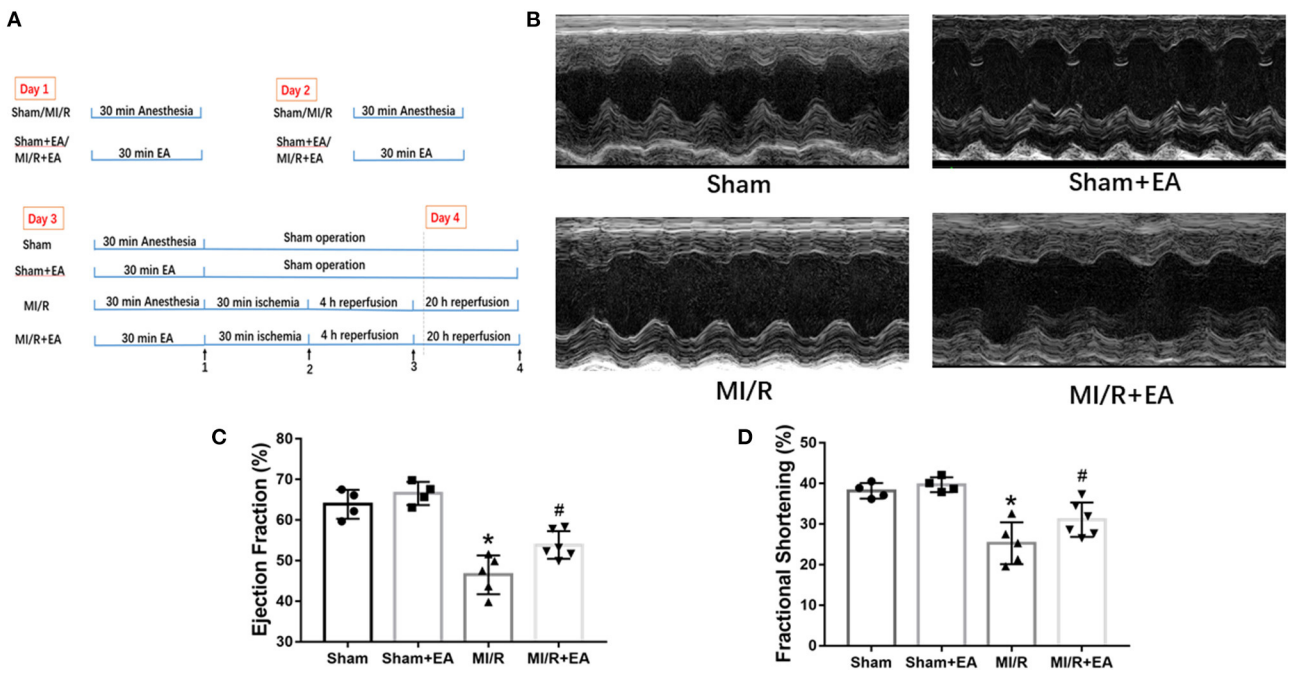

FIGURE 1 | EA pretreatment mitigated MI/R-induced cardiac dysfunction. **(A)** A schematic illustration of the study groups and *in vivo* experimental protocols. Time point 1: ischemic phase. Time point 2: reperfusion phase. Time point 3: heart tissue collection for protein analysis. Time point 4: echo analysis and TTC staining. **(B)** Representative images of M-mode echocardiography in MI/R-induced cardiac injury following EA pretreatment. Images of M-mode echocardiography were recorded from horizontal direction of the heart and then were analyzed to assess cardiac systolic function. **(C)** Ejection fraction. **(D)** Fractional shortening. $n = 4$–6 in each group. EA, electroacupuncture; MI/R, myocardial ischemia/reperfusion; TTC, triphenyltetrazolium chloride. $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. MI/R group.

Cell Culture and Stimulated Ischemia/Reperfusion Model

H9c2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, UT, USA) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO_2 air incubator. The experiments included four groups: (1) the cells in the CON group were cultured in the serum-free DMEM. (2) H9c2 cells in stimulated I/R (SI/R) group were cultured in serum-free DMEM for 12 h and then subjected to the ischemic buffer (10 mM of deoxyglucose, 137 mM of NaCl, 12 mM of KCl, 0.49 mM of $MgCl_2$, 0.9 mM of $CaCl_2{\cdot}2H_2O$, 0.75 mM of sodium dithionate, 20 mM of lactate, and 4 mM of Hepes, pH 6.5) for 2 h. The cells were then cultured in normal DMEM at 37°C in an incubator (5% CO_2, 95% air) for 4 h to establish *in vitro* SI/R model. (3) The cells in XI group (XBP1 inhibitor, 4μ8C) were cultured in the serum-free DMEM and then incubated with 5 μM of 4μ8C for 2 h. (4) The cells in SI/R+XI group were incubated 5 μM of 4μ8C for 2 h and then subjected to ischemic medium for 2 h. Then the cells were cultured in normal DMEM at 37°C in an incubator (5% CO_2, 95% air) for 4 h. The supernatant of cell culture was collected for LDH and CK-MB activities measurements following the protocol described above.

Western Blotting

The proteins were isolated from the heart left ventricles including the infarct zone and broader zone and H9c2 cells for western blotting detection. The proteins were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred on to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA); subsequently, the membrane is incubated with 5% non-fat milk in TBST. Western blotting was then performed with antibodies against XBP1 (Cat. 83418, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), GRP78 (Cat. 3183, 1:1,000; Cell Signaling Technology), p-Akt (Cat. 4060, 1:1,000; Cell Signaling Technology), Akt (Cat. 4691, 1:1,000; Cell Signaling Technology), Bcl-2 (Cat. ab196495, 1:1,000; Abcam, Cambridge, UK), Bax (Cat. 2772, 1:1,000; Cell Signaling Technology), cleaved Caspase 3 (Cat. 9664, 1:1,000; Cell Signaling Technology), and GAPDH (Cat. AT0002, CMC TAG, 1:5,000). After that, the proteins were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized by a ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Then the relative quantification of proteins was presented as the ratio of target proteins to GAPDH.

Statistical Analysis

All data were presented as mean ± SD. Data were analyzed with the GraphPad Prism Software version 7.0 (GraphPad Software, San Diego, CA, USA). Normality analysis of data was performed by the Shapiro–Wilk test. Statistical significance ($P < 0.05$) was estimated by one-way ANOVA followed by Bonferroni correction for *post-hoc t*-test.

RESULTS

Electroacupuncture Pretreatment Protected Cardiac Function Against Myocardial Ischemia/Reperfusion Injury

To evaluate the effects of EA on cardiac function following MI/R injury and EA treatment, echocardiography was performed. M-mode images were obtained to measure LVEF and LVFS so as to evaluate cardiac contractile function. As shown in **Figure 1**, MI/R significantly reduced LVEF and LVFS compared with sham group, while EA pretreatment for 3 consecutive days greatly increased LVEF and LVFS compared with MI/R group. These data demonstrated that MI/R resulted in compromised cardiac function, while EA pretreatment improved cardiac function, which was impaired by MI/R injury. However, cardiac function in EA+sham group was not significantly altered compared with sham group, implying no obvious effects on cardiac function by EA pretreatment in sham-operated mice. These results data together revealed that EA pretreatment elevated MI/R-induced reduction of LVEF and LVFS, thus protecting hearts from MI/R injury.

Electroacupuncture Pretreatment Reduced Myocardial Infarct Size and the Activities of Lactate Dehydrogenase and Creatine Kinase-MB

To further investigate whether EA pretreatment could affect MI/R-induced myocardial infarct size and myocardial cell death, TTC/Evans blue double staining was used to assess infarct size; and LDH and CK-MB activities were determined to evaluate myocardial cell death. Our results showed that MI/R led to a significant increase of myocardial infarct size compared with sham group, while EA pretreatment dramatically reduced myocardial infarct size compared with MI/R group as shown in **Figure 2**. The above result clearly showed that EA pretreatment significantly reduced MI/R-induced myocardial infarct size. Furthermore, the activities of LDH and CK-MB in the serum were increased in response to MI/R injury compared with sham group, while EA pretreatment decreased the activities of LDH and CK-MB in EA+MI/R group compared with MI/R group as shown in **Figure 2**. These data revealed that myocardial cell death caused by MI/R injury was inhibited following EA pretreatment. Taken together, these results indicated that EA pretreatment decreased myocardial infarct size and LDH and CK-MB release to protect hearts from MI/R injury.

Electroacupuncture Reduced Myocardial Ischemia/Reperfusion-Induced Cell Apoptosis and Activated XBP1/GRP78/Akt Pathway

To elucidate the role of cell apoptosis in EA protection against MI/R injury, cell apoptosis among different groups was determined. As shown in **Figure 3**, MI/R significantly enhanced myocardial apoptosis compared with sham group. Moreover, EA pretreatment decreased MI/R-induced cell apoptosis by 27.6%

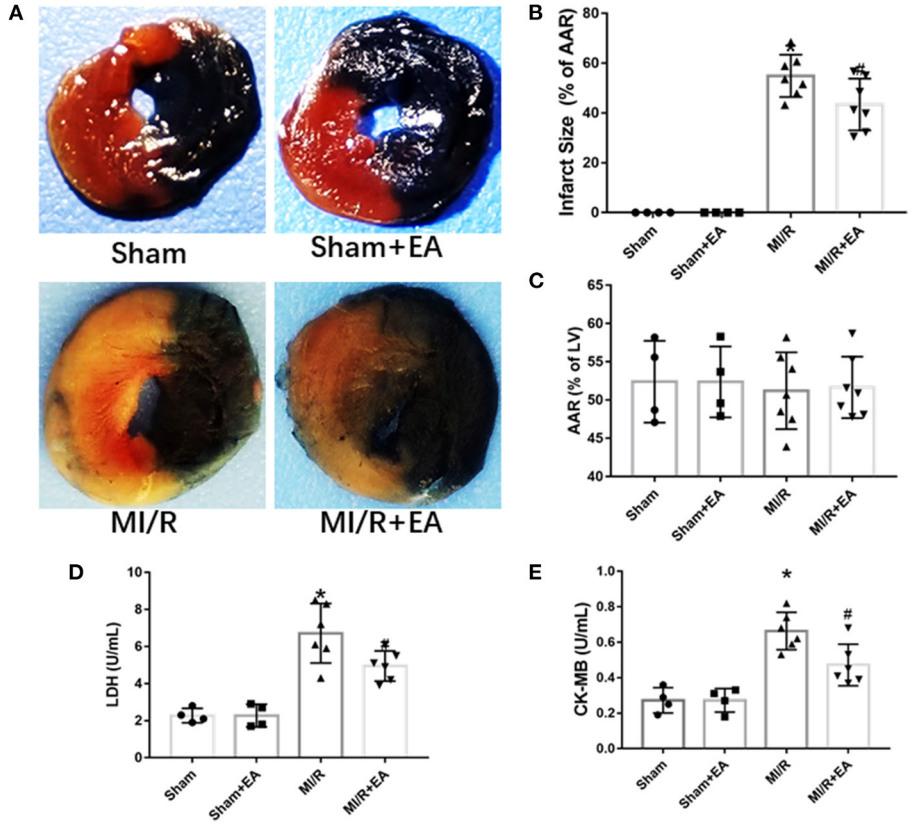

FIGURE 2 | The effects of EA pretreatment on myocardial infarct size, LDH, and CK-MB activities in MI/R-induced cardiac injury. **(A)** Representative images of cardiac slices by TTC/Evans blue double staining in MI/R-induced cardiac injury following EA pretreatment. **(B)** Myocardial infarct size of different groups. **(C)** Myocardial area at risk (AAR) following MI/R procedure and EA treatment. $n = 4$–7 in each group for TTC/Evans blue double staining. **(D)** Serum LDH activity. **(E)** Serum CK-MB activity. $n = 4$–6 in each group for enzyme activity measurements. LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB isoform; EA, electroacupuncture; MI/R, myocardial ischemia/reperfusion; TTC, triphenyltetrazolium chloride. $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. MI/R group.

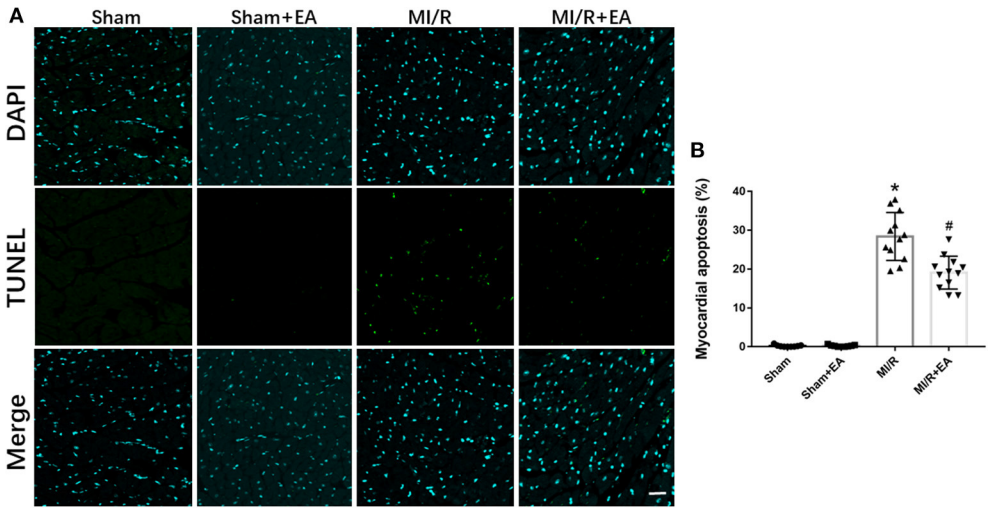

FIGURE 3 | The effects of EA pretreatment on myocardial apoptosis in MI/R-induced cardiac injury. **(A)** Representative images of myocardial apoptosis by TUNEL staining in MI/R-induced cardiac injury following EA pretreatment. **(B)** Myocardial apoptosis of mice from different groups. $n = 3$–4 for each group. Three to four hearts in each group were used for TUNEL staining, and five random fields in three sections of each heart were used for further analysis. Bar, 100 μm. $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. MI/R group. EA, electroacupuncture; MI/R, myocardial ischemia/reperfusion.

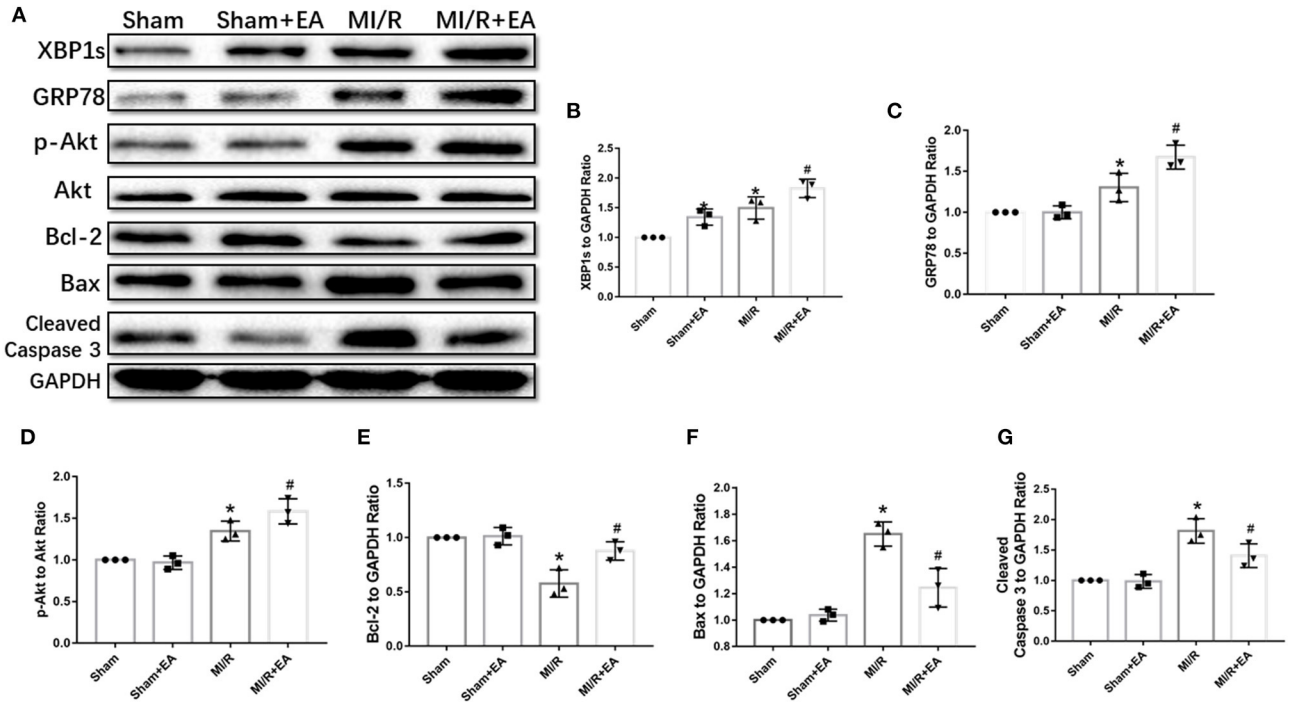

FIGURE 4 | EA pretreatment activated XBP1/GRP78/Akt pathway and reduced apoptotic protein expressions. **(A)** Representative blot images of proteins related to XBP1/GRP78/Akt and apoptotic signaling pathways. **(B–G)** The quantitative analysis of XBP1, GRP78, p-Akt, Bcl-2, Bax, and cleaved Caspase 3 protein expressions. $n = 3$ in each group. $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. MI/R group. EA, electroacupuncture; MI/R, myocardial ischemia/reperfusion.

compared with MI/R group, implying the protective role of EA on reducing cell apoptosis during MI/R injury.

To further investigate the potential molecular mechanisms underlying EA protection against MI/R injury, the expressions of XBP1/GRP78/Akt signaling pathway and apoptotic proteins were assessed. As shown in **Figure 4**, our data demonstrated that MI/R injury resulted in increase of XBP1, GRP78, and phosphorylated Akt (p-Akt) expressions, while the pro-apoptotic proteins including Bax and cleaved Caspase 3 were upregulated compared with sham group. The results showed that the expressions of XBP1, GRP78, and Akt were further increased, and the expressions of Bax and cleaved Caspase 3 were decreased following EA pretreatment. These results indicated that the molecular mechanism of EA protection against MI/R injury was at least partly via activation of XBP1/GRP78/Akt signaling pathway, therefore ultimately reducing cell apoptosis.

Inhibition of XBP1 Exacerbated Stimulated Ischemia/Reperfusion Injury and Cell Apoptosis in H9c2 Cells

To further investigate the role of XBP1-mediated signaling pathway in SI/R-induced cell injury, the inhibitor 4μ8C was used in *in vitro* study. The results in **Figure 5** revealed that XBP1 inhibitor 4μ8C significantly exacerbated SI/R-induced H9c2 cell injury manifested by the increase of LDH and CK-MB activities and the elevation of cell apoptosis compared with the SI/R group. However, 4μ8C alone did not significantly affect the activities of LDH and CK-MB or cell apoptosis in normal conditions. These results showed that XBP1 inhibition could deteriorate SI/R-induced cell injury via increase of cell death.

XBP1 Inhibition Downregulated GRP78/Akt Pathway in Stimulated Ischemia/Reperfusion-Injured H9c2 Cells

To demonstrate the effects of XBP1 inhibition on GRP78/Akt signaling pathway, we further explored the effects of 4μ8C on the XBP1/GRP78/Akt pathway. The western blotting in **Figure 6** demonstrated that the XBP1 inhibitor 4μ8C downregulated XBP1, GRP78, Akt, and Bcl-2 expressions and elevated the expressions of Bax and cleaved Caspase 3 compared with MI/R group. Our *in vitro* results favored the notion that the inhibition of XBP1 could worsen SI/R-induced cell injury via regulation of GRP78/Akt pathway.

DISCUSSION

The present study demonstrated that EA pretreatment attenuated MI/R-induced cardiac dysfunction and mitigated MI/R-induced damage by decreasing serum LDH, CK-MB, and myocardial apoptosis. The underlying molecular mechanism of EA protection was shown to be involved in the activation of XBP1/GRP78/Akt pathway. Further *in vitro* result revealed that inhibition of XBP1 decreased the downstream GRP78 and Akt

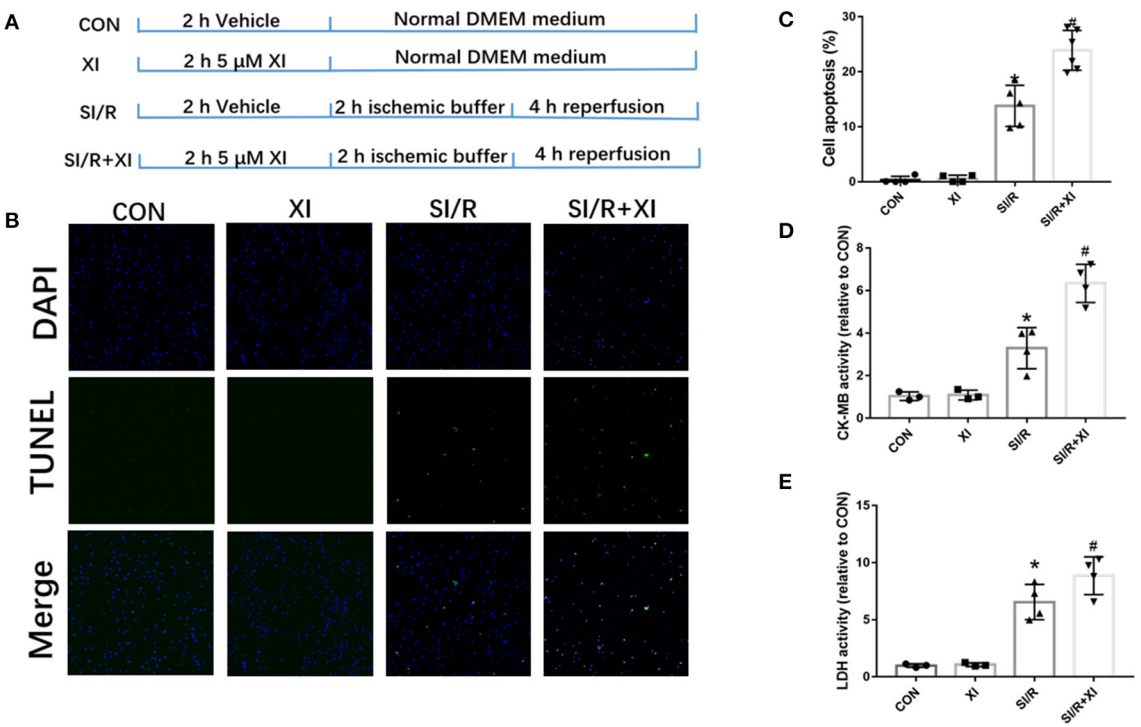

FIGURE 5 | 4μ8C, an XBP1 inhibitor, increased H9c2 cell apoptosis LDH and CK-MB activities *in vitro*. **(A)** A schematic illustration of the study groups and *in vitro* experimental protocols. **(B)** Representative images of cell apoptosis by TUNEL staining in SI/R-induced cell injury following 4μ8C pretreatment. **(C)** The quantitative analysis of cell apoptosis following 4μ8C pretreatment. Eight random fields were selected for quantitation of cell apoptosis for each repeat experiment. **(D)** LDH activity. **(E)** CK-MB activity. $n = 3$–4 in each group. XI, XBP1 inhibitor; LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB; SI/R, stimulated ischemia/reperfusion. Bar, 50 μm. $^{*}P < 0.05$ vs. CON group, $^{\#}P < 0.05$ vs. SI/R group.

expressions and elevated cell apoptosis, implying the importance of XBP1-mediated pathway against MI/R injury.

As a traditional Chinese medical therapy, EA is shown to be a beneficial treatment for several diseases including stress urinary incontinence, knee osteoarthritis, and acute ischemic cerebral apoplexy in clinical studies (23–25). Notably, EA significantly reduced myocardial injury induced by cardiac hypertrophy and ischemic insult in animal models and clinical studies (18, 19). EA pretreatment at Neiguan (PC6) acupoint mitigated cardiac hypertrophy via upregulation of ERK signaling pathway (26). Furthermore, Lujan et al. demonstrated that EA decreased the incidence of I/R-mediated ventricular tachyarrhythmias via lowering cardiac metabolic demand (27). Inhibition of cardiac norepinephrine release and regulation of opioid and PKC-dependent pathways by EA treatment attenuated MI/R injury in a rabbit model (17). Consistent with these previous studies, we observed that EA pretreatment for 3 consecutive days reduced myocardial infarct size, increased LVEF and LVFS, but suppressed the activities of LDH and CK-MB in MI/R injury. Moreover, EA pretreatment decreased the levels of expressions of Bax and Cleaved Caspase 3. These results clearly showed that EA pretreatment attenuated MI/R-induced cardiac contractile dysfunction and myocardial apoptosis.

Accumulating evidence has demonstrated that ER stress was markedly activated in I/R-injured myocardium (28, 29). ER stress is known to occur when the protein synthesis and protein process exceed its capacity in ER lumen under cellular stress. The UPR can be initiated by three signaling pathways including eIF2a-ATF4, IRE1a-XBP1, and ATF6 pathways to maintain ER homeostasis (30). Moreover, melatonin inhibited PERK-eIF2α-ATF4-mediated ER stress to protect cardiac function from MI/R injury (31). It was also revealed that ischemic preconditioning attenuated MI/R-induced injury through suppression of ER stress (32). All these data favored the notion that the inhibition of ER stress would be beneficial to mitigate myocardial ischemic injury. However, these protective effects were not observed by direct inhibition of key molecules in ER stress but the upstream regulators of ER stress. In contrast to these results, acute activation of ER stress by the key molecules involved in ER stress signaling pathway displayed cardioprotective roles in ischemic heart diseases, which was even attributed to the other molecular mechanisms beyond ER stress signaling pathways. ATF6 as a key mediator of one conserved branch of ER stress protected hearts from MI/R injury via inducing the expressions of catalase and protein disulfide isomerase (10, 11). Furthermore, ATF6 binds to the promoter of the protein disulfide isomerase associated 6 (*pdia6*) gene to protect cardiomyocytes against simulated I/R-induced death *in vitro* (33). GRP78 as a signal sensor of ER stress activated by ischemic preconditioning attenuated ischemic injury in cardiomyocytes via activation of Nrf2/HO-1 pathway (34,

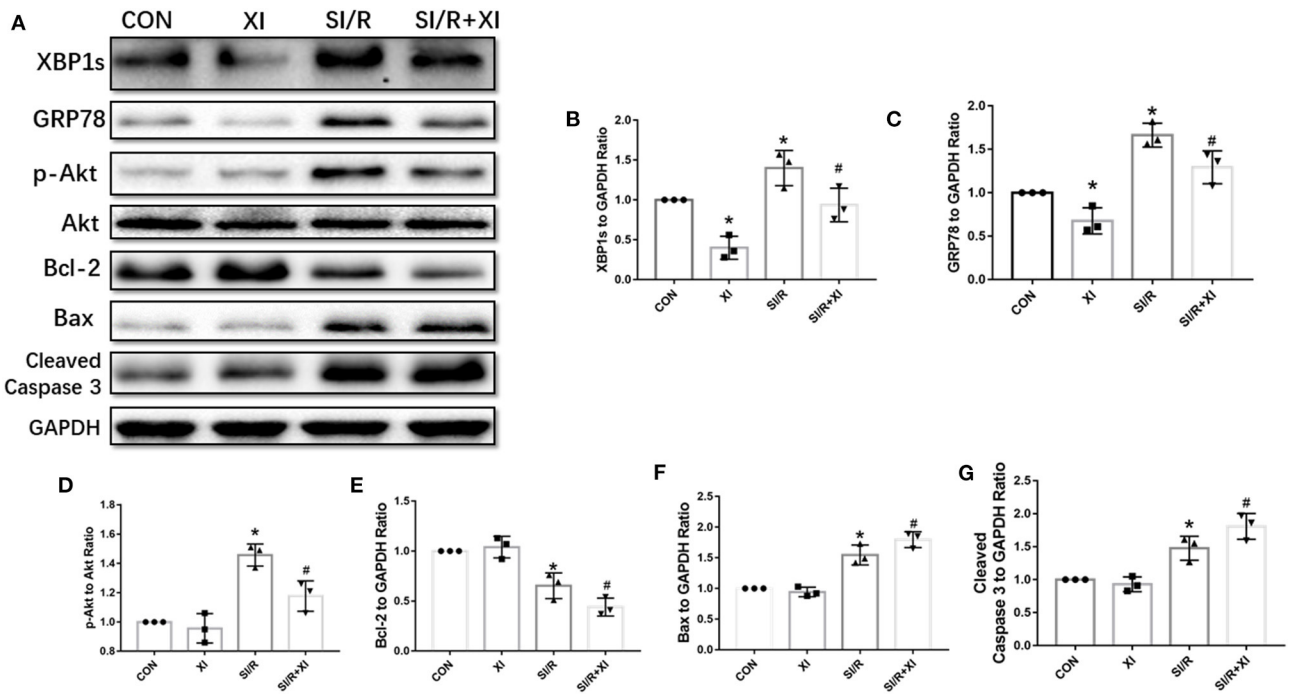

FIGURE 6 | 4μ8C inhibited XBP1/GRP78/Akt pathway and enhanced apoptotic protein expressions *in vitro*. **(A)** Representative blot images of proteins related to XBP1/GRP78/Akt and apoptotic signaling pathways in H9c2 cells. **(B–G)** The quantitative analysis of XBP1, GRP78, p-Akt, Bcl-2, Bax, and cleaved Caspase 3 protein expressions. $n = 3$ in each group. $^{*}P < 0.05$ vs. CON group, $^{\#}P < 0.05$ vs. SI/R group.

35). Cardiomyocyte-specific overexpression of GRP78 protected hearts from MI/R injury *in vivo* and *in vitro* through stimulation of Akt signaling pathway (12). Moreover, this study revealed that GRP78 could interact with phosphatidylinositol 3-kinase and therefore lead to the stimulation of Akt (12). Thus, our present study was designed to investigate whether activation of XBP1 could activate GRP78/Akt signaling pathway to protect hearts from MI/R injury. Our results revealed that EA pretreatment markedly upregulated XBP1 expression and the downstream GRP78 expression during MI/R injury. Then GRP78 further enhanced Akt signaling to reduce MI/R-induced cell apoptosis. Additionally, in an *in vitro* SI/R model, inhibition of XBP1 could significantly reduce cell viability and increase cell apoptosis and the activities of LDH and CK-MB, indicating a vital role of XBP1 in the pathology of MI/R injury.

The key role of Akt signaling in the pathogenesis of MI/R injury has been illustrated in numerous previous studies. As an important anti-apoptotic pathway, promotion of Akt signaling pathway significantly reduced cell apoptosis to protect hearts and cardiomyocytes from I/R injury (36–38). Our data consistently showed that cell apoptosis determined by TUNEL staining and apoptotic protein expressions was significantly reduced by the induction of XBP1/GRP78/Akt axis. The inhibition of XBP1 in H9c2 cells inhibited Akt phosphorylation, thus execrating cell injury and apoptosis. Our study clearly showed that the inhibition of XBP1 worsened SI/R-induced cell injury.

However, there are some limitations in the present study. First, although we demonstrated that EA pretreatment could increase XBP1 expression, the mechanisms and the upstream regulators of XBP1 were still absent and need further exploration. We speculate that EA pretreatment may lead to the overall metabolic changes of the muscle and alter the profile of myokines. Second, the time point of EA treatment was before MI/R surgery. This will limit the clinical use, and the post-ischemia treatment is more reasonable for clinical application. Third, the *in vitro* data can only demonstrate that the XBP1/GRP78/Akt pathway participates in SI/R-induced cell injury since EA cannot directly treat cell *in vitro*.

In summary, the results of this study suggest for the first time that EA pretreatment upregulated XBP1/GRP78/Akt signaling pathway and improved cardiac function during MI/R injury. Clarification of upstream of XBP1 in the pathological process of MI/R injury will be required to better understand the action of EA protection, which may not only contribute to elucidation of the molecular mechanism but have potential clinical use.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee of Air Force Medical University.

AUTHOR CONTRIBUTIONS

LY, JY, and MZ designed and supervised the study and revised the manuscript. NW, JM, YM, and CM conducted the experiments and collected the data. LL and PQ collected and analyzed the data. EG established the animal model. NW and JM wrote the draft. All authors approved the final manuscript.

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frontiers in Cardiovascular Medicine

BRIEF RESEARCH REPORT
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Cardiomyocyte Protection by Hibernating Brown Bear Serum: Toward the Identification of New Protective Molecules Against Myocardial Infarction

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Ischemic heart disease remains one of the leading causes of death worldwide. Despite intensive research on the treatment of acute myocardial infarction, no effective therapy has shown clinical success. Therefore, novel therapeutic strategies are required to protect the heart from reperfusion injury. Interestingly, despite physical inactivity during hibernation, brown bears (*Ursus arctos*) cope with cardiovascular physiological conditions that would be detrimental to humans. We hypothesized that bear serum might contain circulating factors that could provide protection against cell injury. In this study, we sought to determine whether addition of bear serum might improve cardiomyocyte survival following hypoxia–reoxygenation. Isolated mouse cardiomyocytes underwent 45 min of hypoxia followed by reoxygenation. At the onset of reoxygenation, cells received fetal bovine serum (FBS; positive control), summer (SBS) or winter bear serum (WBS), or adult serums of other species, as indicated. After 2 h of reoxygenation, propidium iodide staining was used to evaluate cell viability by flow cytometry. Whereas, 0.5% SBS tended to decrease reperfusion injury, 0.5% WBS significantly reduced cell death, averaging 74.04 ± 7.06% vs. 79.20 ± 6.53% in the FBS group. This cardioprotective effect was lost at 0.1%, became toxic above 5%, and was specific to the bear. Our results showed that bear serum exerts a therapeutic effect with an efficacy threshold, an optimal dose, and a toxic effect on cardiomyocyte viability after hypoxia–reoxygenation. Therefore, the bear serum may be a potential source for identifying new therapeutic molecules to fight against myocardial reperfusion injury and cell death in general.

Keywords: cardiomyocyte, hypoxia-reoxygenation injury, protection, bear serum, hibernation, novel therapeutic strategy

INTRODUCTION

Despite significant advances in the ability to reperfuse ischemic myocardium and save heart tissue from reperfusion injury, ischemic heart disease remains one of the leading causes of death worldwide. Many therapeutic strategies have been studied, in particular methods of maintaining post-ischemic cell survival, the so-called cardioprotective interventions. However, although much has been learned about the methods and mechanisms of cardioprotection, no effective therapy has shown success in clinical translation.

Over the last decade of research in this area, most cardioprotective strategies have been designed to either target and inhibit a crucial cell death pathway or to activate a specific endogenous cardioprotective pathway (1, 2). We believe that the best strategy to improve both survival and quality of life in patients suffering from myocardial infarction is to minimize myocardial death that occurs due to reperfusion injury. It is also becoming clear that in addition to cardiomyocytes, cardioprotection should also target other cardiac or circulating cell types, and blood-cell-free circulating factors including globulins, micro-RNA, cytokines, receptors and adhesion molecules, which may provide direct or paracrine benefits. As such, there is a need to discover and investigate novel therapeutic targets for cardioprotection.

Many species of mammals, birds, and reptiles have evolved a strategy of reduced metabolic rate and energy conservation for prolonged periods by hibernating. During 4–6 months of hibernation, bears (*Ursus* spp.) do not eat, drink, or urinate; and they show minimal activity, yet they appear to retain normal organ function. Moreover, hibernating bears differ from hibernating rodents in that they maintain a higher body temperature [33–35°C (3) vs. <10°C], and they are reported to be shallow hibernators (4); however, they do not periodically arouse during the entire duration of their hibernation period. Such characteristics therefore make hibernating bears good models in a biomedical context.

To conserve energy during hibernation, the bear's oxygen demand is reduced to ∼25% of the active state (5). Cardiovascular adaptations must occur for the myocardium to remain healthy and efficient during a period of extremely low heart rates and cardiac output (5–7). The cardiac adaptations during hibernation are characterized by a profound bradycardia with extreme respiration sinus arrhythmia and a preserved left ventricular ejection fraction, associated with a decrease in left ventricle mass/volume ratio indicating some degree of cardiac remodeling to adapt to the altered hemodynamic state (8–10). Interestingly, when they emerge from their dens in the spring, bears are free from cardiovascular diseases (11, 12), kidney failure (13–15), sarcopenia (16–18), osteoporosis (19, 20), and other deleterious conditions (21, 22). The contrast with physically inactive humans could not be greater (23–29).

Thus, the hibernation phenomenon is more than biologically interesting because understanding how organs cope with the stresses of hibernation could have direct clinical relevance (30) and especially for cardiovascular disease, such as myocardial infarction. Although we cannot rule out the possible role of parasympathetic and sympathetic nervous systems in the regulation of the cardiac function in bears entering or coming out of hibernation (3, 8), it is likely that circulating compounds may contribute to cardioprotection *in vivo*. Indeed, many blood components have already been proposed to be involved in a humoral mechanism of cardioprotection (31). Moreover, this hypothesis is reinforced by our recent demonstration that hibernating bear serum contains circulating components that can inhibit protein catabolism in cultured human muscle cells, with myosin accumulation in myotubes (32). Therefore, as a way to validate if hibernating bear serum actually contains circulating factors that could provide protection against cell death during hypoxia–reoxygenation (HR) injury, the objective of the present study was to evaluate the effectiveness of an acute treatment with bear serum at reoxygenation on the viability of post-ischemic primary mouse cardiomyocytes.

In this study, cardiomyocytes isolated from adult mice were exposed to HR sequence with brown bear (*Ursus arctos*) serum collected in winter and summer periods (WBS and SBS, respectively); and the therapeutic index of bear serum treatment was evaluated by flow cytometry. Our results showed that the addition of WBS was protective against reperfusion injury at the optimal dose of 0.5%. This effect was lost when the dose was reduced to 0.1% and became toxic above 5% of bear serum addition. Importantly, our results highlight that this profile effect seems to be specific to the serum from bear species.

Our study suggests that bear serum seems to be a potential source for identifying new therapeutic molecules to fight against human myocardial reperfusion injury and cell death in general.

MATERIALS AND METHODS

Bear Serum

During the winters and summers of 2016 and 2019, blood samples were collected from the jugular vein of anesthetized free-ranging subadult (2- to 3-year-old) brown bears (nine females and four males) within 20 min after darting, as described previously (32, 33). Serum samples were prepared (3,000 g, 20 min) within 1 h after sampling and then stored at −80°C until the experiment. Both summer and winter mixes were obtained by pooling the same volume of serum for all bears [see ref. (32)].

Hypoxia–Reoxygenation Model

As previously described (34, 35), adult cardiomyocytes were isolated from 8- to 12-week-old C57Bl/6J male and female mice (Charles River, L'Arbresle, France). Rod-shaped calcium-tolerant mouse cardiomyocytes were then subjected to a suspension-simulated hypoxia in a controlled hypoxic chamber (Eppendorf Galaxy 48R; Eppendorf, Hamburg, Germany), induced by nitrogen flushing up to 1% partial O_2 pressure for 45 min, in 1 ml of a Tyrode solution (140 mM of NaCl, 5 mM of KCl, 10 mM of HEPES, 1 mM of $MgCl_2$, and 1.8 mM of $CaCl_2$ at pH 7.4 at 37°C) (36). Reoxygenation was induced at 37°C by the addition of 1 ml of normal culture medium [MEM #21575022 Gibco®, 10% fetal bovine serum (FBS), 10 mM of BDM, 100 U/ml of penicillin, 2 mM of glutamine, and 2 mM of ATP] supplemented with different serum concentrations. Control groups consisted of cell

suspensions without hypoxic stress in a normal culture medium supplemented with different concentrations of each serum.

Cell Death by Flow Cytometry

At the end of the HR protocol, cardiomyocytes were collected for flow cytometry analysis. Propidium iodide (PI; P4864 Sigma-Aldrich, St. Louis, MO, USA), a cell viability probe, was added extemporaneously before acquisition at 1 μg/ml. Flow cytometry experiments were conducted blindly using Fortessa X-20 (BD Biosciences, San Jose, CA, USA). In total, 1,000 events were acquired per tube. PI was excited at 561 nm, and the emission band-pass filter was collected at 620 nm. Cell death was represented by the percentage of positive cells for PI staining.

Statistical Analysis

The data were analyzed with DIVA Software (BD Biosciences) and were quantified and expressed as mean ± SD, where indicated. Differences in means among multiple groups were analyzed using a two-way ANOVA followed by a Tukey's *post-hoc* test (two variables: experimental groups and experimental days). Statistical significance was set to a threshold of $p \leq 0.05$. No data/animals were excluded from the study. Statistics were computed using GraphPad Prism 6.1 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Hypoxic Cardiomyocytes Treated With Bear Serum Exhibit Reduced Cell Death at Reoxygenation

To determine whether bear serum might provide beneficial effects against reperfusion injury, we first mimicked the bear serum dose published by Chanon et al. (32). As shown in **Figure 1A**, control and hypoxic groups were supplemented at the onset of the reperfusion period with 5% of bear or FBS. Our results showed that, in control groups, the addition of 5% bear serum significantly increased cell death averaging 87.31 ± 11.31% and 75.23 ± 17.76% in SBS and WBS groups, respectively, as compared with 40.29 ± 5.77% in the FBS group ($p < 0.05$; **Figure 1B**). After HR, whereas cell death increased up to 76 ± 5.46% in the FBS group, most cardiomyocytes were dead in both SBS and WBS groups, reaching 95.50 ± 5.23% and 92.76 ± 9.01%, respectively. These surprising results suggest that bear serum treatment seems to be toxic for adult cardiomyocytes at a dose of 5%. Next, we chose to establish the dose–response effects of bear serum by reducing its concentration at reperfusion up to 0.1%.

Interestingly, at a dose of 2.5%, the toxicity of bear serum disappeared in control groups averaging 40.40 ± 4.9%, 42.91 ± 7.02%, and 39.90 ± 6.50% cell death in FBS, SBS ($p = 0.958$), and WBS ($p > 0.999$) groups, respectively (**Figure 1C**). However, after HR, although the cell death was not different between the WBS and FBS groups, averaging 84 ± 66% and 77.42% respectively, the addition of 2.5% of SBS still remained toxic, with a cell death averaging 88.96 ± 12.48% as compared with the FBS group (**Figure 1C**).

Our results showed that the addition of 1% bear serum provided no additional effect on cell death, neither in control groups, averaging 38.87 ± 4.17% in SBS ($p = 0.97$) and 40.48 ± 5.58% in WBS ($p = 0.98$) groups, nor in HR groups, averaging 74 ± 8.14% ($p = 0.64$) and 78.6.20% ($p = 0.71$), respectively, vs. FBS (**Figure 1D**). This suggests no advantage or disadvantage of bear serum treatment to the fate of cardiomyocytes in controls or after HR at this dose.

Treatment of control groups with 0.5% bear serum did not modify cell death, with 40 ± 5.56% in SBS group and 39.03 ± 4.68% in WBS vs. 40 ± 5.81% in the FBS group (p = ns; **Figure 1E**). On the other hand, whereas SBS tended to decrease cell death after HR ($p = 0.09$ vs. FBS), a treatment of 0.5% of WBS at reoxygenation significantly reduced cell death, averaging 74.04 ± 7.06% vs. 79.20 ± 6.53% in the FBS group ($p < 0.05$; **Figure 1D**). To rule out a possible imbalance in the protein content between summer and winter serums, we measured the total protein content of each serum mix. As reported in **Supplementary Table 1**, the total protein content was similar in each bear serum mix ranging from 15.95 ± 1.02 to 18.13 ± 1.29 mg/ml (p = ns). Bear serum collected during summer may nevertheless contain substances in concentrations that may provide protection against cell death and that this phenomenon is amplified with the serum collected in hibernating bears. By continuing to reduce the bear serum dose to 0.1%, our results showed no effect on survival of control cardiomyocytes, with death rates ranging from 36.97 ± 6.08% to 38.95 ± 7.60% (**Figure 1F**). The potential cardioprotective effect of bear serum was lost after HR with death rates of 77.54 ± 2.98% in the SBS group, 77.38 ± 6.68% in the WBS group, and 77.94 ± 5.98% in the FBS group ($p > 0.99$; **Figure 1F**).

Altogether, these results suggest that the efficiency of the bear serum against cell death was dependent on both the dose and the phenotype of sampled bears. Thus, our results highlight a potential cardioprotective role of WBS at 0.5%.

The Therapeutic Effect of Serum Treatment on Cardiomyocyte Viability Is Specific of the Serum From Ursid Species

According to the literature, including cardiomyocyte isolation and maintenance (35, 36), FBS is the most used serum in cell culture. To check whether the serum from other animal species could exert the same effects, we repeated the previous experimental protocol with the addition of two different species of adult serum from horse (#H1138, Sigma) and rabbit (#R4505, Sigma). Similar to the bear samples, horse and rabbit adult serums were not decomplemented. Our results showed that the addition of horse or rabbit serum (5, 2.5, 1, 0.5, and 0.1%) did not influence cardiomyocyte viability in control conditions (**Figure 2**). Moreover, it is interesting to note that the absence of FBS also did not affect cell viability in control group averaging 34.09 ± 4.10%, ruling out the possibility that specific growth factors from the serum of subadult bears are involved in the

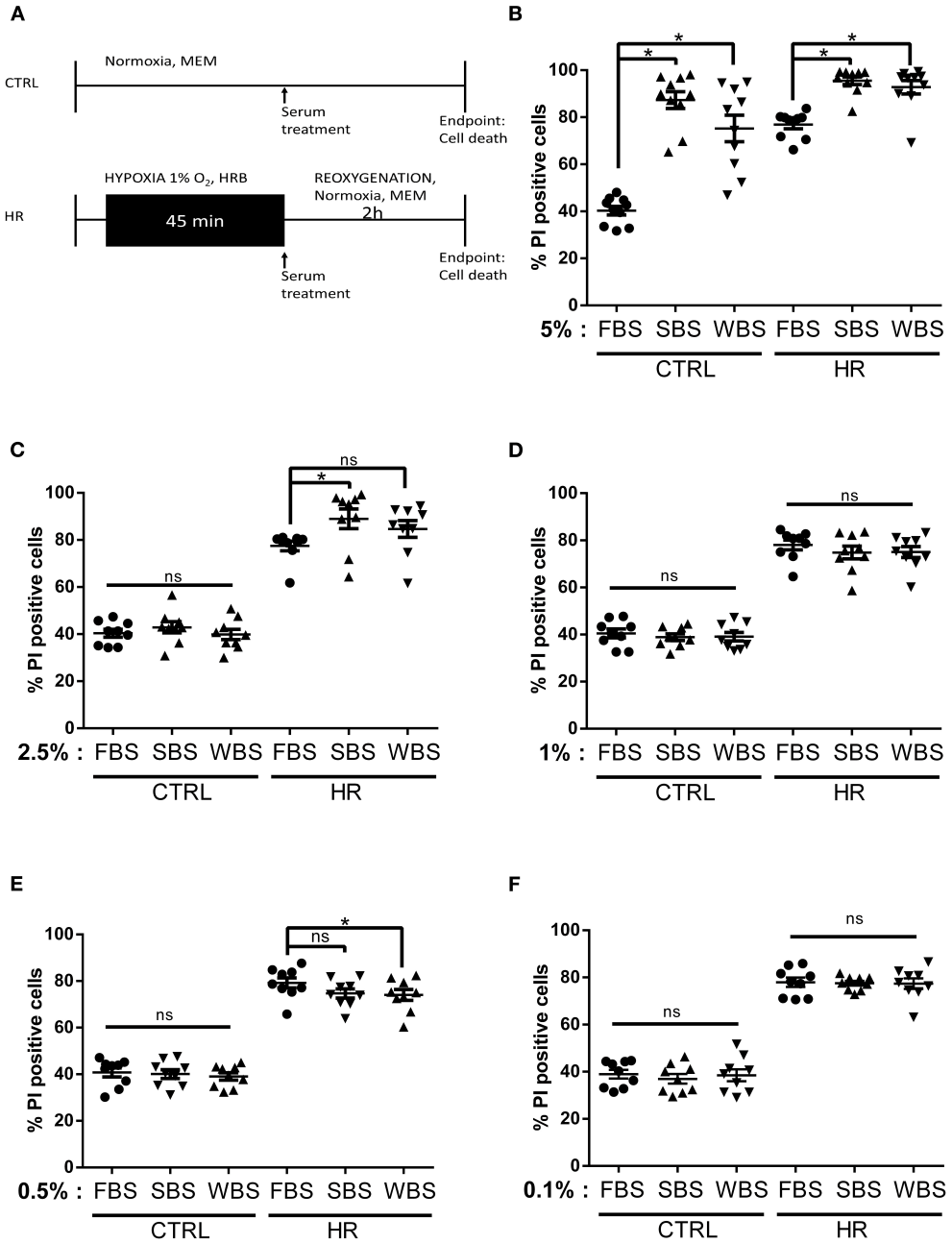

FIGURE 1 | Dose–response effects of bear serum treatment on cardiomyocyte viability: **(A)** Experimental design. Isolated mouse cardiomyocytes underwent 45-min hypoxia followed by 2-h reoxygenation. At the onset of reoxygenation, normoxic and hypoxic cardiomyocytes received serum concentrations as indicated. The percentage of cell death was measured in both normoxic (CTRL) and hypoxic (HR) cells treated with **(B)** 5%, **(C)** 2.5%, **(D)** 1%, **(E)** 0.5%, and **(F)** 0.1% of fetal bovine serum (FBS), summer bear serum (SBS), and winter bear serum (WBS). Mean of propidium iodide (PI) positive cells ± SD, $n = 9$–10 different experimental days with 1,000 events/assay (*$p < 0.05$). Differences in means among multiple groups were analyzed using two-way ANOVA with a Tukey's *post-hoc* test (ns: non significant).

observed effects. Altogether, these results show that, as was the case of fetal serum, the addition of adult horse or rabbit serum did not influence cell viability in our experimental conditions. Although HR stress significantly increased cell death in each HR groups ($p < 0.05$ vs. respecting control group), the addition of different concentrations of adult serums from horse and rabbit did not influence cell death rate ($p =$ ns vs. HR FBS groups) (**Figure 2**). Altogether, these results suggested that the presence of horse or rabbit adult serum in the reoxygenation medium had no impact on the viability of cardiomyocytes after HR. Their use

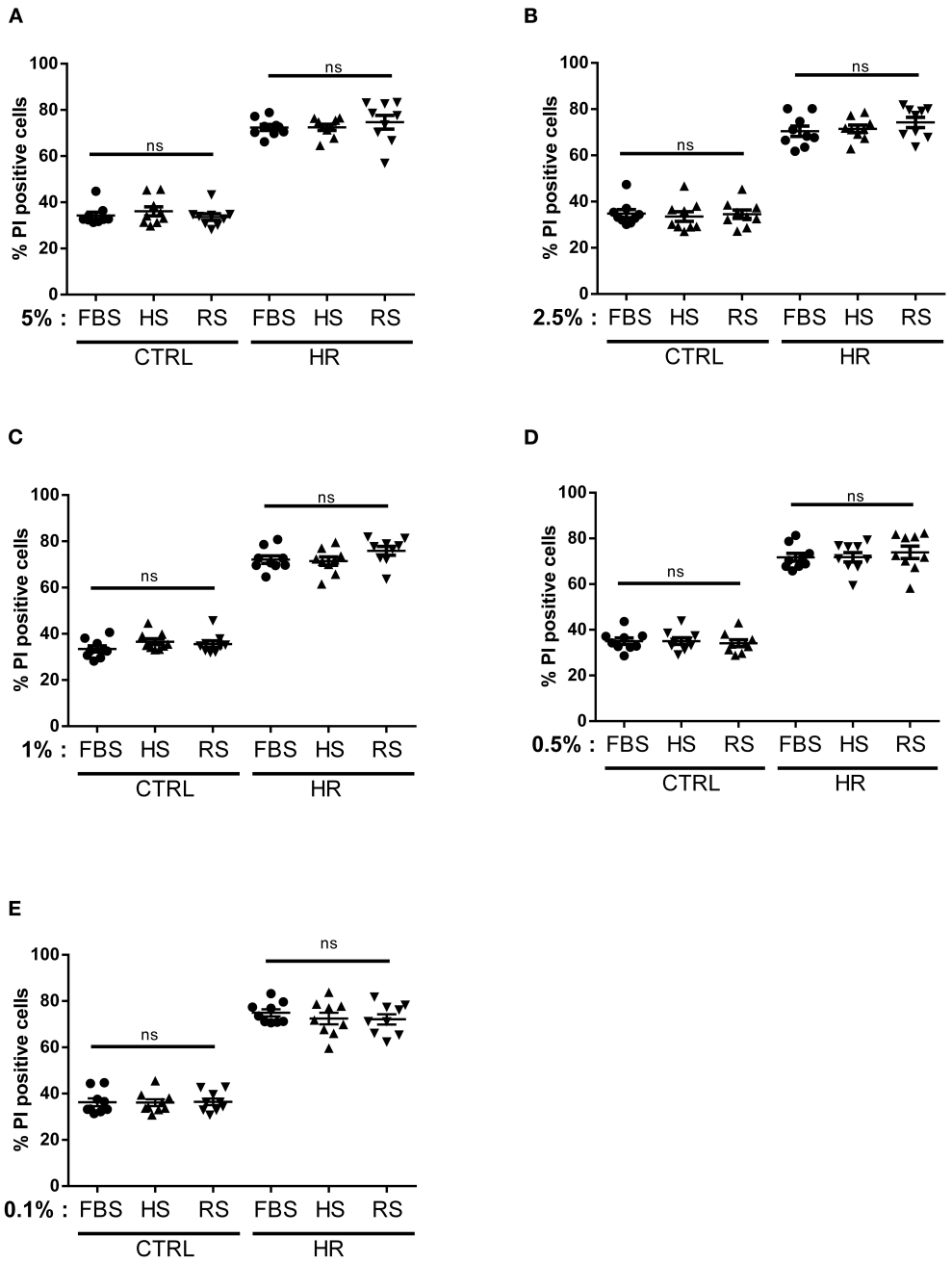

FIGURE 2 | Dose–response effects of adult serum treatment on cardiomyocyte viability: percentage of cell death measured in both normoxic (CTRL) and hypoxic (HR) cells treated with **(A)** 5%, **(B)** 2.5%, **(C)** 1%, **(D)** 0.5%, and **(E)** 0.1% of fetal bovine serum (FBS), horse serum (HS), and/or rabbit serum (RS). Mean of propidium iodide (PI) positive cells ± SD, $n = 9$ different experimental days with 1,000 events/assay. Differences in means among multiple groups were analyzed using two-way ANOVA with a Tukey's *post-hoc* test (ns: non significant).

does not seem more advantageous than that of FBS. Moreover, without any dose–effect relationship of horse and rabbit adult serum on cell viability, our results did not show any threshold, toxic, or efficient effect on cell death. These results reinforced the conclusion that the efficacy profile we have observed in the presence of bear serum is specific to ursid species.

DISCUSSION

In this study, we described that bear serum presented a therapeutic profile from an efficacy threshold to a toxic effect on cardiomyocyte viability after HR. Although 0.5% SBS tended to decrease reperfusion injury, 0.5% WBS

significantly reduced cell death after HR. This cardioprotective effect was lost at 0.1% and became adverse at 5%, which suggests a therapeutic window between 0.1 and 1% of bear serum. Moreover, our results demonstrated that this therapeutic profile was specific to the serum from brown bears, in contrast to horse or rabbit serum. As a new approach to overcome the lack of efficient treatment in clinical cardioprotection, our results suggest that hibernating bear serum might be a source for identifying new cardioprotective molecules.

Accordingly, these data reinforce previous research showing that treatment with serum could provide beneficial effects against some pathologies (37, 38). Indeed, it has been demonstrated that treatment of cells with serum from patients with myocardial infarction prevents inflammation in cardiomyocytes, thereby protecting healthy tissue (37). Others have demonstrated that human serum albumin treatment reduces ischemia–reperfusion injury in skeletal muscle in a rabbit model (38). Altogether, our results add to this that bear serum might contain molecules that confer cardioprotection against cell death during reperfusion injury.

Our data further demonstrated that, although SBS and WBS seem to induce similar responses to cardiomyocyte viability, WBS was more favorable than SBS. Indeed, when mimicking experimental protocols from our group on human myotubes, we were surprised to measure a toxic effect with 5% bear serum treatment, but it is worth noticing that the toxic effect was always lower in the hibernating serum, compared with the summer serum. According to the literature (39–45), the potential cytotoxicity of serum for *in vitro* cells culture involves mainly the activation of the complement, and several hormones and inhibitory growth factors, as well as the toxic effects of polyamines, exosomes, and potential molecules that cause oxidative stress and stimulate pro-inflammatory cytokine release known to trigger apoptosis. We cannot also exclude that high serum concentration may affect cell metabolism in our model. Such mechanisms remain to be investigated in depth in the future.

On the other hand, both serums tended to reduce cell death at reperfusion, but only WBS provided a significant reduction of 6% of cell death, compared with FBS positive controls. We are aware that this decrease is modest, but it is an encouraging result for the development of a new cardioprotection strategy. Moreover, it remains to be determined whether the promising effects that were observed may be affected by the anesthetic agents used to immobilize the bears. Now, we must optimize the protocol and the analysis to decipher the optimal dose between 0.1 and 1% serum treatment, the optimized temperature (because hibernating body bear temperature is reduced to 33–35°C), and to decipher which active molecules are similarly present in SBS and WBS and which molecules are not active to explain the efficacy gap between the seasons.

Several reports, including those of Kim et al. (46), provided the evidence that the composition of serum itself could influence myocardial mRNA, which may provide beneficial or deleterious effects in ischemia–reperfusion models. Among the circulating components that are known to show seasonal regulation in bears (47), fatty acids, whose composition are changed due to prolonged fasting, could play a role (32). Moreover, as described by Zolla (48), future studies are required to identify the therapeutic small molecules that confer cardioprotection *via* bear serum treatment. Finally, it is worth to notice that this novel therapeutic strategy was specific to the serum of Ursidae family origin, since supplementation with adult horse or rabbit serums did not impact cell viability as compared with respective controls.

CONCLUSION

Our results demonstrate that although active molecules have not yet been identified, bear serum and more especially, hibernating bear serum provide specific cardioprotection against reperfusion injury. Our demonstration of the protective effect of serum molecules coming from hibernating animals in non-hibernating animals opens a new therapeutic avenue for identifying cardioprotective molecules with future applications in humans.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Swedish Ethical Committee on Animal Experiment (applications Dnr C3/2016 and Dnr C18/2015), the Swedish Environmental Protection Agency (NV-0741-18), the Swedish Board of Agriculture (Dnr 5.2.18–3060/17), and the French Claude Bernard Lyon 1 University animal research committees #19896-201903212127912.

AUTHOR CONTRIBUTIONS

EL, FB, and LGo: conceptualization and methodology. LGi, CCDS, and LGo: investigation. LGi and LGo: writing—original draft. EL, FB, JS, JA, GG-K, LGi, CCDS, and LGo: writing—review. JS and JA: english editing. FB, EL, GG-K, and LGo: funding acquisition. LGo: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.687501/full#supplementary-material

Supplementary Table 1 | Bear serum characteristics: Description of different bear serum mix, with single bear ID number, Year of collection, Age, Gender and the protein content of each seasonal mix bear serum. Mean of protein content (mg/ml) ± SD.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cardiovascular Medicine

REVIEW
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Cardioprotective Roles of Endothelial Progenitor Cell-Derived Exosomes

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With the globally increasing prevalence, cardiovascular diseases (CVDs) have become the leading cause of mortality. The transplantation of endothelial progenitor cells (EPCs) holds a great promise due to their potential for vasculogenesis, angiogenesis, and protective cytokine release, whose mechanisms are essential for CVD therapies. In reality, many investigations have attributed the therapeutic effects of EPC transplantation to the secretion of paracrine factors rather than the differentiation function. Of note, previous studies have suggested that EPCs could also release exosomes (diameter range of 30–150 nm), which carry various lipids and proteins and are abundant in microRNAs. The EPC-derived exosomes (EPC-EXs) were reported to act on the heart and blood vessels and were implicated in anti-inflammation, anti-oxidation, anti-apoptosis, the inhibition of endothelial-to-mesenchymal transition (EndMT), and cardiac fibrosis, as well as anti-vascular remodeling and angiogenesis, which were considered as protective effects against CVDs. In this review, we summarize the current knowledge on using EPC-EXs as therapeutic agents and provide a detailed description of their identified mechanisms of action to promote the prognosis of CVDs.

Keywords: cardiovascular diseases, EPC-derived exosomes, anti-inflammatory, anti-apoptosis, cardiac fibrosis, cell regeneration

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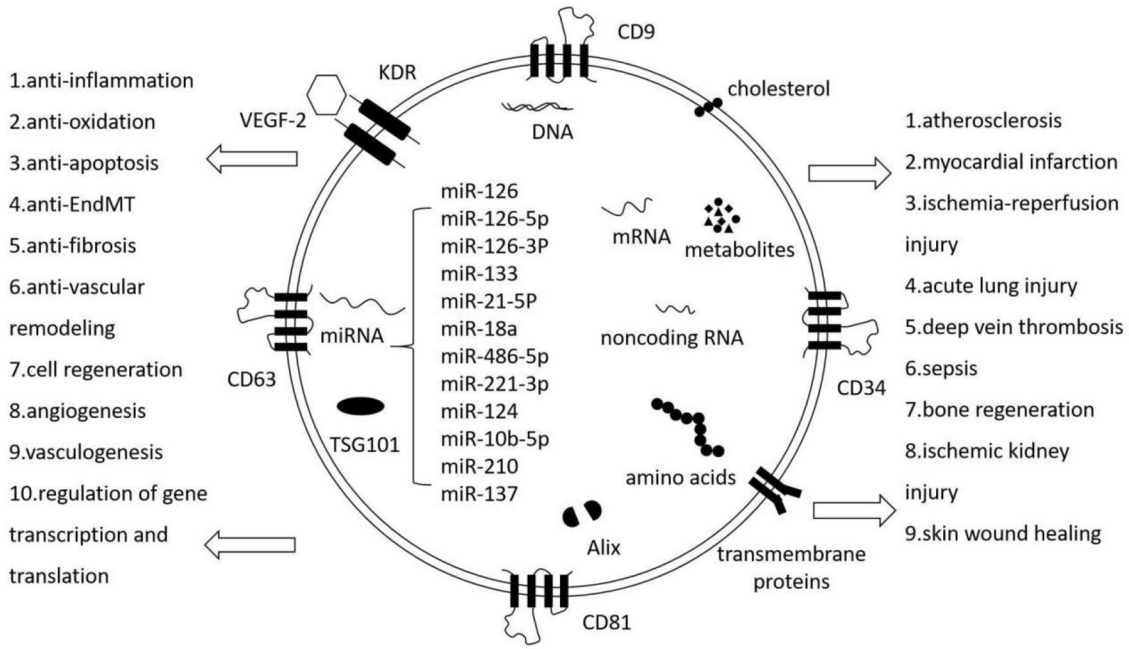
INTRODUCTION

Cardiovascular diseases (CVDs), especially acute myocardial infarction (AMI), are a primary cause of death worldwide, responsible for an estimated 31% of all deaths, and 17.9 million lives each year. Events that contribute to AMI and subsequent complications are multifaceted, such as thrombosis, the activation of renin–angiotensin–aldosterone system (RAAS), cytokine release, oxidative stress, inflammatory effects, endothelial dysfunction, and ventricular remodeling (1–3). Endothelial dysfunction is an early marker of CVDs and the following complications. Many factors such as endothelial nitric oxide synthase (eNOS) uncoupling and inflammatory cytokines reduce endothelial FGFR1 and endothelial glucocorticoid receptor expression and activate transforming growth factor-beta (TGF-β) and Wnt signaling. TGF-β activates Smad proteins and shuttles to the nucleus to interact with Snail, Twist, and Slug to induce endothelial-to-mesenchymal transition (EndMT) (4, 5). Wnt signaling contributes to the disruption of cytokine and chemokine homeostasis, and results in EndMT (6). Mounting evidence indicates that EndMT is involved in CVDs, including pulmonary hypertension, atherosclerosis, and valvular disease. Reperfusion strategies, especially bypass surgery and percutaneous coronary intervention (PCI), are currently the main treatments for AMI, and are accountable for a significant reduction of AMI-related



GRAPHICAL ABSTRACT | EPC-EXs: The hallmarks and pleiotropic functions. EPC-EXs are extracellular vesicles generated by EPCs and they carry lipids, proteins, nucleic acids, metabolites, and some EPC markers. They mediate intercellular communication and affect various aspects of cell biology.

morbidity and mortality. With the extensive development of cell therapies for clinical application, stem cell-based AMI therapy has shown a great promise clinically in regenerating damaged myocardium and enhancing cardiac function in animal models and patients (7, 8). Endothelial progenitor cells (EPCs) are considered as highly potent regenerating cells with strong proliferative ability in response to tissue ischemia or hypoxia. For example, intravenously injected EPCs could incorporate into post-infarct myocardium, differentiate into new blood vessel in the infarct bed (vasculogenesis), and trigger the proliferation of pre-existing vasculature (angiogenesis) (9, 10). In fact, the general consensus is that only a minority of EPCs survive and engraft after transplantation because of potential immunological rejection, chromosomal variation, embolus formation, and so on (11). Nonetheless, EPC transplantation was shown to improve function and prognosis in AMI to an extent, and this was attributed to EPCs secreting paracrine factors and the more recently studied exosomes (12, 13). EPC-derived exosomes (EPC-EXs) were critical paracrine factors in promoting endothelial dysfunction, such as anti-inflammation, anti-oxidation, anti-apoptosis, inhibition of EndMT and cardiac fibrosis, anti-vascular remodeling, and angiogenesis in CVDs. EPC-EXs also showed protective effects in acute pulmonary injury, sepsis, ischemic kidney injury, skin wound healing, and so on. Except for exosomes marker, EPC-EXs also expressed many molecules characteristic of EPCs signatures such as CD34, vascular endothelial growth factor receptor (VEGFR)-2, and kinase insert domain receptor (KDR), and presented functions of EPCs such as enhancing EPCs proliferation, migration, and angiogenic tubule formation as well (**Graphical Abstract**). In this review, we summarize our current knowledge on using EPC-EXs as therapeutic agents for CVDs and discuss the identified mechanisms through which they exert their effects.

EPC-DERIVED EXOSOMES

Characteristics of Exosomes

Extracellular vesicles (EVs), which have a key role in intercellular and even interorganismal communication, include three subpopulations based on intracellular origin and size: microvesicles, exosomes, and apoptosomes. Microvesicles (diameter range of 50 nm−1 μm, and in some cases up to 10 μm) originate from the fission of the plasma membrane and direct outwards budding. Apoptosomes (diameter range of 500 nm−5 μm) resulted from the apoptotic cell disassembly. Compared with other secreted vesicles, exosomes have much better-defined roles in several biological and pathological processes. Exosomes (diameter range of 30–150 nm) carry various lipids and proteins and are abundant in mRNA, DNA, microRNA, long non-coding RNA, and further nucleic acid species (14). In general, exosome biogenesis consists of three steps: the formation of endocytic vesicles from the plasma membrane, the inward budding of the endosomes resulting in intracellular multivesicular bodies (MVBs) that consist of intraluminal vesicles (ILVs), and the release of these MVBs known as exosomes (15). Exosomes have been isolated from breast milk, blood, urine, cerebrospinal fluid, saliva, etc. by differential centrifugation, monoclonal antibody-based methods,

and ultrafiltration. Also, the previous research has demonstrated a workflow to quantitatively analyze proteins of extracellular vesicle subgroups by an optimized method combining polymer-based precipitation and size exclusion chromatography (16). It was reported that various cells release exosomes, including mesenchymal stem cells (MSCs), neurons, T lymphocytes, B lymphocytes, endothelial cells (ECs), EPCs, tumor cells, and others. Since this discovery, numerous investigations have identified exosomes as a means of intercellular communication with a beneficial role in physiological processes, such as immune response, inflammation, and cell regeneration (17–19). They were also implicated in the pathogenesis of atherosclerosis, vascular remodeling, and thrombosis (20), as well as the development and deterioration of diseases such as tumors, Alzheimer's disease, and HIV-1 infection (21–23). Exosomes also contained organ-protective antifibrotic microRNAs, such as miR-29 and let-7s. A previous study has demonstrated that exosome-encapsulated miR-29 attenuated kidney fibrosis by downregulation of YY1 and TGF-β3. Transcription factor YY1 directly upregulated αSMA and collagen. TGF-β3 activated SMAD-based or non-SMAD-based pathways, resulting in fibrosis (24–26). Other non-coding RNAs included circular RNA (circRNiAs), P-element induced Wimpy testis (PIWI)-interacting RNAs (piRNAs), and long non-coding RNA (lncRNAs) except miRNAs. CircRNAs were a class of endogenous non-coding RNAs that formed a closed continuous loop without 5′ caps and 3′ poly tails. CircRNAs were found to regulate the transcription and function of miRNA-target genes and participated in the pathogenesis of multiple CVDs (27). piRNAs were a class of small RNAs that were 24–31 nucleotides in length. piRNAs silenced gene expression by interacting with PIWI proteins and guiding them to silence transposable elements (28). LncRNAs were non-protein-coding RNAs longer than 200 nucleotides. LncRNAs regulated gene expression at transcriptional, post-transcriptional, and epigenetic levels (29). IV-injected exosomes were detected in the spleen, followed by the liver, then the lungs and kidneys, but the brain, heart, and muscle showed lower amounts than others. Also, the curve of exosomes distribution presented a rapid distribution phase followed by a longer elimination phase *via* hepatic and renal routes. Moreover, they demonstrated that systemically injected exosomes can be delivered to tumor sites quickly. It was also reported that there was a difference in the biodistribution of exosomes according to the exosome-producing cells. Exosomes, which as both synthetic nanocarriers and cell-mediated drug delivery systems, enhanced tissue bioavailability and efficacy of relevant drugs (30–32). However, significant gaps remain in the complete understanding of the role of exosomes in diseases.

EPC-Derived Exosomes

Previous studies have demonstrated affirmatively that EPCs are important therapeutic agents in the field of regenerative medicine, with potential utility in both cardiovascular therapies and other tissue engineering applications. Likewise, EPCs also synthesize and secrete functional exosomes that participate in angiogenesis and endothelium repair (33). EPCs-EXs were also found to display various beneficial therapeutic potentials compared to EPCs: regardless of political or ethical questions, they reduce the incidence of infectious diseases and tumor formation; they are available in large quantities, i.e., artificial exosomes can also be produced using clinical-grade synthetic lipids, recombinant proteins, and gene engineering in the future; and they do not induce significant immune responses after repeated transplantation. A comparison of EPCs and EPC-EXs is presented in **Table 1**. Exosomes from EPCs indeed carry a diversity of transcription factors, including exosome markers such as CD63, CD81, CD9, as well as many molecules that are signature characteristics of EPCs, such as CD34, VEGFR-2, and KDR (34). Exosomes from EPCs have also been found to contain lipids, proteins, mRNAs, precursor miRNAs (pre-miRNAs), miRNAs, and so on. Few studies have yet separated the functions of EPC-microvesicles (EPC-MVs) and EPC-EXs completely because of the limitations of isolation and identification methods. Wang et al. presented novel specific and sensitive methods for detecting EPC-MVs/EPC-EXs from cell culture medium and human plasma compared with previous techniques to differentiate exosomes by the combination of microbeads, fluorescence Q-dots, and nanoparticle tracking analysis (NTA) techniques. The average size of EPCs-MVs and EPC-EXs was 120 ± 1000 nm and 154 ± 59 nm by NTA analysis, which were in accordance with previous observations. EPC-specific antibody (CD34, KDR)-conjugated microbeads combined with fluorescence Q-dots were able to isolate and phenotype EPCs-MVs/EPC-EXs from biofluids (35, 36). EPC-EXs were released by abundant external stimuli, such as inflammatory conditioning of parental cells or hypoxia, indicating that the surrounding environment of EPCs would impact on exosome release. For example, EPCs decreased exosome release and downregulated the set of CD63, Alix, and Rab27a genes in response to diabetic-stimulated condition compared to normal condition (37). In another case, exosomes from endothelial colony-forming cells (ECFCs, a type of EPCs) in hypoxia significantly ameliorated cardiac fibroblasts by the reduction of miR-10b-5p, which targeted the fibrotic genes smad ubiquitin regulatory factor 1 (Smurf1) and histone deacetylase 4 (HDAC4), but did not exhibit this role in the normal condition (38). Moreover, moderate exercise could upregulate the levels of EPC-EXs and the amount of carried miR-126, and EPC-EXs hampered ECs apoptosis and angiogenic dysfunction through the modulation of miR-126/SPRED1/VEGF in a HG and hypoxia dual injury rat model; EPC-EXs thus improved the recovery of neurological function by alleviating acute brain cell apoptosis (39, 40). The previous observations suggested that healthy subjects and patients with different diseases released exosomes with different RNA and protein contents into the circulation, which could be measured as biomarkers. There were different levels of $CD34^{+}KDR^{+}$ EPC-EXs at different times during ischemic stroke, which may be used as biomarkers for diseases and indicators for the prognosis of and therapeutic efficacy for ischemic stroke (35).

miRNAs of EPC-Derived Exosomes

It has been clearly evidenced that miRNAs play a critical role in various pathological and physiological processes by

TABLE 1 | Comparison of EPCs and EPC-EXs in cell therapy.

	EPCs	EPC-EXs
Vasculogenesis	+	+
Angiogenesis	+	+
Anti-inflammatory	+	+
Anti-apoptosis	+	+
Cell regeneration	+	+
Anti-fibrosis	+	+
Disease biomarkers	+	+
Tumorigenicity	+	N
Tumor metastasis	+	N
"Capture" stenosis	+	N
Immunological rejection	+	N
Chromosomal variation	+	N
Emboli formation	+	N
Phenotype change	+	N

+, data available; N, No data available.

regulating gene expression at the post-transcriptional level (41). Experimental studies identified that exosomes carry a distinctive repertoire of microRNAs (miRNAs) and other small non-coding RNAs, such as piRNAs, circRNAs, and lncRNAs (42). The proportion of miRNA in exosomes was found to be higher than that in their parent cells (43). There were various modes for cells to selectively sort miRNA into exosomes such as miRNA motif, sumoylated heterogeneous nuclear ribonucleoprotein (hnRNP)-dependent pathway, neural sphingomyelinase 2 (nSMase2)-dependent pathway, and miRNA induced silencing complex (miRISC)-related pathway (44–46). Exosomal miRNAs have been found to be more stable than free miRNAs and therefore had lasting effects on disease-related gene expressions. miRNAs from exosomes are considered important regulators of various of cellular processes involving cell–cell communication, such as cell survival and proliferation (47). EPC-EXs have abundant levels of miRNAs, which are a growing class of non-protein-coding single-strand RNAs consisting an average of only 22 nucleotides. miRNAs are derived from primary miRNAs, which are transcribed by RNA polymerase II from their own non-coding gene or from the introns of protein-coding genes. Primary miRNAs are cleaved into an average of 70-nucleotide-long miRNA precursor (pre-miRNA) by Drosa, which is then excised by Dicer into mature miRNAs that assemble with an argonaute protein to form the miRNA-induced silencing complex (RISC). The selective packaging of miRNAs in EPC-EXs (including EPC-MVs, since many earlier studies did not separate EPC-EXs from EPC-MVs) and their functions were also found important for disease treatment (**Table 2**) (38, 48–63). They are rich in cardioprotective and proangiogenic miRNAs, such as miR-126, miR-133, and miR-486. For instance, the miR-126 gene was indicated to be expressed in combination with its host gene Egfl7, which plays a role in angiogenesis (64). Mounting evidence has revealed that miRNA-126 from EPC-EXs plays a critical role in cardiomyocyte protection, neovascularization, vascular homeostasis, repair, and thus the therapy of various vascular diseases. Sun et al. found that EPC-EXs loaded with miRNA-126 enhanced migration and angiogenesis in EPCs *in vitro* and significantly promoted thrombus resolution in an animal model of venous thrombosis (49). In a murine model of sepsis, miR-126-3p and miR-126-5p from EPC-EXs could maintain vascular homeostasis by reducing LPS-induced upregulation of vascular cell adhesion molecules-1 (VCAM-1) and high-mobility group box protein-1 (HMGB-1) in ECs, thereby reducing lung microvascular endothelial inflammation and dysfunction; the effects were reversed by transfecting with inhibitors of miR-126-3p and 5p (53). Other miRNAs, such as miRNA-18a, miR-21-5p, and miR-133, also participated in promoting endothelial dysfunction and inhibit myocardial fibrosis.

THERAPEUTIC POTENTIAL OF EPC-DERIVED EXOSOMES FOR THE CARDIOVASCULAR SYSTEM

Anti-inflammation and Anti-oxidation

CVDs are closely associated with inflammation, oxidative stress, and redox signaling. Persistent low-grade inflammation was shown to cause immunosenescence within the aging process, which contributed to endothelial dysfunction, atherosclerosis, activating RAAS, cardiac remodeling, and cardiovascular complications (65). Oxidative stress leads to eNOS uncoupling, whose functional manifestation is endothelial dysfunction (66). Thus, it is worth noting that targeted anti-inflammation and anti-oxidation therapy can lower cardiovascular mortality. It is well-established that exosomes of various cellular origin, including EPCs, participate in the inhibition of inflammation response to repair tissue in *in vitro* and *in vivo* models (67, 68). Zhou et al. demonstrated that EPC-EXs treatment significantly attenuated these increases of inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, interferon (IFN), macrophage inflammatory proteins (MIP)-1, MIP-2, monokine induced by gamma interferon (MIG), and interferon gamma-induced protein (IP)-10 (50, 53). One study indicated that ECFC-derived exosomes (ECFC-EXs) were enriched in exosome markers tumor susceptibility gene101 (TSG101) and CD63. Treatment with ECFC-EXs blocked hypoxia/reoxygenation (H/R)-induced increases due to the expression of the proinflammatory protein intercellular cell adhesion molecules-1 (ICAM-1) and endothelial cell apoptosis (69). Interleukin (IL)-10 is an anti-inflammatory cytokine that suppresses macrophage and proinflammatory Th17 T-cell responses by inhibiting the inflammatory cytokines IL-6, IL-12, and IL-23. Another study also demonstrated that EPCs augmented the LPS-induced production of macrophage IL-10 and expression of miRNA-126 and miRNA-125b, which regulated EC function and inflammation, thus promoting the decrease of lung vascular leakage, liver, and kidney injury in sepsis *in vivo* (70). According to various studies, EPC-EXs provided anti-oxidative properties through reducing reactive oxygen species (ROS) production

TABLE 2 | Manuscripts demonstrating the functional effects of miRNA in EPCs-derived microvesicles/exosomes in cardiovascular diseases.

Type	Stimulus	Biological effect	Recipient cell	Relevant molecular mechanism	Ref.
miR-126	–	Promoted proliferation, angiogenesis, migration and acute pulmonary injury	Endothelial cells	Inhibited SPRED-1, activated the RAF/ERK signaling	(48)
	–	Promoted migration, tubulogenic activity, angiogenesis thrombosis resolution and recanalization	EPCs	Inhibited Pcdh7	(49)
	Lipopolysaccharide (LPS)	Reduced permeability, inflammation and pulmonary edema	Alveolar	Decreased HMGB-1, PIK3R2, and VEGFα, increased claudin1, claudin4 and occludin	(50)
	Lentivirus	Reduced apoptosis and promoted proliferation and migration	Osteoblast cells MC3T3-E1	Increased levels of Bcl-2 and p-Erk1/2 protein expression	(51)
	Uncontrolled diabetes healthy controls	Promoted migration, reduced apoptosis, and ROS production	EPCs	Increased VEGFR2	(52)
miR-126-5p, miR-126-3p	Sepsis	Prevented microvascular dysfunction, improved sepsis outcomes	Microvascular	Decreased HMGB-1 and VCAM1	(53)
	–	Improved proliferation, migration, and angiogenic capacity	Endothelial cells	Increased VEGFA, bFGF, TGFB1, and ANG	(54)
miR-133	Hypoxia/reoxygenation	Promoted angiogenesis and inhibited MEndoT of cardiac fibroblasts	Cardiac fibroblasts	Increased CD31, VE-cadherin and vWF, decreased α-SMA, N-cadherin, vimentin, and collagen I, increased YBX-1, SYNCRIP and hnRNPA2B1	(55)
miR-21-5p	Balloon injury	PROMOTED proliferation, migration, angiogenic capacity and EC repair.	Endothelial cells	Suppressed THBS1	(56)
miR-18a	Hypoxia/reoxygenation	Decreased apoptosis, promoted angiogenesis	Aging endothelial cells	Decreased Nox2, increased nitric oxide and eNOS, activated the miR-18a/eNOS/NO pathway	(57)
miR-486-5P	Hypoxia	Blocked apoptosis, reduced ischemic kidney injury	Endothelial cells	Decreased PTEN	(58)
miR-221-3p		Promoted proliferation, angiogenesis, and skin wound healing in diabetic mice	Diabetic skin wounds	Decreased p27, caspase-3, E-selectin, c-Jun N-terminal kinase involved in the AGE-RAGE signaling pathway.	(59)
miR-124	–	Reversed the migration and osteoclastic differentiation, enhanced fracture healing	Bone marrow-derived macrophages	Increased lncRNA-MALAT1 and ITGB1	(60)
miR-10b-5p	Normoxia	Anti-fibrotic and reduced cardiac fibrosis	Cardiac fibroblasts	Decreased HDAC4 and Smurf1	(38)
miR-210	Hypoxia/reoxygenation(H/R)	Reduced H/R-induced endothelial cell apoptosis, ROS overproduction and angiogenic dysfunction, improved mitochondrial function	Endothelial cells	Decreased mitochondrial fragmentation, elevated MMP and ATP level	(61)
	Oxygen-glucose deprivation (OGD)	Promoted proliferation	EPCs	Increased Ca2+ fluctuation	(62)
miR-137	Oxyhemoglobin	Reduced the number of apoptotic neurons	SH-SY5Y cells	Increased COX2 and PGE2	(63)

and enhancing eNOS expression (52, 57). The investigations revealed that EPC-EXs exerted protective effects through the inhibition of inflammation reaction and the promotion of anti-oxidation.

Anti-apoptosis

The apoptosis (programmed cell death) of cells has been previously identified as an important process in a variety of CVDs, including atherosclerosis, heart failure, ventricular

TABLE 3 | Therapy by exosomes/microvesicles for cardiovascular repair in animal *in vivo* models.

Species/type of injury or *in vivo* assay	Dosage	Therapeutic type	Function, highlights	Ref.
C57/BL6 male mice/deep venous thrombosis (inferior vena cava)	300 μg of Exo or miR-126-Exo	Were transplanted into the femoral vein *in situ*.	Promoted the migration and angiogenesis of EPCs, improved thrombus organization and recanalization	(49)
Mice/left anterior descending coronary artery (LAD)	2×10^9 particles(exosomes)	Were transplanted intramyocardially into the left ventricular wall (border zone) at three different locations immediately after left anterior descending ligation	Decreased MI scar size and promoted neovascularization	(90)
Female SD rats/balloon injury (left common carotid artery)	2×10^{12} exosome particles	No data available	Accelerated re-endothelialization of the injured arteries	(91)
Male SD rats/balloon injury (left common carotid artery)	30 μg exosomes	Intravenously injected	Promoted EC repair; inhibited neo-intimal hyperplasia	(92)

remodeling, pulmonary arterial hypertension, and other peripheral arterial diseases (71–73). In hypoxic circumstances, the cell initiates a cascade of events such as energy deprivation, radical formation, and in particular ROS generation that lead to apoptotic cell death (74). Many emerging studies have suggested that EPC-EXs presented cell protective features of anti-apoptosis by modulating miRNAs and a variety of downstream signaling pathways (57, 61). However, the detailed underlying mechanisms of the anti-apoptotic effect of EPC-EXs remain unclear.

Inhibition of Endothelial-to-Mesenchymal Transition (EndMT) and Cardiac Fibrosis

Excessive cardiac fibrosis is a significant problem in nearly all types of CVDs. Cardiac fibrosis originates from fibroblast proliferation and strong activation, and EndMT partially enhances the process of fibrosis in organs including in the heart. EndMT is a process where ECs reduce the expression of endothelial genes/proteins (CD31, VE-Cadherin) and increase the expression of mesenchymal genes/proteins such as alpha-smooth muscle actin (α-SMA), vimentin, Pro-collagen, and fibroblast-specific protein-1 (FSP-1). The TGF-β signaling system activates SMAD proteins from complexes and interacts with key regulators of EndMT: SNAI1, SNAI2, ZEB1, ZEB2, KLF4, TCF3, and TWIST. These interactions culminate in chromatin rearrangements and transcription factor binding to endothelial, mesenchymal, and other relevant gene promoter regions that induce EndMT (5, 75). Signaling molecules involving Wnt/β-catenin, endothelial FGFR1 signaling, mitochondrial protein endothelial SIRT3, and nuclear receptor endothelial glucocorticoid receptor are also the endogenous anti-EndMT molecules and their loss leads to activation of EndMT events in organs. eNOS uncoupling and inflammatory cytokines reduce endothelial FGFR1 and endothelial glucocorticoid receptor expression and activate TGF-β and Wnt signaling. A previous study has demonstrated that loss of SIRT3 in ECs disrupted the EC homeostasis, displayed a higher level of TGFβ-smad3 signaling, and displayed defective metabolism-associated EndMT (76). N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is an endogenous anti-fibrotic peptide, which is associated with fibroblast growth factor receptor1 (FGFR1). Li et al. have investigated that endothelial FGFR1 deficiency in diabetic mice resulted in severe organ fibrosis in both the kidney and heart *via* the induction of AcSDKP-resistant EndMT (77). Mesenchymal-to-endothelial transition (MEndoT) could make fibroblasts obtain the functions of ECs and make them participate in angiogenesis in the cardiac injury area, which could reverse cardiac fibrosis (78). Recent evidence suggested that EPC-EXs promote fibroblast angiogenesis and MEndoT through the intercellular transfer of miR-133, thereby attenuating cardiac fibrosis (41). Another investigation confirmed that EPC-EXs enhanced the proliferation and angiogenesis of cardiac fibroblasts *in vitro* and increased the expression of the EC-specific markers, including CD31 and VEGF-2, and decreased the expression of proteins involved in fibrosis, such as α-SMA, vimentin, collagen I, TGF-β, TNF-α, and HMGB1. Therefore, EPC-EXs promoted the proliferation and angiogenesis of cardiac fibroblasts by inhibiting EndMT and decreasing the expression of HMGB1 (79). EPC-EXs inhibit the progression of cardiac fibrosis by mediating homeostasis of EndMT and MEndoT.

Cell Regeneration, Anti-vascular Remodeling, and Angiogenesis

Vascular remodeling, which is a typical pathological characteristic of various CVDs, such as atherosclerosis, hypertension, pulmonary hypertension, and myocardial hypertrophy (80–82), is a critical target in the treatment of CVDs. Therapeutic angiogenesis offers another promise to improve blood supply in ischemic CVDs (83, 84). It was indicated that human EPC-EXs enhanced the proliferation and migration of endothelial cells *in vitro* and promoted vascular repair in rat models of balloon injury by upregulating ECs function *in vivo* (85). In another rat model of balloon-induced carotid artery injury, it was demonstrated that the administration of EPC-EXs potentiated re-endothelialization after endothelial damage probably through inhibiting thrombospondin-1 (THBS1) and delivering miR-21-5p (56). Chen et al. demonstrated that EPC-EVs (including EPC-MVs and EPC-EXs) enhanced peri-infarct angiogenesis and hemodynamics after MI (86). All of these

scholars paid attention to the importance of anti-vascular remodeling and angiogenesis. Meanwhile, more effort should be made to study the molecular mechanisms of anti-vascular remodeling and angiogenesis by EPCs-EXs.

Function of Gene-Modified EPC-EXs

With extensive ongoing research on EPC-EXs, many researchers have focused on the superiority of EPC-EXs combined with gene transfer for therapeutic angiogenesis and vasculogenesis. Many studies targeted on consequences of ACE2 transduced EPC-EXs on ECs *in vitro* and neovascularization *in vivo*. The studies found that ACE2 gene transfer of EPC-EXs decreased the apoptosis of injured ECs, ROS production, mitochondrion fragmentation, Nox2 and Nox4 expression, and increased ECs function, MMP, and ATP levels through downregulating Nox2 and upregulating eNOS *in vitro* (57, 87). Furthermore, Wang et al. further explored the effect of combining EPC-EXs with ACE2 gene transfer in a C57BL/6 mice model of intracerebral hemorrhagic stroke (ICH) *in vivo*. They found a statistically significant decrease of hemorrhage volume for the ACE2-EPC-EXs rather than the EPC-EXs group. ACE-EPC-EXs also improved neurological deficit and BBB permeability, alleviated brain edema, downregulated the expressions of TNF-a and NFrB and upregulated the IrBa level (88). miR-126 has been a further target agent for gene modification to enhance the vasculogenic properties of EPC-EXs (49, 51, 89). For instance, transfer of miR-126 by EPC-EXs reduced apoptosis and promoted the proliferation and migration of MC3T3-E1 cells *in vitro* (51). Transfer miR-126 of EPC-EXs decreased infarct volume, increased cerebral blood flow (CBF) and cerebral microvascular density (MVD), promoted angiogenesis and neurogenesis, and downregulated cleaved caspase-3 and VEGF2 more significantly than EPC-EXs in a db/db type II diabetic mice model of middle cerebral artery occlusion (MCAO) surgery for inducing ischemic stroke *in vivo* (89). Sun et al. demonstrated that EPC-EXs loaded with miR-26 promoted thrombus and recanalization by elevating Pcdh7 mRNA expression (49). To this end, the transfer of single genes and multiple genes to enhance EPC-EXs function, as well as the understanding of the underlying molecular and cellular mechanisms should be researched more extensively.

THERAPEUTIC EFFECTS OF EPC-DERIVED EXOSOMES IN CVDs

EPC-EXs play an essential role in EPCs-based therapies of CVDs, including atherosclerosis, MI, and reperfusion injury. EPCs-EXs were shown to exhibit a therapeutic effect similar to EPCs transplantation, and some investigations also demonstrated their effects of promoting the prognosis of CVDs in animal models *in vivo* (49, 90–92) (**Table 3**).

Atherosclerosis

Atherosclerosis is a serious vascular disease characterized by endothelial dysfunction, inflammation, and the formation of plaques. The latter contain lipids, extracellular matrix, mesenchymal cells, and immune cells. The process of EndMT makes ECs acquire the markers and functions of mesenchymal cells and thus can act as a source of mesenchymal cells in atherosclerotic plaques (93). Several prior studies reported that EPC-EXs had the crucial functions of promoting ECs dysfunction, reducing oxidative stress, elevating eNOS expression (57), and inhibiting EndMT (78). In a mouse model of atherosclerosis treated with EPC-EXs, the atherosclerotic plaques abundantly decreased. Anti-atherosclerosis processes might include the regulation of miRNAs expression of EPC-EXs; decrease of the levels of oxidative stress factors malondialdehyde (MDA) and superoxide dismutase (SOD) and the inflammatory factors ICAM-1, IL-8, and C-reactive protein (CRP); and the change of high K^+ solution- and Phe-induced vasoconstriction and endothelium-dependent vasodilation in the thoracic aorta (94).

Myocardial Infarction (MI)

MI, a detrimental consequence of acute coronary occlusion, is featured by inflammation, the apoptosis of cardiomyocytes and oxidative stress, which induce vasodilatation and increase neovascularization. In the past decades, various therapeutic strategies have been tested to find a more effective treatment for CVDs. Notably, cell therapy has gradually become an attractive and effective treatment method for CVDs (95–97). With the emergence of studies on EPC-EXs, exosome secretion by various cell types, including cardiomyocytes (CMs), ECs, fibroblasts, and circulating progenitor cells (CPCs), have been demonstrated to provide protective prognosis in MI. In a mouse MI model, EPC-EXs were injected intramyocardially into the left ventricular wall (border zone) at three different locations immediately after left anterior descending ligation. In the IL-10 knockdown group, EPC-EXs were enriched in inflammation-related proteins featuring a two- to fourfold increase in Integrin Linked Kinase (ILK) expression, and were shown to activate the NF-κB pathway in recipient cells and enhance inflammatory response by upregulating inflammatory genes, while wild-type EPC-EXs showed the opposite effects. Wild-type EPC-EXs also improved the left ventricular cardiac function, significantly reduced cardiomyocyte apoptosis, decreased MI scar size and promoted neovascularization compared with IL-10 knockdown EPC-EXs (90). In another rat model of MI, it was revealed that EPC-EVs (including EPC-MVs and EPC-EXs) (injections around the border zone of the infarcted area) delivered into the ischemic myocardium *via* an injectable hydrogel enhanced peri-infarct angiogenesis and myocardial hemodynamics, and the therapeutic efficiency and efficacy of myocardial preservation was greatly increased by a shear-thinning gel (86).

Reperfusion Injury

Ischemia–reperfusion injury (I/RI) might result from increasing mortality and morbidity by irreversible structural damage and organ dysfunction in a large number of diseases, such as MI, stroke, and transplantation (98). The production of free radicals in I/RI is attributed to myocardial injury, which has three forms: myocardial stunning, reperfusion arrhythmia, and myocardial necrosis. Many investigations have deepened our insight into the mechanisms and therapeutic strategies for

myocardium I/IR, including the role of exosomes (99, 100). EPC-EXs were found to reduce apoptosis, ROS overproduction, and angiogenic dysfunction; decrease mitochondrial fragmentation; elevate MMP and ATP level; and improve mitochondrial mfn2 and drp1 dysregulation in endothelial cells (61). ACE2-EPC-EXs exhibited greater anti-oxidative and anti-apoptotic effects on aging ECs than on young ECs subject to H/R injury through carrying miR-18a and subsequently downregulating the Nox2/ROS pathway (57). Further experiments should also be conducted to examine the protective roles and mechanisms of EPC-EXs in I/RI *in vivo*.

Perspectives and Future Direction

Rapid detection of CVDs is the cornerstone of improving prognosis and preventing further comorbidities and complications. The possibility to isolate and characterize EPC-EXs from bodily fluids makes them very attractive diagnostic markers. Many exosome-based cancer diagnostic kits have been developed quickly in recent years. However, in the field of cardiovascular medicine, EPC-EXs as diagnostic markers are still an unexplored world that we are committed to pioneer. In addition, it is more attractive to use EPC-EXs as a therapeutic drug rather than conventional EPC transplantation. However, the same composition of exosomes expresses various pathophysiological functions under different microenvironments *in vivo*; how to preserve the biological activity of cytokines, proteins, and miRNAs in exosomes and deliver them to target sites is a big challenge for us now. The biodistribution, as well as the long-term effects and safety of administered EPC-EXs, would need to be explored and controlled.

As mentioned above, the promise and excitement surrounding EPC-EXs in CVDs can be manifested daily by previously reported studies. Although the field of EPC-EXs has much to be developed, the exploration and specific application of EPC-EXs and potential treatment will continue to be a rapidly advancing focus for cardiovascular researchers. Exosome-based approaches could "take EPCs out of cell therapy."

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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PPARβ/δ Is Required for Mesenchymal Stem Cell Cardioprotective Effects Independently of Their Anti-inflammatory Properties in Myocardial Ischemia-Reperfusion Injury

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Myocardial infarction ranks first for the mortality worldwide. Because the adult heart is unable to regenerate, fibrosis develops to compensate for the loss of contractile tissue after infarction, leading to cardiac remodeling and heart failure. Adult mesenchymal stem cells (MSC) regenerative properties, as well as their safety and efficacy, have been demonstrated in preclinical models. However, in clinical trials, their beneficial effects are controversial. In an experimental model of arthritis, we have previously shown that PPARβ/δ deficiency enhanced the therapeutic effect of MSC. The aim of the present study was to compare the therapeutic effects of wild-type MSC (MSC) and MSC deficient for PPARβ/δ (KO MSC) perfused in an *ex vivo* mouse model of ischemia-reperfusion (IR) injury. For this purpose, hearts from C57BL/6J mice were subjected *ex vivo* to 30 min ischemia followed by 1-h reperfusion. MSC and KO MSC were injected into the Langendorff system during reperfusion. After 1 h of reperfusion, the TTC method was used to assess infarct size. Coronary effluents collected in basal condition (before ischemia) and after ischemia at 1 h of reperfusion were analyzed for their cytokine profiles. The dose-response curve for the cardioprotection was established *ex vivo* using different doses of MSC (3.10^5, 6.10^5, and 24.10^5 cells/heart) and the dose of 6.10^5 MSC was found to be the optimal concentration. We showed that the cardioprotective effect of MSC was PPARβ/δ-dependent since it was lost using KO MSC. Moreover, cytokine profiling of the coronary effluents collected in the eluates after 60 min of reperfusion revealed that MSC treatment decreases CXCL1 chemokine and interleukin-6 release

compared with untreated hearts. This anti-inflammatory effect of MSC was also observed when hearts were treated with PPARβ/δ-deficient MSC. In conclusion, our study revealed that the acute cardioprotective properties of MSC in an *ex vivo* model of IR injury, assessed by a decreased infarct size at 1 h of reperfusion, are PPARβ/δ-dependent but not related to their anti-inflammatory effects.

Keywords: myocardial infarction, reperfusion injury, inflammation, PPAR, mesenchymal stem cells, cardioprotection

INTRODUCTION

Acute myocardial infarction (AMI) is the leading cause of cardiovascular mortality worldwide and a provider of heart failure (1). Prompt revascularization of the culprit artery with primary coronary angioplasty or thrombolysis is associated with deleterious side effects called ischemia-reperfusion (IR) injury due to abrupt restoration of blood flow and oxygen.

The release of DAMPS (*Damage-associated molecular patterns*) from dead cells, in concert with the activation of the complement cascade and reactive oxygen species (ROS), triggers an acute pro-inflammatory response at the onset of AMI that activates the resident immune cells of the heart. Reperfusion exacerbates this inflammatory response to eliminate necrotic cells and repair the infarcted myocardium (2). Interleukin 1 (IL-1), and interleukin 6 (IL-6) are the major cytokines that mediate this short but strong inflammatory burst, which contributes to cell death and irreversible IR injury (2). At the site of the injury, many cell types, including cardiomyocytes, vascular cells, fibroblasts, and immune cells, are involved in this inflammatory response. Necrotic cardiomyocytes in the infarcted area provide the main stimulus for the post-infarction inflammatory response through the release of DAMPs. In the border zone, surviving cardiomyocytes, once activated by IL-1, Toll Like Receptor (TLR) ligands, and ROS, will produce and secrete cytokines such as IL-6 (3), TNFα (4), and chemokines such as CXCL1 (KC/GRO) and MIP-2 (5) to trigger inflammatory activation. Endothelial cells, the most abundant non-cardiomyocytes in the heart, when activated by TNFα, produce CXCL1 (6). In addition to a local inflammatory response, myocardial cells sense tissue necrosis and trigger the post-infarction inflammatory response, stimulating the release and recruitment of bone marrow (BM)-derived leukocytes. However, the relative contribution of cardiomyocyte-derived inflammatory mediators in the progression and extension of post-infarction inflammation remains unknown.

Previous studies have shown that the application of ischemic postconditioning (PostC) i.e., repeated brief episodes of IR in the myocardial tissue applied at the onset of reperfusion, was able to specifically inhibit IR injury (7, 8). PostC, considered a gold standard strategy for cardioprotection in animal models of AMI, was reported to be mediated by multiple intracellular cascades leading to anti-apoptotic and anti-inflammatory effects resulting in cardioprotection (7, 9, 10). Various targets have been identified in PostC signaling pathways, however, no product of potential clinical utility, including anti-inflammatory drugs, has emerged from all candidates identified as cardioprotective in preclinical studies (11, 12). This suggests that other strategies with pleiotropic mechanisms of action are clearly needed (13).

Preclinical studies have shown that MSC-based therapy improves myocardial functional recovery after AMI by promoting endogenous cell survival, proliferation, and angiogenesis. In addition, MSC exert pleiotropic effects, including reduction of inflammation and apoptosis through their ability to release bioactive molecules (14, 15). Based on these promising results, MSC were then tested in clinical trials that demonstrated their safety and promising efficacy in phase I and II, but yielded inconclusive results in phase III trials (16). Indeed, no significant long-term beneficial effects in AMI patients has been reported based on recent meta-analyses (17, 18).This failure to translate preclinical results into human clinical trials could be attributed to, in part, trial design differences, the source and dose of MSC used, and the route and timing of MSC injection (18). To bridge the gap between preclinical and clinical studies, the development of "preconditioning" methods to improve MSC therapeutic potential has been widely investigated (19) mainly focusing on the enhancement of their anti-inflammatory properties. For example, MSC treated with IGF-1 before transplantation into the ischemic heart reduce the production and expression of proinflammatory cytokines, including TNFα, IL-1β, and IL-6 and improve cardiac functions (20). Although promising, this approach of enhancing the anti-inflammatory properties of MSC to improve their therapeutic potential in AMI has been poorly investigated.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors expressed in three different isoforms, PPARα, PPARβ/δ, and PPARγ, which heterodimerize with the retinoid X receptor (RXR) and act as transcriptional regulators after ligand binding. Peroxisome proliferator-activated receptor isoforms exert multiple functions depending on tissue ligands and cofactors (21). PPARβ/δ, a proangiogenic member of the PPAR family, is ubiquitously expressed (22–24) in contrast to PPARα, which is mainly detected in brown adipose tissue, intestine, heart, liver, kidney, and PPARγ, which is expressed in immune cells, intestine, white, and brown adipose tissue. The potent anti-inflammatory actions of PPARβ/δ on several immune cells including macrophages have been previously reported. Indeed, the capacity of IL-4 and IL-13 to direct macrophages to an M2-like anti-inflammatory phenotype in mouse adipose tissue and liver depends on PPARβ/δ expression (25–27). Recently, in an experimental model of the auto-immune and inflammatory disorder in mice, "collagen-induced arthritis (CIA)," we demonstrated that PPARβ/δ expression level could

predict the immunoregulatory potential of MSC and that its inhibition increased their immunoregulatory and therapeutic activities (28).

Given that reperfusion injury is associated with acute inflammation, we hypothesized that inactivation of PPARβ/δ might impact the cardioprotective properties of MSC during IR injury associated to local inflammation (26). In mouse models, in order to get closer to the classical clinical conditions of MSC administration (29–37), the local injection of MSC at the acute phase, avoiding the systemic route is prefered. Thus, in the present study, we explored the contribution of PPARβ/δ in the acute local cardioprotective effect mediated by MSC during reperfusion in an *ex vivo* mouse model of isolated heart subjected to IR injury.

MATERIALS AND METHODS

Ethics

Studies involving animals were reviewed and approved by the Institute's SBEA (*Structure Bien-être Animal*) commitee in accordance with the European directive 2010/63/EU and the French Ministerial Order of February 01, 2013.

Animal Housing and Care

Experiments were performed in C57BL/6J mice (Charles River laboratory) in accordance with the European Communities Council directive of November 1986 and in accordance with the Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication 8th Edition, 2011). All mice were maintained under controlled environmental conditions (22 ± 2°C, 12 h light /12 h dark cycle) in the Institute's animal facility.

Ex vivo Experiments

Male mice were anesthetized with an intraperitoneal injection (IP) of ketamine (14 mg/kg, Imalgène®; *Merial*), xylazine (14 mg/kg, Rompun®; *Bayer*) followed by an injection of pentobarbital (IP; 76.6 mg/kg; *Sanofi-Aventis*). The anesthetized mice received 250 U heparin (IP) in order to prevent blood clot formation. After sternotomy, the heart was excised, cannulated through the ascending aorta, and quickly mounted on the Langendorff perfusion system. Prewarmed Tyrode's solution (NaCl 140 mM, KCl 5.4 mM, MgCl 1 mM, Hepes 5 mM, glucose 5.5 mM, $CaCl_2$ 1.8 mM, pH 7.4) was perfused at constant pressure (70 mmHg) and temperature (37°C).

Ischemia-Reperfusion Protocol

On the Langendorff perfusion system, the heart was perfused with prewarmed Tyrode solution for 15 min (stabilization). Global ischemia was obtained by stopping the perfusion flow (*no-flow*) for 30 min. A reperfusion step (60 min) was achieved by restoring the flow. Mesenchymal stem cells treatment (prepared in Tyrode solution) was applied during reperfusion as a non-recirculating perfusate. The control condition (IR) was obtained using only Tyrode. A positive control of cardioprotection was obtained by applying an ischemic postconditioning stimulus, comprising three cycles of 1 min ischemia-1 min reperfusion at the onset of reperfusion (PostC group). Perfusates containing coronary effluents were collected at the apex of the heart at both 10 min of the stabilization (basal) phase and at 15, 30, and 60 min after the onset of reperfusion and were stored at −80°C for further experiments.

Infarct Size Measurement

At the end of the IR protocol, the heart was harvested from the apparatus. The left ventricle (LV) was embedded in agar (4% w/v), and transversely sliced (1 mm) with a vibratome. To reveal tissue viability, slices were incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC, *Sigma-Aldrich*) for 15 min at 37°C. After a fixation step (4% paraformaldehyde, 48 h), each slice was photographed from each side. The infarct area was quantified by planimetric measurements with *ImageJ* software.

MSC Culture

Isolation, amplification, and characterization of murine MSC were performed as previously described (33). Briefly, BM was flushed out from the long bones of *Ppard*$^{fl/fl}$sox2cre^{tg} PPARβ/δ-deficient mice and their wild-type littermates (*Ppard*$^{fl/+}$) kindly provided by Gerhard Krönke laboratory (Institute of Rheumatology and Immunology, Erlangen, Germany) (38) to isolate KO MSC and MSC, respectively. Cells were cultured in minimal essential medium (MEM)-α containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 ng/ml human basic fibroblast growth factor (bFGF) at a density of 0.5×10^6 cells/cm^2. Phenotypic and functional characterization of MSC has been performed previously (28). To confirm the effects of PPARb/d inactivation, mesenchymal stem cells were pre-incubated 24 h with 5 μM of PPARβ/δ selective antagonist GSK0660.

MSC Labeling With CM-DiI

The stock solution of the fluorescent cell-tracer CM-DiI (Molecular Probes) was reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 1 μg/μl. Mesenchymal stem cells were collected and suspended at the concentration of 1×10^7 cells/10 μg CM-DiI in 5 ml PBS. Cells were incubated at 37°C for 5 min followed by 15 min at 4°C, in the dark. Unincorporated fluorescent dye was then removed by centrifugation at 300 g for 5 min and two washes with PBS were performed. Labeled cells were resuspended in Tyrode's solution and maintained at 4°C prior being injected into the myocardium.

Cytokine Level Quantification

For quantification of cytokine levels, coronary effluents from perfused hearts were collected under basal conditions (*t*0), after 10 min of stabilization, and after IR injury at 15, 30, and 60 min after the onset of reperfusion. The different perfusates were stored at −80°C until the assay was performed using the Meso Scale Discovery (MSD) V-Plex Plus Proinflammatory Panel 1 (mouse) kit at the "Plateforme de Protéomique Clinique de Montpellier" according to the manufacturer's protocol. As compared to other methods, MSD is the most suitable assay for samples with low endogenous levels of the cytokines although cytokine's levels can be below the limits of detection

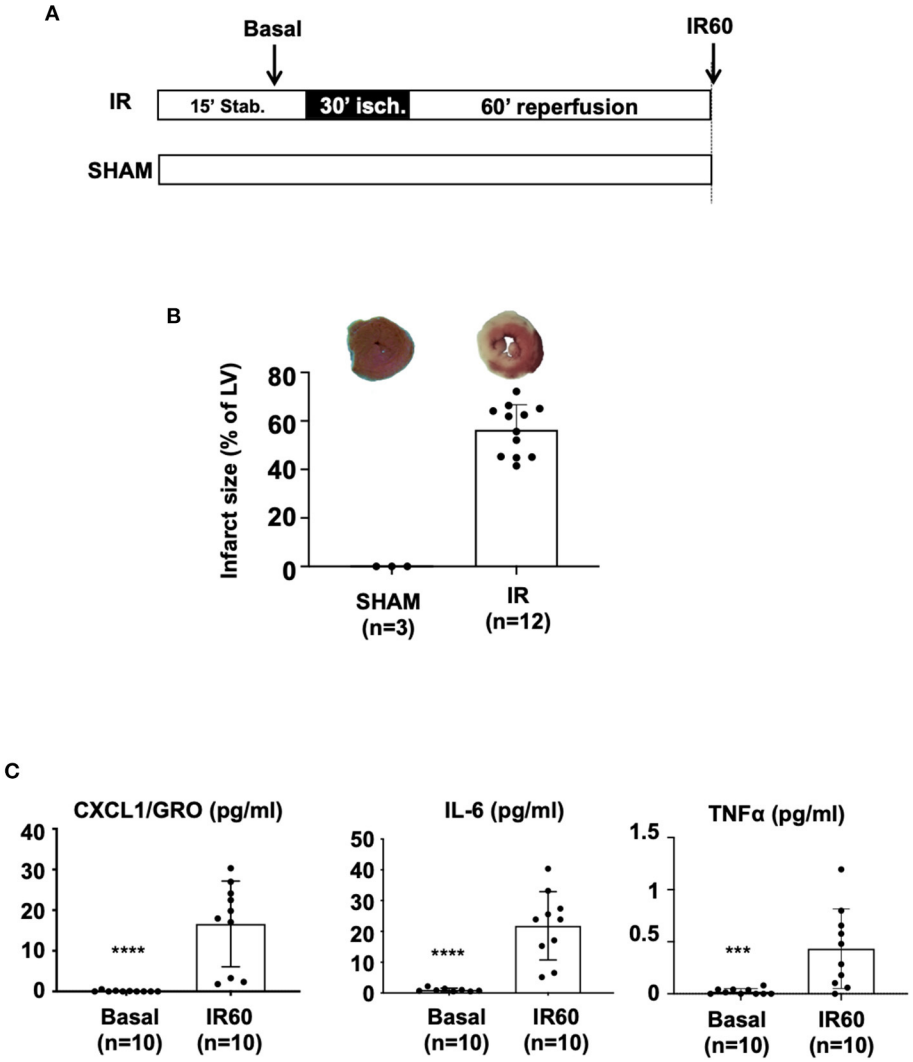

FIGURE 1 | C57BL/6J mouse hearts were mounted on a Langendorff system and subjected to IR injury. **(A)** The *ex vivo* protocol included a 15 min-stabilization period, followed by 30 min of global ischemia achieved by stopping the flow in the aorta (no-flow). Reperfusion was achieved by restoring the Tyrode infusion during 60 min. For the SHAM condition, the heart was perfused throughout the protocol without any ischemic induction. At the end of the protocol, infarct size was measured using the TTC-staining method. Coronary effluents were collected at two time points during the *ex vivo* protocol: during stabilization to evaluate the "basal" level of cytokine release and at the end of the reperfusion phase to evaluate the "IR60" cytokine production after IR injury. **(B)** Scatter plots and bars (mean ± SD) were represented for infarct size (in % of LV) in IR ($n = 12$) and SHAM ($n = 3$) hearts. Representative pictures of TTC-stained LV slices were shown for each group. **(C)** Scatter plots with bars (mean ± SD) are presented for quantification of cytokines within coronary effluents collected before ischemia (Basal) and after 60 min of reperfusion after the IR protocol (IR_{60}) using the Meso Scale Discovery (MSD) V-Plex Plus Proinflammatory Panel 1 (mouse) kit. Statistical analysis was performed using the Mann-Whitney test. For CXCL1 (pg/ml), **** was noted for $p < 0.0001$, for IL-6 (pg/ml), **** was noted for $p < 0.0001$ and for TNFα (pg/ml), *** was noted for $p = 0.0008$.

of this immunoassays. The V-Plex Plus Proinflammatory Panel 1 includes IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO (CXCL1), IL-10, IL-12p70, and TNFα. When the concentration of a cytokine was undistinguishable from background (i.e., below the limit of detection), the sample was considered negative for that cytokine.

Immunochemistry

At the end of *ex vivo* experiments, LV were fixated in 4%-PFA and embedded in paraffin. Each LV was cut from apex to base (sections of 4 μM each 150 μM). The paraffin-embedded sections were deparaffinized then rehydrated through an alcohol gradient. Left ventricle sections were incubated with a primary anti α-actinin antibody (1:100, mouse monoclonal; *Sigma-Aldrich*). Cell nuclei were stained with Hoechst (*Life technologies SAS*) and endothelial cells with Isolectin B4 (FITC Conjugate; *Sigma-Aldrich*). After incubation with primary antibodies, sections were washed in PBS, and then incubated (3 h) with secondary antibodies (1:2,000, *Jackson ImmunoRes Laboratories*, Inc.). Primary and secondary antibodies were

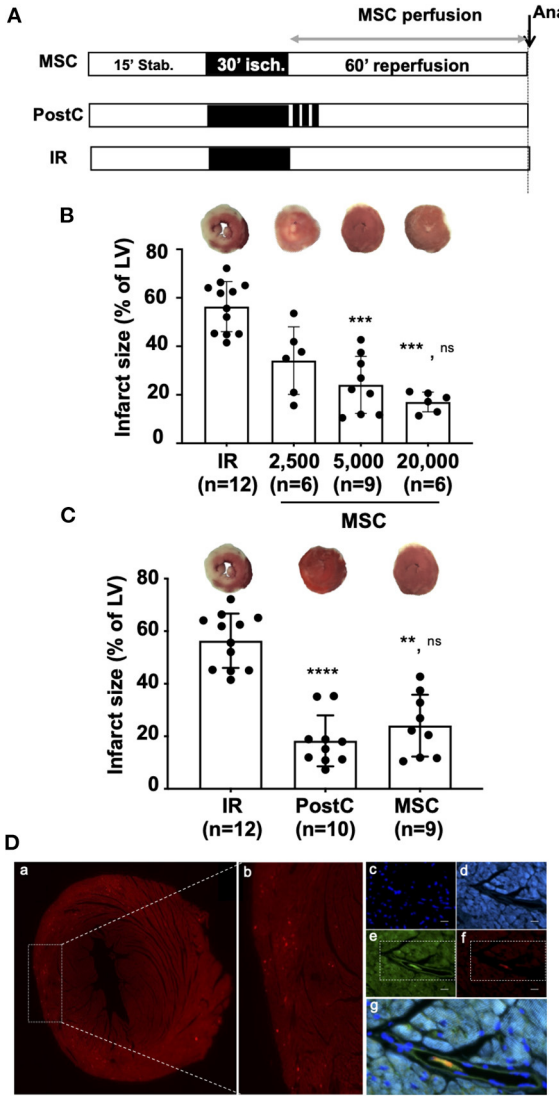

FIGURE 2 | (A) Isolated hearts perfused *ex vivo* on the Langendorff system were submitted to perfusion protocol similar to that described in **Figure 1A**. In the MSC group, reperfusion was achieved with a solution of MSC cells prepared in a Tyrode buffer at various concentrations (2,500; 5,000; or 20,000 cells/mL). For the PostC group, a postconditioning stimulus comprising three cycles of 1 min ischemia-1 min reperfusion was applied at the onset of reperfusion. In the control condition (IR), hearts were reperfused with Tyrode solution alone (control condition). Histological analysis was performed at the end of the protocols for infarct size measurement and immunochemistry. **(B)** Scatter plots and bars (mean ± SD) were represented for infarct size (in % of LV) in IR ($n = 12$), MSC 2,500 cells/ml ($n = 6$), MSC 5,000 cells/ml ($n = 9$), and MSC 20,000 ($n = 6$). Representative pictures of TTC-stained LV slices were shown for each group. Statistical analysis was performed using Kruskal-Wallis with the Dunn's *post hoc* test for multiple comparison. Statistical significance is noted *** for $p = 0.0009$ (MSC 5,000 vs. IR), *** for $p = 0.0003$ (MSC 20,000 vs. IR) and ns for $p > 0.99$ (MSC 20,000 vs. MSC 5,000). **(C)** Scatter plots and bars (mean ± SD) were represented for infarct size (in % of LV) in IR ($n = 12$), PostC ($n = 10$), and MSC (5,000 cells/ml, $n = 9$). Representative pictures of TTC-stained LV slices were shown for each group. Statistical analysis was performed using Kruskal-Wallis with the Dunn's post hoc test for multiple comparison. Statistical significance is noted **** for $p > 0.0001$ (PostC vs. IR), ** for $p = 0.0018$ (MSC vs. IR) and ns for $p > 0.99$ (PostC vs. *(Continued)*

FIGURE 2 | MSC). **(D)** Representative pictures of microscopic observations among 12 (data not shown) for an MSC-treated heart section **(a)** and **(b)** corresponding enlarged immunostaining images (Original magnification: x40 oil immersion) showing **(c)** cell nuclei (DAPI), **(d)** alpha-actinin, **(e)** microvessels (isolectin B4), **(f)** DI-I labeled MSC, and **(g)** merge allowing to show that MSC are located in the microvessels after 60 min of reperfusion (same time point of infarct size evaluation).

diluted in PBS containing 3% BSA and 0.1% Triton X100. Stained sections were mounted in Mowiol (*Biovalley*). Images were obtained with a Zeiss Axioimager Z3 fluorescent microscope after observation of six different sections of the LV harvested on $n = 2$ hearts treated by MSC labeled with CM-DiI and analyzed using ImageJ and Adobe Photoshop to prepare the final figures.

Statistical Analysis

Data expressed as mean ± SD values were compared among groups using non-parametric Mann-Whitney (two groups) and Kruskal-Wallis (multiple comparison) methods. *P*-values < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$(****) were considered statistically significant. Analysis and graphical representation were performed using Graph-Pad Prism^{TM} software (GraphPad).

RESULTS

Induction of a Pro-inflammatory Response in Isolated Perfused Heart Subjected to Ischemia-Reperfusion Injury *Ex vivo*

For this study, we have developed an *ex vivo* model of global ischemia followed by reperfusion (IR protocol) to evaluate the short-term therapeutic effects of MSC. Isolated hearts were mounted and perfused on a Langendorff system and subjected to 30 min of global ischemia (no-flow) followed by 60 min of reperfusion (see protocol in **Figure 1A**). The first part of the study was devoted to the characterization of our model. Hearts after myocardial IR injury were characterized by an infarct size with a mean value of 56.4% ± 10.3 expressed as percentage of the LV (**Figure 1B**).

We therefore asked whether induction of IR injury was associated with excessive release of pro-inflammatory cytokines in coronary effluents collected 60 min after the onset of reperfusion (time point at which infarct size was evaluated) compared with the basal level before ischemia (collected during stabilization). Of the 10 cytokines quantified using the MSD VPlex Plus Proinflammatory, seven including IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70 were not considered because of their undetectable levels. However, the concentration of three cytokines, KC/GRO (CXCL1), IL-6, and TNFα, were showed to be significantly increased in the coronary effluents of the hearts after IR injury (after ischemia and 60 min of reperfusion) compared with the basal conditions (collected at 10 min stabilization, basal) (**Figure 1C**). Overall, these results reveal that myocardial IR injury of C57BL/6

mouse hearts subjected *ex vivo* to 30 min of ischemia followed by 1 h of reperfusion is associated with the release of pro-inflammatory cytokines.

MSC Exerted a Potent Cardioprotective Effect When Administered During Reperfusion in an *Ex vivo* Model of Global Ischemia

To reduce IR injury-related inflammation and thus infarct size, we used two well-known cardioprotective strategies, ischemic PostC and MSC-based therapy, to compare for the first time their effects in an *ex vivo* mouse model. Mesenchymal stem cells were administered during reperfusion in isolated hearts subjected to 30 min of global ischemia as described in the protocol shown in **Figure 2A**. Different concentrations of MSC in the perfusion solution were tested (**Figure 2B**). The dose-response curve was established using different concentrations of MSC and showed that 5,000 cells/mL (6.10^5 cells/heart) and 20,000 cells/ml (24.10^5 cells/heart) induced cardioprotective effects by decreasing infarct size (24.01% ± 11.8, $n = 9$ for MSC 5,000 vs. 56.4% ± 10.3, $n = 12$ for IR; $p = 0.0009$, and 17.0% ± 4.1; $n = 6$ for MSC 20,000 vs. 56.4% ± 10.3, $n = 12$; $p = 0.0003$) as opposed to 2,500 cells/ml (3.10^5 cells/heart), which had no significant beneficial effect (34.1% ± 13.9, $n = 6$ for MSC 2,500 vs. 56.4 % ± 10.3, $n = 12$ for IR; $p = 0.1571$). The concentration of 5,000 cells/ml was chosen for all experiments in our study because it was the minimum concentration giving maximum cardioprotection in the *ex vivo* mouse model. Furthermore, we demonstrated that reperfusion with MSC at 5,000 cells/ml (6.10^5 cells/heart) reduced infarct size to the same extent as ischemic PostC, taken as a positive control in our experiments (18.3 % ± 9.7, $n = 10$ for PostC vs. 24.1% ± 11.8, $n = 9$ for MSC 5,000; $p > 0.99$). Immunohistological analysis revealed that *ex vivo* perfused MSC labeled with CM-DiI were 100% co-localized with Isolectin B4-positive endothelial cells in coronary microvessels after 1 h of reperfusion (**Figure 2D**).

Altogether, these results demonstrate that 6.10^5 MSC/heart provide a similar cardioprotection to that of PostC, considered as a positive control in our study.

The Potent Cardioprotective Effect of Both Ischemic PostC and MSC Is Associated With a Decrease in Pro-inflammatory Cytokines in the Injured Myocardium

To determine whether the beneficial effect of PostC or MSC administration on IR injury was associated with the regulation of the immune response, we quantified pro-inflammatory cytokines in coronary effluents collected at 15, 30, and 60 min after the onset of reperfusion (**Figure 3A**). Among the 10 cytokines quantified by the MSD approach, CXCL1 (KC/GRO), IL-6, and TNFα, were detected with significant levels only at 60 min of reperfusion in the samples from the untreated IR hearts. IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-10, and IL-12p70 were not detected. None of the 10 cytokines were detectable at 15 and 30 min post-reperfusion (data not shown). In addition, the levels of CXCL1

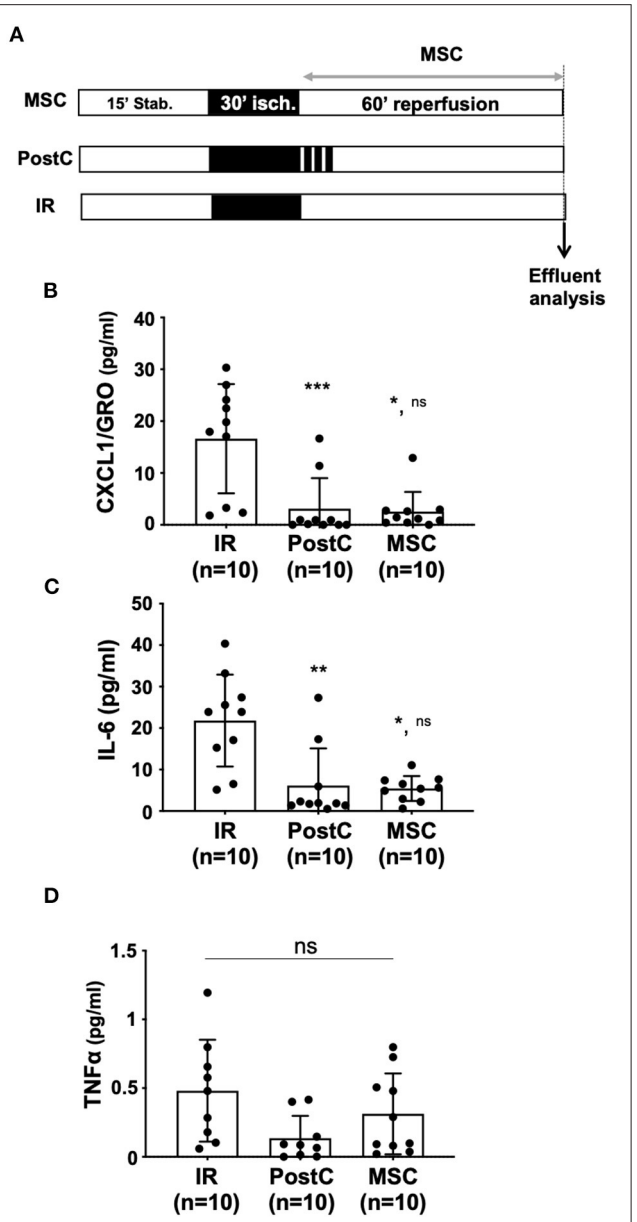

FIGURE 3 | (A) Isolated hearts perfused *ex vivo* on the Langendorff system were submitted to the perfusion protocol similar to that described in **Figure 1A**. In the MSC group, reperfusion was achieved with a MSC Tyrode solution (5,000 cells/ml). For the PostC group, a postconditioning stimulus comprising three cycles of 1 min ischemia-1 min reperfusion was applied at the onset of reperfusion. In the control condition (IR), hearts were reperfused with Tyrode solution alone (control condition). Coronary effluents were collected at the end of the reperfusion phase to evaluate cytokine production after IR, PostC or MSC protocols. **(B–D)** Scatter plots with bars (mean ± SD) are presented for quantification of cytokines within coronary effluents collected after 60 min of reperfusion using the Meso Scale Discovery (MSD) V-Plex Plus Proinflammatory Panel 1 (mouse) kit. Statistical analysis was performed using the Kruskal-Wallis test followed by the Dunn's post test. **(B)** For CXCL1 (pg/mL), *** was noted for $p = 0.0006$ (PostC vs. IR), ** for $p = 0.018$ (MSC vs. IR) and ns for $p > 0.999$ (MSC vs. PostC). **(C)** For IL-6 (pg/ml), ** was noted for $p = 0.0021$ (PostC vs. IR), * for $p = 0.0287$ (MSC vs. IR) and ns for $p > 0.999$ (MSC vs. PostC) and **(D)**: for TNFα (pg/ml), ns was noted for $p = 0.059$.

(KC/GRO) (**Figure 3B**) were significantly lower in coronary effluents from hearts treated by PostC or MSC (16.61 pg/ml ± 10.53, $n = 10$ for IR vs. 3.10 pg/ml ± 5.9, $n = 10$ for PostC; $p^{***} = 0.0006$, and vs. 2.56 pg/ml ± 3.79, $n = 10$ for MSC; $p^{*} = 0.018$). Similar results were obtained for IL-6 (21.83 pg/ml ± 11.08, $n = 10$ for IR vs. 6.16 pg/ml ± 8.95, $n = 10$; $p^{**} = 0.021$, and vs. 5.43 pg/ml ± 3.02, $n = 10$ for PostC; $p^{*} = 0.029$ for MSC; **Figure 3C**). For TNFα, there was a slight reduction of the effluent levels upon PostC treatment (without reaching significativity), which was not observed in the MSC group ($p^{ns} > 0.99$; **Figure 3D**).

This result indicates that the protective effects of both PostC and MSC were associated with a potent anti-inflammatory effect assessed by quantification of pro-inflammatory cytokines in coronary effluents.

PPARβ/δ Is Involved in the Cardioprotective Effects Mediated by MSC Against IR Injury

Recently, we showed that PPARβ/δ is pivotal for the MSC immunoregulatory and therapeutic functions in an experimental model of arthritis (28). However, the role of PPARβ/δ on the cardioprotective activity of MSC and the relevance of PPARβ/δ to the anti-inflammatory properties of MSC in the inflamed myocardium have never been addressed. To determine whether PPARβ/δ is essential for the cardioprotective properties of MSC, we compared the effect of MSC isolated from PPARβ/$\delta^{-/-}$ deficient mice (KO MSC) and those obtained from their PPARβ/$\delta^{+/+}$ control littermates (MSC). Isolated hearts were perfused during reperfusion with solutions containing MSC at the optimal dose of 6.10^5cells/heart (see protocol **Figure 4A**). Under these conditions, the drastic decrease in infarct size induced by MSC (24.1% ± 11.8, $n = 9$ for MSC vs. 56.4% ± 10.3, $n = 12$ for IR; $p^{**} = 0.001$) was abolished when KO MSC were infused into isolated hearts after the ischemic insult (48.4% ± 25.4, $n = 13$ for KO MSC vs. 56.4% ± 10.3, $n = 12$ for IR; $p^{ns} = 0.75$ and 48.4% ± 25.4, $n = 13$ for KO MSC vs. 24.1% ± 11.8, $n = 9$ for MSC; $p^{*} = 0.029$) (**Figure 4B**). A similar absence of cardioprotective effet was observed after the infusion of MSC pharmacologically inactivated for PPARβ/δ using GSK0660, a selective antagonist of PPARβ/δ (data not shown). To determine whether the loss of therapeutic effect of MSC in response to PPARβ/δ knockdown was associated with a loss of their ability to reduce inflammation in infarcted myocardium, we quantified pro-inflammatory cytokines within coronary effluents collected 60 min after the onset of reperfusion. Quantification of cytokines by MSD was performed in coronary effluents from hearts treated with either MSC or KO MSC and compared with those of untreated hearts. We demonstrated that PPARβ/δ knockdown in MSC did not alter their anti-inflammatory potential as revealed by the measured levels of CXCL1 (2.56 pg/ml ± 3.79, $n = 10$ for MSC vs. 3.73 pg/ml ± 1.65, $n = 8$ for KO MSC, $p^{ns} = 0.28$; **Figure 4C**) and IL-6 (5.43 pg/ml ± 3.02, $n = 10$ for MSC vs. 7.65 pg/ml ± 5.04, $n = 8$ for KO MSC, $p^{ns} > 0.99$; **Figure 4D**) in coronary effluents collected during reperfusion. There was no difference in the TNFα levels among groups ($p^{ns} = 0.60$; **Figure 4E**).

DISCUSSION

Our study evaluated, for the first time, the role of PPARβ/δ in MSC-induced cardioprotective effects in an *ex vivo* mouse model of myocardial IR injury. The rationale comes from data recently reported from our laboratory showing a pivotal role of these receptors for the immunoregulatory and therapeutic functions of MSC in an experimental model of arthritis (28). We first demonstrated that the *ex vivo* protocol of global ischemia (30 min) followed by 60 min-reperfusion used in that study induced a proinflammatory response assessed by an increase in the level of TNFα, IL-6, and CXCL1 in coronary effluents collected at 60 min of reperfusion. We then identified the optimal dose of MSC, 6.10^5 cells/heart, to provide significant cardioprotection in the *ex vivo* mouse model with minimal cell concentration. Of note, at this selected dose of MSC, we observed similar cardioprotection to that of ischemic postconditioning (PostC) taken as a positive control in our study. Indeed, both treatments reduced the pro-inflammatory response of IR injury and decreased infarct size with the same efficacy. Moreover, this study revealed that the acute cardioprotective properties of MSC during the first hour of reperfusion are PPARβ/δ-dependent but not related to their anti-inflammatory effects on the release of CXCL1 and IL-6 in coronary effluents.

Numerous studies have demonstrated in isolated perfused hearts (*ex vivo*) the cardioprotective effect of PostC or various pharmacologic drugs applied at the onset of reperfusion. Considering most *in vivo* studies, the protocol is quite similar since PostC or protective drugs are applied at the onset of reperfusion and infarct size measured often at 1 h reperfusion, an effect maintained at 24 h or at several months of reperfusion (39). This means that major local events occur very early during reperfusion. These rapid local events, which are triggered during reperfusion, include inflammatory processes and/or paracrine effects of secreted molecules such as cytokines by endogenous or exogenous cells. The *ex vivo* model is well-suited to identify these pathways, as short-term protection is critical for long term protection.

Despite its benefical effects in AMI patients, reperfusion therapy induces local and systemic inflammation, termed sterile inflammation, which will enhance the initial inflammatory response triggered to clear necrotic cells after AMI and to repair the infarcted myocardium. To develop and evaluate innovative therapeutic approaches, we designed an *ex vivo* model that provides strong IR injury assessed by 56% infarct size (expressed as percentage of the LV) and the release of pro-inflammatory cytokines at significant levels in coronary effluents, as reported *in vivo* in patients with acute coronary syndrome (40). This inflammatory response is a potential therapeutic target to improve the post-AMI clinical state because it plays a crucial role in determining infarct size and subsequent left ventricular remodeling. To protect the myocardium, many therapeutic strategies targeting indirectly or directly the proinflammatory response after AMI have been tested. In this context, approaches to decrease the levels of inflammatory cytokines and chemokines in animal models have been developed and used with promising results regarding

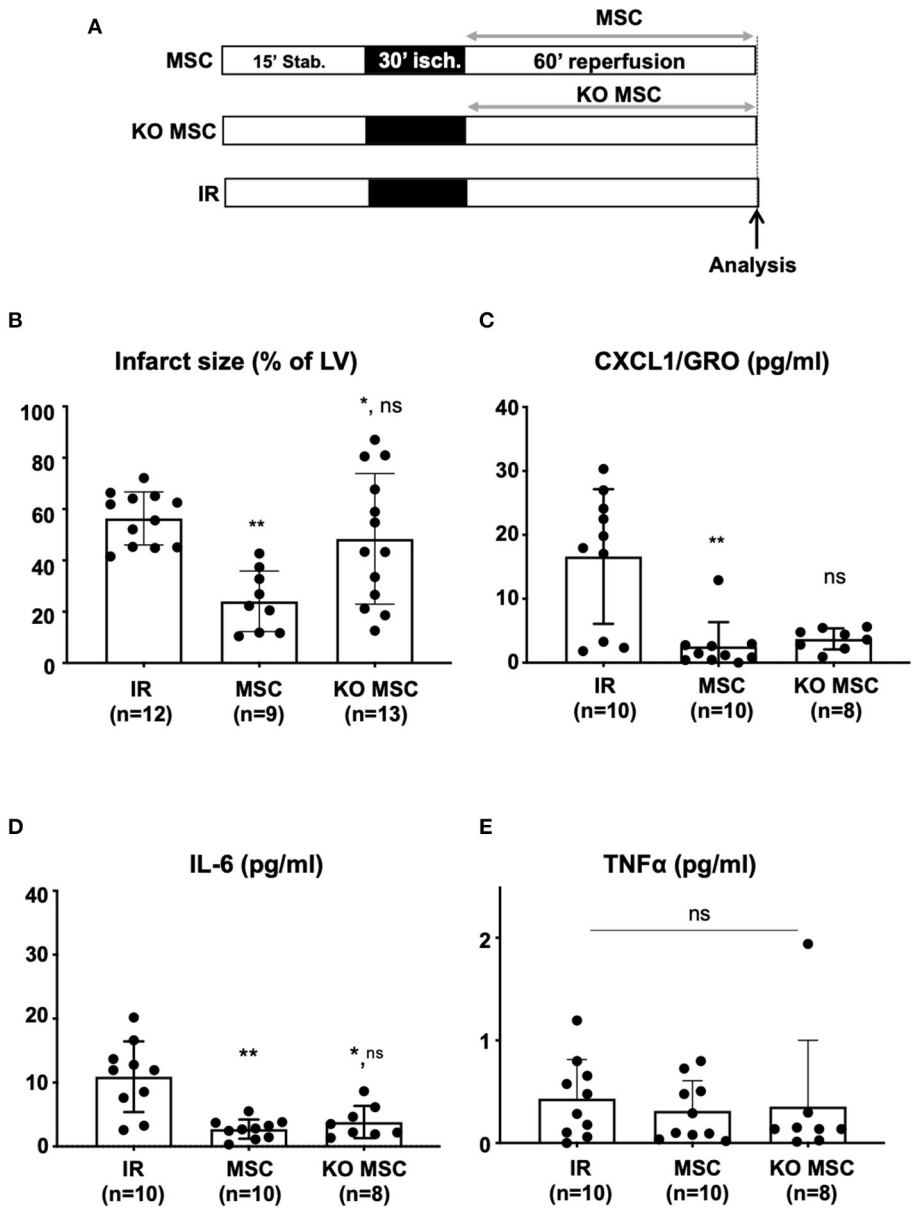

FIGURE 4 | (A) Isolated hearts perfused *ex vivo* on the Langendorff system were submitted to perfusion protocol similar to that described in **Figure 1A**. Reperfusion was achieved with a Tyrode solution alone in the IR group or with a Tyrode solution prepared at a concentration of 5,000 cells/ml with MSC (MSC group) and with KO MSC (KO MSC group). At the end of the protocol, infarct size analysis was performed on the isolated heart **(B)** and cytokine production was analyzed **(C–E)** using the Meso Scale Discovery (MSD) V-Plex Plus Proinflammatory Panel 1 (mouse) kit. Statistical analysis was performed using the Kruskal-Wallis test with the Dunn's post test. **(B)** Scatter plots and bars (mean ± SD) were represented for infarct size (in % of LV) in IR ($n = 12$), MSC (5,000 cells/ml, $n = 9$), and KO MSC (5,000 cells/ml, $n = 13$). Statistical significance is noted ** for $p = 0.001$ (MSC vs IR), * for $p = 0.029$ (KO MSC vs. IR), and ns for $p = 0.075$ (KO MSC vs. MSC). **(C–E)** Scatter plots with bars (mean ± SD) are presented for quantification of TNFα, CXCL1, and IL-6 within coronary effluents collected at 60 min after the onset of reperfusion from untreated hearts (IR), hearts treated with wild-type MSC (MSC) and MSC deficient for PPARβ/δ (KO MSC) using the Meso Scale Discovery kit. Statistical analysis was performed using the Kruskal-Wallis test. **(C)** For CXCL1 (pg/ml), ** was noted for $p = 0.0017$ (MSC vs. IR), ns was noted for $p = 0.34$ (KO MSC vs. IR) and for $p = 0.28$ for (KO MSC vs. MSC); **(D)** For IL-6 (pg/ml), ** was noted for $p = 0.0021$ (MSC vs. IR), * for $p = 0.028$ (KO MSC vs. IR), and ns for $p > 0.999$ (KO MSC vs. MSC); **(E)** for TNFα (pg/ml), ns was noted for $p = 0.60$.

the control of inflammation (2). However, most clinical trials based on the use of broad-spectrum immunosuppressive drugs such as corticosteroids or immunosuppressants in AMI patients have shown no benefit on infarct size or clinical outcomes (2). Since rapid activation of this inflammatory response is a consequence of an inappropriate activation of the innate immune system triggered by DAMPs release, targeting key components of the innate immune system appeared as a promising approach for limiting IR injury (41). Thus, more refined strategies have been proposed instead of fully suppressing

the immune system. In this context, promising results have been obtained although they have been mitigated by negative results as reported for inhibitors of IL-1 (42, 43). Targeting inflammation in AMI patients is quite challenging because innate immunity has been reported to contribute to myocardial repair and remodeling (44). This makes it difficult to determine the right dose and timing of administration of therapeutic agents to avoid compromising innate immunity-induced cardiac repair. Mesenchymal stem cells-based therapy in this context appears as an interesting strategy in creating local inflammation permissive to regeneration.

Ischemic postconditioning, considered the gold standard of cardioprotection against IR injury, has been reported to activate a myriad of intracellular cascades leading to inhibition of regulated cell death and also an anti-inflammatory response to induce a strong cardioprotective effect at 1 h of reperfusion (9). In the *ex vivo* model of IR injury used in the present study, the PostC cardioprotective strategy reduced both infarct size by 75.1% (PostC vs. IR, p^*) and IL-6 and CXCL1 levels by 71.8 and 91.4% compared with IR, respectively, in coronary effluents from isolated hearts. Taken together, these results suggest that regulation of the inflammatory response is associated with cardioprotection against IR injury mediated by PostC.

Therefore, global therapies such as PostC, have emerged as promising approaches to treat multifaceted ischemic heart disease and restore cardiac function. Indeed, considering the pleiotropic effects of MSC that include their immunoregulatory, antifibrotic, and anti-apoptotic capabilities, MSC-based therapy could counteract the three main pathogenic axes of AMI and thus have been considered a breakthrough in this incurable disease with unmet medical needs. Safety and efficacy were evaluated by assessment of adverse events and the improvement of the left ventricular ejection fraction (LVEF) and mortality rate, respectively. Although the results obtained were marked by significant heterogeneity, MSC injection did not appear to be associated with acute adverse events but induced an improvement in LVEF in patients. No significant differences in mortality were reported. Other outcomes of interest were rarely studied, which limits the conclusions. In our study, MSC exerted a cardioprotective effect similar to that provided by PostC, considered a positive control in our study. Indeed, infarct size was decreased by 69.3% using MSC-based cell therapy (PostC vs. MSC, p = ns). In addition, MSC treatment (6.10^5 cells/heart) had a strong anti-inflammatory effect, as assessed by MSD quantification of IL-6 and CXCL1, which were decreased by 75.1 and 84.6%, respectively.

In this context, we tested the therapeutic role of MSC knockdown for PPARβ/δ previously described by our laboratory to exhibit an enhanced ability to inhibit both T-cell proliferation, *in vitro*, and arthritic development and progression in CIA *in vivo* compared with naive MSC (28). When primed with TNFα and IFNγ, MSC deficient for PPARβ/δ express increased levels of mediators of MSC immunosuppression, including VCAM-1, ICAM-1, and nitric oxide (NO), compared with their wild-type counterparts (28).

In the present study, we observed that knockdown of PPARβ/δ in MSC did not alter their anti-inflammatory properties when injected into the infarcted myocardium. Indeed, both wild-type and PPARβ/δ KO MSC significantly decreased pro-inflammatory cytokine levels within coronary effluents after 60 min of reperfusion after an ischemic insult. Therefore, the loss of MSC therapeutic effect in the myocardium subjected to IR injury reported here for PPARβ/δ KO MSC could be attributed to an impairment of other functions of MSC pivotal for their beneficial effect in AMI or to a reduction of their survival in perfused hearts. We have recently shown that PPARβ/δ modulation impacts on MSC metabolism (45). Indeed, inactivation of PPARβ/δ promoted the metabolic switch of MSC from oxidative phosphorylation to glycolysis (45). This is in agreement with the role of PPARβ/δ previously described in energy-demanding cells and tissues in which PPARβ/δ promotes fatty acid oxidation that leads to increased ATP production contributing, not only to cell survival but also to cell protection and maintenance of its function (46).

Therefore, it is tempting to anticipate that PPARβ/δ deficiency in MSC could affect their survival rate and reduce their engraftment once injected *in vivo* since PPARβ/δ has been described to promote survival of several cell types, including cancer cells and cardiomyocytes (47, 48) [for review see (49)]. In endothelial cells, anti-apoptotic role of PPARβ/δ has been reported and the underlying mechanisms were related to endothelial 14-3-3 upregulation (50, 51). Similarly, in the cardiomyoblast cell line H9c2, activation of PPARβ/δ by the selective agonist, GW501516, was described to protect the heart from H_2O_2-induced cell death (52). PPARβ/δ is highly expressed by MSC (28) but its role on MSC anti-apoptotic and cardioprotective properties has never been investigated. Moreover, further studies are required to identify key mediators regulated by PPARβ/δ and involved in the acute cardioprotective effect of MSC.

In conclusion, our study shows for the first time that PPARβ/δ plays a key role in the acute local cardioprotective effect of MSC against myocardial IR injury *ex vivo*. Moreover, PPARβ/δ knockdown does not affect the anti-inflammatory properties of MSC assessed in isolated hearts after 1 h of reperfusion. Altogether, these results highlithing the crucial role of PPARβ/δ in MSC cardioprotective properties pave the way toward the development of novel strategies for MSC-based therapy for AMI patients. Further *in vivo* studies will be required to evaluate the contribution of the peripheral immune system to the inflammatory response of IR injury and the subsequent cardiac remodeling.

STUDY LIMITATION

This study had some limitations because the coronary flow was not assessed in our *ex vivo* experiments performed on a conventional Langendorff system (glass coils and tubes) in contrast to previous studies in our laboratory performed with a fully integrated system (discarded here because cells adhered to the long polyethylene tubes) (53, 54). This technical limitation could introduce biases in the quantification of cytokines in the perfusates because the concentration of circulating molecules depends on the coronary flow.

To interpret results on **Figure 1C**, we can refer to data from the literature obtained with the same *ex vivo* model of 30-min global ischemia followed by 60 min of reperfusion showing that coronary flow at 60 min of reperfusion is about 80% of baseline (55). In this context, the decrease in coronary flow at 60 min of reperfusion could contribute to the increase in the measured levels of cytokines in the perfusates compared with basal conditions. Our interpretation was toward an increased release of proinflammatory cytokines in our IR model, consistent with the widely reported pro-inflammatory effects of IR injury (2) even *in vivo* in patients with acute coronary syndrome (40). Considering the results presented in **Figure 3C**, we observed decreased amounts of cytokines (CXCL1 and IL-6) in the PostC condition vs. IR, whereas Maruyama et al. have reported similar values of coronary flow values at 60 min of reperfusion in both IR and PostC conditions (55). On the basis of these results, we can therefore suggest that the decrease in cytokine levels at IR60 observed after a PostC (see **Figure 3C**) may result mainly from a decrease in the release of cytokines vs. IR, in accordance with the anti-inflammatory effect of PostC described *in vivo* (10, 56). However, we cannot exclude an improvement in the coronary flow upon PostC, as described for many other cardioprotective strategies evaluated in our laboratory (53, 54) or others (57), which could also contribute to the reduction in measured amounts of cytokines.

Considering MSC effects (**Figures 3**, **4**), the authors are confident in their interpretation of the results showing a decrease in cytokine release vs. IR, consistent with the anti-inflammatory effect of MSC widely described in the literature (14, 15). Furthermore, these results are corroborated by those showing reduced levels of IL-6 in the serum of pigs subjected to myocardial IR injury *in vivo* (58) and, more importantly, by other data showing decreased protein production and gene expression of inflammatory cytokines (including IL-6) that are not dependent on the coronary flow (20). In addition, it can be assumed that the coronary flow might be decreased when MSC are administered during reperfusion because of their relatively large size in small-diameter coronary vessels, as suggested by *in vivo* data showing microvascular obstruction after intracoronary injection of MSC (59–61), especially with high doses of MSC (44.10^6 cells) leading to an increase in intravascular resistance, a subsequent decrease in coronary flow, and also to microinfarction (62, 63). However, this deleterious effect does not seem to occur here in our experiments because (i) the mean values of cytokine levels assessed in the MSC group were close to those obtained for PostC (**Figures 3B,C**), (ii) the dose of MSC was relatively low (6.10^5 cells/heart), and (iii) a drastic decrease in infarct size was observed upon MSC treatment (**Figures 2B,C, 4B**).

Overall, despite the absence of coronary flow assessment, the results on decreased cytokine concentration presented here seem to be related to the anti-inflammatory effects of PostC and MSC treatment against IR injury.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Studies involving animals were reviewed and approved by the Institute's SBEA (Structure Bien-être Animal) committee in accordance with the European directive 2010/63/EU and the French Ministerial Order of February 01, 2013.

AUTHOR CONTRIBUTIONS

FD and SB-L designed the all project and the experiments. CJ, CP, JN, and AV contributed to design the experimental protocols and interpret the data for the work. CS, CB, GT, RC, PL-C, and NN performed the experiments and analyzed the results. FD and SB-L wrote the manuscript with the input of CJ, JN, AV, SK, and CP. All authors revised and gave final approval of the manuscript.

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Potential Therapies to Protect the Aging Heart Against Ischemia/Reperfusion Injury

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Despite important advances in the treatment of myocardial infarction that have significantly reduced mortality, there is still an unmet need to limit the infarct size after reperfusion injury in order to prevent the onset and severity of heart failure. Multiple cardioprotective maneuvers, therapeutic targets, peptides and drugs have been developed to effectively protect the myocardium from reperfusion-induced cell death in preclinical studies. Nonetheless, the translation of these therapies from laboratory to clinical contexts has been quite challenging. Comorbidities, comedications or inadequate ischemia/reperfusion experimental models are clearly identified variables that need to be accounted for in order to achieve effective cardioprotection studies. The aging heart is characterized by altered proteostasis, DNA instability, epigenetic changes, among others. A vast number of studies has shown that multiple therapeutic strategies, such as ischemic conditioning phenomena and protective drugs are unable to protect the aged heart from myocardial infarction. In this Mini-Review, we will provide an updated state of the art concerning potential new cardioprotective strategies targeting the aging heart.

Keywords: cardioprotection, aging, ischemia/reperfusion injury, mitochondria, senolytics

INTRODUCTION

Although the available treatments of acute myocardial infarction (MI) have improved in the past decades, the consequent heart failure is consistently rising and thus, effective protection of the heart to preserve ventricular function and reduce myocardial remodeling is still an unmet need (1). Several years of research have established that cardiomyocytes have signaling pathways that, upon activation, can reduce the damage elicited by ischemia/reperfusion (I/R) injury, such as the Reperfusion Injury Salvage Kinases (RISK) and the Survivor Activating Enhancement Factor (SAFE) (2–4). There are numerous cardioprotective strategies that can be induced by different ischemic conditioning procedures and drugs with successful results in preclinical studies (5). Nonetheless, translation of cardioprotection from bench to bedside has been highly

challenging (6). Remote ischemic conditioning (RIC) is an important example of this. This cardioprotective maneuver has been thoroughly established as a potent therapeutic strategy in multiple preclinical studies, as well as small clinical trials (5). However, the CONDI-2/ERIC-PPCI trial showed solid evidence that RIC does not provide protection after 1 year in patients with ST-elevation myocardial infarction treated with primary percutaneous coronary intervention (7). It is currently proposed that this discrepancy between preclinical and clinical settings may be attributed to factors such as comorbidities and comedications (8), as well as sex and age (9, 10).

In the context of myocardial infarction and cardioprotection, aging is increasingly attracting attention, due to its rising prevalence. Over the past decades, life expectancy has been steadily increasing (11). It has been estimated that by 2050, a quarter of the global population will be over 65 years old, with the exception of Africa (12). Importantly, although life expectancy has increased, disease-free lifespan has not been markedly increased as compared with lifespan, thereby increasing the burden of age-related diseases, such as cancer, neurodegenerative and cardiovascular diseases (11). Indeed, aging is considered a significant risk factor for cardiovascular diseases (13, 14) and particularly, for MI (15). Accordingly, chronic low-grade inflammation associated with aging is known to promote endothelial and vascular smooth muscle dysfunction, thus exacerbating atherosclerosis and increasing the risk of plaque rupture and thrombus formation (16).

The hallmarks of myocardial aging may account for the reduced tolerance against myocardial I/R injury in preclinical studies (17) and thus, its understanding may enable the development of new and effective therapies to reduce cardiac damage after MI in the context of aging. This mini review aims to discuss potential cardioprotective approaches in the aging heart such as the use of senolytics, as well as therapeutic strategies that aim to decrease mitochondrial damage and reduce inflammation.

THE AGING CARDIOMYOCYTE

One of the key features of the aging heart is mitochondrial dysfunction, characterized by increased mitochondrial fragmentation, ROS production and mitochondrial permeability transition pore opening, as well as reduced mitochondrial biogenesis and electron transport chain activity (17–19). Furthermore, senescent cardiomyocytes also undergo DNA instability, mostly related with telomere shortening and mutations of nuclear and mitochondrial DNA (20). Altered proteostasis is another important feature of aging cardiomyocytes, whereby a reduction in protein synthesis/turnover is associated with a decreased efficiency of the ubiquitin-proteasome system and autophagy (21). The main features of the aged cardiomyocyte are summarized in **Figure 1A**. In addition, Ruiz-Meana et al. provide an in depth analysis and discussion of the aged cardiomyocyte in the context of cardioprotection (17).

POTENTIAL CARDIOPROTECTIVE THERAPIES IN THE AGED HEART

Senolytics: Cardioprotective Potential in the Aged Heart

Senescent cells increase in aged tissues, which has been associated with the progression of age-related diseases (22). In this context, senescence markers are augmented in aged cardiomyocytes (23), which has been linked to higher risk of cardiovascular diseases (24). Senolytics are agents that can selectively target pro-survival proteins of senescent cells, inducing cell death. Regarding the heart, there are three major senolytics that have been widely studied *in vivo* and *in vitro*; Dasatinib, Quercetin, and Navitoclax (24). These senolytics have been shown to improve vascular function (25, 26). Importantly, a study performed by Walaszczyk et al. showed that oral administration of navitoclax to aged mice before *in vivo* myocardial infarction reduced morality, as well as age-related myocardial remodeling and improved left ventricular function (27).

Despite the beneficial effects of senolytics on a preclinical level, there are only two clinical studies showing that these drugs can decrease senescent cells in humans (28, 29). Justice et al., showed that a combination of Dasatinib + Quercetin in patients with idiopathic pulmonary fibrosis can improve physical function (29), while Hickson et al. observed that administration of Dasatinib + Quercetin in patients with diabetic kidney disease elicited a decrease in senescent cells of adipose tissue (28). Despite showing efficacy in treatment of myocardial infarction in the aged heart, Navitoclax has yet to be tested in humans with cardiovascular diseases. Overall, senolytics are a promising pharmacological approach, which appears to effectively reduce senescent cells in humans, but additional preclinical evidence is warranted before they can be translated into the clinical setting of cardioprotection in the elder population.

Currently, there are ongoing efforts to identify new or old compounds that can decrease the number of senescent cells (30). For instance, cardiac glycosides (CGs), have been recently identified as potential senolytic compounds that can successfully decrease the number of senescent cells in the context of cancer in old mice (31) and senescence-induced lung fibrosis (32) in mice. Both of these studies showed that the senolytic effect exerted by CGs depends on the inhibition of Na^+/K^+ ATPase pump (31, 32). Nonetheless, despite the novelty of using these old drugs to clear senescent cells, there are currently no studies showing whether CGs can protect the adult or aged myocardium from I/R injury and thus, this research field is still in preliminary stages in the context of cardioprotection. Moreover, Ouabain is a cardiac glycoside that has been shown to impair growth of proliferative cells in human epithelial cells in a context of oncogene-induced senescence (33) and thus, the specificity of these drugs for senescent cells must be thoroughly evaluated before considering their use in MI.

Recently, an interesting non-pharmacological approach has been investigated. Amor et al. showed that the urokinase-type plasminogen activator receptor (uPAR)-a cell-surface marker that is overexpressed in senescent cells- is a target for chimeric antigen receptor (CAR) T cells, which can clear senescent

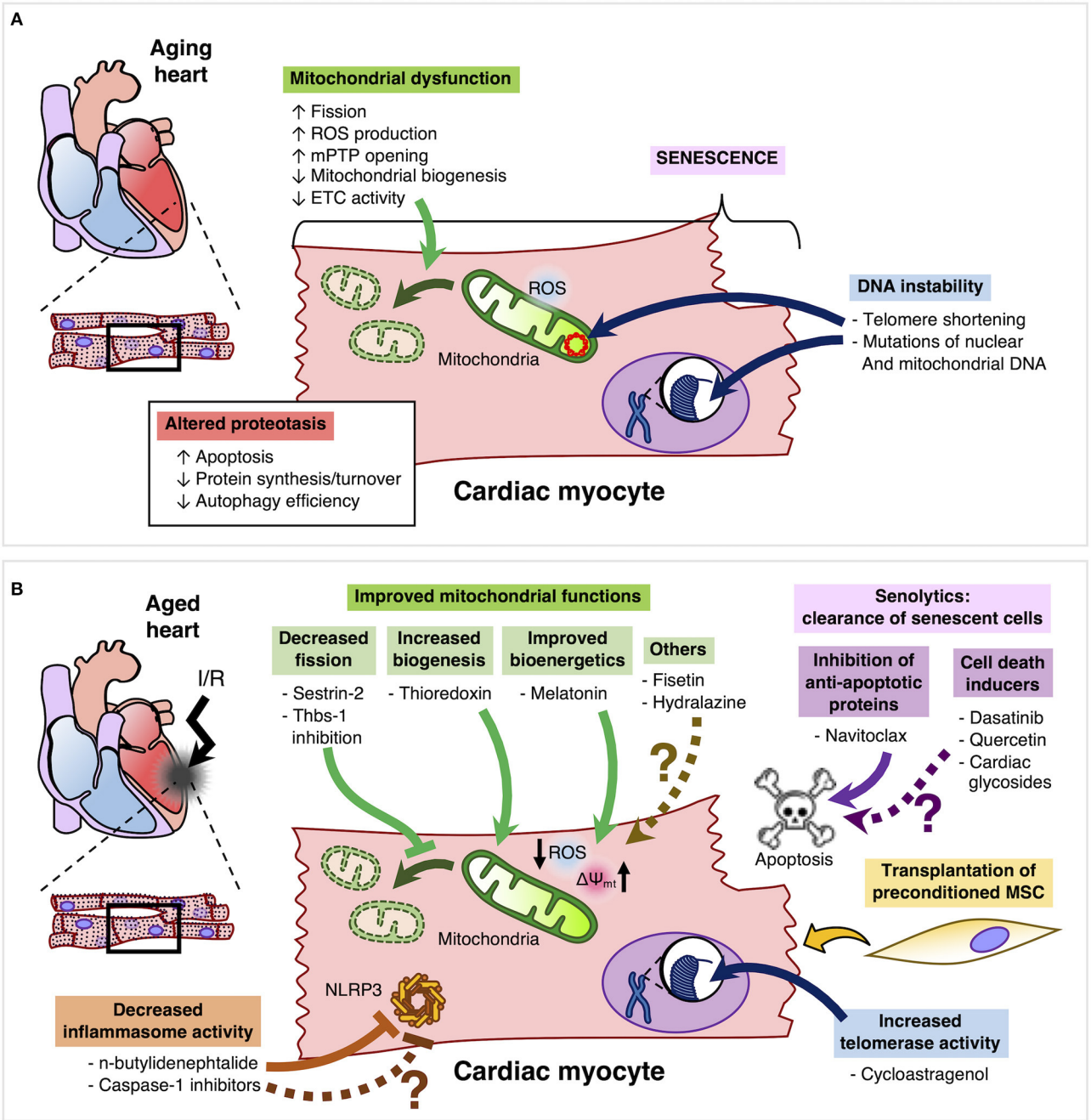

FIGURE 1 | (A) Aging cardiomyocytes are mainly characterized by mitochondrial dysfunction, senescence, altered proteostasis and DNA instability. **(B)** Improvement of mitochondrial dysfunction in aged cardiomyocytes may be achieved by inhibition of Thbs-1 and Sestrin-2, which can decrease Drp-1-mediated mitochondrial fission. Moreover, thioredoxin can promote mitochondrial biogenesis and melatonin can improve bioenergetics by reducing ROS production and improving mitochondrial membrane potential. Other compounds such as Fisetin and hydralazine can confer cardioprotection by reducing mitochondrial dysfunction, but whether these compounds can effectively limit the infarct size and improve left ventricular function of the aging heart remains to be elucidated. Senolytics can clear senescent cells by inhibiting anti-apoptotic proteins. However, only Navitoclax has been tested in the aged heart. However, other senolytics such as Dasatinib, Quercetin, and cardiac glycosides are yet to be evaluated in the settings of cardioprotection and aging. Furthermore, activation of telomerase in order to preserve telomere length and integrity is a promising candidate for cardioprotection in the aging heart. Another emergent target is to inhibit the inflammasome by compounds such as n-butylidenephtalide. Finally, the transplantation of preconditioned mesenchymal cells may have an important therapeutic role in the protection of the aged myocardium.

cells in mice both *in vitro* and *in vivo* in the context of lung adenocarcinoma and liver fibrosis (34), thus providing a promising proof of concept of therapeutic agents for the treatment of senescence-associated diseases, such as MI of the aging heart. However, concerns have been raised over the adverse effects of this therapy. The most frequently reported side effects are cytokine release syndrome and neurotoxicity, but cardiovascular toxic effects, such as systolic dysfunction, arrhythmias, or hypotension have also been reported and thereby, a thorough assessment of cardiac imaging and evaluation of reliable biomarkers is needed to identify at-risk population and establish the safety of this therapy (35).

Role of Mitochondria in Cardioprotection of the Aged Heart

The mitochondria have been identified as an important target to reduce myocardial ischemia/reperfusion injury in the aged heart (36). The mitochondria in aging cardiomyocytes shows elevated ROS production, higher fragmentation and reduced biogenesis, thus producing mitochondrial dysfunction, which can contribute to the increased susceptibility of the aged heart to ischemic injury (17). Therefore, therapies targeting the mitochondria are an attractive area of research in cardioprotection.

It has been reported that Thrombospondin-1 (Thbs-1), which is known to induce ROS production after I/R, regulates Drp-1 dependent mitochondrial fission after MI in both young and old mice (37). Thus, inhibition of Thbs-1 levels after I/R may reduce mitochondrial fission and ROS production, thereby protecting the aging heart against I/R injury. Furthermore, it has been shown that overexpression of thioredoxin -a cytosolic redox protein with reducing properties- in aged mice subjected to *in vivo* I/R, improved mitochondrial function and biogenesis, as well as reducing the infarct size and left ventricular dysfunction (38). Another potential target for cardioprotection is Sestrin2. This protein is induced by stress and forms a complex with AMP-activated protein kinase (AMPK), thus promoting autophagy. Importantly, its cellular levels drop with age, and it's been shown that a Sestrin2-KO promotes mitochondrial fission and damage after myocardial I/R in mice (39). Moreover, overexpression of Sestrin2 in aged mice hearts improved the heart's response to MI (39). Finally, melatonin is a molecule secreted by the pineal gland in humans. It exerts antioxidant and anti-inflammatory properties that may play a cardioprotective role (40). A study in aged rats showed that administration of melatonin and nicotinamide mononucleotide reduces the infarct size and improves left ventricular function after I/R injury by reducing mitochondrial ROS and improving mitochondrial membrane potential (41). Moreover, it has been suggested that melatonin protects against I/R injury via the RISK and SAFE pathways (42). Interestingly, a small clinical trial has provided proof of concept that the timing of administration of melatonin in infarcted patients undergoing percutaneous coronary intervention is key for its effectiveness (43). However, this observation needs to be confirmed in large randomized controlled clinical trials.

There are multiple strategies and treatments that target the mitochondria to achieve cardioprotection that have not been yet studied in the context of aging. We will discuss two of them: fisetin and hydralazine. Fisetin is a flavonoid found in many fruits with senolytic properties (44). Recently it's been shown that it can also confer protection in I/R settings. A preliminary study showed that fisetin can inhibit H9c2 cardiomyoblast apoptosis after hypoxia/reoxygenation, as well as to reduce mitochondrial ROS production and inhibit the activation of caspases 3 and 9 (45). In addition, adult rats pre-treated with fisetin and subjected to *ex vivo* I/R showed a reduced infarct size, reduced mitochondrial oxidative stress and improved mitochondrial structure and function by a mechanism that appears to involve inhibition of GSK3β activity (46). Hydralazin, is an FDA approved drug currently used as an anti-hypertensive agent and in the treatment of chronic heart failure. Recently, it has been described that it can confer cardioprotection in mice using *in vivo*, *ex vivo* and *in vitro* experimental models of I/R injury via inhibition of Drp-1-mediated mitochondrial fission (47). These two molecules have in common their capacity to protect the heart from I/R injury by improving mitochondrial function and thus, these therapeutic agents are promising candidates to protect the aging heart from MI. Therefore, future studies should explore this possibility.

Inflammasome: Importance of NLPR3 in Aging

During MI, a pathogen/antigen-independent inflammatory response, known as sterile inflammation, takes place (48). Due to the rupture in the cellular structure that occurs during MI, damage-associated molecular patterns (DAMPS) mediators are released and are recognized by pattern recognition receptors (PRRs), which in turn mediate the initiation of the inflammatory response (49). NLPR3 inflammasome is a multiprotein complex formed by the activation of PRRs, thereby increasing the production and release of proinflammatory cytokines via activation of caspase-1 (50). NLPR3 has attracted attention as a mediator of damage produced by I/R injury (51). Mastracola et al. tested the role of NLPR3 in C57B1 male mice fed with a high fat high fructose diet (HFHF) using an *ex vivo* I/R model. The authors found that the HFHF diet upregulated NLPR3 protein content, and this elevation downregulated the RISK/HIF-2alpha pathways (51–53). Furthermore, NLPR3 has also been proposed as a target in the aging myocardium. Lee et al. has explored this hypothesis in a recent study where they use *n*-butylidenephthalide as a preconditioning agent and tested the capacity of human adipose–derived stem cell (hADSC) engraftment in the recovery of infarcted myocardium of young and old Wistar rats (54). The study showed NLPR3 activity and ROS production was significantly increased in old rats after MI, as compared with young rats. Transplantation of hADSC reduced NLPR3 activation and ROS levels in both young and old rats, but this response was significantly more effective in young rats. The authors discovered that the NLPR3 inflammasome mediates the difference of the response to the engraftments in old and young rats. They also reported that n-butylidenephthalide reversed the complex microenvironment that impedes engraftment success (54).

Interestingly, pharmacological inhibition of caspase-1 reduced the infarct size in isolated rat hearts (55). Also, caspase-1 inhibition was also shown to provide additional protection when combined with remote ischemic preconditioning in rats subjected to *in vivo* myocardial infarction (56). This study is particularly interesting, since these experiments were performed using a co-medication model, consisting of co-administration with an opioid agonist, heparin and a platelet-inhibitor to mimic a clinical setting, thereby highlighting the translational value of this cardioprotective therapy (56). This promising evidence merits further research to evaluate whether this approach may have a significant impact in the cardioprotection of the aged heart.

Telomerase Activity in Aging and Cardioprotection

Telomeres are repeated hexanucleotide sequences at the end of eukaryotic chromosomes. Their presence is associated with DNA protection during cell division. Division of the cell as well as oxidative stress shortens these structures, leading the cell to a senescent state or apoptosis (57). Telomere length has been associated with coronary artery disease and therefore, it has been proposed as a biomarker for cardiovascular diseases (58). Telomerase is a key regulator of telomere length and integrity (20) and as such, has gained attention for its potential benefits in age-related cardiovascular diseases. For instance, absence of telomerase has been associated with increased susceptibility to ischemic injury (59). By the same token, overexpression of telomerase can confer cardioprotection in mice hearts (60). Furthermore, overexpression of growth differentiation factor 11 (GDF11) in mice subjected to *in vivo* I/R injury showed a reduced infarct size, activation of telomerase, longer telomeres and increased mitochondrial biogenesis (61). In a clinical context, Gupta et al. have published a pilot study that proposes telomere length as a screening tool for patients with acute myocardial infarction (62). Their study revealed that telomere length was reduced in young patients without risk factors who underwent MI, as compared with young patients without MI (62). Moreover, Maier et al. have published the design of a pilot trial that uses the telomerase activator TA-65MD, which is a purified and encapsulated form of cycloastragenol, in elderly patients with acute coronary syndrome (63). The aim of this study is to assess whether this therapy can reverse inmmunosenescence, which is associated to the pathophysiological progression of coronary artery disease. The results to this trial are scheduled to be published in 2021 (63). Although more preclinical data is required to accurately assess whether this therapeutic strategy can reduce cardiac damage in aging, telomerase appears to be an essential player in cardioprotection.

Other Potential Therapies to Protect the Aged Myocardium From I/R Injury

In addition to the aforementioned strategies, there are other approaches that may wield cardioprotective effects in aging conditions. An interesting study has suggested that necroptosis may contribute to ischemic susceptibility in the aging heart and that metformin reduces necroptosis elicited by alterations of autophagy during myocardial aging (64). Another potential therapeutic target may be the JAK/STAT pathway. It has been reported that inhibition of JAK in old mice has been linked with reduced senescence-associated secretory phenotype and frailty (65). Moreover, deletion of Kcne4 in old mice has been found to sex-specifically induce arrhythmias by a mechanism that involves defective signaling of the RISK/SAFE pathway mediated by testosterone (66). Furthermore, it has been observed that the JAK/STAT pathway is activated in aging humans, which is associated with chronic inflammation and increased cardiovascular risk (67). However, whether targeting the JAK/STAT pathway may restore the effectiveness of protective strategies in the context of MI remains to be elucidated.

Aging has also been described to alter mesenchymal stem cell function, which impairs their cardioprotective effects (68). It has been recently identified that miR-155-5p can regulate mesenchymal stem cell senescence (69). This study demonstrated that inhibition of miR-155-5p reduced senescence in aged mesenchymal stem cell, thus improving their ability to protect the aged heart from MI in mice (69).

Finally, a study by Crewe et al. revealed that adipocytes can secrete small extracellular vesicles that can transport damaged mitochondria, which can precondition the heart by inducing a short period of mitochondrial oxidative stress, which eventually leads to an antioxidant effect that can effectively protect the heart from MI (70). While this effect has not been evaluated in the aged heart, protection elicited by transport of mitochondria by small extracellular vesicles via hormesis may be an effective therapy, given the importance of this organelle in the aging myocardium.

Conclusions and Future Perspectives

The aged heart is more susceptible to I/R injury. Moreover, multiple cardioprotective maneuvers have been shown to be ineffective under these conditions, highlighting the need to develop therapies that can provide clinical efficacy in the reduction of infarct size. Clearance of senescent cells by the senolytic drug navitoclax has shown clear protection of aged hearts from MI, but the current evidence is still preliminary and more senolytics need to be tested to establish the real potential of these agents. Moreover, the clinical evidence regarding the use of these drugs is still in early stages, especially since there are no studies testing their effects in MI patients. Cardioprotective approaches targeting the mitochondria in the aged heart are promising, although the current evidence is mainly centered in preclinical studies, suggesting that the translational value of these therapies remains to be tested in clinical settings. Similarly, targeting of the inflammasome or telomerase activity are research areas of interest, but further studies are certainly required to evaluate whether these therapeutic strategies can be translated from bench to bedside. Currently, cardioprotection in aging can be tested using primary cells, *ex vivo* and *in vivo* animal models to evaluate the effect of protective drugs or conditioning maneuvers (17). The use of cell lines may be useful to assess stress-induced premature senescence and thus generate a preliminary proof-of-concept regarding a potential senolytic therapy, but while this allows to avoid the use of

animals, this approach only elicits a senescence-like phenotype (71). Therefore, this model may have a low translational value. Regarding cardioprotection research, preclinical models are solid in the early stages, but clinical studies are strong in the follow-up process (72). In this context, Heusch has proposed to use integrative large animal models and study the whole process: from acute myocardial infarction to a 12 months follow-up (72) and this idea is certainly valid for the study of cardioprotection in aging as well. Furthermore, it's crucial to consider the importance of generating reliable evidence to drive cardioprotection research forward by adhering to proposed guidelines to ensure rigor and reproducibility, such as the ones presented by Bøtker et al. (73). Finally, while the potential therapies we have reviewed are promising (**Figure 1B**), it is important to acknowledge a lack of studies in this field. For instance, while aging can impair the cardioprotective effect of conditioning strategies in preclinical studies (8) a retrospective study showed that age did not disrupt cardioprotection in coronary artery bypass graft patients with or without RIC (74). Thus, while the evidence showing aging as a confounding factor in cardioprotection is thorough, clinical evidence is mainly obtained from retrospective secondary analyses (8), highlighting the distance between bench and bedside, which is barrier that needs to be overcome to protect the elderly from myocardial infarction.

AUTHOR CONTRIBUTIONS

MD-V, ÚZ-C, AR-R, NH-Z, IP, RB-S, and JR participated in the conception and design of the manuscript and made significant contributions to the analysis of the evidence discussed. All authors wrote the manuscript and gave the final approval of the submitted manuscript.

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Sodium-Glucose Co-transporter-2 Inhibitor of Dapagliflozin Attenuates Myocardial Ischemia/Reperfusion Injury by Limiting NLRP3 Inflammasome Activation and Modulating Autophagy

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Background: The sodium-glucose co-transporter-2 (SGLT-2) inhibitor dapagliflozin improves cardiovascular outcomes in patients with type 2 diabetes in a manner that is partially independent of its hypoglycemic effect. These observations suggest that it may exert a cardioprotective effect by another mechanism. This study explored the effects of dapagliflozin on myocardial ischemia/reperfusion injury in a mouse model.

Materials and Methods: For the *in vivo* I/R studies, mice received 40 mg/kg/d dapagliflozin, starting 7 days before I/R. Evans Blue/TTC double-staining was used to determine the infarct size. Serum levels of cTnI, CK-MB, and LDH were measured. Inflammation, autophagy protein expression, and caspase-1 activity changes were measured at the protein level. Primary cardiomyocytes were used to investigate the direct effect of dapagliflozin on cardiomyocytes and to verify whether they have the same effect as observed in *in vivo* experiments.

Result: A high dose of dapagliflozin significantly reduced infarct size and decreased the serum levels of cTnI, CK-MB, and LDH. Dapagliflozin also reduced serum levels of IL-1β, reduced expression of myocardial inflammation-related proteins, and inhibited cardiac caspase-1 activity. The treatment restored autophagy flux and promoted the degradation of autophagosomes. Relief of inflammation relied on autophagosome phagocytosis of NLRP3 and autophagosome clearance after lysosome improvement. 10 μM dapagliflozin reduced intracellular Ca^{2+} and Na^{+} in primary cardiomyocytes, and increasing NHE1 and NCX expression mitigated dapagliflozin effects on autophagy.

Conclusion: Dapagliflozin protects against myocardial ischemia/reperfusion injury independently of its hypoglycemic effect. High-dose dapagliflozin pretreatment might

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limit NLRP3 inflammasome activation and mediate its selective autophagy. Dapagliflozin directly acts on cardiomyocytes through NHE1/NCX.

Keywords: sodium-glucose cotransporter-2 inhibitor, myocardial ischemia/reperfusion injury, autophagy, inflammasome, NLRP3

INTRODUCTION

Ischemic heart disease (IHD) is the primary cause of morbidity and mortality worldwide (1), imposing tremendous pressures on the healthcare system and economic development (2). It is estimated that by 2030, IHD will be the second leading cause of death worldwide (3). The primary treatment for IHD is coronary reperfusion (percutaneous coronary intervention, thrombolysis, and coronary bypass surgery) to restore the blood supply to the ischemic myocardium (4). However, sudden reperfusion may cause additional damage (5), commonly known as myocardial ischemia/reperfusion injury (I/R). Prevention of the injury after myocardial reperfusion is a challenge.

Dapagliflozin (DAPA), a sodium-glucose co-transporter-2 (SGLT-2) inhibitor, is a new type of antidiabetic medication that reduces blood glucose by inhibiting SGLT-2 in the proximal tubules of the kidney (6). Unlike other hypoglycemic drugs, dapagliflozin rarely causes hypoglycemia, even in non-diabetic patients (7). Studies suggested that SGLT-2 inhibitors are associated with significantly improved cardiovascular outcomes in patients with type 2 diabetes (T2DM) (8), including improving myocardial structure (9) and cardiac function (10), inhibition of cardiac inflammation (10), reduction of oxidative stress (11) and myocardial cell apoptosis (10), protection of mitochondrial function (9) and maintaining ion balance in isolated cardiomyocytes (12). For these reasons, in patients without T2DM who suffer from cardiovascular diseases like heart failure, SGLT-2 inhibitors might benefit (13, 14). We hypothesized that SGLT-2 inhibitors would meliorate myocardial ischemia/reperfusion injury.

Activation of inflammation and release of inflammatory cytokines aggravate myocardial I/R injury (15). Inflammasomes are an essential component of the inflammatory response, leading to the production of pro-inflammatory cytokines, such as NLRP3-related inflammasomes, which are composed of NLRP3 (NACHT, LRR, and PYD domains containing protein three), procaspase-1, and ASC (apoptosis-associated speck-like protein containing a CARD). Inflammasomes may activate caspase-1 to cleave IL-1β (16). Autophagy is a self-protection mechanism in eukaryotes under normal conditions (17). In autophagy, cytoplasmic components are transferred to lysosomes for degradation. Autophagy has been reported in myocardial I/R injury (18). In addition, autophagy might eliminate inflammatory stimuli and degrade NLRP3-related inflammasomes to retard inflammation (19). The inflammasomes are ubiquitinated, recruited to the autophagic adapter protein P62, and then transported to autophagosomes to complete the degradation process (20). It is worth noting that autophagy and inflammation are double-edged swords for cardiac I/R: they can decrease or increase cardiac I/R depending on their degree of activation. This study aimed to determine whether dapagliflozin would protect against myocardial I/R injury and reduce cardiac inflammation stimuli and if this result could be achieved by modulating autophagy.

MATERIALS AND METHODS

Animals

All related operations involving animals follow the "Guidelines for Care and Use of Laboratory Animals" (NIH Publication No.85-23, revised 1996) published by the US National Institutes of Health (Bethesda, MD). And approved by the Animal Care and Use Committee at the Wenzhou Medical University (wydw2020-0634). All Male C57/BL6 mice (6–7 weeks old and 20–25 g weight) were purchased from the SLAC Laboratory Animal Center of Shanghai (Shanghai, China).

Myocardial Ischemia/Reperfusion Model

The myocardial I/R model was operated as our previous study (21). In detail, the mice were first anesthetized with isoflurane and intubated. Then a thoracotomy was performed to expose the heart in the third or fourth rib. The left anterior descending coronary artery was ligated with a 7-0 silk thread to cause myocardial ischemia. After 30 min of ischemia, the silk thread was untied and the blood was reperfused for 4 h. Mice in the sham group underwent the same operation but were not ligated. The operation uses an animal anesthesia machine/ventilator from RWD Life Science, the pressure is adjusted to 0.4 after isoflurane perfusion, flow rate two, stroke volum (μl) 300, strokes/min 180. The isoflurane pumping was stopped immediately after the start of reperfusion, and the air was maintained until awakening.

High-Throughput Sequencing

We selected a total of 6 mouse heart samples, of which three were treated by sham operation, and three were ischemia for 30 min and reperfused for 4 h. After the samples were collected, they were transported to Beijing on dry ice in accordance with the biological sample storage requirements, and Beijing Novogene completed high-throughput second-generation sequencing.

Animal Grouping and Pretreatment

After all C57/BL6 mice were reared to 7 weeks, they were randomly divided into five groups: (a) Sham group-mice were subjected to sham operation and treated with vehicle (saline); (b) I/R group- mice were subjected to I/R intervention and treated with vehicle (saline); (c) I/R+CQ group-mice were subjected to I/R intervention after intraperitoneal injection of chloroquine and treated with vehicle (saline); (d) I/R+DAPA group-mice were subjected to I/R intervention and treated with dapagliflozin (40 mg/kg/day); (e) I/R+ DAPA+CQ group-mice were subjected to I/R intervention after intraperitoneal injection of chloroquine

and treated with dapagliflozin (40 mg/kg/day). Treatment with dapagliflozin (MCE, China, 40 mg/kg/day) or vehicle by gavage was started 7 days before the cardiac I/R intervention. The dose of chloroquine (Sigma, USA) is 10 mg/kg, which is injected intraperitoneally 10 min before each operation, which has been mentioned in our previous article (21).

Detection of Myocardial Infarction Size and Risk Area

Evans Blue/Triphenyltetrazolium chloride (TTC, Sigma, USA) staining was used to detect the myocardial infarct size induced by I/R injury. The coronary artery was ligated again, and the abdominal aorta was injected with 200 μL of 2% Evans Blue, the heart was immediately taken out, placed in a mold for sectioning, and then incubated in 1% TTC staining solution at 37°C for 30 min, after finishing overnight in 4% paraformaldehyde and filming. The images were analyzed using Image Pro software (Media Cybernetics, Inc. Bethesda, MD), and infarct size was calculated using a method as previously described (21), infarct size was compared when area at risk that were similar among groups.

Serum Biochemical Analysis

Blood was taken 4 h after reperfusion, and the serum was separated by centrifugation at 7,500 rpm for 15 min, and immediately detected or temporarily frozen at −80°C. Serum TnI concentration was measured using an enzyme-linked immunosorbent assay (ELISA) measurement of Troponin I kit (Elabscience, E-EL-M0086c). Serum LDH activity was measured using a Lactate dehydrogenase (LDH) assay kit (Nanjing jiancheng, China). Serum creatine kinase-MB (CK-MB) was measured with a creatine kinase MB isoenzyme Assay Kit (Nanjing jiancheng, China).

Detection of Secreted IL-1β

Serum concentrations and cell culture supernatant medium level of IL-1β were measured by the corresponding ELISA kits (Elabscience, E-EL-M0037) according to the manufacturer's instructions.

Detection of Caspase-1 Activities

The cardiac activity of caspase-1 was calculated via Colorimetric Assay Kit (Solarbio, China). First, the total protein was extracted according to the instructions and formulated into a 2 mg/ml system.

Myocardial Tissue and Cellular Immunofluorescence

Immunofluorescence staining is used in myocardial tissues and cells. Use frozen tissue sections or 4% paraformaldehyde-fixed cells, rupture the cell membrane with 0.3% Triton for 10 min, block with 5% BSA for 1 h at room temperature, and then incubate with the corresponding primary antibody at 4°C overnight. Incubate with the corresponding secondary antibody for 1 h in the dark the next day, and finally add DAPI to stain the nucleus. After completion, add anti-fluorescence quenching solution and take pictures under the microscope (Leica, Germany).

Cardiomyocyte Primary Culture (PCCMs)

Isolate primary cardiomyocytes from the heart of C57/BL6 mice born within 1–3 days. The specific method can refer to the study by Ehler et al. (22). The detailed steps for isolation of primary cardiomyocytes are written in the **Supplementary Material**.

Hypoxia/Re-Oxygenation (H/R) Model

These PCCMs were then used to simulate the H/R model (22). Hypoxic conditions was performed in a hypoxic incubator with an N_2 concentration of 94% and a O_2 concentration of 1% and a CO_2 concentration of 5% for 6 h in medium deprived of serum. Then PCCMs were replaced with normal medium and put into ordinary incubators (CO_2 concentration of 5% and O_2 concentration of 21%) for 18 h to simulate reoxygenation injury. For *in vitro* experiments, DAPA(10 μM) was administered for 1 h (23) and CQ was administered for 24 h at 10 μM (22) in advance. Before starting H/R, replace with a new medium.

Cell Viability and Toxicity Detection

The PCCMs were plated in 24-well plates and treated separately. After processing, use the Cell Counting Kit-8 (Beyotime, China) to measure the number of viable cells according to the manufacturer's instructions. At the same time, the upper medium of PCCMs after treatment was tested for cytotoxicity with LDH Assay Kit.

Hoechst 33342/PI Staining Assay

Hoechst 33342/PI (Beyotime, China) has been reported to be used to label dead or apoptotic cells. We seeded PCCMs on cell slides in 24-well plates. After completing the corresponding treatments, we added Hoechst 33342 and PI working solution in order according to the manufacturer's instructions. Finally, we added the anti-fluorescent quenching solution and took it under a fluorescent microscope for observation.

Lysotracker Red Stain

Lysotracker Red can be selectively retained in the acidic lysosomes, thereby achieving specific fluorescent labeling of lysosomes (24). In our experiments, we used Lysotracker Red (Beyotime, China) to detect the number of intact lysosomes in an H/R model simulated by PCCMs.

Co-immunoprecipitation

PCCMs lysates were centrifuged for 10 min at 14,000 g at 4°C. 500 μL of the lysate (1000 μg of total protein) was added to the monoclonal primary antibody of the target protein at 4°C overnight. The next day, agarose beads were added and incubated for 3 h. The agarose beads were washed 3 times and then mixed with SDS for western blot analysis. The above reagents were all purchased from absin (abs955, China).

Fluo-4AM and SBFI AM Measurement

First, the PCCMs are grouped and processed as before. After the H/R treatment, dilute Fluo-4AM (Beyotime, China) and SBFI AM to working concentrations according to the instructions, add

them to the culture medium, incubate at 37°C for 30 min, wash with PBS and then put them back into the cell incubator for 30 min, and select the 334/505 wavelength for detection.

Transfection Protocol

For overexpression plasmid transfection, 1 μg plasmid was added in 500 μl of Opti-MEM, followed by addition of 3 μl of Lipofectamine RNAiMAX in 500 μl of Opti-Mem and incubated for 5 min. The two mixtures were pooled and incubated further for 10 min at RT. The respective transfection mixture was then added to the cells and mixed by gently swirling the plate. The plate was then incubated at 37°C for 18 h in a 5% CO2 incubator. After incubation, media was added to the cells, and the cells were further incubated until harvest i.e., 48 h from the beginning of transfection.

Western Blot Analysis (WB)

Myocardial tissue/cell total protein preparation and concentration determination kits are from Beyotime (Shanghai, China). Proteins of different molecular weights were separated on SDS-PAGE gels, transferred to 0.22 μm PVDF membranes, and blocked with skimmed milk powder then incubated with primary antibodies of NLRP3 (A12694, ABclonal), Caspase-1 (22915-1-AP, proteintech), ASC (abs135824, absin), IL-1β (16806-1-AP, proteintech), Cleaved-IL-1β (A1112, ABclonal), Atg-5 (12994T, CST), Beclin-1 (3738S, CST), P62 (23214S, CST), LC3B (ab243506, abcam), LAMP1 (3243S, CST), LAMP2 (66301-1-Ig, proteintech),CTSD (21327-1-AP, proteintech), CTSB (12216-1-AP, proteintech), and GAPDH (10494-1-AP, proteintech) at 4°C overnight. After incubating with the corresponding secondary antibody the next day, protein bands were scanned by a ChemiDoc MP device (Bio-Rad, Hercules, CA, USA). Finally, ImageJ software (NIH, USA) was used for analysis.

Statistical Analysis

Data were presented as mean ± SD, and were analyzed using SPSS version 21.0 (IBM, Armonk, NY, USA). All the data we used were normally distributed. A two-tailed unpaired Student's *t*-test was conducted to compare two experimental groups or one way analysis of variance (ANOVA) followed by Duncan's T3 multiple-range test was used for the comparison of more than two groups. $P < 0.05$ were considered as statistically significant.

RESULTS

Dapagliflozin Treatment Attenuates Myocardial Ischemia/Reperfusion Injury *in vivo* and *in vitro*

We first evaluated the effect of DAPA on myocardial I/R injury by assessing myocardial infarct size. After DAPA treatment, the area of myocardial infarction in mice was significantly decreased for area at risk that were similar among groups (**Figures 1A–C**). DAPA significantly decreased cTnI, CK-MB, and LDH levels after I/R (**Figures 1D–F**).

Coincidentally, we also found that DAPA treatment significantly improved cell viability of PCCMs after hypoxia/re-oxygenation injury (H/R). The optimal concentration of DAPA was 10 μM (**Figure 1I**). Subsequent treatment with 10 μM DAPA reduced LDH in the cell supernatant (**Figure 1J**) and a significant decrease in myocardial cell death (**Figures 1G,H**).

Dapagliflozin Treatment Attenuates the Inflammatory Response in Myocardial I/R Injury

Many factors cause myocardial I/R injury; to explore which factor is the main factor in our model, we performed high-throughput sequencing on the I/R injury area. Sequencing results suggest that inflammatory response is the central part of the biological process (**Figure 2A**).

We speculated that DAPA treatment would reduce the inflammatory response in I/R injury. First, we detected inflammatory proteins in the tissues and found that DAPA treatment significantly reduced the expression levels of Pro-IL-1β and Cleaved-IL-1β (**Figures 2B–D**). We also detected the secretion level of IL-1β in the serum of mice. It was found that DAPA treatment also reduced the secretion of IL-1β in the serum of I/R mice (**Figure 2E**). Coincidentally, DAPA also improved H/R injury of PCCMs *in vitro*. DAPA treatment decreased expression of Pro-IL-1β and Cleaved-IL-1β *in vitro* (**Figures 2F–H**) and reduced the secretion of IL-1β in the PCCMs culture medium (**Figure 2I**).

Dapagliflozin Can Inhibit the Assembly and Activation of NLRP3 Inflammasome

Through sequencing, we have found that the inflammatory response dominates in our I/R model, and DAPA treatment inhibits this inflammatory response. In order to explore how inflammation is activated here, we performed KEGG pathway analysis on the sequencing results and screened the top 10 using "count number" as the standard and found that the signaling pathways directly related to inflammation are "NF-kappa B" and "NOD-like receptor" (**Figure 3A**). And through screening with "fold enrichment" as the standard, it is found that "NOD-like receptor" is more important (**Figure 3B**).

So we selected NLRP3 inflammasome as the verification target. By detecting the level of NLRP3 inflammasome-related protein, we found that after DAPA treatment, the expression of NLRP3 and ASC were reduced (**Figures 3C–F,J,K**), and the production of Cleaved-caspase-1 was also significantly inhibited (**Figures 3C,D,G,H,L,M**). On the other hand, the enzyme activity of caspase-1 activated during I/R injury also decreased significantly after DAPA treatment (**Figure 3I**).

Dapagliflozin Treatment Restores Autophagy Flux Following Ischemia/Reperfusion Injury *in vivo* and *in vitro*

Recently, autophagy has been implicated in I/R (25) and may be related to inflammation (19). We analyzed the expression of autophagy-related proteins by Western blot analysis to determine

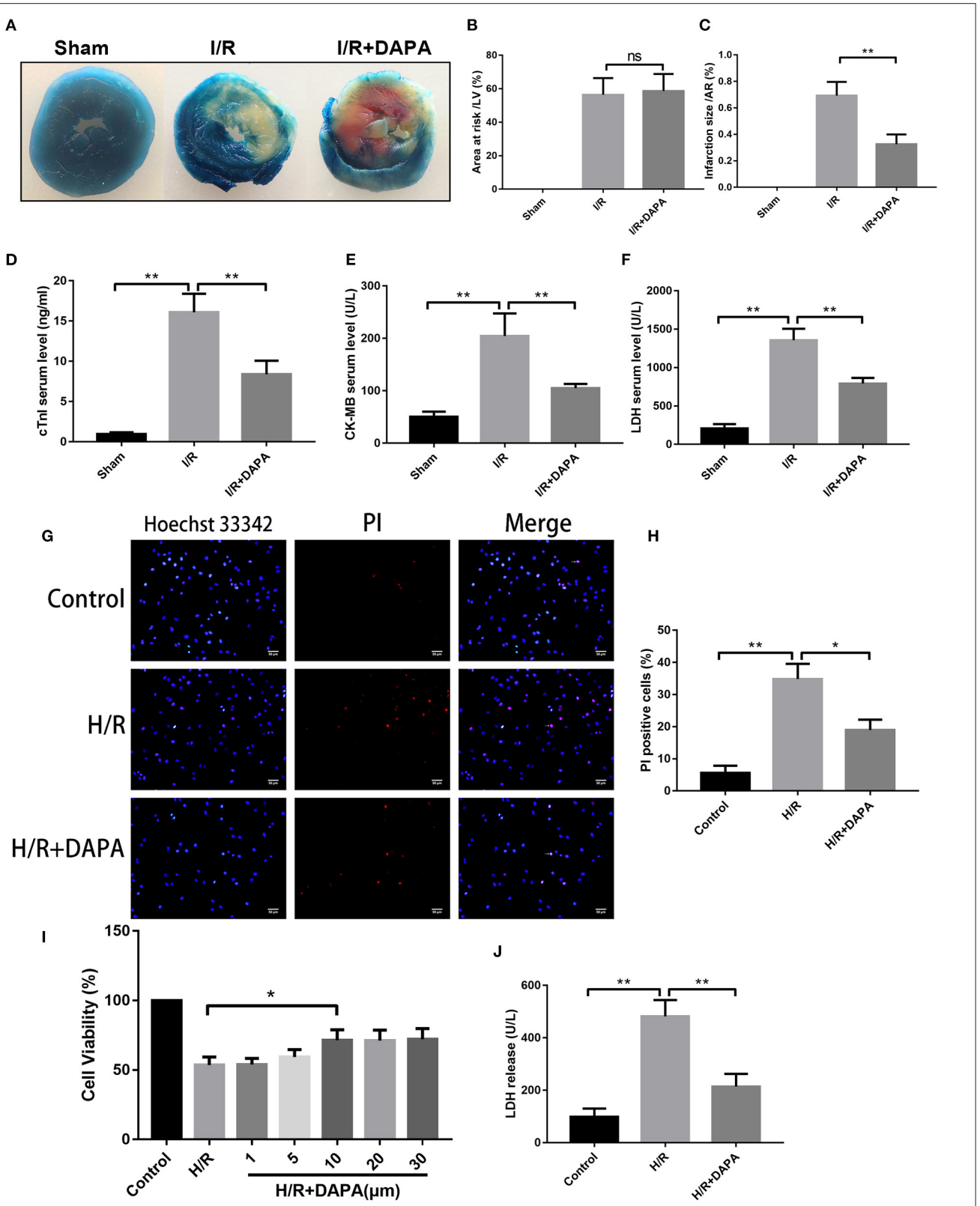

FIGURE 1 | Dapagliflozin treatment attenuates myocardial ischemia/reperfusion injury *in vivo* and *in vitro*. **(A)** 6-7w mice were administered DAPA or vehicle control, established I/R model, and measured the infarct size of each group using TTC. **(B,C)** Comparison of the myocardial infarction area in each group, $n = 3$. **(D–F)** cTnI, CK-MB, and LDH levels in mouse serum, $n = 9$. **(G)** Red indicates PI staining and blue indicates Hoechst staining. **(H)** PI staining positive ratio, $n = 3$. **(I,J)** Lactate dehydrogenase (LDH) and cell viability assay, $n = 9$. Data are means ± SDs, $^{*}P < 0.05$; $^{**}P < 0.01$.

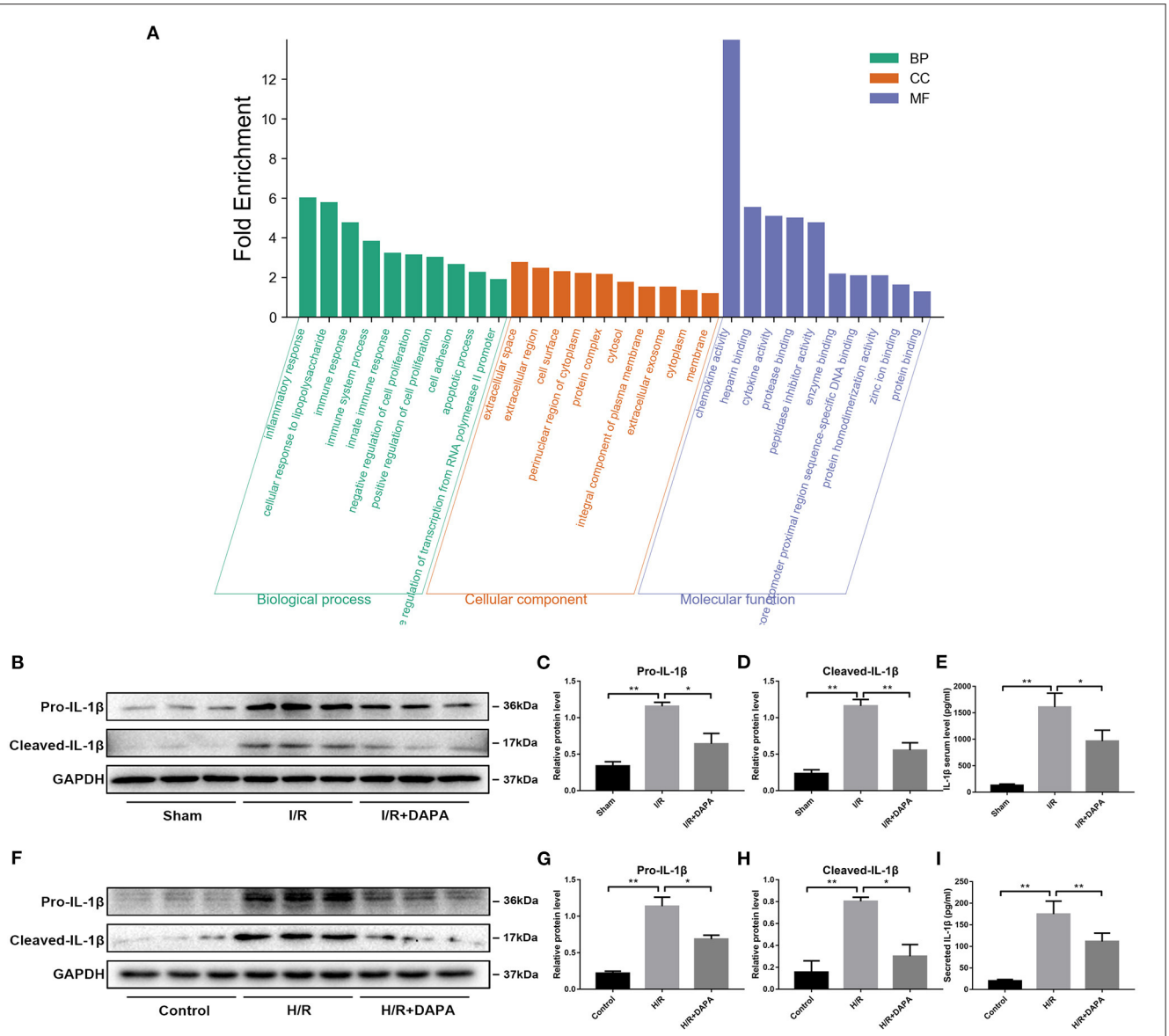

FIGURE 2 | Dapagliflozin treatment attenuates the inflammatory response in myocardial I/R injury. **(A)** GO function enrichment after I/R myocardial high-throughput sequencing, fold change ≥1.5 and adjusted $p < 0.05$, $n = 3$. **(B)** Western blot detection was performed from LV protein extracts from each group *in vivo*. **(C,D)** Western blot analysis of Pro-IL-1β and Cleaved-IL-1β *in vivo*, $n = 3$. **(E)** Serum IL-1β levels, $n = 9$. **(F)** Western blot detection was performed from PCCMs protein extracts from each group *in vitro*. **(G,H)** Western blot analysis of Pro-IL-1β and Cleaved-IL-1β *in vitro*, $n = 3$. **(I)** Secretion of IL-1β from cell supernatants, $n = 9$. Data are means ± SDs, $^{*}P < 0.05$; $^{**}P < 0.01$.

the effect of DAPA on autophagy in myocardial I/R injury. The results showed that LC3B-II, a representative protein of autophagy, was significantly increased in I/R, accompanied by an increase of Atg-5 and Beclin-1, which are related to autophagosome production (26). P62, a ubiquitinated protein that may indicate autophagic flux (26), was also increased (**Figures 4A–E,G–K**). The formation of autophagosomes was increased, and the destruction of autophagosomes was reduced, which led to the accumulation of autophagosomes.

DAPA treatment reduced the expression of LC3B-II, Atg-5, beclin-1, and P62 (**Figures 4A–E,G–K**). We next explored whether the reduction of autophagosomes after DAPA treatment was due to inhibition of autophagosome production, increased destruction, or both. To show that this phenomenon occurs in myocardial cells, we co-stained LC3 and cTnT (cardiac-specific markers) and measured their relative concentrations by immunofluorescence (**Figure 4F**). The results were consistent with those of Western blot assays. When PCCMs were stained for LC3 and imaged by immunofluorescence microscopy, the results were the same: pre-treatment of DAPA reduced the accumulation of autophagosomes (**Figure 4L**).

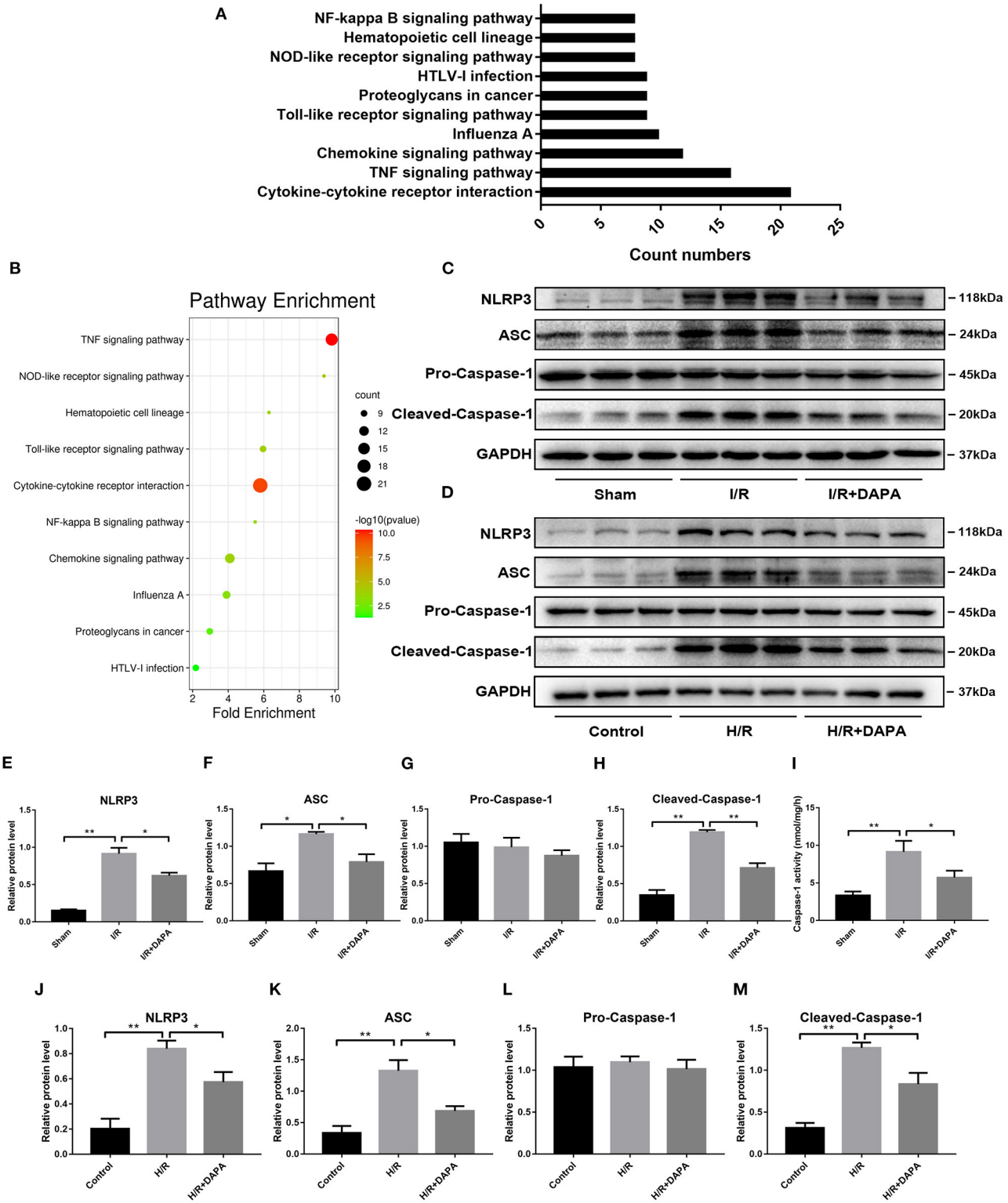

FIGURE 3 | Dapagliflozin can inhibit the assembly and activation of NLRP3 inflammasome. **(A,B)** KEGG pathway enrichment after I/R myocardial high-throughput sequencing, fold change≥1.5 and adjusted $p < 0.05$, $n = 3$. **(C)** Western blot detection was performed from LV protein extracts from each group *in vivo*. **(D)** Western blot detection was performed from PCCMs protein extracts from each group *in vitro*. **(E–H)** Western blot analysis of NLRP3, ASC, Pro-Caspase-1, Cleaved-Caspase-1 *in vivo*, $n = 3$. **(I)** Caspase-1 activity in myocardium tissues, $n = 9$. **(J–M)** Western blot analysis of NLRP3, ASC, Pro-Caspase-1, Cleaved-Caspase-1 *in vitro*, $n = 3$. Data are means ± SDs, $^{*}P < 0.05$; $^{**}P < 0.01$.

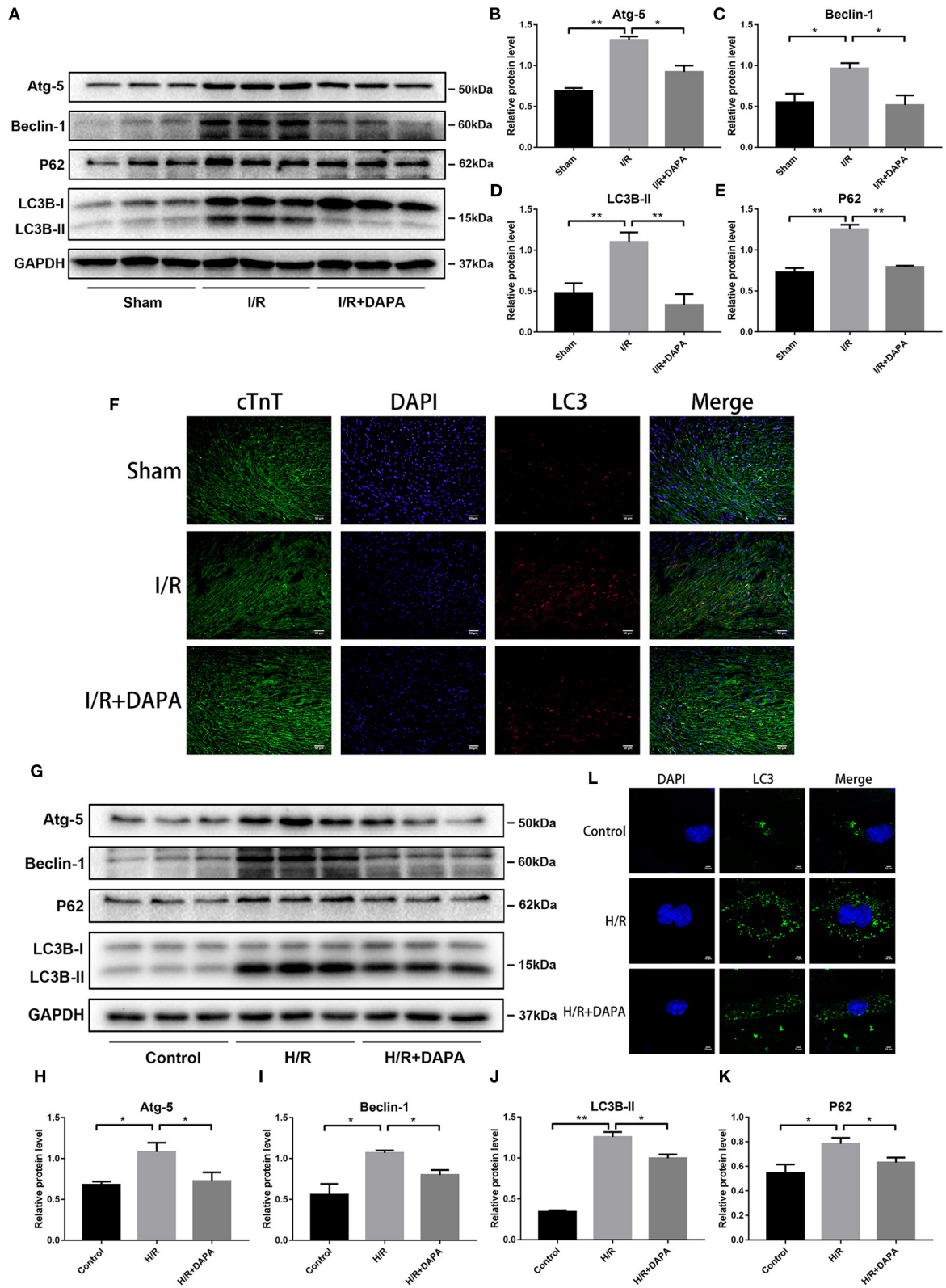

FIGURE 4 | Dapagliflozin treatment restores autophagy flux following ischemia/reperfusion injury *in vivo and in vitro*. **(A)** Western blot detection was performed from LV protein extracts from each group *in vivo*. **(B–E)** Western blot analysis of Atg-5, Beclin-1, LC3B-II and P62 *in vivo*, $n = 3$. **(F)** LC3 immunofluorescence staining to detect autophagosome formation *in vivo*, $n = 3$. **(G)** Western blot detection was performed from PCCMs protein extracts from each group *in vitro*. **(H–K)** Western blot analysis of Atg-5, Beclin-1, LC3B-II and P62 *in vitro*, $n = 3$. **(L)** LC3 immunofluorescence staining to detect autophagosome formation in PCCMs, $n = 3$. DAPA was pretreated for 1 h at 10 μM *in vitro*. Data are means ± SDs, $^{*}P < 0.05$; $^{**}P < 0.01$.

Moreover, the expression of P62 was reduced after the addition of DAPA. P62 is a selective autophagy receptor and is degraded by autophagy; thus, increased levels of p62 reflect the inhibition of autophagy (27). Combined with our previous results, we speculated whether there is a relationship between this blocked autophagy flux and increased inflammation and whether hindering autophagosome degradation affects the inflammation change in I/R injury.

Dapagliflozin Mediated Cardiomyocyte Protection Depends on the Expected Clearance of Autophagosomes *in vivo*

One of the normal elements of autophagy flux, the successful degradation of autophagosomes, requires the participation of normally-functioning lysosomes (28). We used chloroquine (CQ) to disrupt lysosomal function and block autophagosome degradation (22).

After CQ exposure, we first detected autophagy-related proteins by Western blotting to determine whether CQ inhibition was achieved. Compared with the DAPA group, the DAPA + CQ group showed a significant increase in LC3B-II (**Figures 5A,B**), indicating an increase in autophagosome accumulation. Similarly, in the DAPA + CQ group, levels of P62 were significantly higher than those in the DAPA group (**Figures 5A,C**). Therefore, we inferred that the degradation of autophagosomes was reduced after the addition of CQ. We concluded that the use of CQ in our model successfully inhibited the degradation of autophagosomes. We also examined whether this phenomenon occurred in myocardial cells. Using tissue immunofluorescence technology, we found that the results on both sides were consistent (**Figure 5D**). We also discovered that in the comparison between the I/R group and the I/R + CQ group, the expression of LC3B-II and P62 in the I/R + CQ group was further enhanced. Blocked degradation of autophagosomes when I/R occurred appeared to be incomplete.

We then asked whether I/R damage changed after using CQ. We observed that damage in the DAPA + CQ group increased following the addition of CQ. Compared with the DAPA group, the DAPA + CQ group increased the myocardial infarction area (**Figures 5E–G**). Similarly, we tested myocardial infarction markers in the serum, including cTnI, CK-MB, and LDH, and found that the treatment effect of the DAPA group was reversed by CQ (**Figures 5H–J**). Compared to the I/R group, the I/R + CQ group results were exacerbated to varying degrees (**Figures 5E–J**). Therefore, the elimination of autophagosomes plays a more critical role in myocardial I/R injury. Impaired clearance will increase I/R damage. Conversely, promoting the elimination of autophagosomes can reduce I/R injury, and DAPA may play an important role here.

What is the relationship between clearance of autophagosomes and I/R inflammation? When autophagosome clearance was blocked by CQ, inflammation in the DAPA + CQ group was significantly greater than that in the DAPA group. The most direct manifestation was the increased expression of pro-IL-1β and cleaved-IL-1β (**Figures 5K,P,Q**). Serum IL-1β secretion increased after CQ administration (**Figure 5R**). The expression of NLRP3 and ASC also changed similarly as inflammation (**Figures 5K,L,M**). With cleaved-caspase-1 (**Figures 5K,N,O,S**), which represents the protein that inflammasomes activate for further function, inflammation in the I/R + CQ group was further exacerbated than the I/R group. The observations suggest that clearance of autophagosomes in I/R injury is also closely related to the occurrence and progression of inflammation.

Combining these observations, we found that DAPA may play a role by restoring autophagy flux and autophagosome degradation, which was damaged by I/R injury.

Dapagliflozin Mediated Cardiomyocyte Protection Relies on Normal Clearance of Autophagosomes in PCCMs

Next, we explored whether these results would occur in isolated myocardial cells using CQ and monitoring marker molecules related to autophagy. As shown in **Figures 6A–D**, when the pathway of autophagosome degradation was inhibited, the number of autophagosomes in PCCMs increased. Coincidentally, under H/R conditions, autophagosome clearance was blocked incompletely, which helps to clarify whether the changes *in vivo* and *in vitro* were consistent.

We tested whether damage after H/R injury changed after CQ inhibited autophagosome clearance in PCCMs. The addition of CQ further aggravated the damage of H/R, which can be seen from the comparison between the H/R and H/R + CQ groups (**Figures 6G,H**). Conversely, when comparing the DAPA + CQ group with the DAPA group, CQ eliminated the improvement attributable to DAPA (**Figures 6G,H**). Hoechst/PI double-stained PCCMs also showed the same change in the percentage of PCCMs deaths (**Figures 6E,F**). To further explore whether H/R injury and DAPA-related changes are related to inflammation, we examined the expression of inflammation-related proteins. As expected, CQ reversed the DAPA induced reduction of inflammation and aggravated H/R-induced inflammation, manifested by upregulation of pro-IL-1β and cleaved-IL-1β protein levels (**Figures 6I,N,O**) and increased IL-1β secretion in the cell supernatant (**Figure 6P**). In addition, observing the NLRP3 inflammasome index, the results showed that after CQ suppressed autophagosome clearance, levels of NLRP3, ASC, and cleaved-caspase-1 increased (**Figures 6I–M**).

These observations suggest that H/R-impaired autophagosome clearance cause damage, which DAPA can partially offset by promoting autophagosome clearance.

Dapagliflozin Mediated Cardiomyocyte Protection Is Related to Selective Degradation of NLRP3 by Autophagosomes in PCCMs

How does DAPA reduce I/R-induced inflammation and improve I/R injury by promoting autophagy? Autophagy can remove toxic waste, including some compounds that affect excessive inflammation (29). In addition, inflammasome components, including NLRP3, can be cleared by autophagy (19). Based on

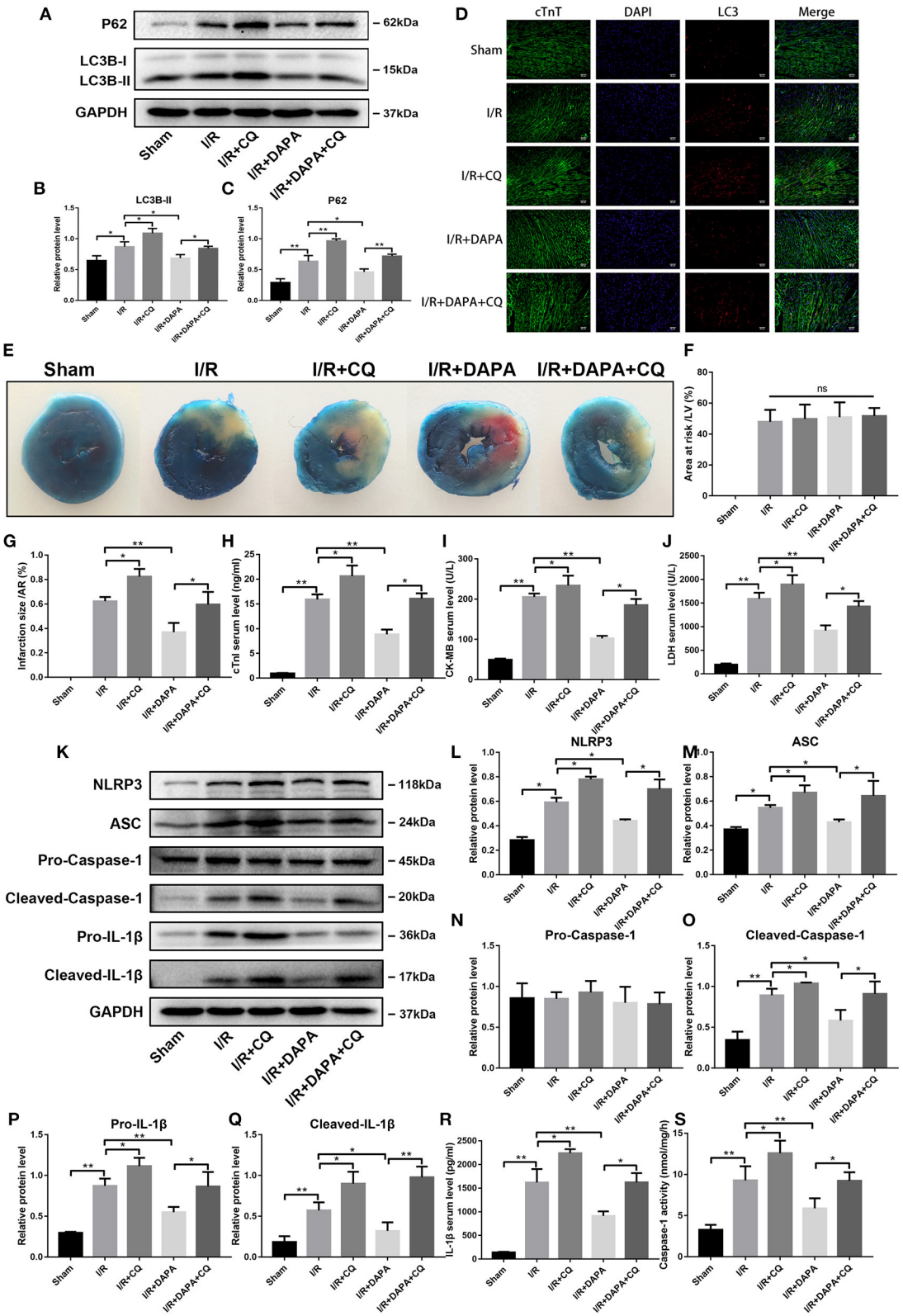

FIGURE 5 | Dapagliflozin mediated cardiomyocyte protection depends on normal clearance of autophagosomes *in vivo*. **(A)** Western blot detection was performed from LV protein extracts from each group *in vivo*. **(B,C)** Western blot analysis of LC3B-II and P62 *in vivo*, $n = 3$. **(D)** LC3 immunofluorescence staining to detect autophagosome formation *in vivo*, $n = 3$.**(E)** C57/bl6 mice were administered DAPA, CQ or vehicle control, established I/R model, and measured the infarct size of

(Continued)

FIGURE 5 | each group using TTC. **(F,G)** Comparison of the myocardial infarction area in each group, $n = 3$. **(H–J)** cTnI, CK-MB, and LDH levels in mouse serum, $n = 9$. **(K)** Western blot detection was performed from LV protein extracts from each group *in vivo*. **(L–Q)** Western blot analysis of NLRP3, ASC, Pro-Caspase-1, Cleaved-Caspase-1, Pro-IL-1β and Cleaved-IL-1β *in vivo*, $n = 3$. **(R)** Serum IL-1β levels, $n = 9$. **(S)** Caspase-1 activity in myocardium tissues, $n = 9$. Data are means ± SDs, $^*P < 0.05$; $^{**}P < 0.01$.

these reports, we believe that autophagosomes in our model system clear NLRP3 and that DAPA may play an important role.

We used co-immunoprecipitation and fluorescence co-localization to investigate this phenomenon further. Through Western blot analysis, we observed an interaction between P62 and NLRP3, possibly promoted by DAPA, in the H/R and H/R + DAPA groups (**Figures 7A–D**). Fluorescence results identified co-localization of autophagosome marker LC3 and inflammasome marker NLRP3 (**Figure 7E**). These findings suggest that eliminating autophagosomes may remove the inflammasome component NLRP3, and the application of DAPA may accelerate the phagocytosis of NLRP3 by autophagosomes.

Dapagliflozin Accelerates Autophagosome Clearance by Reducing Lysosomal Destruction in PCCMs

Autophagosomes phagocytose the inflammasome component NLRP3, and although DAPA may strengthen this process, it is insufficient to degrade the autophagosomes adequately. We have shown that DAPA can promote the clearance of autophagosomes in I/R, but the successful clearance of autophagosomes requires normal lysosomes for proper degradation. Disruption of lysosomal function or impaired lysosomal degradation capacity will affect autophagy flux (28).

To determine if DAPA improved the function of lysosomes in I/R, we used Lysotracker red probe for acid staining to mark normal lysosomes. As shown in **Figure 7J**, the number of lysosomes with an acidic environment decreased in the H/R group but increased in the H/R + DAPA group. In addition, we detected some indicators of lysosomes at the protein level, such as LAMP1, which closely tracks lysosome numbers, and LAMP2, a critical protein of autophagosome-lysosome fusion (18), that showed reduced expression in the H/R group but were upregulated in the H/R + DAPA group (**Figures 7F–H**). CTSD (Cathepsin D) and CTSB (Cathepsin B), typical lysosomal labeled proteases, are involved in the degradation of autophagosomes (30). We found that CTSD expression was downregulated in the H/R group but increased after DAPA treatment (**Figures 7F,I**). We used LAMP2 to co-stain with CTSB and observed a decrease in fluorescence in H/R; the overlap between LAMP2 and CTSB was also reduced (**Figure 7K**). These results suggest that (a) the lysosomal membrane permeability changed in H/R, resulting in a decrease in the number of lysosomes with a typical acidic environment, and (b) some cathepsins in the lysosome were released into the cytoplasm. Although these factors may be responsible for reducing autophagosome degradation, DAPA appears to attenuate this damage.

Dapagliflozin Protects Cardiomyocytes by Inhibiting NHE1 and NCX

Studies have suggested that SGLT-2i can act on NHE1 (sodium hydrogen ion transporter 1) on cardiomyocytes and inhibit its activity (12), thereby producing physiological effects; others believe that it is myocardial protection produced by NCX (sodium-calcium ion transporter) effect (31). We used Auto Dock Tools to perform molecular simulations on dapagliflozin and NHE1 (PDBID: 3v5s) and NCX (I-TASSER) and found that dapagliflozin and NHE1, and NCX have binding ability (**Figures 8A–E**). Further, we observed that after DAPA treatment, Ca^{2+} and Na^{+} in PCCMs have been effectively alleviated.

We used plasmids to upregulate the activities of NHE1 **Figures 8F,G** and NCX in PCCMs to prove whether DAPA's intervention is related to these two receptors. The results showed that as the activity of NHE1 increased during H/R injury, the assembly and activation of NLRP3 inflammasomes also increased (**Figures 8H–N**), indicating that the increased activity of NHE1 eliminated the treatment brought by DAPA. Similarly, the upregulation of NCX activity also showed the above results (**Figures 8O–U**).

DISCUSSION

Our current research provides new insights into the role of DAPA in protecting against myocardial I/R injury in mice. We found: (a) Impaired autophagosome clearance during myocardial I/R in mice, which increases the inflammatory response and aggravates myocardial damage; (b) DAPA attenuates the enlargement of infarct size by restoring autophagy flux and inhibiting inflammation; (c) DAPA provides cardioprotection by promoting the degradation of the inflammasome component NLRP3 during autophagy; (d) DAPA promotes autophagosome degradation by reducing lysosomal damage and protecting lysosomal function; and (e) DAPA acts directly on cardiomyocyte NHE1/NCX (**Figure 9**).

Dapagliflozin, empagliflozin, and canagliflozin are SGLT-2 inhibitors. SGLT-2 is mainly expressed in the kidney but not in the heart (32). Numerous reports suggest that SGLT-2 inhibitors may inhibit inflammation, especially in patients with T2DM. In the early stages of diabetes, long-term use of empagliflozin can inhibit macrophage infiltration, reduce oxidative stress, and suppress inflammation (11). Studies suggested that empagliflozin can directly act on the myocardium through NHE1 while acting on the myocardium through blood sugar levels (33). Dapagliflozin and canagliflozin may alter macrophage polarization and inhibit the production of pro-inflammatory factors such as IL-1β, IL-6, and TNF-α (34). In addition, SGLT-2 inhibitors may reduce the production of

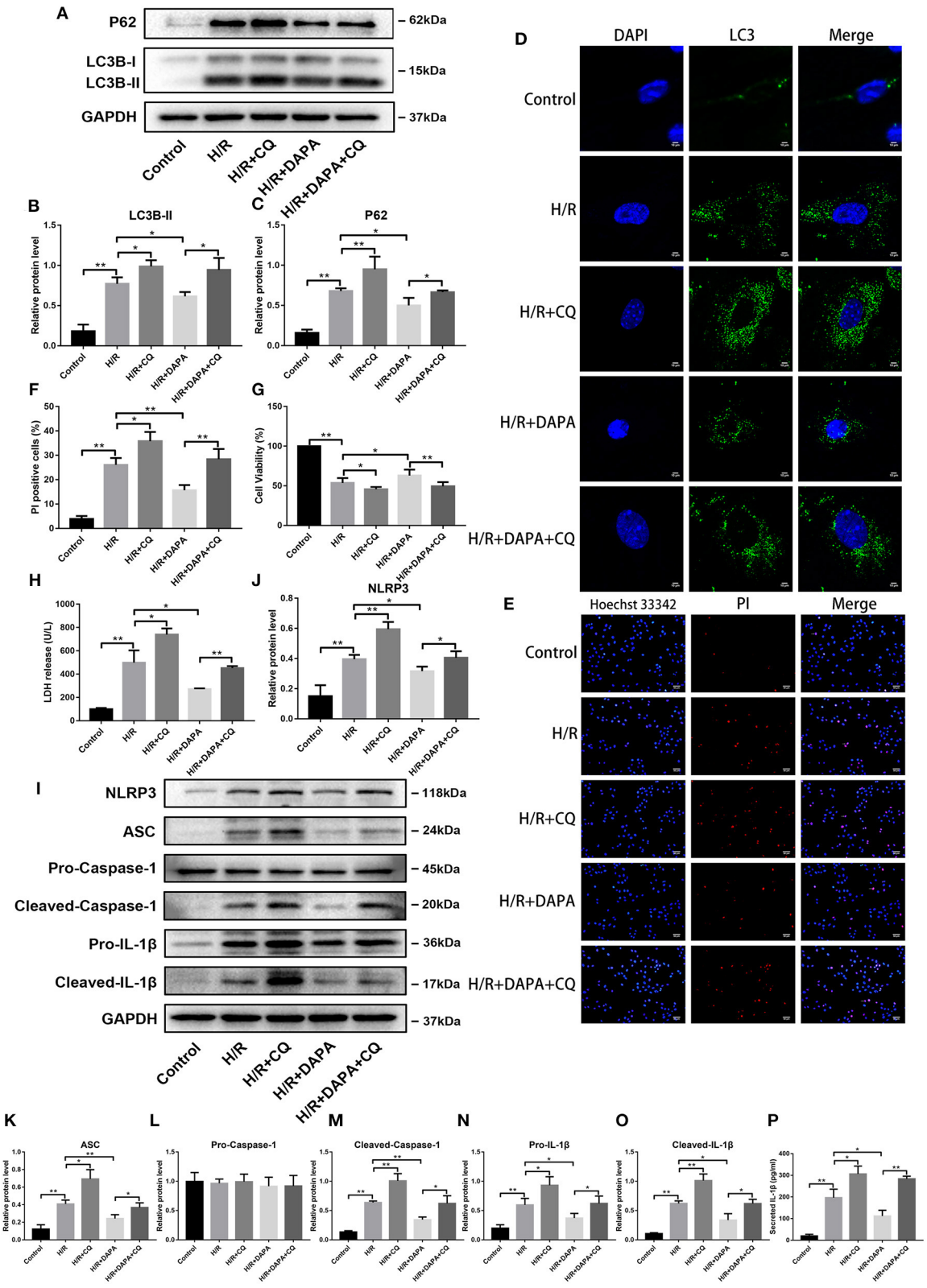

FIGURE 6 | Dapagliflozin mediated cardiomyocyte protection relies on normal clearance of autophagosomes in PCCMs. **(A)** Western blot detection was performed from PCCMs protein extracts from each group *in vitro*. **(B,C)** Western blot analysis of LC3B-II and P62 *in vitro*, $n = 3$. **(D)** LC3 immunofluorescence staining to detect autophagosome formation in PCCMs, $n = 3$. **(E)** Red indicates PI staining and blue indicates Hoechst staining. **(F)** PI staining positive ratio, $n = 3$. **(G,H)** Lactate

(Continued)

FIGURE 6 | dehydrogenase (LDH) and cell viability assay, $n = 9$. **(I)** Western blot detection was performed from PCCMs protein extracts from each group *in vitro*. **(J–O)** Western blot analysis of NLRP3, ASC, Pro-Caspase-1, Cleaved-Caspase-1, Pro-IL-1β and Cleaved-IL-1β *in vitro*, $n = 3$. **(P)** Secretion of IL-1β from cell supernatants, $n = 9$. DAPA was pretreated for 1 h at 10 μM. Data are means ± SDs, $^{*}P < 0.05$; $^{**}P < 0.01$.

NLRP3/ASC inflammasome, thereby suppressing inflammatory responses (10), independent of lowering blood glucose.

Recent studies indicate that SGLT-2 inhibitors may work by acting on autophagy. For example, empagliflozin can increase the LC3B II/I ratio and decrease the expression of P62 (35). However, some studies have noted that SGLT-2 inhibitors can upregulate autophagy-related ubiquitinated protein P62 (36). In our *in vivo* and *in vitro* studies, dapagliflozin downregulated P62 and reduced the expression of LC3B II. It is not yet known what caused this difference. It is not clear how SGLT-2 inhibitors regulate autophagy, but autophagy activation is often related to nutritional deprivation, involving AMPK (37) (adenosine monophosphate-activated protein kinase), SIRT1 (38) (sirtuin-1), HIF-1α (39), and HIF-2α (40) (hypoxia-inducible factors). Coincidentally, there have been many reports of interactions between SGLT-2 inhibitors and these proteins. For example, canagliflozin and empagliflozin increase SIRT1 expression (41), and changes in SIRT1 will affect levels of HIF-2α (42). Empagliflozin can activate AMPK and HIF-1α (43). These exciting experiments may suggest that there are more links between SGLT-2 inhibitors and autophagy. Some studies have noted that empagliflozin is inefficient at activating AMPK at a lower concentration (23), even if this concentration is available in some studies. We speculated that the activated AMPK protein had been degraded (44) at the time of our measurement due to the different injury times, which produced the opposite result.

The typical inflammatory response is mediated by activated caspase-1, and the assembly of NLRP3 inflammasome is the prerequisite for caspase-1 to be activated. Following the production of inactive IL-1β by pro-inflammatory stimulation, inactive IL-1β must undergo inflammasome treatment before it can mature and be secreted. Many stimuli can induce inflammation and activation of the NLRP3 inflammasome, including I/R (15), corroborating our findings. In our study, the inflammatory factors pro-IL-1β and cleaved-IL-1β have been changing along with inflammasome-related indicators. Autophagy may be used to control the elimination of unnecessary and excessive inflammation (20). One possible mechanism for reducing inflammation is the degradation of inflammasomes and related components through the action of P62. Our research also demonstrated the effect of autophagy on inflammation. When we inhibited the elimination of autophagosomes, inflammation increased, but promoting the degradation of autophagosomes reduced the inflammatory response. Furthermore, we showed that autophagosomes could degrade the inflammasome-constituent protein NLRP3. When this core component, which is used to assemble inflammasomes, is eliminated, there is less processing to promote the maturation of inflammatory cytokines.

Treatment with dapagliflozin significantly improved cell death, possibly in many forms. Studies have mentioned that dapagliflozin improves cardiomyocyte apoptosis and mitochondrial function (45). It has been reported that dapagliflozin affects energy metabolism, and then affects death outcome through autophagy flux (46), or improves inflammation-related cell apoptosis (10), inhibits STAT3 pathway, etc (34). Most of the researches involve the changes of SGLT-2i on cell energy metabolism, and it is speculated that it may be related to Na+/Ca2+. Although we are only involved in inflammation at present, inflammation can also induce cell death, such as pyroptosis (47). What's more, the NLRP3 inflammasome is also the main regulator of pyroptosis in I/R injury. On the other hand, most of the current researches are still focused on the chronic treatment of drugs. Although some studies mention that the acute treatment of SGLT-2i can also bring improvement (45, 48), it has been found that SGLT-2i needs to be used before ischemia occurs or in the middle of ischemia can save the death of myocardium. This undoubtedly increases the difficulty of using drugs for treatment. It may be a better choice that early preventive use for high-risk groups.

There are several limitations to our research. First, we cannot rule out the effects of other mechanisms that protect I/R damage in addition to the mechanisms examined here, such as the secretion level of insulin/glucagon. Second, although we focused on eliminating autophagosomes, whether the induction of autophagy can be further induced or inhibited will require further study. Furthermore, our results suggest that DAPA processing promotes the labeling of NLRP3 by P62, but it is not clear how this step is completed. Fourth, since cardiomyocytes do not express SGLT-2, we searched for NHE1 and NCX as new targets for dapagliflozin through molecular docking, but this requires more precise interaction to prove their connection, such as SPR (Surface Plasmon Resonance) technology and gene knockout and overexpression *in vivo*. Finally, treatment with 40 mg/kg/d DAPA was approximately 20x higher than the allometric-adapted dose used in human clinical trials. The 10 μM DAPA in our cellular studies is 20x higher than the maximal concentration of DAPA observed in human serum. Therefore, we cannot exclude that our results are primarily caused by these high dosages and are challenging to translate to human conditions. Further research is necessary to examine this finding in more depth. For example, biological materials should be used to transport dapagliflozin; therefore, the heart is treated with high doses of medications alone.

In summary, DAPA can improve I/R damage, reduce infarct size, and improve cardiac function in non-diabetic mice. The protective effect is accomplished, at least in part, by the selective autophagy degradation of the inflammasome component NLRP3, reducing the maturation and secretion of

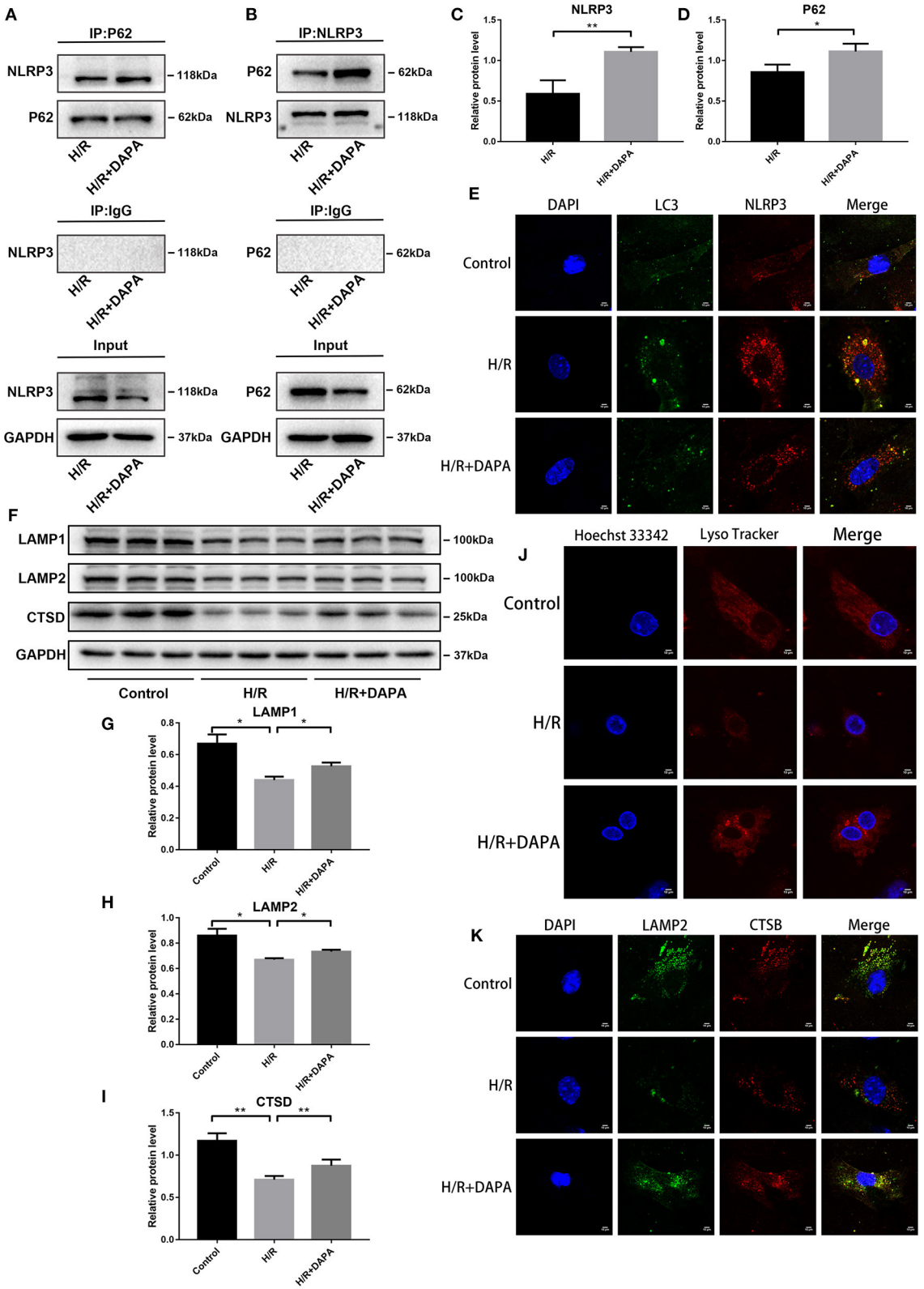

FIGURE 7 | Dapagliflozin improves lysosomal function and accelerates the selective degradation of NLRP3 in PCCMs. **(A,B)** Combination of NLRP3 protein with P62 was identified by Co-IP. **(C,D)** Western blot analysis of Co-IP, $n = 3$. **(E)** Fluorescence co-localization of LC3 and NLRP3, $n = 3$. **(F)** Western blot detection was performed from PCCMs protein extracts from each group *in vitro*. **(G–I)** Western blot analysis of LAMP1, LAMP2 and CTSD, $n = 6$. **(J)** Lyso Tracker Red staining detected lysosomes in PCCMs, $n = 3$. **(K)** Fluorescence co-localization of LAMP2 and CTSB, $n = 3$. DAPA was pretreated for 1 h at 10 μM. Data are means ± SDs, $^{*}P < 0.05$; $^{**}P < 0.01$.

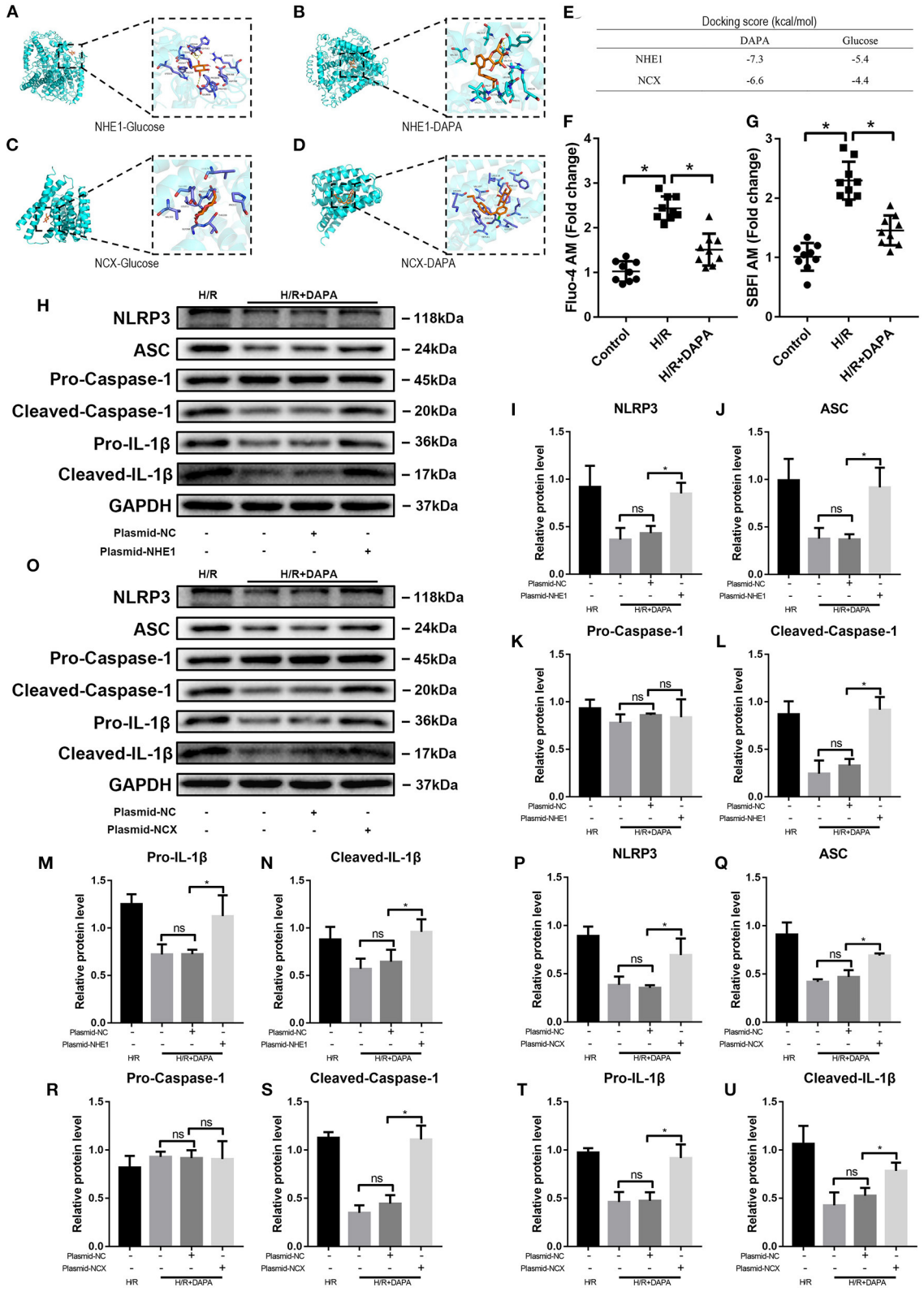

FIGURE 8 | Dapagliflozin protects cardiomyocytes by inhibiting NHE1 and NCX in PCCMs. **(A–D)** Molecular docking with Auto Dock Tools. **(E)** Docking score. **(F)** Ca^{2+} concentration change in PCCMs, $n = 9$. **(G)** Na^{+} concentration change in PCCMs, $n = 9$. **(H–N)** Western blot detection was performed from PCCMs protein extracts from each group with overexpression plasmid-NHE1 *in vitro*, $n = 3$. **(O–U)** Western blot detection was performed from PCCMs protein extracts from each group with overexpression plasmid-NCX *in vitro*, $n = 3$. DAPA was pretreated for 1 h at 10 μM. Data are means ± SDs, $^{*}P < 0.05$; $P < 0.01$. ns: not statistically.

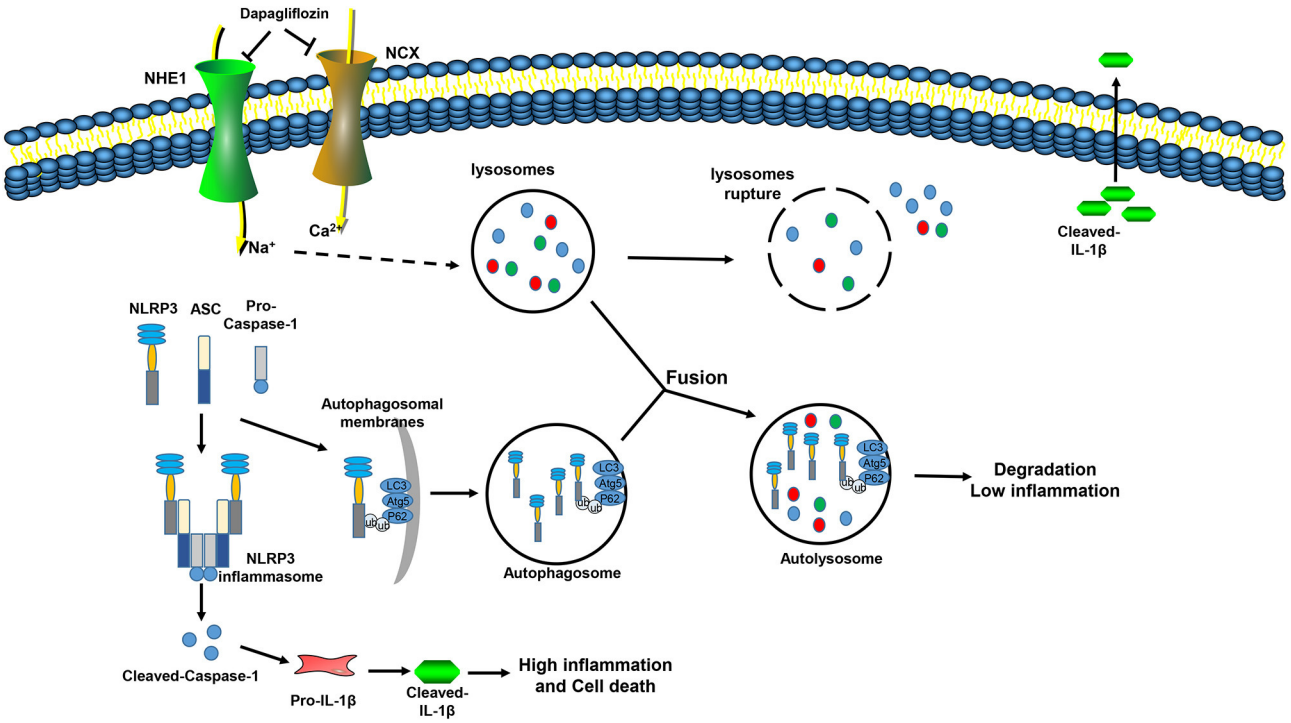

FIGURE 9 | Mechanism model of dapagliflozin reducing myocardial I/R injury. During I/R injury, Na^+ and Ca^{2+} overload in myocardial cells leads to increases in the permeability of the lysosomal membrane, cathepsin in the lysosome leaks out, autophagosome clearance are inhibited, and the NLRP3 inflammasome is assembled and activated, which aggravates the inflammation and promotes cell death. Dapagliflozin inhibits the activity of NHE1 and NCX in the myocardium, relieves Na+, Ca2+ overload, improves lysosome function, promotes autophagosome degradation, promotes the interaction of P62 and NLRP3, inhibits inflammasome assembly, inhibits inflammation activation, reduces inflammatory damage, and reduces cell death.

inflammatory factors. At the same time, although SGLT-2 does not exist in cardiomyocytes, dapagliflozin can directly act on NHE1/NCX in the myocardium.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee at the Wenzhou Medical University (wydw2020-0634).

AUTHOR CONTRIBUTIONS

Y-WY: designed the experiment, completed the research, analyzed the experimental data, and wrote the manuscript. J-QQ, K-YH, LQ, SL, Y-BW, F-NR, LW, and Y-YZ: participated in the experiments. Y-JX and K-TJ: participated in experimental design and manuscript revision. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.768214/full#supplementary-material

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REVIEW
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Therapeutic Peptides to Treat Myocardial Ischemia-Reperfusion Injury

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Cardiovascular diseases (CVD) including acute myocardial infarction (AMI) rank first in worldwide mortality and according to the World Health Organization (WHO), they will stay at this rank until 2030. Prompt revascularization of the occluded artery to reperfuse the myocardium is the only recommended treatment (by angioplasty or thrombolysis) to decrease infarct size (IS). However, despite beneficial effects on ischemic lesions, reperfusion leads to ischemia-reperfusion (IR) injury related mainly to apoptosis. Improvement of revascularization techniques and patient care has decreased myocardial infarction (MI) mortality however heart failure (HF) morbidity is increasing, contributing to the cost-intense worldwide HF epidemic. Currently, there is no treatment for reperfusion injury despite promising results in animal models. There is now an obvious need to develop new cardioprotective strategies to decrease morbidity/mortality of CVD, which is increasing due to the aging of the population and the rising prevalence rates of diabetes and obesity. In this review, we will summarize the different therapeutic peptides developed or used focused on the treatment of myocardial IR injury (MIRI). Therapeutic peptides will be presented depending on their interacting mechanisms (apoptosis, necroptosis, and inflammation) reported as playing an important role in reperfusion injury following myocardial ischemia. The search and development of therapeutic peptides have become very active, with increasing numbers of candidates entering clinical trials. Their optimization and their potential application in the treatment of patients with AMI will be discussed.

Keywords: myocardial infarction, ischemia-reperfusion injury, therapeutic peptide, pharmacological treatment, cardioprotection

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INTRODUCTION

Epidemiology

According to the World Health Organization (WHO), cardiovascular diseases (CVD) are the number one cause of death worldwide representing 31% of all global deaths and 18.6 million lives per year (1). CVD's burdens are predicted by WHO to stay at the first rank until 2030 due to the aging of the population and the increasing prevalence of diabetes and obesity.

Acute myocardial infarction (AMI) among CVD represents the first cause of mortality worldwide (15.9 million/year). Since more than two decades, myocardial ischemia-reperfusion injury (MIRI) has been investigated resulting in important progress in both the knowledge of the mechanisms underlying cell death and in improved interventional procedures. However, MIRI is still associated with significant mortality

and morbidity since 30% of infarcted patients die and 25–50% of survivors develop heart failure (HF) representing huge societal costs.

Furthermore, experts predict that the global burden of cardiovascular disease will grow exponentially over the next few years because of the increased prevalence of diabetes and due to the long-term effects of the current COVID-19 pandemic (2, 3).

Myocardial Ischemia-Reperfusion Injury

AMI is a consequence of the complete coronary artery occlusion occurring at the site of a plaque rupture, exposing its inner core and thus promoting thrombus formation. Criteria for AMI (types 1, 2, and 3 MI) are based on the presence of a myocardial injury with clinical evidence of acute myocardial ischemia and detection of a rise and/or fall of cardiac troponin (cTn) values, associated with at least one of the following symptoms: myocardial ischemia, new ischemic electrocardiographic (ECG) changes, development of pathological Q wave, new regional wall motion abnormality or detection of a thrombus [see the universal definition (4)].

For all types of MI, rapid restoration of blood flow in the ischemic myocardium leading to myocardial reperfusion, either by thrombolysis or angioplasty, has become the "cornerstones" of treatment for AMI (5, 6). All recommendations agree that reperfusion therapy should be performed in patients within the first 12 h of infarction to limit infarct size (IS), improve survival and prevent post-ischemic HF (7). However, sudden blood flow restoration leads to fatal damage to cardiac cells *via* the activation of various intracellular cascades (8). Since the initial description of this phenomenon by Jennings et al. (9) near 40 years ago, our understanding of the mechanisms of reperfusion injury has increased considerably. The pathogenicity is linked mainly to regulated cell death (RCD) under the control of numerous biochemical and cellular processes such as a burst of reactive oxygen species (ROS), loss of ionic homeostasis, mitochondrial dysfunction, and inflammation (10). Lethal reperfusion injury, additional to ischemic injury, culminates in apoptotic death of cardiac cells that were viable immediately before myocardial reperfusion. Because the adult heart fails to have quantities of endogenous stem cells for cardiac regeneration (11), dead cardiomyocytes are lost forever. At the moment, no pharmacological treatment is available to prevent reperfusion injury (12, 13).

Modes of Cell Death During MIRI

The mechanisms contributing to IR injury are multifactorial and highly integrated (10). The existence of such mechanisms triggered by reperfusion and leading to deleterious side effects including cell death can explain the persistence of significant mortality despite early patient management and the development of chronic HF in a significant proportion of reperfused AMI (14). Indeed, reperfusion triggers cascades of biochemical and metabolic events that aggravate changes generated during ischemia. Studies in animal models suggest that reperfusion lesions are responsible for 25 to 50% of the final IS (15).

Cells can die from accidental cell death (ACD) (such as necrosis), which is usually triggered by unexpected injury or attack, escaping from any controlled molecular mechanisms (16, 17). Qualitatively, necrosis is the major mechanism of cardiomyocyte death during ischemia as a direct consequence of oxygen deprivation leading to severe injury. In contrast, during reperfusion, cardiomyocytes die from regulated cell death (RCD) mechanisms involving genetically defined effector molecules and precise signaling cascades such as apoptosis and necroptosis (8).

Apoptosis

Characterized by cell shrinkage, chromatin condensation, and distinctive blebbing (budding) of the plasma membrane, occurs *via* the intrinsic (**Figure 1**) or extrinsic (**Figure 2**) pathways converging into Caspase-activation and DNA fragmentation as a common result (18). Specific DNA fragmentation is mainly detected in the myocardium after reperfusion and not during ischemia, suggesting that its activation is specifically triggered at the onset of reperfusion, depending on the recovery of ATP production (19, 20). This is supported by a major IS reduction observed after the administration of inhibitors of pro-apoptotic mediators at early reperfusion (21, 22). Moreover, the gold standard of cardioprotection, ischemic postconditioning, inhibits apoptosis as does the ischemic preconditioning from which it is derived (23–25). Cardioprotection results from anti-apoptotic strategies (18) such as peptides targeting the First Apoptosis Signal (FAS) death-dependent apoptotic receptor (26, 27), or the mitochondrial permeability transition pore (mPTP) such as Cyclosporine-A (CsA) with however inconsistent preclinical and clinical results (28).

Necroptosis

In cardiac pathology, necroptosis has been identified as a lytic form of RCD leading to the release of proinflammatory intracellular molecules (29, 30). Even if necroptosis is morphologically similar to necrosis, this pathway could also depend on Caspase-8 activity and therefor, be pharmacological modulable (i.e., inhibited by necrostatin-1). Necroptosis is triggered by oxidative stress or TNFα (Tumor Necrosis Factor), FasL (FAS Ligand), and TRAIL (TNF-Related Apoptosis-Inducing Ligand) cytokines activating death receptors (**Figure 3**) (31). Necroptosis is triggered only if Caspase-8, responsible for the cleavage of RIPK1 (Receptor-Interacting serine/threonine-Protein Kinase 1), is inhibited. Phosphorylated RIPK1 and RIPK3 together with MLKL (Mixed Lineage Kinase Domain Like Pseudokinase) form the necrosome leading to phosphorylation and oligomerization of MLKL, which translocates to the plasma membrane to induce membrane rupture (32).

Autophagy-Dependent Cell Death

Autophagy is an evolutionary process to maintain cell homeostasis based on the degradation of intracellular materials and components within the lysosomal compartment of eukaryotic cells (17). Because of the elimination of misfolded/dysfunctional proteins or organelles, autophagy was believed to be a cytoprotective catabolic mechanism of substrate recycling for ATP generation and cell survival. Autophagy is activated by ATP-depletion and subsequent AMPK

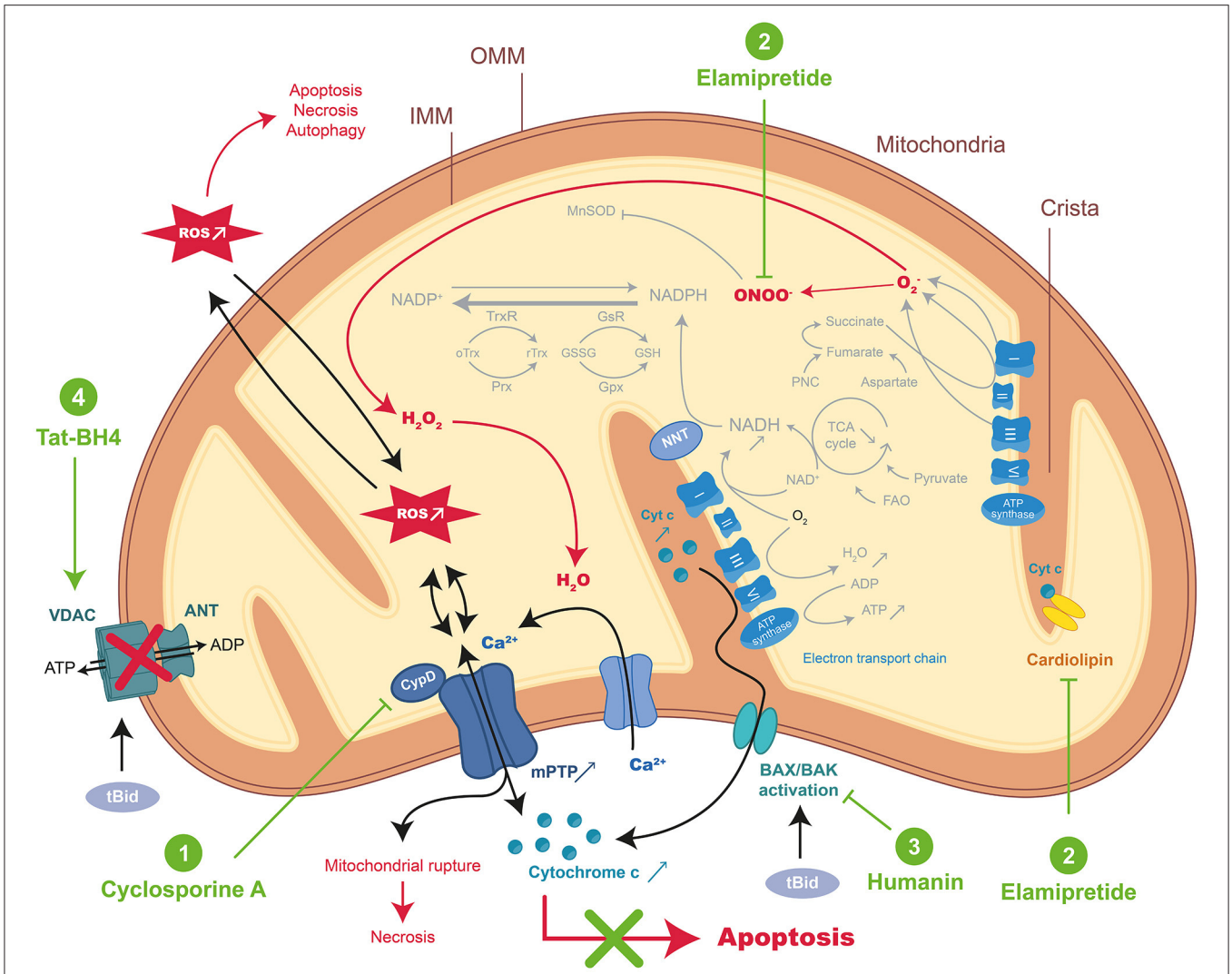

FIGURE 1 | Schematic representation of mitochondrial-dependent apoptosis and mode of action of the therapeutic peptides during MIRI. During acute myocardial ischemia-reperfusion injury (MIRI), reactive oxygen species (ROS) burst and mitochondrial Ca^{2+} overload activate regulated cell death (RCD) resulting in apoptosis or necrosis through the mitochondrial permeability transition pore (mPTP) opening. Excessive ROS induced important changes in normal mitochondrial structure and function resulting in the disorder of mitochondrial metabolic function. Therapeutic peptides reducing intrinsic apoptosis during MIRI were highlighted in green: (1) Cyclosporine A (CsA), (2) Elamipretide, (3) Humanin, and (4) Tat-BH4. CypD, cyclophilin D; NNT, nicotinamide nucleotide transhydrogenase; FAO, fatty acid β-oxidation; Prx, peroxiredoxins; Gpx, glutathione peroxidase; GsR, glutathione reductase; Trx, thioredoxin; TrxR, thioredoxin reductase; GSH, glutathione; GSSG, oxidized glutathione; PNC, purine nucleotide cycle; tBid, truncated form of BH3 Interacting domain Death agonist; BAX, BCL2 associated X Apoptosis regulator; BAK, BCL-2 Antagonist/Killer 1; OMM, outer membrane, IMM, inner membrane.

(AMP-dependent Protein Kinase) activation, calcium overload, and ROS, which are found during prolonged ischemia, IR, and HF (33, 34). A high level of Beclin-1 is critical for early autophagosome formation and its activity can be reduced by BCL-2 (B-cell lymphoma-2) or BCL-XL (B-cell lymphoma extra-large). Beclin-1 is cleaved by Caspases, showing the existence of a crosstalk between autophagy and apoptosis (35). More recently, autosis was described as a new form of autophagy responsible for continuous cardiomyocyte death in the late phase of reperfusion although cell death processes should be completed within 2 h of reperfusion (36, 37).

Inflammation

Necrotic cardiomyocytes in the infarcted area provide the main stimulus for post-infarction inflammatory response through the release of DAMPs (Damage-Associated Molecular Patterns) in concert with complement cascade and ROS activation, mobilizing the resident immune cells of the heart at the onset of AMI. Neutrophil infiltration, innate immunity activation as well as cell-mediated damage are pathological mechanisms of inflammation-related IR injury observed after MI as exemplified through experimental and clinical studies (38). In the context of AMI, the pro-inflammatory response, rapidly orchestrated

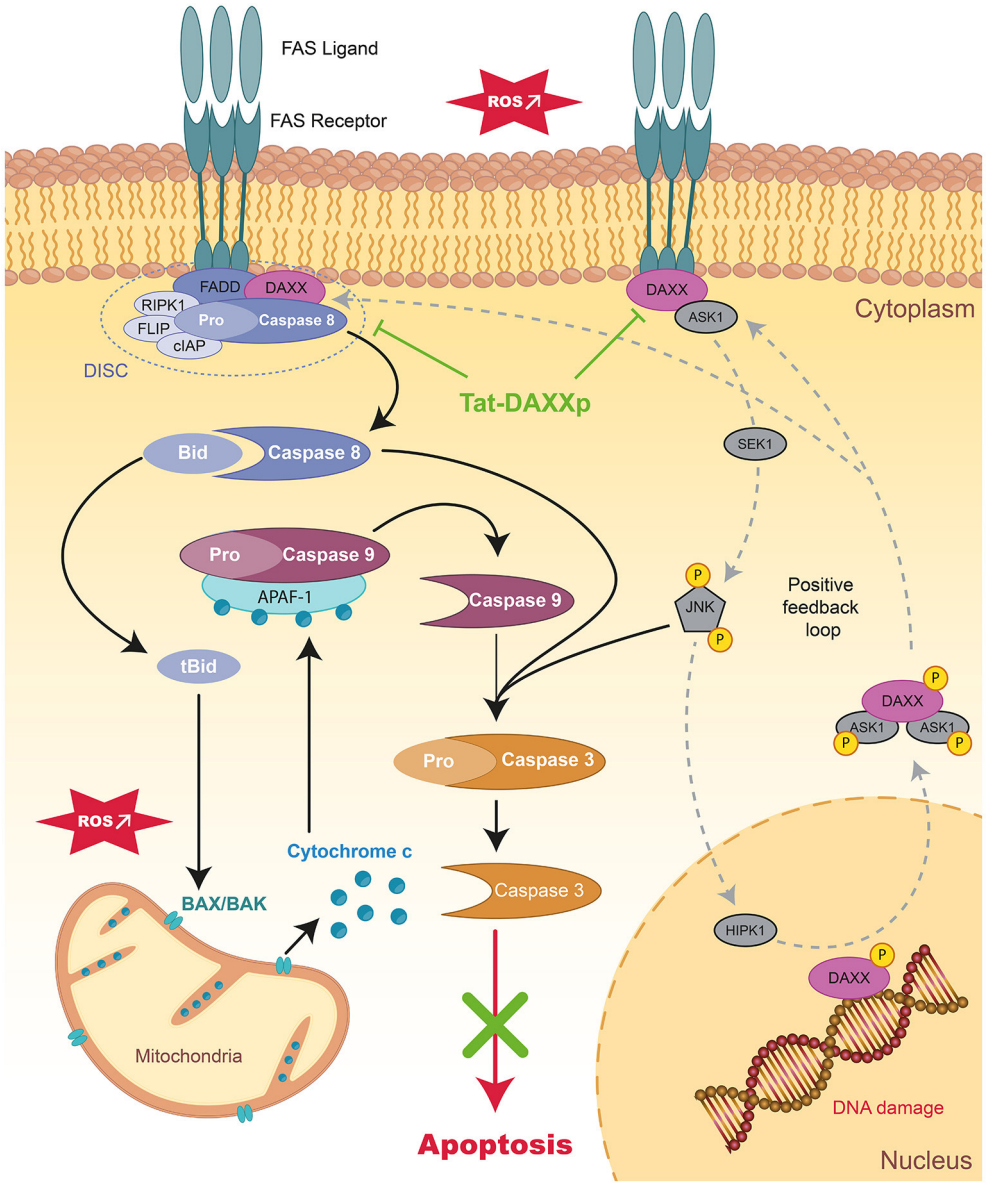

FIGURE 2 | Schematic representation of extrinsic apoptotic pathways and mode of action of therapeutic peptides during MIRI. Schema presenting the signaling apoptotic cascades activated during MIIR involving receptor-dependent pathways in the myocardium. The positive feedback loops regulating DAXX nucleic-cytoplasmic ratio is showed in dark gray. Tat-DAXXp treatment administered at the onset of reperfusion (highlighted in green) can inhibit both the extrinsic and intrinsic pathways. FAS, First Apoptosis Signal; FADD, Fas-Associated protein with Death Domain; DAXX, Death-domain associated protein-6; DISC, death-inducing signaling complex; RIPK1/3, Receptor-interacting serine/threonine-protein kinase 1/3; FLIP, FLICE-inhibitory protein; cIAP, cellular inhibitor of apoptosis proteins 1; ASK1, Apoptosis Signal regulating Kinase 1; JNK, c-Jun N-terminal Kinase; HIPK, homeodomain-interacting protein kinase.

during ischemia to remove the necrotic cells and repair the infarcted myocardium, is exacerbated following reperfusion leading to cardiomyocyte death and MIRI within 6 h and 24 h post-reperfusion. TLR2, TLR4, TLR9 (Toll-Like Receptors) and NLRP3 in the NLRP3-ASC-Caspase-1 inflammasome contribute to this strong but short inflammatory burst following reperfusion through IL-1 (Interleukin-1), IL-6, and active Caspase-1 mediators *via* the NF-κB pathway (**Figure 4**) (39). Inflammation is deeply involved in the pathophysiology of MIRI but also in fibrosis formation and in post-infarct remodeling leading to HF (40). Because inflammation contributes to IS and cardiac remodeling, it is a major predictor of adverse events after AMI (41, 42).

Therapeutic Peptides as a Novel Approach for Treating MIRI?

Important chemical development of solid-phase peptide synthesis allowed the rise of not only small but also larger

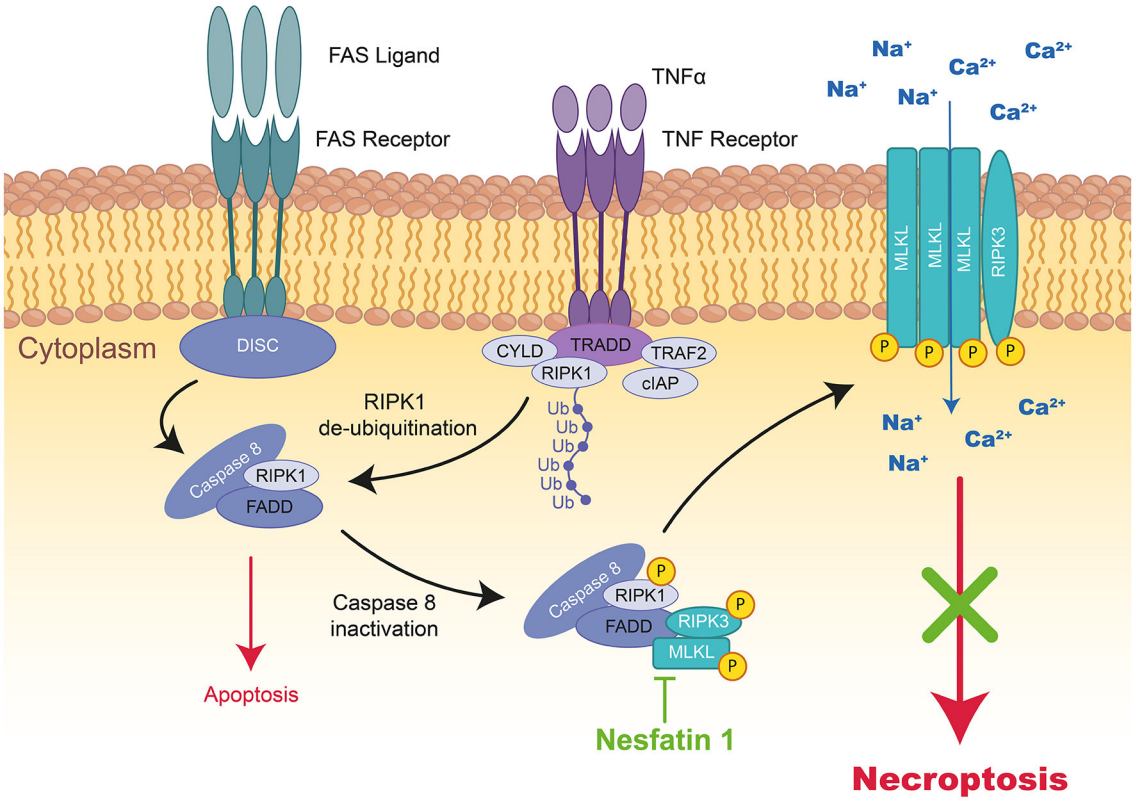

FIGURE 3 | Schematic representation of necroptosis and the mode of action of Nesfatin 1 therapeutic peptide during MIRI. TNFα activates the TNF receptor, which induces the formation of a complex formed by TRADD, TRAF2, RIPK1, CYLD, and cIAP1 at the cytoplasmic membrane. In the absence of cIAP1, RIPK1, FADD, and Caspase-8 form cytosolic DICS complex, Caspase-dependent pathways are activated inducing apoptosis. However, by Caspase-8 inactivation, RIPK1 interacts with RIPk3 and MLKL to form a third complex inducing necroptosis. The kinase of RIPK1 phosphorylates RIPK3 and MLKL resulting in their translocation to the plasma membrane, where the complex mediates membrane permeabilization. The therapeutic peptide Nesfatin-1 (highlighted in green) can reduce RIPK1, RIPK3, and MLKL expression and therefore necroptosis. TNF, tumor necrosis factor; TRADD, tumor necrosis factor receptor type 1-associated death domain; TRAF2, TNF receptor-associated factor 2; RIPK1/3, receptor-interacting serine/threonine-protein kinase 1; CYLD, lysine 63 deubiquitinase; cIAP1, cellular inhibitor of apoptosis protein 1; MLKL, mixed lineage kinase domain like pseudo kinase.

synthetic peptides. In this review, peptides will be defined as molecules containing no more than 30 amino acids, to discriminate them from proteins or antibodies, which constitute a large field within the pharmaceutical industry. Peptides are attractive therapeutic molecules based on their favorable pharmacokinetic profile, good solubility, low toxicity/mitogenicity, and furthermore due to the unlimited possibility of introducing modifications to improve their stability/binding affinity (43). It is then not surprising that therapeutic approaches using peptides have become an emerging market in the pharmaceutical industry over the past decades and that today more than 60 peptide drugs are Food Drug Administration approved and that many more are studied in clinical and preclinical trials (44). The global peptide therapeutics market accounted for $28.15 billion in 2019 and is expected to reach $66.76 billion by 2027 growing at a CAGR of 11.4% during the forecast period (45).

As a consequence, a large variety of bioactive peptides that target processes of apoptosis, necroptosis, inflammation, and autophagy in MIRI have been identified and characterized (46). The next chapter will be focused on therapeutic peptides administrated at the onset of reperfusion (pharmacological post conditioning) which is the only relevant clinical application since pharmacological preconditioning is incompatible with AMI therapy. Furthermore, we have mainly focused on those validated in *ex vivo* and *in vivo* IR animal models.

THERAPEUTIC PEPTIDES REDUCING APOPTOSIS TO TREAT MIRI

Apoptotic mechanisms are dependent on ATP production, which means that this mechanism is not activated in ischemic conditions but is specific to the reperfusion phase (19). Indeed, apoptotic cascades pre-activate during ischemia are fully executed during reperfusion (DNA fragmentation) (47). Accordingly, numerous *in vivo* studies have reported

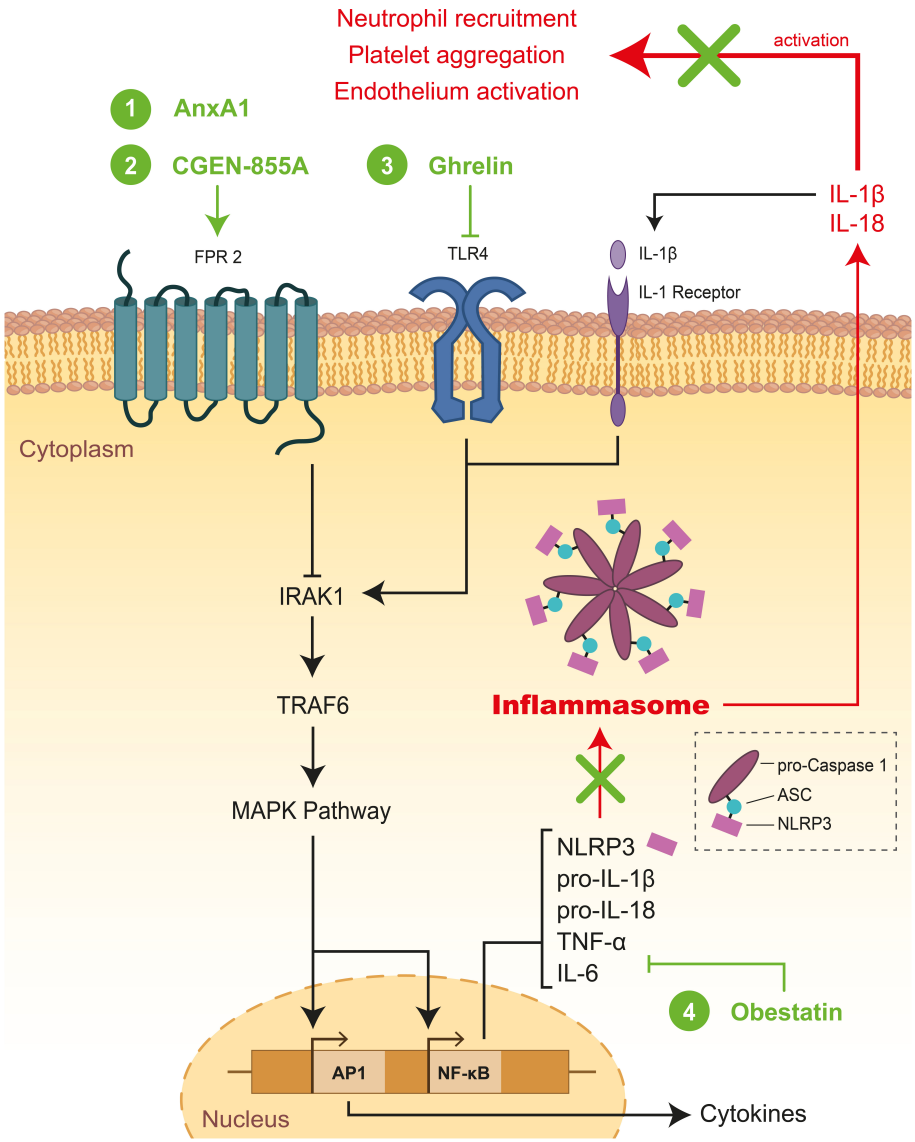

FIGURE 4 | Schematic representation of inflammation and mode of action of therapeutic peptides during MIRI. FPR, TLR4, or IL1 receptors activate the MAPK pathway through IRAK1 and TRAF6. Afterward, the MAPK pathway activates the expression of NLRP3 which formed the inflammasome together with pro Caspase-1 and ASC (Apoptosis-associated speck like protein containing a Caspase recruitment domain) contributing to the strong but short inflammatory burst. Secretion of mature forms of IL-18 and IL-1β activate neutrophil recruitment, platelet aggregation, and endothelium activation. Therapeutic peptides reducing inflammation during MIRI were highlighted in green: (1) AnxA1, (2) CGEN-855A, (3) Ghrelin, and (4) Obestatin. FPR, Formyl peptide receptor; TLR4, Toll-Like Receptor 4; IL1, Interleukin 1; IRAK1, Interleukin 1 Receptor Associated Kinase 1; TRAF6, TNF receptor-associated factor 6; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3.

positive results for specific anti-apoptotic interventions as cardioprotective strategies against MIRI (22, 23, 26, 48–50) because apoptosis, through the intrinsic (mitochondrial) (17) and extrinsic (death receptor-dependent) (17, 26) pathways, is a reversible process and can be manipulated to allow cardiomyocyte survival during AMI. All therapeutic peptides described below are summarized in **Table 1** including the used experimental conditions (doses, administration modes, animal models, and potent effects), and those evaluated in clinical trials are listed in **Table 2**.

Therapeutic Peptides Acting on the Intrinsic Apoptotic Pathway

The intrinsic or mitochondrial pathway is activated upon intracellular stress such as calcium overload or damaged DNA and is characterized by the irreversible permeabilization of the mitochondrial outer membrane under the control of proteins with BCL-2 homology domain and a transmembrane segment (111). The formation of pores in the outer membrane by oligomerization of BAX (BCL2 associated X Apoptosis regulator) and BAK (BCL-2 Antagonist/Killer 1) is regulated by activating

BH3-only proteins such as Bid (BH3 Interacting-Domain death agonist) and by sensitizers such as BAD (BCL2 associated Agonist of cell Death) that sequester the anti-apoptotic proteins (BCL-XL) previously bound to direct activators.

Moreover, in addition to its activation by Caspase-8 or granzyme B, Bid is engaged in response to death receptor stimulation, allowing crosstalk between the intrinsic and extrinsic pathways (112). The permeabilization of the outer membrane leads to the release of pro-apoptotic factors into the cytoplasm, endonuclease G that will cleave DNA (Caspase-independent mechanism) or SMAC (Second Mitochondrial Activator of Caspases) and cytochrome C (CytC) inducing *in fine* DNA fragmentation and cell apoptosis (113). Mitochondrial respiratory chain with reduced CytC leads to mitochondrial ATP synthesis dysfunctions and to the dissipation of the mitochondrial transmembrane potential ($\Delta\psi m$), which in turn triggers the opening of the mPTP (**Figure 1**) (114). mPTP is a protein complex whose molecular identity remains not fully elucidated. Several proteins have been reported to be part of this complex such as VDAC (Voltage-Dependent Anion Channel) located in the outer membrane and ANT (Adenine Nucleotide Translocator) spanning the inner membranes, whereas others are described as protein regulators, such as the mitochondrial matrix chaperone cyclophilin D (CypD) (114, 115). The mPTP formation and opening occur at the onset of reperfusion (116) upon (i) oxidative stress when the respiratory chain is suddenly exposed to oxygen, (ii) Ca^{2+} ion accumulation due to rapid mitochondrial membrane potential restoration, and (iii) neutralization of acidic pH as H^{+} ions compete with Ca^{2+} ions that bind to the mPTP trigger site (117). Furthermore, mPTP allows the passage of small molecules (<1.5 kDa) into the mitochondrial matrix (118) and will contribute to the permeabilization of the mitochondrial inner membrane.

mPTP opening has been proposed as the key driver of MIRI because the concentration of the endogenous potentiators of the mPTP (e.g., calcium and ROS) increased during this phenomenon whereas inhibitors (e.g., Cyclosporine A) reduced IS (119). However, a major limitation of mPTP inhibiting is the lack of knowledge on mPTP-forming proteins and how they are activated by calcium and ROS.

Cyclosporine A

Cyclosporine A (CsA) is a natural cyclic 11-mer peptide, isolated from fungus *Tolypocladium inflatum*, widely used to down-regulate immune system activity and therefore the risk of organ rejection after allogeneic organ transplant. CsA is able to block mitochondrial calcium efflux in isolated mitochondria and to inhibit the Ca^{2+}-dependent mPTP opening in the inner membrane of heart mitochondria (120, 121). CsA (122) and the analogs NIM811 (123) and Debio-025 (124) bind mitochondrial CypD, preventing the mPTP pore opening, and have been described as promising drugs for the cardioprotection against MIRI (**Figure 1**) (119). Indeed, CsA has provided encouraging results in many animal IR models (51–58) (for details see **Table 1**) and also in a proof-of-concept study in patients (98). More recently, large-scale clinical trials were performed to evaluate CsA long-term cardioprotective effects (CIRCUS, CYCLE, CYRUS) (99–101) (**Table 2**). Unfortunately, published results have shown no evidence of long-term protection, reduced mortality, or prevention of early multiple organ failure. The controversial findings obtained in clinical studies were attributed to the differences in CsA formulations since it is a class II compound with extremely low aqueous solubility (6.6 g/mL) and high lipophilicity (Log P = 3). However, even if both formulations have been described to have similar pharmacokinetics (125), Sandimmune used in CYCLE and CYRUS trials did not show the expected efficacy previously reported in the proof-of-concept trial by Piot et al. (98, 100, 101). The same negative results were obtained using CicloMulsion®, a lipid emulsion of CsA, in the large-scale CIRCUS clinical trial (99).

More recently, new formulations using PLGA-, squalene- or lipid-based nanoparticles were developed to increase cell permeability of the therapeutic CsA but no preclinical study was yet performed (126–128). Also, combined administration of polymeric nanoparticles encapsulating CsA or pitavastatin (organic compound) targeting mPTP opening and monocyte-mediated inflammation, respectively, has been reported to be more efficient than a single administration of encapsulated CsA (129) even if CsA impacts *per se* the immune response after myocardial IR (130).

Elamipretide

The small cell-permeable Szeto-Schiller peptide (also known as SS-31, MTP-131, Bendavia, or Elamipretide) was developed for targeted delivery of antioxidants to the inner mitochondrial membrane (131). This 4-mer SS peptide can scavenge hydrogen peroxide or peroxynitrite and inhibit lipid peroxidation through its structural motif alternating aromatic residues and basic amino acids (**Figure 1**). Therefore, this SS-31 peptide provides significant protection against MIRI as shown by higher contractile force levels as well as increased heart beating rates, and prevents myocardial stunning when administered upon reperfusion in the *ex vivo* guinea pig heart (59). As a mechanism of action, SS-31 could interact with phospholipids (i.e., cardiolipin) on the inner mitochondrial membrane maximizing membrane shape to improve the electron transport chain function and minimize the production of mitochondrial-derived ROS (**Figure 1**).

Later on, Kloner and co-workers demonstrated that Elamipretide reduced myocardial IS in different IR models (60). For example, post-ischemic Elamipretide administration decreases IS in an *ex vivo* guinea pig IR model in the same way as in the *in vivo* sheep IR model where it was infused during the last 30 min of ischemia. Surprisingly, the authors could not show any cardioprotection in an *in vivo* rabbit IR model. More recently, Allen and colleagues demonstrated that Elamipretide (10 μM) administrated at the onset of reperfusion in an *ex vivo* rat IR model can improve mitochondrial function by aggregating cardiolipin (61).

Based on these promising experimental data, a multicenter study (EMBRACE STEMI) was performed to evaluate Elamipretide as an adjunct therapy to percutaneous coronary intervention for STEMI. The drug injected to patients before

TABLE 1 | Therapeutic peptides used in different IR animal models mentioned in this review.

Peptides	Other names	Experimental models	Administration	Species	Mechanisms	References
MITOCHONDRIA-DEPENDENT APOPTOSIS						
Cyclosporine A	Ciclosporine A, CsA, CycloMulsion, Sandimmune	*in vivo* 30 min I/3 h R	10 mg/kg, i.v.—5 min before R	Rabbit	Reduced IS	(51)
		in vivo 30 min I/2 h R	10 mg/kg, i.v.—at the onset of R	Mouse	Reduced IS	(52)
		in vivo 25 min I/2 h R	5 mg/kg, i.v.—5 min before R	Rat	Reduced IS	(53)
		in vivo 90 min H/2 h R	5 mg/kg, i.v.—5 min before R	Pig	Reduced IS	(54)
		in vivo 40 min I/3 h R	10 mg/kg, i.v.—5 min before R	Pig	No reduction in IS	(55)
		in vivo 45 min I/2 h R	10 mg/kg, i.v.—3 min before R	Pig	No reduction in IS	(56)
		in vivo 40 min I/4 h R	2.5 mg/kg, i.v.—7 min before R	Pig	No reduction in IS	(57)
		in vivo 60 min I/3 h R	10 mg/kg, i.v.—15-10 min before R	Pig	Reduced IS and microvesicular damage, better LV function	(58)
Elamipretide	SS-31, MTP-131, Bendavia	*ex vivo* 30 min global I/3 h R	1 μmol/L—during the whole R	Guinea pig	Improved contractile force, increased heart beating rates	(59)
		ex vivo 20 min global I/2 h R	post-ischemic administration 1 nM during R	Guinea pig	Reduced IS	(60)
		in vivo 30 min I/180 min R	at the onset of R–0.10 mg/kg/h i.v. during 20 min then 0.05 mg/kg h i.v. during 160 min	Rabbit	No significant effect on IS	
		in vivo 60 min I/180 min R	0.05 mg/kg/h i.v.—during the 30 min before R	Sheep	Reduced IS, protection against the no-reflow phenomenon	
		ex vivo 20 min global I/120 min R	10 μmol/L i.v.—at the onset of R	Rat	Improve mitochondria function by aggregating cardiolipin	(61)
Humanin	S14G-humanin	*in vivo* 45 min I/24 h R	2 mg/kg i.c.—at the onset of R	Mouse	Reduced IS and increased EF	(62)
		in vivo 30 min I/2 h R	252 μg/kg i.v.—15 min before R	Rat	Reduced IS, arrhythmia, and cardiac mitochondrial dysfunction	(63)
		in vivo 60 min I/48 h R	2 mg/kg i.v.—10 min before R	Pig	Reduced IS	(64)
Tat-BH4	/	*in vivo* 40 min I/24 h R	1 mg/kg i.v.—at the onset of R	Mouse	Reduced IS and apoptosis	(65)
Tat-V1-Cal	/	*in vivo* 30 min I/2 h R	1 mg/kg i.v.—5 min before R	Rat	Reduced IS	(66)
AID-Tat	/	*in vivo* 30 min I/12 weeks R	10 μmol/L i.c.—at the onset of R	Rat	Reduced IS and supported contractility	(67)
RECEPTOR-DEPENDENT APOPTOSIS						
Tat-DAXXp	TD	*in vivo* 40 min I/24 h R	1 mg/kg i.v.—at the onset of R	Mouse	Reduced IS and apoptosis	(26)
		in vivo 40 min I/6 months R	1 mg/kg i.v.—at the onset of R	Mouse	Reduced fibrosis, increase EF	(27)
Tat-FADDp	TF	*in vivo* 40 min I/24 h R	1 mg/kg i.v.—at the onset of R	Mouse	Reduced IS and apoptosis	(68)

(Continued)

TABLE 1 | Continued

Peptides	Other names	Experimental models	Administration	Species	Mechanisms	References
INFLAMMATION						
ANP	ANP1-28	*ex vivo* 15 min I/15 min R	0.1 μmol/L—at the onset of R	Rat	Better cardiac and corona flow recovery	(69)
	Carperitide	*in vivo* 60 min I/6 h R	0.2 μg/kg/min i.v.—15 min after I till the end of R	Dog	Reduced IS, increased blood flow, decreased left ventricular systolic pressure, and end-diastolic pressure	(70)
		in vivo 90 min I/6 h R	0.1 μg/kg/min i.v.—10 min before I till 1 h of R	Dog	Reduced IS	(71)
	Urodilatin (URO)	*ex vivo* 40 min I/1 h R	0.05 μmol/L—first 15 min of R	Rat	Higher LV pressure	(72)
		in vivo 47 min I/2 h R	10 ng/kg/min i.v.—during the first 25 min of R	Pig	Reduced IS	
BNP	/	*ex vivo* 30 min I/90 min R	10 nmol/L—5 min before R till 15 min of R	Rat	Reduced IS	(73)
	/	*in vivo* 30 min I/4 h R	0.03μg/kg min i.v.—15 min before R till the end of R	Rat	Reduced IS, decreased LDH, and CK levels	(74)
	RhBPN	*in vivo* 40 min I/24 h R	0.035 mg i.p.—after IR one injection/d for 3 d	Mice	Reduced IS and CD4+T cell proliferation	(75)
DNP	Lebetin 2 (L2)	*ex vivo* 30 min I/90 min R	200 nmol/L—5 min before R till 15 min of R	Rat	Reduced IS	(73)
		in vivo 30 min I/2 h R	100 ng/g i.p.—5 min before R	Mouse	Reduced IS	(76)
		in vivo 35min I/2 d or 14 d R	25 ng/g i.p.—5 min before R	Rat	Reduced IS, collagen content, and enhanced M2-like macrophages	
CNP	/	*ex vivo* 25 min I/2 h R	30 nmol/L—during the first 30 min of R	Rat	Reduced IS and coronary perfusion pressure (CPP)	(77)
VNP	Vasonatrin	*in vivo* 30 min I/4 h or 6 h R	100 μg/kg, i.v.—10 min before R	Rat	Reduced IS, Reduced LV systolic and end-diastolic pressure	(78)
Ac2-26	N-terminus of Annexin-1	*in vivo* 25 min I/2 h R	1 mg/kg i.v.—at the onset of R	Rat	Reduced IS, Myeloperoxidase (MPO) activity, and IL-1b levels	(79)
		in vivo 40 min I/24 h R	1 mg/kg i.v.—at the onset of R	Mouse	Reduced IS, cTnI (24 hR), inflammation (48 h R), fibrosis, and apoptosis (7-days R)	(80)
$AnxA1_{2-50}$		*in vivo* 25 min I/2 h R	5 μg/mouse i.v.—at the onset of R	Mouse	Reduced IS and plasma levels of cTnI, CCL5, ILβ	(81)
$CR\text{-}AnxA1_{2-50}$						
$CR\text{-}AnxA1_{2-48}$		*in vivo* 25 min I/2 h R	5 μg/mouse i.v.—at the onset of R	Mouse	Reduced IS and plasma CCL5 concentration	(82)
CGEN-855A	/	*in vivo* 25 min I/2 h R	2 mg/kg i.v.—immediately after R	Mouse	Reduced IS, cTnI and PMN	(83)
		in vivo 30 min I/3 h R	2 mg/kg i.v.—5 min before R	Rat		
DS-IkL	/	*in vivo* 45 min I/24 h R	30 μM 100 μL i.v.—immediately after R	Mouse	Reduced IS and cTnI concentration	(84)

(Continued)

TABLE 1 | Continued

Peptides	Other names	Experimental models	Administration	Species	Mechanisms	References
Ghrelin	/	*ex vivo* 30 min global I/30 min R	10,000 pM—during R	Rat	Increased coronary flow, heart rate, left ventricular systolic pressure and left ventricular end-diastolic pressure	(85)
		in vivo 30 min I/24 h R	8 nmol/kg i.v.—at the onset of R	Rat	Reduced IS, inhibition of the TLR4, NLRP3, and Caspase-1 expression	(86)
Obestatin	/	*in vivo* 30 min I/24 h R	50 nM/kg—LV injection at the R	Rat	Reduced IS	(87)
		ex vivo 30 min I/2 h R	75 nM—during the first 20 min of R	Rat	Reduced IS	(88)
NECROPTOSIS						
Nesfatin-1	/	*in vivo* 30 min I/24 h R	20 μg/kg i.p.—prior R	Rat	Reduced IS and EF, Reduced Beclin-1 and Caspase-3 expression	(89)
		in vivo 30 min I/24 h R	20 μg/kg i.p.—prior R	Rat	Reduced expression of RIPK1, RIPK3, MLKL, ROCK1, and ROCK2 proteins	(90)
		in vivo 30 min I/2 h R	100 pmol/L—during the first 20 min of R	Rat	Reduced IS, ERK1/2 activation	(91)
AUTOPHAGY						
HBSP	Helix B surface peptide	*in vivo* 45 min I/2 h R	90 μg/kg, i.p.—5 min before R	Mouse	Reduced IS, decreased cardiomyocyte apoptosis	(92)
OTHERS						
GLP-1	Glucagon-like peptide 1; [GLP-1(7–36)amide]; Exenatide	*ex vivo* 45 min global I/2 h R	0.3 nM—at the onset of R	Rat	Improve LV pressure, no effect on IS	(93)
		in vivo 30 min I/2 h R	4.8 pmol/kg/min—perfusion during the procedure	Rat	Reduced IS	(94)
Apelin-13/-36	/	*ex vivo* 35 min global I/35 min R	1,000 nM Apelin-13/1,000 nM Apelin-36—at the onset of R	Rat	Reduced IS (Apelin-13 = 40%/Apelin-36 = 26%)	(14)
		in vivo 30 min I/120 min R	0.1 mg/kg Apelin-13/0.27 mg/kg Apelin-36, i.v.—at the onset of R	Mouse	Reduced IS (Apelin 13 = 43%/Apelin 36 = 33%)	
Apelin-13	/	*in vivo* 45 min regional I/24 h R	0.1 μg/kg—at 5 min after R	Mouse	Reduced IS, decreased apoptosis	(95)
Elabela	Apela; Toddler	*in vivo* 30 min regional I/4 h R	0.7 mg/kg, i.v.—at 5 min of R	Rat	Decreased apoptosis, fibrosis, and oxidative stress	(96)

H, hypoxia; I, ischemia; R, reperfusion; i.p, intra peritoneal injection; i.v, intra venous injection; i.c., intra coronary injection; IS, infarct size; LV, left ventricle.

reperfusion was safe and well-tolerated but not associated with a decreased IS as assessed by creatine kinase-myocardial band (CK-MB) quantification (97).

Humanin

Humanin (HN) is a mitochondrial-derived polypeptide (24-mer) encoded by mtDNA that regulates mitochondrial functions under stress conditions and protects cells against various situations such as diabetes mellitus, cardiovascular and neurodegenerative diseases mainly through anti-apoptotic effects leading to sequestration of BAX and Bid (132) (**Figure 1**). Muzumdar and colleagues have shown a significant reduction in IS after an intracardiac administration of HNG (S14G-humanin with a point mutation) at the onset of reperfusion in mice subjected to MIRI. HNG cardioprotection was associated with a significant increase in AMPK and endothelial nitric oxide synthase phosphorylation as well as to attenuation of BAX and BCL-2 levels (62).

Later, Thummasorn and co-workers have demonstrated on rats subjected to MIRI that administration of HNG 15 min before reperfusion decreased IS and arrhythmia (63). Sharp and colleagues confirmed these results in a large animal model

TABLE 2 | Therapeutic peptides used in clinical trials mentioned in this review.

Peptides	Clinical trial	Administration	Nb of patients	Results	References
Elamipretide	EMBRACE	0.05 mg/kg/h, between 60–15 min before PCI and for 1 h following reperfusion	297	**no IS reduction (CK-MB quantification)**	(97)
Cyclosporine A	/	2.5 mg/kg, catheter in the antecubital vein, <10 min before direct stenting	57	IS reduction	(98)
	CIRCUS	2.5 mg/kg, i.v., 12 h within symptom onset	970	**not better than placebo**	(99)
	CYCLE	2.5 mg/kg, i.v., 6 h within symptom onset	410	**no effect on ST-segment resolution or hs-cTnT, no improved clinical outcomes or LV remodeling up to 6 months**	(100)
	CYRUS	2.5 mg/kg, i.v., asap after the onset of ACLS	6,758	**do not prevent early multiple organ failure**	(101)
Carperitine	/	0.085 μg/kg/min i.v. for 65 h	3,777	better outcome	(102)
	J-WIND	0.025 μg/kg/min i.v. for 3 days	1,216	Reduced IS, increased LV EF, decreased reperfusion injury, **severe hypotension**	(103)
	AVCMA	0.0125–0.025 mg/kg i.v.	111	higher plasma BNP level, reduced blood pressure, **hypotension**	(104)
Nesiritide	/	0.01–0.03 μg/kg	862	**Increased risk of death after treatment**	(105)
	/	≤0.03 g/kg/min i.v.	1,269	**Increased renal disfunction**	(106)
Exenatide	/	25 μg/250 mL i.v. 15 min before intervention and maintained 6 h	172	Reduced IS, larger salvage index	(107)
	/	20 μg during PCI and 10 μg twice daily during 48 h	58	Reduced IS, improved LV function	(108)
	/	10 μg/h 30 min and 0.84 μg/h 72 h	191	**No benefit**	(109)
	COMBAT-MI	18 μg/180 mL i.v. 15 min before intervention and maintained 6 h combined with RIC procedure	222	**No benefit**	(110)

i.v, intra venous injection; IS, infarct size; LV, left ventricle; PCI, percutaneous coronary intervention; negative outcomes of clinical trials were highlighted in bold.

of MIRI but these effects were abrogated when ischemic time duration was prolonged from 60 to 75 min (64). Thus, although HNG cardioprotection translates beyond different animal models, further clinical studies are needed to validate HNG therapy for a clinical application.

Tat-BH4

Mitochondrial dysfunction and permeability mPTP opening are regulated in part by the voltage-dependent anion channel of the outer mitochondrial membrane (VDAC), which is itself controlled by pro- and anti-apoptotic BCL-2 family members (**Figure 1**) (133). Based on this fact, Roberta Gottlieb's group designed a peptide corresponding to residues 4–23 of BCL-XL protein conjugated to the protein transduction domain of HIV TAT (TAT-BH4) to develop a cardioprotective therapeutic strategy (134). Indeed, TAT-BH4 preconditioning attenuated CK release and reduced IS in IR rat hearts (15 min before I), demonstrating the role of mitochondria and pro-apoptotic BCL-2 proteins in the process of cell death.

To develop a more physiological-relevant therapeutic application, our group has analyzed the effects of the BH4 peptide injected intravenously at the onset of reperfusion in an *in vivo* murine MIRI model. Among the four formulations of BH4 with various cell-penetrating peptides [CPP: Tat, (RXR)4, Bpep and Pip2b] tested, we observed a decrease of ∼47% in IS and ∼60% in apoptosis *in vivo* either with Tat-BH4 or Pip2b-BH4 when administered intravenously 5 min before reperfusion (65).

Other Therapeutic Peptides Inhibiting the Mitochondrial Pathway

V1-cal

Hurt and co-workers have determined that TRPV1 (Transient Receptor Potential Vanilloid 1), a non-selective calcium (ion) channel activated in cellular pain insults including hypoxia, regulates mitochondrial membrane potential and MIRI (66). By using an 11-mer peptide decoy V1-cal coupled to the Tat CPP (135), the authors revealed a substantial reduction in IR injury by inhibiting the inducible calcineurin-TRPV1 interaction in an *in vivo* MIRI rat model.

AID-Tat

Viola and colleagues have tested an 18-mer peptide directed against the alpha-interacting domain (AID) of the alpha 1c subunit of L-type calcium channel vectorized by Tat (AID-Tat peptide), which has been shown to attenuate the increase in mitochondrial membrane potential and metabolic activity after activation of the channel (136). Later, they showed that AID-Tat peptide was able to reduce IS in rat hearts exposed to IR injury *ex vivo* when administered immediately after reperfusion (67). AID-Tat peptide was reported to significantly decrease IS and improve

cardiac contractility up to 12 weeks post-MI in rats *in vivo* as a result of a decrease in metabolic demand during reperfusion.

Therapeutic Peptides Inhibiting the Extrinsic Apoptotic Pathway

For several years, we and several other laboratories have confirmed that the death receptor-dependent apoptotic (or extrinsic) pathway is activated during IR injury (137, 138) since elevated FasL levels were found in the blood of AMI patients activating the FAS death-receptor pathway and triggers the downstream apoptotic signaling pathway (20). DAXX (Death-domain associated protein-6) protein acting as downstream FAS receptor adapter appears to play a key role in IR injury in various organs including the heart (50, 139, 140). The different roles of the DAXX protein depend on its subcellular localization: (i) anti-apoptotic in the nucleus and (ii) pro-apoptotic in the cytosol upon the Apoptosis Signal regulating Kinase 1 (ASK1)-shuttling triggered by various stimuli such as oxidative or ischemic stresses (141).

Therefore, we have focused on the development of a therapeutic peptide targeting the FAS:DAXX interaction as a new treatment against MIRI. We designed a 16-mer interfering peptide DAXXp by SPOT synthesis vectorized with the Tat CPP resulting in the conjugated ***Tat-DAXXp*** peptide (26). Our study showed that Tat-DAXXp (1 mg/kg, i.v. 5 min before reperfusion) treatment resulted in 48%-decreased IS in a murine IR model when assessed after 24 h reperfusion. Tat-DAXXp cardioprotection was achieved through the inhibition of both extrinsic and intrinsic apoptotic pathways (**Figures 1**, **2**) and the activation of pro-survival cascades. More impressively, Tat-DAXXp showed the same cardioprotection in a 6-month follow-up study using the same drug/ischemic protocol (27). In brief, Tat-DAXXp treatment decreased by 70% plasma cTnI concentration and mortality assessed at 24 h post-MI, and furthermore, increased ejection fraction (24%) compared to the non-treated control group during the 6-month follow-up. At the end of the protocol, histological analysis revealed a 54%-decreased left ventricular fibrosis content compared to non-treated mice. Remarkably, Tat-DAXXp was still efficient after a 30-min delayed administration after reperfusion showing a wide therapeutic time window of cardioprotection.

In conclusion, targeting the extrinsic pathway with Tat-DAXXp peptide at the onset of reperfusion revealed potent upstream cardioprotection in a murine model of MIRI validating this peptide as a promising candidate for therapeutic application since it promotes both cell survival and improves cardiac contractile function.

THERAPEUTIC PEPTIDES REDUCING INFLAMMATION TO TREAT MIRI

Formyl Peptide Receptor Binding Peptides

Annexin A1 (AnxA1) is a 37 kDa glucocorticoid-regulated protein known to regulate the termination of inflammation and to have a therapeutic potential in IR injury (142). Its N-terminal peptide Ac2-26 was shown to bind the formyl peptide receptor (FPR) family which inhibits neutrophil adhesion, migration, and infiltration (81, 143, 144). In 2001, the group of Perretti has reported that ***Ac2-26*** (1 mg/kg, i.v.) administrated at the onset of reperfusion or during 60 min, revealed significant cardioprotection associated with lower myeloperoxidase activity and IL-1β levels a rat IR model (79). More recently, Qin and co-workers have confirmed a significant reduction in inflammation (48 h post R) associated with decreased IS, fibrosis, and apoptosis (7-days post R) in a murine IR model (80).

In parallel, Perretti and colleagues worked on several longer and modified Annexin A1 peptides called $AnxA1_{2-50}$, CR-$AnxA1_{2-50}$ (81), and CR-$AnxA1_{2-48}$ (82) displaying cardioprotective properties leading to reduced IS and decreased systemic concentration of the Chemokine C-C motif ligand 5 in a murine IR model. Mechanistically, the three peptides act as new Lipoxin A4 receptor agonists impacting phagocyte responses resulting in protective actions.

Knowing that also agonists of formyl-peptide receptor-like 1 displayed cardioprotective effects in IR models, Hecht and co-workers have developed the ***CGEN-855A*** peptide (83) providing cardioprotection in both murine and rat IR models and displaying anti-inflammatory activity as revealed by polymorphonuclear neutrophil inhibition.

Based on the selectin binding sequence of EC-SEAL (145), Dehghani and colleagues have created the 7-mer ***DS-IkL peptide*** using the one-bead-one-compound combinatorial library to incorporate unnatural amino acids coupled to the negatively charged proteoglycan dermatan sulfate (DS) known to interact with P-selectin (84). DS-IkL localized at regions of vascular inflammation can reduce IS and cTnI levels in a murine IR model. Mice treated with DS-IkL at the onset of reperfusion and additionally 24 h later showed reduced neutrophil extravasation, macrophage accumulation, fibroblast, and endothelial cell proliferation, and fibrosis compared to the non-treated mice.

Other Inflammation-Inhibiting Peptides

Ghrelin is an octanoylated, 28-mer peptide, which is mainly generated in the stomach and also in small amounts in other organs such as the heart (146). The first evidence of a cardioprotective effect of Ghrelin administered at the onset of reperfusion was obtained in an *ex vivo* rat model of MIRI showing the reduced myocardial release of lactate dehydrogenase (LDH) and myoglobin as well as the depletion of myocardial ATP (85). In a IR mouse model, Ghrelin was reported to reduce IS and inflammation when administered for 3 days before AMI (147). In a more relevant model of MIRI, Wang et al. showed that one-shot Ghrelin administration (8 nmol/kg, i.p.) at the onset of reperfusion protected the rat heart against IR injury by inhibiting oxidative stress and inflammation *via* TLR4/NLRP3 signaling pathway (86).

Obestatin a 23-mer peptide issued from the carboxy-terminal part of proghrelin (ghrelin derives from the amino-terminal part of the same precursor) was reported to protect cardiomyocytes from MIRI *in vitro* and *in vivo* (87, 88,

148). Obestatin administrated by local injection in the left myocardium at the onset of reperfusion was able to reduce IS by ∼24% in a rat IR model and to decrease mRNA levels of TNF-α, IL-6, ICAM-1, and iNOS in rat cardiomyocytes after reperfusion (87). Nearly in parallel, Penna and colleagues observed a ∼50%–decreased IS after the administration of 75 nM Obestatin during the 20 first min of reperfusion in rats (88).

THERAPEUTIC PEPTIDES REDUCING NECROPTOSIS TO TREAT MIRI

To our best knowledge, very few peptides were identified as therapeutic treatment inhibiting necroptosis.

The only peptide recently reported is ***Nesfatin-1***; a new energy-regulating peptide displaying a pivotal role in the modulation of cardiovascular functions and protection against MIRI (89). A previous *ex vivo* study on rats revealed that Nesfatin-1 administration in the first 20 min of reperfusion decreases IS by the same extent as ischemic postconditioning through the activation of the pro-survival kinase ERK1/2 (91). In a rat MI model, Nesfatin-1 intraperitoneal injection provided a 50% IS reduction associated with a reduction in Beclin-1 (autophagy) and Caspase-3 (apoptosis) expression. Later on, this group demonstrated that only a high dose of Nesfatin-1 (20 μg/kg) was able to inhibit the expression of RIPK1, RIPK3, MLKL, ROCK1, and ROCK2 proteins (necroptosis and necrosis) in the same rat IR model (90).

THERAPEUTIC PEPTIDES REDUCING AUTOPHAGY TO TREAT MIRI

The anti-apoptotic and pro-angiogenic effects of erythropoietin (EPO) have prompted a growing interest as a therapeutic molecule for the treatment of AMI and HF. Despite promising results in animal MI models where EPO reduces IS and maintains ventricular function (149), clinical studies have revealed controversial results and both safety and tolerability problems.

In 2008, Brines and colleagues designed an 11-mer peptide issued from the helix B of the EPO receptor beta-common chain subunit (= helix B surface peptide, HBSP) (150). *In vivo* studies in MI models have confirmed that HBSP protects the heart from ischemic damage in the same way as EPO (151). Further on, Lin et al. demonstrated that HBSP pretreatment attenuated diabetic cardiomyopathy by inhibiting AMPK-dependent autophagy (152). Another study reported that the protective effect of HBSP against IR injury (i.p. 90 μg/kg, 5 min before reperfusion) is based on its inhibitory effect on cell autophagy (92). Furthermore, HBSP treatment in a hypoxia/reoxygenation-induced apoptosis model on H9c2 cells revealed an inhibition of the autophagy-related proteins (LC3II/LC3I) expression and an enhanced expression of phosphorylated phosphoinositide 3-kinase (PI3K) (153).

OTHER THERAPEUTIC PEPTIDES TO TREAT MIRI

GLP-1 and GLP-1 Agonists

Glucagon-Like Peptide 1 [GLP-1, also known as GLP-1(7–36)amide] was reported to exert biological actions in the cardiovascular system. Pharmacological postconditioning with GLP-1 has been found effective to reduce IS *in vivo* in rats subjected to IR (94, 154). In isolated mouse hearts, administration of GLP-1(9–36)amide (0.3 nM) induced a 32% IS decrease associated with PI3K-protein Kinase B (PKB)/Akt- and ERK1/2-dependent mechanisms (155).

The first clinical trial (172 patients) evaluating Exenatide, a GLP-1 receptor agonist used as an antidiabetic drug (25 μg/250 mL saline 15 min before and 6 h after reperfusion) revealed promising results in IS reduction (107). Woo et al. showed in addition to IS reduction an improvement of left ventricular function at 6 months post-MI in 58 patients treated by exenatide 20 μg during the percutaneous coronary intervention (PCI) and 10 μg twice daily during 48 h post-MI (108). Roos et al. did not confirm the same cardioprotective effects in their cohort of 191 patients despite a prolonged treatment duration (10 μg/h for 30 min followed by 0.84 μg/h for 72 h) (109). Cardioprotection was observed with another agonist, Liraglutide, showing a reduced necrotic area (156) and improved left ventricular ejection fraction after PCI (157). The mechanisms of action of the GLP-1 receptor agonist modulates myocardial metabolism and hemodynamic effects including peripheral, pulmonary, and coronary vasodilatation, mimicking ischemic preconditioning (158).

A recent clinical trial COMBAT-MI combining remote ischemic conditioning (RIC) and exenatide administration shows that neither RIC nor exenatide, or their combination, were able to reduce IS in STEMI patients when administered as an adjunct to primary percutaneous coronary intervention (110).

Apelin and Derived Peptides

Apelin (APLN) is the endogenous ligand for the G-protein-coupled apelin receptor (APJ receptor) (159) synthesized as a 77-amino acid prepropeptide further processed into C-terminal fragments Apelin-36, Apelin-19, Apelin-17, and Apelin-13. The adipocytokine Apelin plays a critical role in cardiovascular hemostasis. Secreted in myocardial cells and coronary endothelium, its expression is increased during myocardial damage (160). Since the lack of Apelin was reported to compromise functional recovery of the injured heart, Apelin and its derived peptides were administered as therapeutic molecules. Simpkin et al. demonstrated for the first time that pharmacological postconditioning with Apelin-13 and Apelin-36 peptides protects the heart against IR injury *in vivo* through the RISK pathway activation and by delaying the mPTP opening, resulting in a 43% and a 33%-decreased IS, respectively (161). Additionally, Apelin-16 was shown to increase the contractility of reperfused rat hearts (*ex vivo*) *via* the activation of pro-survival kinases (PKC and ERK1/2) (162).

In obese mice (High-fat diet model), pharmacological postconditioning with Apelin-13 was reported to decrease infarct

size, prevent apoptosis and mitochondrial damage induced by IR injury (95). A new endogenous ligand of the Apelin-APJ axis (Elabela also called Toddler or Apela) allowing to protect against IR-induced fibrosis, apoptosis, and oxidative stress *via* the PI3K/AKT signaling pathway has been identified (96).

VASOACTIVE THERAPEUTIC PEPTIDES

Atrial and Brain Natriuretic Peptides

The natriuretic peptide (NP) system consists of at least two distinct endogenous peptides: atrial natriuretic peptide (ANP) and brain (or B-type) natriuretic peptide (BNP). Due to the endocrine function of the heart, these peptide-hormones are secreted inducing specific signals *via* c-GMP coupled receptors. Besides different functions (e.g., lipolysis, lipid oxidation, mitochondrial respiration), NPs play an important role in cardiac vascularization reducing arterial blood pressure as well as sodium reabsorption (163).

NPs were recognized as cardioprotective compounds for MIRI in different animal models based on data showing that ***ANP*** administration reduced IS, increased blood flow, and decreased both left ventricular systolic and end-diastolic pressures in dogs subjected to myocardial IR (70). More recently, IS reduction by ANP was confirmed by Asanuma et al. in a more severe IR dog model (71). Similar results were obtained with ***BNP*** in a rat IR model showing reduced IS and decreased LDH and CK levels compared to untreated animals (74). Thereafter, Li et al. using a recombinant BNP (RhBNP) demonstrated the attenuation of inflammatory infiltration and CD4+ T cell proliferation function in addition to IS reduction (75).

In 2003, hANP treatment was reported to limit IR injury on a small cohort of 19 AMI patients (164). The subsequent study revealed that an ANP infusion during >48 h allows preventing LV remodeling in 50 patients with first anterior AMI (165). Afterward, ANP cardioprotective effects were confirmed in AMI patients by a large multi-center randomized trial (J-WIND—Japan-Working Groups of Acute Myocardial Infarction for the Reduction of Necrotic Damage) (166). Patients treated by human ANP had a reduced IS (-14.7%) assessed by a CK release, an increased LVEF (5.1%), a reduced IR injury (25.9%), and more importantly, decreased risks of cardiac death or HF compared to the control group (103, 166, 167).

In Japan and the US, ***Carperitide*** (28-mer synthetic ANP) or ***Nesiritide*** (23-mer synthetic BNP) have been approved as a treatment for acutely decompensated HF. However, adverse events were observed such as relevant hypotension (102–104) or increased mortality (168) and worsened renal function for Nesiritide (105, 106).

Derivates of Natriuretic Peptides

Urodilatin

Urodilatin an ANP homolog was used as a pharmacological postconditioning in MIRI models. For example, the cyclic 31-mer peptide showed an increased LV pressure in a rat *ex vivo* IR model when applied during the first 15 min of reperfusion and, furthermore, a reduced IS in a pig *in vivo* MIRI model after an intravenous administration during the first 25 min of reperfusion (72).

C-type Natriuretic Peptide

C-type natriuretic peptide (CNP) is a 22-mer peptide, structurally related to but genetically distinct from ANP and BNP. Isolated rat hearts subjected to MIRI revealed smaller IS and a reduced coronary perfusion pressure when treated with CNP during the first 30 min of reperfusion (30 nmol/L) (77).

Lebetin 2

Lebetin 2 (L2), a 38-mer peptide snake venom-derived NP isolated from *Macrovipera lebetina,* has the advantage to be more stable compared to human NPs. L2 perfused to rat hearts *ex vivo* reduced IS similarly to BNP (73). Interestingly, the same authors showed some years later that L2 has strong and prolonged cardioprotective effects in post-MI (mouse and rat IR models) mainly due to modulation of the inflammatory response as evidenced by enhanced M2-like macrophage detection (76).

Vasonatrin Peptide

Vasonatrin peptide (VNP) is an artificial 22-mer chimeric peptide issued from ANP and CNP showing more potent diuretic, natriuretic, and vasorelaxant properties compared with other NPs (169). This peptide was able to attenuate MIRI in diabetic rats (administrated 10 min before R) as demonstrated by reduced LV systolic and end-diastolic pressure as well as decreased Caspase-3 activity and plasma CK/lactate dehydrogenase (LDH) quantities (78).

CONCLUSIONS

Cardiovascular diseases including AMI ranks first in worldwide mortality and according to WHO, they will stay at this rank until 2030. Currently, despite promising results in animal models, there is no pharmacological treatment, which could be administrated in adjunct to reperfusion therapy to inhibit its adverse effects known as reperfusion injury. Differences between preclinical animal MI models and the clinical scenario in patients, including age, comorbidities, and cotreatments could be an explanation (170, 171). Other reasons could be related to the limited comprehension of the underlying pathophysiology and the absence of specific biomarkers to clearly identify MIRI.

Finally, the therapeutic time-window for the application of pharmacological therapies plays a critical role to provide a successful treatment of reperfusion injury. The burst of cell death induced by reperfusion after prolonged ischemia can only be prevented if the administration of cardioprotectants occurs at the onset of reperfusion and preferably before reopening of the culprit coronary artery (26, 172, 173). However, even if the time-window of drug administration should be before reperfusion, further investigations are needed to define how long the protective therapy must be applied to fully prevent MIRI.

PERSPECTIVES

Since MIRI is a complex interplay of different pathways, a strategy involving multiple targets should be considered in the development of pharmacological drugs. Some of the pathways currently being targeted are the different apoptotic pathways, microvasculature circulation, inflammation, platelets, mitochondrial dynamics, and RISK/SAFE pathways leading to cell survival and improved cardiac function (174).

After disappointing results in clinical assays (2014–2016), academic research has forwarded the development of novel therapeutic molecules such as highlighted by more than 1,000 PubMed publications in 2021 (searching keywords: "myocardial ischemia-reperfusion," "therapy," "2021").

To succeed in cardioprotection, other aspects of reperfusion injury besides infarct size should be considered in particular microvascular injury since heart function is ensured by both cardiomyocytes and vascular cells (cell ratio 50:50) (174) and early ventricular arrhythmias with a lower contribution.

Additionally, considering the development of new therapeutic peptides (or other pharmacological drugs), special attention should be devoted to the improvement of targeting the ischemic zone (or subcellular localization) within the infarcted heart to maximize local drug concentration and reduce side effects. Recently, Zhang and co-workers showed higher mitochondrial integrity, lower apoptosis of cardiomyocytes, and reduced myocardial IS by encapsulating CsA in PEGylated nanoparticles with mitochondria-targeting [CsA@PLGA-PEG-SS31] (127). Another example is the cyclic heart homing sequence [CSTSMLKAC] grafted on porous silicon nanoparticles revealing an improved accumulation within the heart (up to three-fold) (175).

Taking together, future development of pharmacological drugs to treat AMI patients should be characterized by a drug cocktail or a pleiotropic drug acting specifically on (i) different pathways or (ii) different cell types, or by (iii) an improved tissue or subcellular targeting. The combination of these strategies should provide advantages for future clinical outcomes.

AUTHOR CONTRIBUTIONS

PB and SB-L contributed to conception and design of the manuscript. CFR, KK, and PB performed the selection of the therapeutic peptides. PB wrote the first draft of the manuscript. CFR, KK, EJ, JN, SB-L, and PB wrote sections of the manuscript. EJ performed the graphical design of the figures. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Promising Therapeutic Candidate for Myocardial Ischemia/Reperfusion Injury: What Are the Possible Mechanisms and Roles of Phytochemicals?

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Percutaneous coronary intervention (PCI) is one of the most effective reperfusion strategies for acute myocardial infarction (AMI) despite myocardial ischemia/reperfusion (I/R) injury, causing one of the causes of most cardiomyocyte injuries and deaths. The pathological processes of myocardial I/R injury include apoptosis, autophagy, and irreversible cell death caused by calcium overload, oxidative stress, and inflammation. Eventually, myocardial I/R injury causes a spike of further cardiomyocyte injury that contributes to final infarct size (IS) and bound with hospitalization of heart failure as well as all-cause mortality within the following 12 months. Therefore, the addition of adjuvant intervention to improve myocardial salvage and cardiac function calls for further investigation. Phytochemicals are non-nutritive bioactive secondary compounds abundantly found in Chinese herbal medicine. Great effort has been put into phytochemicals because they are often in line with the expectations to improve myocardial I/R injury without compromising the clinical efficacy or to even produce synergy. We summarized the previous efforts, briefly outlined the mechanism of myocardial I/R injury, and focused on exploring the cardioprotective effects and potential mechanisms of all phytochemical types that have been investigated under myocardial I/R injury. Phytochemicals deserve to be utilized as promising therapeutic candidates for further development and research on combating myocardial I/R injury. Nevertheless, more studies are needed to provide a better understanding of the mechanism of myocardial I/R injury treatment using phytochemicals and possible side effects associated with this approach.

Keywords: myocardial ischemia/reperfusion injury, phytochemicals, pharmacology, mechanisms, therapeutic implication

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INTRODUCTION

AMI remains the world's leading cause of morbidity and mortality (1). Of all the deaths, adverse acute ischemic events, such as ST-elevation myocardial infarction (STEMI), are the main triggers (2). In recent years, most of the endeavors in the processing of STEMI have been focused on guaranteeing the prompt coronary revascularization of the culprit artery and exploitation of pharmacological regimens for further preservation of the coronary blood flow (3, 4). Early primary percutaneous coronary intervention (pPCI; within 2 h since symptoms onset) has proved effective in reducing ischemia time to improve the outcomes of patients with STEMI (5); however, the cardiomyocytes begin to die having experienced a long-term ischemic environment. Even though reperfusion proves effective in limiting this process, it causes a spike of further cardiomyocyte injury (known as "reperfusion injury") that contributes to final IS (6), which remains a crucial determinant of prognosis and is bound with hospitalization of heart failure as well as all-cause mortality within the following 12 months. While the ischemic injury increases with the severity and the duration of blood flow reduction, reperfusion injury achieves its maximum with a moderate amount of ischemic injury (7). Therefore, the addition of adjuvant intervention to limit cardiomyocyte death during myocardial I/R injury has become necessary.

The exact mechanisms of how the homeostasis of cardiac cells is impaired during myocardial I/R injury are not fully understood (8). Pathological changes, such as calcium overload, inflammation, apoptosis, neurohumoral activation, autophagy, and oxidative stress, are considered to be of equal contribution to I/R injury (9). Phytochemicals, the secondary metabolites and natural components of herbs, mainly composed of non-nutritive bioactive compounds, have long been recognized as promising therapeutic candidates for novel drugs (10). They are synthesized only in specific plant cells and do not take part in the energy metabolism nor the catabolic or anabolic ones (11). More than 10,000 phytochemicals have been discovered so far, including saponins, polyphenols, carotenoids, terpenes, and alkaloids, while many remain unknown (12). In recent years, they have attracted more attention as modulators of many cellular signaling pathways and by the ability of health improvement (13). For example, metabolic disorders, such as cardiovascular disease, cancer, and obesity, may benefit from many phytochemicals (14). Research and clinical studies have demonstrated the compounds' biological effects, such as antioxidant, anti-inflammatory, and cytotoxic activities, suggesting that these natural products may be potential to alleviate the myocardial I/R injury (15, 16).

This review provides a concise summary of the efforts of former researchers on how phytochemicals alleviate myocardial I/R injury and highlights the evidence of their cardioprotective-related mechanisms. We aim to present new insight into the development of potential treatments for myocardial I/R injury.

THE MECHANISM OF MYOCARDIAL I/R INJURY

Many pathways that induce cell death, also known as apoptosis, programmed necrosis, or necroptosis, are initiated by myocardial I/R injury, involving several signaling pathways (9). Therefore, it is necessary to find improved protective strategies to prevent myocardial I/R injury, of which the related mechanisms have been widely studied. A growing number of pieces of research, both *in vitro* and *in vivo*, have proved phytochemicals to be potently cardioprotective on myocardial I/R injury, mainly by restraining irreversible cell death caused by apoptosis, autophagy, and necrosis *via* preventing calcium overload, oxidative stress, and inflammation (17) (**Figure 1**).

Apoptosis

Apoptosis exists in several cellular organisms. Stimulation of apoptotic pathways leads to cell death in ischemic heart cells (18). Generally, the high level of reactive oxygen species (ROS) can be lethal for I/R-injured cells and attributed to cardiomyocyte apoptosis (19). It is proved that during myocardial I/R injury, apoptosis-related genes, such as signal transducer and activator of transcription 3(STAT3), B-cell lymphoma-C(BCL-C), and B-cell lymphoma-xL(Bcl-xL) in the myocardial tissue, are rearranged (20). There is evidence that the increased expression of these genes produces protective proteins against apoptotic pathways and reduces physiotherapy (21). Ca^{2+} signaling, which can be modulated and synchronized by mitochondria, is an essential part of apoptosis. Accumulation of Ca^{2+} in mitochondria leads to apoptosis (22). In porcine models of chronic myocardial ischemia and hibernation, autophagy-enhanced cardiomyocytes were negative for apoptosis, while apoptotic cells were negative for autophagy, suggesting that autophagy plays a protective role against apoptosis in this model (23). mTORC1 can sense cellular

Abbreviations: AMI, acute myocardial infarction; STEMI, ST-elevation myocardial infarction; pPCI, primary percutaneous coronary intervention; IS, infarct size; I/R, ischemia-reperfusion; NADH+, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; BCL-C, B-cell lymphoma-C; Bcl-xL, B-cell lymphoma-xL; mTOR, mammalian target of rapamycin; Cyt c, cytochrome c; Bcl-2, B-cell lymphoma-2; Bax, BCL2-associated X; H/R, hypoxia-reoxygenation; VSMC, vascular smooth muscle cell; 3-MA, 3-methyladenine; HMGB1, high-mobility group box protein 1; GSK-3β, glycogen synthase kinase-3β; PI3K, phosphatidylinositol-3-OH kinase; Akt, protein kinase b; ATP, adenosine triphosphate; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; BRCA1, breast cancer type 1 sensitive protein; NLRP3, nod-like receptor protein 3; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; Nrf2, nuclear factor-erythroid 2-related factor 2; STIM1, stromal interaction molecule1; Sal-B, salvianolatic acid B; 6-G, 6-Gingerol; CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase; ERK, p-extracellular signal-regulated protein kinase; MEK, (p)-mitogen-activated protein kinase kinase; CG, Calycosin-7-O-β-D-glucoside; NO, nitric oxide; HKL, honokiol; TAB, tournefolic acid B; ER, endoplasmic reticulum; AMPK, adenosine monophosphate-activated protein kinase; JAK2, Janus kinase 2; SalA, salvinolic acid A; SA, salvinolic acids; EGCG, epigallocatechin-3-gallate; JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinase; BCF, bauhinia championii flavone; GAS, gastrodin; AS-IV, astragaloside IV; ISBA, isovaleroylbinankadsurin A; RISK, reperfusion injury salvage kinase; GLA, glaucocalyxin A; GB, ginkgolide B; BBR, berberine; ERS, endoplasmic reticulum stress; SOD, superoxide dismutase; HSP70, heat shock proteins 70; PMS, plantamajoside; DATS, diallyl trisulfide; EE, eleutheroside E; TLR4, toll-like receptor 4; RAPA, rapamycin; SHR, spontaneous hypertension rat; TCM, traditional Chinese medicine.

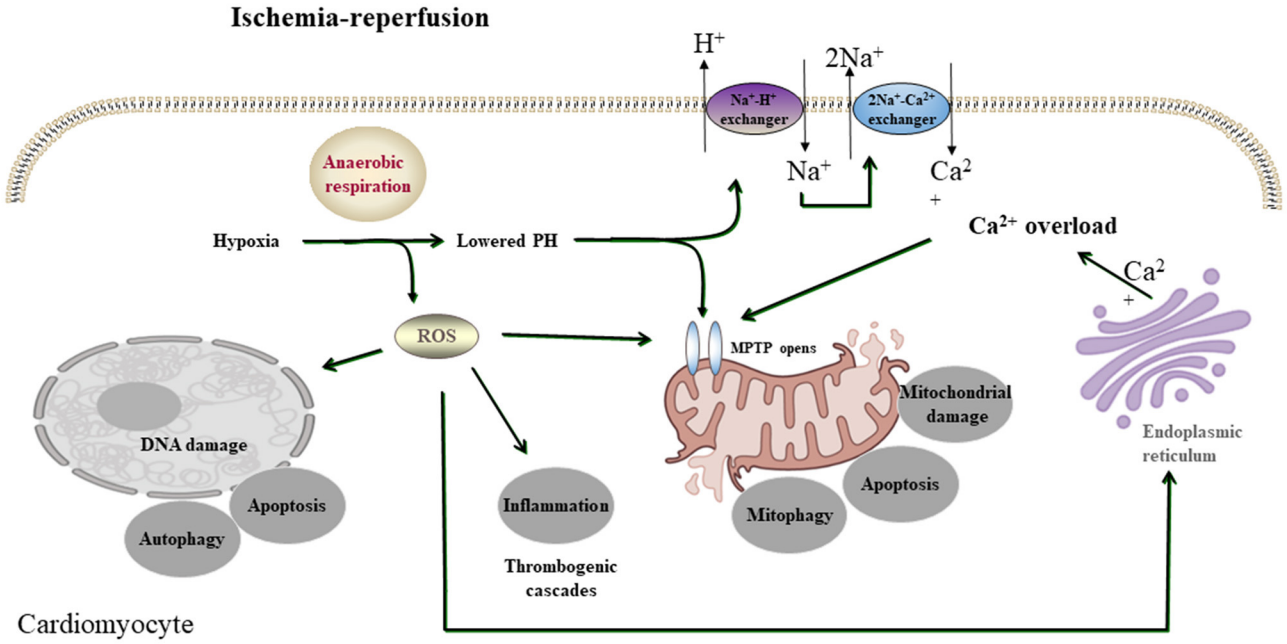


FIGURE 1 | A simplified scheme of the mechanism of acute myocardial I/R injury. During acute myocardial ischemia, ischemic cardiomyocytes switch to anaerobic metabolism to provide ATP. However, this results in the Na+-H+ exchanger to extrude H+ and results in intracellular Na+ overload, which activates the 2Na+-Ca2+ exchanger to function in reverse to extrude Na+ and leads to intracellular Ca2+ overload. The endoplasmic reticulum also markedly reduces Ca2+ reuptake, which exacerbates intracellular Ca2+ overload. Ca2+ can also induce MPTP opening. During reperfusion, the influx of oxygen fuels production of ROS (oxygen paradox). Other sources of ROS include xanthine oxidase (endothelial cells) and NADPH oxidase (neutrophils). ROS can damage virtually every biomolecule found in cells, promote the opening of mPTPs, and activate inflammatory and thrombogenic cascades to exacerbate cell injury.

nutrient status (24) and inhibits myocardial I/R injury. Growth factor receptor-bound protein 1(GRb1) treatment antagonizes the inhibitory effect of mTORC1 (25). B-cell lymphoma-2(Bcl-2) has the potential to inhibit apoptosis, mitochondria disruption, the following cytochrome c(Cyt c) release, and, finally, caspase activation (26). Pretreating with Eupatilin can increase Bcl-2 expression, decrease BCL2-associated X(Bax), and cleaved caspase-3 expression induced by hypoxia-reoxygenation (H/R) in H9c2 cells (27).

Autophagy

Autophagy is essential to maintain cellular homeostasis. But its effects on myocardial I/R injury are paradoxical (17). Autophagy is characterized by the formation of a cup-shaped pre-autophagosomal double-membrane structure, which surrounds cytoplasmic material and closes to form the autophagosome (19). Autophagosome clearance, which can cause autophagy acceleration and cardiomyocyte death, is damaged during I/R injury (28). The knocking out of Beclin1 heterozygous eliminates myocardial I/R-induced autophagosome formation, as well as reduces myocardial infarction and cell death (29). Likewise, 3-methyladenine (3-MA) reduces autophagy caused by I/R *via* prohibiting autophagy and increases survived cells (30). Hesperidin can inhibit excessive autophagy by triggering the PI3K/Akt/mammalian target of the rapamycin (mTOR) pathway. Hesperidin was found to be capable of reinforcing p-PI3K, p-Akt, and p-mTOR levels and downregulating LC3II and Beclin1, whereas its specific inhibitor, LY294002, obviously invalidated all the effects mentioned above (31).

On the contrary, autophagy has been widely reported to be beneficial to myocardial I/R injury. The recovery of myocardium function after I/R benefits from a high level of autophagy. However, the depletion of adenosine triphosphate (ATP) is possibly the reason for autophagosome-lysosome pathway impairment during ischemia, which correlates with permanent injury in contractile function (32). With enhanced autophagy, apoptosis decreases in cardiac myocytes, same as autophagy in apoptotic cells in the porcine model of chronic myocardial ischemia and hibernating myocardium. Therefore, there is a deduction that cells are protected by autophagy against apoptosis in this model (33). Also, glucose deprivation-mediated cell death can be promoted when autophagy is inhibited (29). It can be concluded that when autophagy is upregulated, cells are likely to survive during I/R. Resveratrol is found to alleviate I/R injury of the myocardium in diabetic patients by promoting programmed cell death and *via* upregulating Beclin 1 and LC3-II (34).

Ca^{2+} Overload

When there is myocardium hypoperfusion, affected cardiomyocytes switch to use less oxygen, leading to lactate, H^+, and nicotinamide adenine dinucleotide ($NADH^+$) accumulation and cytosolic pH decrease. To reestablish acid-based balance, the plasmalemma Na^+/Ca^{2+} exchanger is activated. Then, the extracellular H+ ions accumulated during ischemia raise

the proton gradient across the plasmalemma and further result in cytosolic Ca^{2+} increase (9). In addition, under physiological conditions, inactive calpains compete with their endogenous inhibitor calpastatin and are stored in cellular cytosol (35). Calpain is activated by the elevation of intracellular calcium levels, and its conformational changes promote the intracellular translocation of Ca^{2+}, where phospholipids close Ca^{2+} channels, activating downstream proteins or diminishing the Ca^{2+} threshold for calpain activation (36). Myocardial ischemia/reperfusion injury is associated with a calcium homeostasis imbalance (37). *In vivo* studies showed an increment in intracellular Ca^{2+} concentration caused by ischemia/reperfusion in isolated perfused mammalian hearts (36). Reperfusion leads to rapid alterations in ion flux and alters the state of ion exchange, resulting in intracellular calcium overload (38). Increased calcium overload plays a key role in apoptosis, cell cycle, and differentiation, modifying cardiomyocyte function.

Oxidative Stress

Oxidative stress is the result of an imbalance between oxidants and anti-oxidants. When the blood supply in an ischemic area is reestablished, the influx of oxygen produces excessive ROS, which is harmful to the ischemic area. This phenomenon is called the oxygen paradox, meaning that reperfusion after ischemia can result in injury rather than protection. This is because ROS modifies the metabolism in cells and tissues, leading to dysfunction or even cell death (39). ROS is the reason why I/R is deleterious for cells and tissues (40). Thus, oxidative stress reduction may combat I/R injury, and further investigations are needed. NF-E2-related factor 2(Nrf2), a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, promotes the detoxification of pro-oxidative stressors. The Nrf2 signaling pathway plays a critical role in protecting the ischemic myocardium from myocardial I/R injury. Nrf2 deficiency mice show increased oxidative stress as well as an aggravated cardiac injury during I/R (41).

Inflammation

Inflammation is a strong shield to protect the body, but dysfunctional inflammation has much to do with the pathogenesis of many diseases. Leukocyte infiltration can be activated in the infarcted myocardial region *via* a complex inflammatory pathway to protect unaffected regions (42). Evidence shows that, in a heart, reperfused areas can be harmed by an excessively activated inflammatory reaction (43). Nuclear factor kappa-light-chain enhancer of the activated B cells (NF-κB) signaling pathway is crucial to cardiac I-R injury. NF-κB interacts with the nucleus by regulating more than 200 genes, among which some produce inflammatory cytokines, which ultimately lead to excessive inflammation. H9C2 cells, which are damaged by hypoxia through BRCA1/ROS-regulated NLRP3 inflammasome/IL-1β and NF-κB/TNF-α/IL-6 pathways, can be improved by Paeonol (44). When intercellular macromolecular proteins aggregate, they are called inflammasomes, which promote the maturation of inflammatory cytokines (45). NLRP3 consists of NLRP3, ASC, and caspase-1 precursor (Pro-Casp-1) (46) and is the most widely studied inflammatory pathway for now. Artemisinin can reduce the oxidative stress reaction due to its NLRP3-regulating ability (47).

CARDIOPROTECTIVE PHYTOCHEMICALS ATTENUATING MYOCARDIAL I/R INJURY

We thoroughly illustrated the phytochemicals proved to possess protective effects in the heart against I/R injury. Because of their variety, phytochemicals have been classified into phenols, saponins, lignans, terpenes, alkaloids, quinones, polysaccharides, carotenoids, coumarin, and other compounds for a better summary in this review.

Phenols

Phenolic compounds constitute one of the most ubiquitous groups of plant metabolites and are an integral part of both human and animal diets (48). Among numerous natural phytochemicals used to prevent myocardial I/R injury, phenolic compounds are particularly important because of their unique properties. Although, these compounds were first known for their antioxidant properties, several studies over the years have shown that they can exert protective effects against myocardial I/R injury. The mechanisms underlying these potential benefits include the regulation of different cell signaling pathways and gene expression.

Paeonol

Paeonol (2′-hydroxy-4′-methoxyacetophenone), isolated from the plant *Moutan Cortex*, was found to possess broad pharmacological effects on treating atherosclerotic lesions. This is associated with alleviating endothelial injury, ameliorating inflammation and oxidative stress, repressing platelet activation and aggregation, inhibiting vascular smooth muscle cell (VSMC) proliferation and migration, as well as lowering blood lipids (49–53). Pretreating with paeonol can significantly improve the hypoxia-reoxygenation (H/R) damage and the BRCA1 expression of H9C2 cells through the BRCA1/ROS-regulated NLRP3 inflammasome/IL-1β and NF-κB/TNF-α/IL-6 pathways. It may be a candidate drug for treating myocardial I/R injury (44).

Oridonin

Oridonin is a wide-studied flavonoid compound extracted from *Isodon rubescens (Hemsl.) H.Hara*, and it has a multitargeting anticancer effect (54). Lu et al. demonstrated that it exerts cardioprotective effects by reducing I/R-induced inflammatory injury. Pretreating with oridonin also reduced oxidative stress and downregulated the NLRP3 inflammasome pathway. These recent findings have shown the molecular mechanism of its alleviating myocardial I/R injury. Applying oridonin could help prevent and treat myocardial I/R injury (55).

Baicalin

Baicalin is a flavonoid compound isolated from the roots of *Scutellaria baicalensis Georgi*. It proves effective in treating diseases like cancer, osteoarthritis, hepatitis, and nephritis (56–58). It was reported that baicalin exerted antioxidant,

anti-apoptotic, and anti-inflammatory properties (59). Studies demonstrated that baicalin was protective for rat cardiomyocytes through downregulating H/R-induced injury (60). It was demonstrated by Kong et al. that baicalin reduced I/R damage in the heart by its antioxidant and paracrine effects (61). Luan et al. demonstrated that by regulating the Akt/NF-κB signaling pathway, baicalin downregulated myocardial apoptosis and inflammation (62). Liu et al. reported that LV functions were improved and myocardial apoptosis was suppressed by baicalin *via* suppressing the CaSR/ERK1/2 signaling pathway in myocardial I/R injury rats (63). Xu et al. reported the protective effect of baicalin, *via* the JAK/STAT pathway, on myocardial I/R injury. In addition, baicalin reduced cardiomyocytes damage, downregulated cell death caused by I/R, and inhibited inflammation response in the heart by interfering with macrophages (64).

Resveratrol

The natural compound resveratrol was mainly extracted in fruits, such as peanut, grape, and berry. It has been demonstrated that resveratrol downregulates the pathological progression in many disease models, such as cancer or diabetes mellitus (65–67). Currently, resveratrol has been demonstrated to carry a potentially cardioprotective property against myocardial I/R injury *via* regulating inflammatory, angiogenesis, energy metabolism, mitochondrial function, and cardiomyocyte apoptosis (68, 69). Compared with vehicles, resveratrol significantly reduced the size heart infarction area in small animal studies both *in vivo* and *ex vivo*. Neither the reperfusion time nor the route of administration affects the effects of resveratrol (70). Resveratrol also exerts protection on myocardial post-I/R damage through inhibiting stromal interaction molecule1 (STIM1)-mediated store-operated Ca^{2+} accumulation (71), upregulating of Beclin-1 and LC-3II expression to induce autophagy (34) and regulating phosphorylation levels of proteins relative to the PI3K/Akt/e-NOS pathway (72). Concluding from the available data, resveratrol presents a significant limiting effect against myocardial I/R damage.

Polydatin

Polydatin, isolated from *Reynoutria japonica Houtt.*, is another monocrystalline compound like resveratrol. The difference between them is at position C-3, where polydatin has the substitution of a glucoside group instead of a hydroxy group (73, 74). Polydatin exerts several pharmacological properties, such as anti-inflammatory, antioxidant (75), and alleviation of cardiac remodeling induced by pressure overload (74). Ling et al. reported that this compound aggravated autophagy and inhibited cell death during I/R or H/R, and co-treatment with adenovirus carrying short hairpin RNA for Beclin 1 and 3-MA, an autophagic inhibitor, would reverse this effect. Polydatin-treated mice showed a significantly reduced IS in heart tissue and a better heart function, compared with vehicle-treated mice, whereas these effects could be partly antagonized by 3-methyladenine (3-MA). These findings showed that polydatin treatment after infarction lowered myocardial I/R damage by enhancing autophagy to clear impaired mitochondria and to downregulate ROS and apoptosis (76).

Salvianolic Acid B

Salvianolic acid B (Sal B), derived from *Salvia miltiorrhiza Bunge.*, is a water-soluble compound (77). Sal B exerts multiple effects, such as reducing inflammatory factor expression, inhibiting cell death, and alleviating oxidative stress (78, 79). Former evidence has shown that Sal B can alleviate oxidative stress, modulate calcium overload, promote endothelial function, stabilize mitochondrial membrane potential, and upregulate microRNA-30 (80), making it protective against myocardial I/R injury. A recent study has revealed that Sal B could alleviate myocardial I/R damage dose-dependently, promote cardiac function, decrease myocardial infarction size, reduce myocardial injury marker expression, inhibit inflammatory responses, increase PI3K/Akt expression, and decrease high-mobility group box protein 1(HMGB1) expression. The mechanism is that Sal B ameliorated myocardial I/R damage by promoting PI3K/Akt and decreasing the release of HMGB1 in rats (81).

6-Gingerol

6-Gingerol (6-G), a main component of gingerols and a phenolic compound isolated from *Curcuma longa L.*, exerts antioxidative, antiapoptotic, and anti-inflammatory effects (82). Sampath et al. demonstrated that 6-G could prevent atherosclerosis *via* reducing cell death caused by excess oxidative stress (83). El-Bakly et al. found 6-G significantly protected cardiomyocytes by inhibiting cell death *via* alleviating oxidative stress and doxorubicin-induced myocardial damage (84). Lv et al. reported that pretreating with 6-G remarkably promoted cardiac function and decreased IS and I/R-induced creatine kinase-MB levels. 6-G alleviates myocardial I/R injury by reducing I/R-induced cardiomyocyte cell death and upregulating the PI3K/Akt signaling pathway. This evidence proved that 6-G may be a candidate drug for alleviating myocardial I/R injury (85).

Oleuropein

Oleuropein, a glycoside compound, is of antispasmodic effects, and can also reverse arrhythmia. In the rabbit isolated heart, it increases the coronary blood flow by 50% (86). Oleuropein can also lower blood pressure as it strongly inhibits the angiotensin-converting enzyme, as a result of its highly reactive 2,3-dihydroxy glutaraldehyde structure. A study reported that oleuropein protected the heart from myocardial I/R injury. In a myocardial I/R rat model, oleuropein reduced CK-MB and lactate dehydrogenase (LDH) levels as well as infarction size in the heart. Oleuropein also inhibited the caspase-3 pathway and reduced p53, p-IκBα protein, p-extracellular signal-regulated protein kinase (ERK), and phosphorylated (p)-mitogen-activated protein kinase kinase (MEK) expression. This evidence proved that by regulating the MEK/ERK/STAT3 signaling pathway, oleuropein inhibits myocardial I/R in rats (86).

Calycosin-7-O-β-D-Glucoside

Calycosin-7-O-β-D-glucoside (CG) is a major isoflavone extracted from *Astragalus mongholicus Bunge*, which has been proved to exert anti-inflammatory (87) and antioxidant abilities

(88). Studies demonstrated that CG could decrease the size of cerebral infarction in the process of cerebral I/R injury, maintain the stability of the blood-brain barrier, and reduce the I/R-induced neuronal injury (89). *In vitro* and *in vivo* experiments showed that CG activated the JAK2/STAT3 pathway and upregulated the secretion of IL-10, and, therefore, can protect cardiomyocytes from I/R-induced cell death (90).

Puerarin

Puerarin (7,4'-dihydroxy-8-C-glucosylisoflavone) is an isoflavone of broad pharmacological abilities (91), including treating cardiovascular and cerebrovascular diseases, which can be a potential drug in alleviating I/R injury (92). Puerarin lowers the lipid peroxidation level, and aldose reductase activity decreases superoxide ion radicals and protects endothelial cells (93). Studies showed that puerarin remarkably shrinks the myocardial infarction size and increased pressure in the left ventricular in rats with diabetes mellitus suffering from myocardial I/R. Puerarin significantly reduced oxidative stress, inflammation, and NF-κB protein expression. Furthermore, puerarin raised the levels of VEGFA and Ang-I, as well as increased nitric oxide (NO) production, caspase-3 activity, and phosphorylated-endothelial NO synthase protein expression. These findings illustrated that puerarin protected cardiomyocytes and served to reduce myocardial I/R damage (94).

Hesperidin

Widely found in citrus fruits, hesperidin is a flavanone glycoside with a molecular formula of C28H34O15 and a molecular weight of 610.57 Da (95). Hesperidin has been found to possess broad biological effects, including antioxidant, anti-cancer, radio-protective, anti-inflammatory, and anti-allergic, properties (96–99). Gandhi et al. reported that hesperidin reduced arrhythmias and apoptosis caused in myocardial I/R injury, also reduced inflammation and oxidative stress, decreased excessive autophagy, and promoted the PI3K/Akt/mTOR pathway (31, 100).

Luteolin

Luteolin is a flavone widely presented in several plants. Former experiments reported that Lut protected the cardiomyocytes from I/R damage by decreasing microRNA-208b-3p expression and inhibiting the PI3K/Akt pathway (101, 102), and partly reversing the low expression and activity of SERCA2a in the injured area (103). Other evidence demonstrated that it modulated SERCA2a by SUMOylation at lysine 585 (104). These studies demonstrated that luteolin prevents the heart from suffering from I/R injury.

Honokiol

Honokiol (HKL) is isolated from *Magnolia Officinalis Rehder and E.H. Wilson*, which has long been used as a herb in traditional Chinese medicine. It is known for its effect of treating various vascular diseases, including ischemia and infarction (105). HKL was reported to be able to alleviate cerebral I/R injury *via* relieving oxidative stress and downregulating inflammatory reaction (106). Early evidence also proved that HKL could limit the infarct area and reduce arrhythmia in rats with AMI (107), in which its antioxidative and antiapoptotic abilities played critical roles. What is more, HKL could also regulate the SIRT1/Nrf2 signaling pathway, which was also important for its cardioprotective effects (108). Tan et al. demonstrated that post-treating with HKL reduced myocardial I/R injury and promoted autophagic flux in C57BL/6 mice (109).

Tournefolic Acid B

Tournefolic acid B (TAB) is a newly discovered compound isolated from *Clinopodium chinense (Benth.) Kuntze*, a traditional Chinese herbal medicine with modern pharmacological effects, such as anti-inflammatory, antitumor, antiradiation, and lowering blood glucose. Yu et al. reported that TAB significantly prevented the heart from being damaged by I/R injuries by suppressing ER stress and oxidative stress through inhibiting PI3K/AKT pathways. *In vitro* and *ex vivo* experiments, both supported this conclusion, meaning that TAB likely inhibited cell apoptosis by resisting oxidation-endoplasmic reticulum stress *via* activating the PI3K/AKT pathway (110).

Orientin

Orientin, one of the major active flavonoids of *Persicaria orientalis (L.) Spach*, is a traditional Chinese herb. It was reported to exert broad pharmacological properties, including anti-oxidant, anti-inflammation, and anti-apoptosis (111). Former experiments have demonstrated that orientin protected myocardium from I/R damage probably by reducing cell death (112). Evidence showed that the protective effect of orientin against myocardial I/R damage is partly regulated through subtle induction of autophagy, which involves the AMPK-mTORC1 signaling pathway and the phosphorylation of Beclin 1/Bcl-2 interaction in ER (113).

Icariin

Icariin, a natural flavonoid glucoside, is of broad pharmacological properties (114). Studies proved that icariin had antioxidant, antidepressant, anti-inflammatory, neuroprotective, and male sexual function improvement effects *in vitro* (115–119). In congestive heart failure rats, icariin promoted left ventricular function and attenuated cardiac remodeling *via* down-regulating matrix metalloproteinase-2 and−9 activity and inhibited cardiomyocyte death (119). Previous experiments have proved that myocardial function was protected by icariin from myocardial I/R damage in rats. It reduced IS, decreased I/R injury, and inhibited its remodeling. These properties of icariin are associated with lower blood indicators CK, IMA, and LDH levels in the serum and upregulated PI3K/Akt/eNOS pathway in rats' ischemic tissue, making it a candidate drug for preventing and resisting I/R injury in the early stage (120).

Curcumin

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a natural compound isolated from the roots of *Curcuma longa L.*, exerts wide pharmacological activities, including antioxidant, anti-inflammatory, and anticarcinogenic abilities in several rodent models. Previous experiments have suggested that curcumin is protective against some cardiovascular pathological conditions leading to heart

failure (121, 122). Curcumin can improve heart function and ameliorate heart damage because it reduces oxidative stress and cell death, specifically by activating the phosphorylation of JAK2 and STAT3, increasing the myocardium Bcl-2/Bax expression and inhibiting caspase-3 (123).

Salvianolic Acid A

Salvianolic acid A is a water-soluble compound of *Salvia miltiorrhiza Bunge*, which is known to exert broad effects, including antioxidant, anticarcinogenic, anti-fibrotic, anti-inflammatory, and anti-platelet aggregation (124, 125). A previous study reported that, in diabetic rats, the JNK/PI3K/Akt signaling pathway correlated with myocardial I/R injury, and Sal A improved the recovery of heart function and prevented cell death following I/R damage in this model. This study provided critical evidence of the molecular mechanisms relating to Sal A's cardioprotective effects on I/R-injured diabetic rats (126).

Astilbin

Astilbin, a flavonoid compound extracted from the roots of *Smilax china L.*, which has been long used in the traditional Chinese medicine clinical practice, has been found to have anti-hepatic, anti-arthritic, and anti-renal injury effects (127–129). Researchers have reported that in the early stages of STZ-induced diabetes rats, Astilbin resulted in a better heart function recovery caused by myocardial I/R damage *via* constraining inflammation and reducing HMGB1, phosphorylating NF-κB in ischemic myocardial tissue (130).

Eupatilin

Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone), which comes from the species of Artemisia plants, is a flavonoid of bioactive properties. Increasing studies have demonstrated that eupatilin exerts anti-allergic, anti-oxidant, anti-tumor, and anti-inflammatory activities (131–133). Experiments proved that eupatilin alleviated myocardial I/R damage *via* decreasing ROS and cell death by activating the Akt/glycogen synthase kinase-3β(GSK-3β) signaling pathway. Eupatilin is of therapeutic usage in treating myocardial I/R injury (27).

Syringic Acid

Syringic acid (SA), a natural O-methylated trihydroxy benzoic acid isolated from *Dendrobium nobile Lindl.*, possesses broad biological activities, such as anti-oxidant, anti-tumor, and anti-inflammatory properties (134, 135). SA was found to prevent I/R injury. Tokmak et al. (136) reported that pretreating with SA in the spinal cord could reduce oxidative stress and neuronal degeneration induced by I/R. SA also ameliorated renal I/R injury (122). Liu et al. verified that SA exerted cardioprotective activities against myocardial I/R damage *via* activating the PI3K/Akt/GSK-3β signaling pathway and inhibiting the mitochondria-induced cell death (137).

Epigallocatechin-3-Gallate

Epigallocatechin-3-gallate (EGCG), the most widely studied catechin extracted from green tea leaves, was reported to reduce cardiovascular risk (138) through anti-inflammation and antioxidant activities, lowering serum cholesterol levels and reducing atherosclerosis (139, 140). Also, EGCG pretreatment limits IS caused by ischemia in the rat heart (141). Studies verified that giving rats EGCG together with reperfusion protected their hearts from regional myocardial I/R damage by activating pro-survival kinases, involving PI3K-Akt/GSK-3β and inhibiting cell death pathway p38 and JNK but not involving the ERK pathway (142).

Icariin

Icariin was found in *Epimedium brevicornu Maxim.*, and it is a major compound of the herb Yin Yang Huo in traditional Chinese medicine. Its effects include anti-inflammation, antidepression, and antineoplastic properties. It also improves male sexual function, enhances bone healing, and protects neurons (143). Icariin postconditioning could attenuate myocardial I/R injury in the experimental rat model by activating the PI3K/Akt pathway and reducing cell death (144).

Troxerutin

Troxerutin, also known as vitamin P4, a derived natural bioflavonoid, owns wide biological properties including anti-oxidation and anti-inflammation (145). Pretreatment with this flavonoid extracted from Sophora japonica and Dimorphandra gardneriana (146) could decrease the occurrence of arrhythmias induced by I/R in diabetic and healthy rat hearts. Studies proved that the possible mechanism of its cardioprotective abilities may be the downregulating of inflammatory cytokines and inflammatory reactions in the heart (147).

Tilianin

Tilianin proves effective in upregulating NO synthase expression and NO production. It also acts as an anti-inflammatory ingredient (148). A study demonstrated that Tilianin exerted a significant protective effect on myocardial I/R-injured rat hearts (149). Studies verified that Tilianin pretreatment ameliorated the myocardial infarction and I/R damage in rats *via* the preservation of mitochondrial functions. The underlying mechanisms of Tilianin's cardioprotective activities may be mitochondrial preservation and cell apoptosis inhibition (150).

Isoquercitrin

Isoquercitrin is a natural compound present in vegetables, herbs, and flowers (151). It has been discovered that isoquercitrin reduces inflammatory, allergic reactions, and oxidative stress. (152). Isoquercetin was reported to reserve mitochondrial function and inhibit Cyt release induced by I/R injury in H9C2 cells (153). These findings verified that isoquercetin can be a candidate drug with cardiovascular protective effects in the treatment of myocardial I/R injury.

Vitexin

Vitexin (apigenin-8-C-β-D-glucopyranoside) is a flavonoid derived from *Acer rubrum L.*, *Anthurium andraeanum,* and *Cucumis sativus L.* (154). Early studies have shown the hypotensive property and anti-inflammatory ability of vitexin. Recent pieces of research demonstrated vitexin's potential application for treating diseases like cancer (155). *In vivo* studies verified that vitexin was protective against myocardial

I/R damage in rat heart, of which the mechanism could be related to its antioxidation ability and lowering of the levels of inflammatory cytokines by inhibiting the expression of NF-κB and TNF-α, as well as the upregulating phospho-ERK and downregulating phospho-c-Jun expression (156).

Apigenin

As a member of the non-mutagenic flavone subclass, Apigenin, isolated from the leaves of *Apium graveolens L.*, exhibits low levels of toxicity. Previous studies have revealed Api's broad bioactive effects, including antiviral, antibacterial, anti-carcinogenic, antioxidant, and anti-inflammatory (157). Studies showed that Api could inhibit the p38 MAPK signaling pathway to protect the myocardium from I/R damage (158).

Bauhinia Championii Flavone

Bauhinia championii flavone (BCF) is extracted from the stem of *Phanera championii Benth.*, of which the extract promotes blood circulation, reduces inflammatory and oxidative stress, and prevents platelet aggregation (159). Jian et al. reported BCF's protective properties of myocardium suffering from I/R damage in rats. The underlying mechanisms may depend on its ability to inhibit lipid peroxidation and activate the anti-oxidative system, its anti-inflammatory property by downregulating inflammatory levels by inhibiting signaling pathways, such as TLR4/NF-κB. It could also inhibit Bax/Bcl-2 ratios and caspase-3 activation (160).

Gastrodin

The phenolic glycoside gastrodin (GAS) is a monomeric component derived from *Gastrodia elata Blume* and has a variety of properties. It has long been used in treating cerebrovascular and cardiovascular diseases (161). Previous studies have demonstrated that GAS could reduce oxidative stress, lower inflammatory levels, and elevate hypoxia tolerance (162). Neighboring mitochondria and cardiomyocytes could be protected by the pretreatment of GAS *via* promoting autophagic flux and eliminating dysfunctional mitochondria (163).

Pinocembrin

The flavonoid pinocembrin, mainly found in *Propolis*, possesses antibacterial, anti-oxidant, and anti-inflammatory properties (164, 165). Pretreating with pinocembrin reduced cardiac arrhythmia in I/R rats through the enhancement of Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase and the upregulation of Cx43 and Kir2.1 protein expression levels. *Via* upregulating gap junction- or ion channel-related gene or protein expression, cellular gap junction connexin function and IK1 current were restored, and cardiac arrhythmia was suppressed by correcting the P-R intermittent period, QRS duration, intracellular transmission velocity (166).

Silibinin

Silibinin, a polyphenolic flavonoid, is the main active component extracted from the seed of silybum marianum (milk thistle) or artichoke (cynara scolymus) (167). Previous studies have reported silibinin confers protective advantage in improving both liver and cerebral function after I/R, which raises concern about the role of silibinin against reperfusion injury in other tissues, especially in myocardium (168, 169). Chen et al. demonstrated silibinin reduces cardiomyocytes apoptosis, attenuates mitochondrial impairment and endoplasmic reticulum (ER) stress, alleviates ROS generation, neutrophil infiltration, and cytokines release (170). Furthermore, silibinin plus BAY 11-7082 (a selected NF-κB inhibitor) do not provide incremental benefits in improving myocytes apoptosis, oxidative stress, and inflammation in comparison with NF-κB signaling inhibition only. Thus, silibinin could prevent myocardial I/R injury by inhibiting cardiomyocytes apoptosis, reducing ER stress and oxidative stress, and modulating inflammatory response *via* deactivation of the NF-κB signaling pathway.

Saponins

Saponins are known as surface-active compounds that are widely distributed in the plant kingdom (171). They comprise a non-polar aglycone or non-saccharide moiety, coupled with polar mono or oligosaccharides. Saponins mainly include four-ring triterpene saponins and five-ring triterpene saponins. In recent years, many studies have shown that the saponins extracted from herbal are great protective of myocardial I/R injury *in vivo* and *in vitro*. The mechanisms are diverse and mainly involve regulating energy metabolism and calcium homeostasis, and inhibiting oxidative stress and inflammation (172).

Polyphyllin I

Polyphyllin I (PPI) is a steroidal saponin extracted from the roots of *Paris polyphylla*. PPI's anti-cancer effect *via* inhibiting the proliferation and growth of tumor cells has been proved in previous studies, making it an anti-cancer drug candidate (173–175). A recent study has shown that PPI could prevent myocardial I/R injury, decrease myocardial death, and reduce the inflammation response and oxidative stress after I/R. Also, PPI activates NF-κB p65. Therefore, it can be deduced that PPI is protective of myocardial I/R injury in rats (176).

Ginsenoside Rb1

Ginseng (*Panax ginseng C.A.Mey.*) can improve the immune system (177). In many cases, Ginsenoside Rb1 (GRb1) represents ginseng saponins of Panax ginseng C. A. Mey. Recent animal studies have shown that GRb1 is protective of many myocardial diseases, including myocardial I/R injury (178). *In vivo* and *ex vivo* studies have demonstrated GRb1 reduced myocardial I/R injury, and the underlying mechanism is by reducing both CK-MB and Trop l levels after I/R. GRb1 improves cardiac function as well as increases Bcl-2 expression by activating the phosphorylation of mTOR, inhibiting apoptosis-related proteins Bax and cleaved-caspase 3 (25).

Gypenoside

Chinese doctors have been using G. pentaphyllum [*Gynostemma pentaphyllum* (*Thunb.*) Makino] to treat diseases for hundreds of years (179). The main component of G. pentaphyllum is gypenoside (GP), which is known for its antitumor, anti-inflammatory, and anti-oxidative effects (180, 181). To our interest, GP also exhibits protective property against I/R injury. Qi et al. proved that GP protected I/R-injured cerebral neuronal

(182). Chang et al. reported the mechanism of this protective effect, which is by lowering the miR-143-3p level through the activation of the AMPK/Foxo1 pathway. The changes in signaling pathways eventually resulted in the improving condition of I/R-induced OGD/R in H9c2 cells in rat myocardial tissue. No studies before had unveiled gypenosides' effect on miR functions, which means this new finding could be used as a novel strategy for myocardial I/R injuries (183).

Astragaloside IV

As one of the main active components extracted from *Astragalus membranaceus Bunge.*, the lanolin alcohol-shaped Astragaloside IV (AS-IV) is a tetracyclic triterpenoid saponin with high polarity, which has antiapoptotic effects. It has been demonstrated that AS-IV possessed anti-ischemic properties against cerebral I/R injury, pulmonary and cardiovascular disease, diabetic nephropathy, and liver fibrosis (184, 185). The pharmacologic effects of AS-IV include regulating the calcium balance, antioxidative stress, anti-inflammatory, anti-apoptosis, antifibrotic, anti-diabetes, immunoregulation, and cardioprotective effect *via* different signaling pathways (184–186). A review of AS-IV on animal studies demonstrated that AS-IV's cardioprotective effect of antioxidant, anti-apoptosis, and anti-inflammatory in acute myocardial I/R injury depended largely on improving the circulation and upregulation of angiogenesis (187). A previous study reported that AsIV treatment attenuated myocardial I/R injury *via* inhibition of Toll-like receptor 4- and nuclear factor-κB-mediated inflammatory responses and subsequent myocardial apoptosis in a rat model (188). Further study demonstrated that AsIV treatment attenuated myocardial injury, reduced cardiomyocyte apoptosis, decreased $[Ca^{2+}]_i$, inhibited CaSR expression, and increased ERK1/2 phosphorylation levels. These findings not only provided the underlying mechanisms of the cardioprotective effect of AsIV but also further demonstrated the pivotal role of CaSR in myocardial I/R injury (189).

Ginsenoside Rg3

Ginsenoside Rg3 can improve cardiac functions by mitochondria dynamic remodeling and increasing the number of mitochondria (190). It can also attenuate myocardial I/R injury by regulating Akt/endothelial NO synthase (191). Ginsenoside Rg3 exhibits anti-apoptosis and anti-inflammation properties, which is the underlying mechanism of heart function impairment induced by I/R (192).

Ginsenoside Rb3

Ginsenoside Rb3, a component isolated from Panax ginseng (*Panax ginseng C. A. Meyer*), is drawing increasing attention in the treatment of cardiovascular diseases, including myocardial I/R injury. It has been found out that ginsenoside Rb3 exhibited a protective effect on neurons on the I/R injury model *in vitro* by inhibiting cell death and inflammatory cytokines (193). It was found by Ma et al. that ginsenoside Rb3's protective effect partly depended on inhibiting the NF-κB pathway, meaning that ginsenoside Rb3 can be a potential treatment for myocardial I/R injury (194).

Platycodin D

Platycodin D is among the main saponins of *Platycodon grandiflorus* (195). It possesses a variety of effects, including antiinflammation, anti-atherosclerotic, and anti-oxidant (196). Studies showed that Platycodin D could protect the heart from H/R-induced, Akt/Nrf2/HO-1 pathway-mediated oxidative stress, cell damage, as well as cell apoptosis (197).

Lignans

Lignans are a large class of natural compounds comprising two phenyl propane units. Lignans have been found rich in fruits, seeds, and vegetables, and received widespread interest due to their various biological activities, including antioxidant, antitumor, antibacterial, antiviral, insecticidal, fungistatic, estrogenic, and antiestrogenic activities (198).

Isovaleroylbinankadsurin A

Isovaleroylbinankadsurin A (ISBA) is a dibenzocyclooctadiene lignan extracted from S*chisandra Chinensis (Turcz.) Baill.* (199). ISBA possesses more than anti-inflammatory, anti-oxidant, and anti-tumor abilities (200, 201). It was reported that ISBA protected I/R-injured cardiomyocytes in models both *in vitro* and *in vivo*. Apoptosis induced by H/R injury was significantly inhibited *via* the mitochondrial-dependent pathway by ISBA. ISBA's protective effect on cardiomyocytes was mainly by activating the reperfusion injury salvage kinase (RISK) pathway. What is more, ISBA remarkably promoted the cellular anti-oxidative capacity by activating the RISK pathway, and, therefore, reduced oxidative damage induced by I/R injury by inhibiting the ROS generation, which proved ISBA's potential to be as a candidate drug for cardiovascular diseases (202).

Schisandrin B

Schisandrin B (Sch B) is also derived from the fruit of *Schisandra chinensis (Turcz.) Baill.*, a common herb of traditional Chinese medicine (203). Sch B exerts hepatoprotective, anticancer, antioxidant, and antiinflammatory abilities (204). Zhang et al. reported that the pretreatment of Sch B on cardiomyocytes could decrease the size of the infarct area, promote the antioxidant ability, inhibit the ERS-induced apoptosis, and protect the myocardium (205).

Sauchinone

Sauchinone is also extracted from *Schisandra Chinensis (Turcz.) Baill.* (206), which protects cardiomyocytes from I/R injury in rats. Previous studies have shown it can inhibit p38, JNK, and other cell apoptosis signaling pathways (207).

Terpenes

Terpenes represent one of the largest groups of plant secondary metabolites, with ∼55,000 different structures (208). Depending on the number of linked isoprene units, the resulting terpenes are classified into hemi-, mono-, sesqui-, di-, sester-, tri-, sesquar-, tetra-, and polyterpenes. For many decades, it has been suggested that terpenes and terpenoids are potential chemopreventive and therapeutic agents for various diseases (209).

Glaucocalyxin A

Glaucocalyxin A (GLA) is derived from *Isodon japonicus var. glaucocalyx* (*Maxim.*). *H.W.Li* exerts wide bioactive effects, including inhibiting platelet aggregation (210), suppressing the immune system, protecting DNA damage, and cytotoxic activity (211). Previous studies have demonstrated that GLA restored heart function, decreased infarction size, and inhibited apoptosis signaling pathways in mice hearts injured by myocardial I/R. These abilities of GLA may associate with its anti-platelet effect and the reduction of microvascular thrombosis. With decreased bleeding risk, GLA may be a potential therapy for alleviating myocardial RI during cardiac revascularization (212). Another study demonstrated that by activating the Akt/Nrf2/HO-1 pathway, GLA protected H9c2 cells against H/R-induced damage. Therefore, GLA might be a candidate drug for preventing and treating MI (213).

Artemisinin

Artemisinin, isolated mainly from *Artemisia annua L.*, is a sesquiterpene lactone compound with a peroxisome bridging group structure. Recent pieces of research have reported that it was not only anti-malaria but also an anti-tumor agent. Artemisinin can promote cell death, block cell cycle, prevent angiogenesis, and tumor metastasis (214). Its protective effect against I/R injury is mainly due to the activation of the NLRP3 inflammasome pathway, but preconditioning with artemisinin preconditioning could significantly suppress NLRP3 inflammasome activation. On the rat I/R injury model, artemisinin could reduce ROS and inflammation induced by I/R injury, therefore promoting myocardial recovery, including reducing the size of myocardial infarction and inhibiting cardiomyocyte apoptosis and autophagy (47).

Geniposide

Geniposide (C17H24O10, GP), one of the major components of the fruit of *Gardenia jasminoides J. Ellis* and is found in nearly 40 species of herbal plants, is also a well-studied iridoid glycoside (215). There have been a growing number of studies on the bioeffects of geniposide over the past few decades. It has been proved to be antidiabetic, antioxidant, antithrombotic, analgesic, hepatoprotective, neuroprotective, anti-inflammatory, antidepressant, cardioprotective, immune-regulatory, and antitumoral (216). It reduced the myocardial infarct area and apoptosis and promoted heart function. *In vitro* studies demonstrated, in H9c2 cells, GP enhanced the cell viability and prevented apoptosis during H/R. Both *in vivo* and *in vitro* experiments demonstrated that GP downregulated the expression of proteins related to autophagy and prevented autophagosome accumulation. Rapamycin administration could reverse these effects. In summary, GP protected cardiomyocytes from I/R damage and inhibited autophagy by activating AKT/mTOR signaling pathways (217).

Ginkgolide B

Isolated from the leaves of Ginkgo, Ginkgolide B (GB) is a diterpene lactone compound and has a strong effect on inhibiting platelet aggregation (218). Its anti-inflammatory, antioxidant, and anti-apoptotic properties have made it protective of stroke, both ischemic and hemorrhagic (219–221). In hydrogen peroxide-treated H9c2 cells, they pretreated with GB-activated PI3K/Akt/mTOR signaling pathway and upregulated the phosphorylation levels of Akt and mTOR and, therefore, inhibited cell apoptosis (222). *Via* activating the A20-dependent NF-κB signal pathway, GB could also ameliorate I/R-induced inflammatory damage both *in vivo* and *in vitro* (223). Thus, GB protected against myocardial I/R injury by inhibiting ER stress-induced apoptosis *via* the PI3K/AKT/mTOR signaling pathway. This finding suggests GB may be a promising therapy in treating I/R injury (224).

Araloside C

Araloside C is among the major triterpenoid compounds derived from *A. elata* and was found to significantly promote heart function (225). Araloside C was proved to protect the myocardium from I/R damage by inhibiting ROS generation as well as Ca2+ overload. It is demonstrated that such cardioprotective effect is due to its ability to combine with the Hsp90 protein and interact with the ATP/ADP-binding domain of Hsp90 (226).

Triptolide

Isolated from *Tripterygium wilfordii Hook.f.*, triptolide possesses neuroprotective, anti-tumor, and anti-inflammatory abilities (227). Triptolide can alleviate cerebral and hepatic I/R injuries in experiments (228, 229), and it could promote heart condition and reduce inflammation and oxidative stress induced by I/R in rats. Such protective effects of the heart may relate to triptolide's influences on the Nrf2/HO-1 defense pathway (230).

Alkaloids

Alkaloids, a class of nitrogen-containing basic organic compounds found in nature, are varied and complex. Alkaloids are mainly plants, but some are also found in animals. Alkaloids have an extensive pharmacological function. These significant biological activities often play a therapeutic role in Chinese herbal medicine management.

Berberine

Derived from several medicinal plants, such as *Coptis Chinensis Franch.* and *Berberis vulgaris L.*, berberine (BBR) is an alkaloid with broad bioactive effects and has been used for treating many diseases (231). It showed antiapoptotic and antiinflammatory abilities both in cell and animal experiments (232). Huang et al. reported it enhanced H/R-induced cell viability and reduced I/R-induced IS and autophagy in cardiomyocytes (233). Also, BBR decreased CK-MB, LDH, and cTnI serum levels by decreasing myocardial cell death and promoting mitochondrial functions (234). Besides, BBR protects neurons by modulating cell death (235). It protects the myocardium from myocardial I/R injury *via* promoting proliferation, attenuating apoptosis *via* the mitophagy-mediated HIF-1α/BNIP3 pathway (236).

Galanthamine

Galanthamine protects neurons *via* activating the cholinergic pathway in the heart to prevent ischemic injury (237) and is

important in promoting heart fitness and limiting IS (238). A previous study reported that it reduced cardiac dysfunction and alleviated endoplasmic reticulum stress (ERS)-related cell death induced by I/R *via* downregulating the expression of CHOP, Cleaved caspase 12, and caspase 3, as well as upregulating the expression of CADD34 and BiP in rats. It could also mitigate I/R-induced myocardial fibrosis in rats by inhibiting the expression of α-SMA and Collagen I. It was demonstrated that the mechanism of its cardioprotective and apoptosis-inhibiting effects were suppressing AMPK/Nrf2 pathways (239).

Matrine

The quinolizidine alkaloid compound Matrine, extracted from *Sophora flavescens Aiton*, which has been used as a herb in China, possesses antivirus, antitumor, antiallergic, anti-inflammatory, and antifibrotic effects (240–243). It activates the JAK2/STAT3 pathway and the upregulation of HSP70 expression. Guo et al. reported that the compound remarkably increased cell viability suppressed by H/R by decreasing lactate dehydrogenase and inhibiting creatine kinase activity *in vitro*. Also, it was proven to reduce CK-MB and TnI levels in the blood and decrease the size of the infarcted area in the heart as well as the I/R-induced apoptotic index of cardiomyocytes *in vivo*. It alleviated myocardial I/R damage by increasing HSP70 expression *via* activating the JAK2/STAT3 signaling pathway (244).

Palmatine

Palmatine, a natural quaternary protoberberine in the class of isoquinoline alkaloids, possesses pharmacological effects, such as anti-inflammatory and antioxidant (245–247). A previous study demonstrated that it could protect cardiomyocytes from I/R damage in rats. Its possible mechanism is relieving oxidative stress and regulating inflammatory mediators (248).

Capsaicin

Capsaicin is extracted from capsicum plants, such as *Capsicum annuum L.* and is widely used in food, medicine, and pharmacy (249). Pretreating with this compound protects H9c2 cells from H/R injury by upregulating 14-3-3η expression, modulating Bcl-2 and Bax expression and activity, reducing ROS generation, limiting mPTP opening, inhibiting caspase-3 activity, and, ultimately, suppressing cardiomyocytes apoptosis (250).

Quinones

Quinones are mainly divided into four types: benzoquinone, naphthoquinone, phenanthraquinone, and anthraquinone. They naturally occur in bacteria, fungi, animals, and plants and have a variety of pharmacological effects. They are produced in organisms and are utilized as electron-transfer agents, pigments, and in defense mechanisms (251).

Sodium Tanshinone IIA Sulfonate

Among the derivatives of tanshinone, IIA is sodium tanshinone IIA sulfonate (STS), a major lipophilic constitute of *Salvia miltiorrhiza Bge* (252). Studies have shown that it was cardioprotective against several cardiovascular diseases and neuroprotective against neural dysfunction (253–255). Previous studies demonstrated that it also showed pharmacological actions including anti-oxidative stress and anti-inflammation (256, 257). The study provides some evidence that this compound was significantly protective in treating myocardial I/R injury in rats. Its antioxidant ability partly improves heart condition (258).

Shikonin

Shikonin is isolated from *Lithospermum erythrorhizon Siebold and Zucc.*, which has been used for treating several inflammatories and infectious conditions (259). It has been reported that it exerted anti-inflammatory, antibacterial, antiviral, and antioxidant activities (260). The potential merits of pretreating it in H/R-induced cardiomyocyte apoptosis are partly regulated through activating the PI3K/Akt signaling pathway (261).

Polysaccharides

Polysaccharides are widely distributed as natural ingredients in vegetables and fruits. Several studies have shown that the polysaccharides improve cardiovascular diseases through various mechanisms, such as anti-oxidative stress, regulating metabolism, anti-inflammatory, anti-cancer, and immunity-booster properties (262).

Fucoidan

Fucoidan is a sulfated polysaccharide molecule that has been known for its anticancer abilities. It is isolated mainly from the cell wall of different species of brown algae (*Phaeophyta*), a varied group of organisms (263). Omata et al. reported that, in the rat myocardial I/R injury model, there is a limited myocardial-IS and inhibited neutrophil accumulation, and one of the possible mechanisms could be the blockade of P-selectin-mediated neutrophil rolling on the vessel wall (264). Li et al. reported fucoidan improved left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and the contractility index in the rat myocardial I/R injury model, and could regulate the inflammation response *via* HMGB1 and NF-κB inactivation in I/R-induced myocardial damage (265).

Carotenoids

Carotenoids are naturally found in the natural ingredients, particularly in fruits, vegetables, and algae. At present, more than 750 kinds of carotenoids have been identified, of which there are about 100 in edible foods. Carotenoids exhibit several biological and pharmaceutical benefits, such as anti-inflammatory, anti-cancer, and immunity-booster properties (266).

Lycopene

Lycopene is a natural compound whose antioxidant effects have been widely studied (267). Previous studies demonstrated that low levels of circulating lycopene are related to a higher risk of cardiovascular diseases (268, 269). It was proved that pretreatment with 1-μM lycopene before reoxygenation remarkably decreased cardiomyocytes apoptosis induced by H/R. Moreover, IV injection of 1-μM circulating lycopene significantly decreased the risk of MI during *in vivo* I/R in mice and effectively inhibited the oxidation of fatty acid and the activation of JNK signaling during reperfusion (270).

Retinol Palmitate

Retinol palmitate, an analog of vitamin A, exhibits effective peroxyl radical scavengers *via* suppressing peroxidation (271). It has been proved to exert the ability to promote neuronal differentiation, neural patterning, and axonal growth, making it potentially neuroprotective against cerebral I/R damage (272). It could also limit myocardial IS and inhibit cellular apoptosis *via* the downregulation of proapoptotic-related proteins expression and the upregulation of SOD-related proteins expression. Tao et al. suggested that pretreating with retinol palmitate effectively protected the heart from myocardial I/R injury through balancing intracellular oxidants and antioxidants (273).

Coumarin

Coumarin compounds represent an important type of naturally occurring and synthetic oxygen-containing heterocycles with a typical benzopyrone framework. This type of special benzopyrone structure enables its derivatives to readily interact with a diversity of enzymes and receptors in organisms through weak bond interactions, thereby exhibiting wide potentiality as medicinal drugs compounds, inclusive of analgesic, anticoagulant anti-inflammatory, antimicrobial, antineoplastic, antioxidant, and immunomodulatory effects (274).

Osthole

Osthole is a compound mainly derived from *Cnidium monnieri (L.) Cusson* and *Angelica pubescens Maxim.*, and has been used as tonics and aphrodisiacs in clinical practice of traditional Chinese medicine for many years (275). Modern pharmacological studies demonstrated that it possessed antitumor, anti-hepatic, anti-allergic, anti-inflammatory, anti-apoptotic, and estrogen-like effects (276–278). Wang et al. reported that this compound was beneficial to functional recovery after myocardial I/R injury by increasing SOD, GPx, and CAT activities, and decreasing lipid peroxidation products, MDA, and 4-HNE in the damaged heart tissues. The mechanism behind these effects was related to decreasing the expression of the pro-inflammatory factors, increasing anti-inflammatory cytokines, as well as lowering HMGB1, phosphorylated IκB-α, and NF-κB proteins (279).

Esculetin

The natural coumarin compound Esculetin (6,7-dihydroxy coumarin) possesses antioxidant, anti-inflammatory, anti-nociceptive, and anti-tumor activities (280, 281). It also protects against I/R injury. In H/R-stimulated H9c2 cells, esculetin promotes cell viability and reduces lactate dehydrogenase (LDH) release. It also reduces ROS and cell death, following H/R injury through the JAK2/STAT3 pathway (282).

Others

Plantamajoside

Plantamajoside (PMS) is a phenylpropanoid glycoside extracted from *Plantago Asiatica L.* with a long history in food and medical application (283). PMS exhibits anti-inflammatory and antioxidant properties (284, 285). Because of its protective effects against cadmium-induced renal injury and its anti-inflammatory and antifibrotic effects, it has been used to treat many diseases (286, 287). In the *in vitro* I/R model, a previous study investigated the protective effects of PMS on H/R-stimulated oxidative stress, inflammation, and apoptosis in H9c2 cells. PMS attenuated myocardial I/R damage by reducing the inflammatory response, oxidative stress, and apoptosis through Akt/Nrf2/HO-1 and NF-κB signaling pathways (288).

Diallyl Trisulfide

Garlic (*Allium sativum* L.) has long before been recognized as beneficial for several diseases. One of its main bioactive compounds is diallyl trisulfide (DATS), also known as allitridin or 4,5,6-trithia-1,8-nonadiene. DATS is a natural, stable, and safe component that attracts H2S donors for *in vivo* studies with an eye to clinical relevance (289). Jeremic et al. reported that DATS consumption could improve heart functions and prevent the oxidative and histoarchitectural variation in the heart suffering from *ex vivo* induced I/R heart injury (290).

Eleutheroside E

Eleutheroside E (EE) is a bioactive component of *Eleutherococcus senticosus* (*Rupr. and Maxim.*) *Maxim*. It significantly alleviates physical fatigue and promotes endurance performance, protects against neuritic atrophy and neuron apoptosis, and inhibits inflammatory gene expression (291). Previous studies have shown that treating with EE remarkably limited H/R-induced damage in heart tissue by reducing oxidative stress, inactivating NF-κB, and modulating metabolic responses. Moreover, EE reprograms metabolic action. This evidence proved EE to be potentially valuable in treating H/R-injured heart tissue and emphasized the relationship between EE's protection and metabolic reprogramming (292).

Salidroside

Salidroside is a bioactive compound with anti-inflammatory, anti-cancer, anti-oxidant, and anti-fatigue effects (293, 294). A previous study demonstrated that the mechanism of Sal's protective effects against myocardial I/R damage was related to the inhibition of the TLR4/NF-κB signaling pathway, inflammatory response, and cardiomyocyte apoptosis (295).

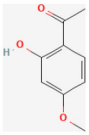
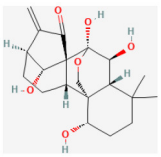
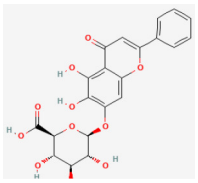
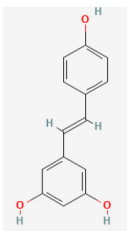
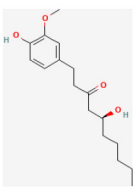
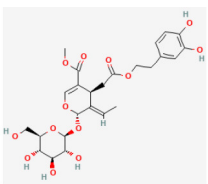
Glycyrrhizin

Glycyrrhizin, one of the most effective ingredients of the root extraction of *Glycyrrhiza glabra L.*, is consisted of glucuronic acid and glycyrrhetinic acid and possesses anti-allergic, anti-oxidant, anti-ulcer, anti-viral, anti-cancer, and immunomodulatory effects (296, 297). It also protects the liver and stabilizes the cell membrane. This compound has been broadly used in Europe and the Middle East (298). It was reported that, in rats, glycyrrhizin triggered HMGB1 and the blocked p38 and JNK pathways, ultimately reducing myocardial I/R damage by attenuating oxidative stress, iNOS, and inflammatory reactions *in vivo* (299).

Cornuside

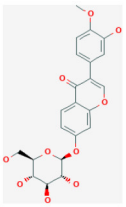
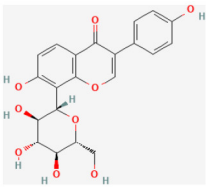
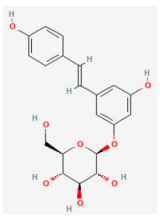
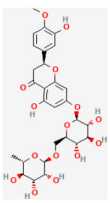
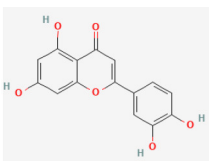
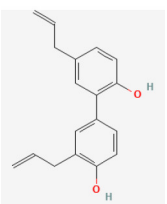
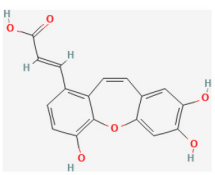
Cornuside is a secoiridoid glucoside derived from the fruit of *Cornus officinalis Siebold and Zucc.*, which has long been used for attenuating inflammation and promoting blood circulation. It has been found that the crude extract of this fruit had pharmacological effects including, anti-neoplasm,

TABLE 1 | The mechanisms of phytochemicals against myocardial I/R injury from experimental studies.

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Phenols					
Paeonol		Hypoxia for 2 h/reoxygenation for 2 h in H9c2 cells	10 μmol/L for 18 h beforeH/R	Suppressed oxidative stress, down-regulated inflammatory responses (BRCA1/ROS-regulated NLRP3 inflammasome/IL-1β and NF-κB/TNF-α/IL-6 pathways)	(44)
Oridonin		LAD ligation ischemia for 0.5 h/reperfusion for 24 h in C57BL/6 mice	10 mg/kg oral for 7 days before I/R	Suppressed oxidative stress, down-regulated inflammatory responses (NLRP3 inflammasome pathways)	(55)
Baicalin		LAD ligation ischemia for 45 min/reperfusion for 180 min in SD rat	20, 60, 120 mg/kg oral for 14 days before I/R	Protected against inflammation through reducing the phosphorylation of JAK2/STAT3 and decreasing the levels of iNOS and IL-1β	(64)
		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat / hypoxia for 6 h/reoxygenation for 4 h in Primary rats' cardiomyocytes	100 mg/kg oral for 14 days befor I/R/10 μmol/L for 30 min before H/R	Inhibited apoptosis (CaSR/ERK1/2 signaling pathway)	(63)
		LAD ligation Ischemia for 30 min/reperfusion for 120 min in SD rat	50, 100, 200 mg/kg oral before I/R	Inhibited apoptosis, and inflammation (activated PI3K/Akt but suppressed NF-κB signaling pathways)	(62)
Resveratrol		LAD ligation ischemia for 30 min/reperfusion for 120 min in C57BL/6 mice/hypoxia for 3 h/reoxygenation for 3 h in Neonatal rat ventricular cardiomyocytes	50 mg/kg oral for 14 days before I/R/10, 30, 50 μmol/L for 24 h before H/R	Exerted anti-apoptosis and inhibited Ca2+ accumulation (STIM1 pathway)	(71)
		LAD ligation ischemia for 30 min/reperfusion for 120 min in diabetic SD rat	20 mg/kg injection for 7 days before I/R	Inhibited oxidative stress (upregulated SIRT1 and downregulated GSK3β, contributing to improving the expression of Nrf2)	(303)
		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	10 mg/kg oral for 287 days before I/R	Activated autophagy (upregulated Beclin 1/LC3-II), and inhibited inflammatory responses (TNF-α, IL-6)	(34)
		Hypoxia for 24 h/reoxygenation for 24 h in cardiomyocytes	40 μmol/L for 24 h before H/R	Suppressed myocardial apoptosis (inhibited PI3K/AKT/e-NOS pathway)	(72)
6-Gingerol		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	6 mg/kg injection for 30 min before I/R	Inhibited cardiomyocyte apoptosis (upregulated the expression of PI3K/Akt signaling pathway)	(85)
Oleuropein		LAD ligation ischemia for 30 min/reperfusion for 180 min in SD rat	20 mg/kg oral for 2 days before I/R	Inhibited apoptosis and inflammation *via* decreasing NF-κB expression and IKB-α degradation pathway	(86)

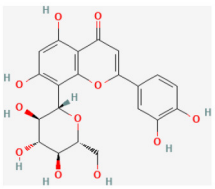
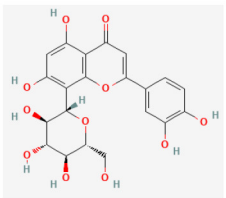
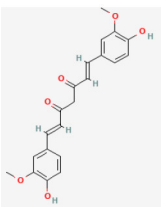
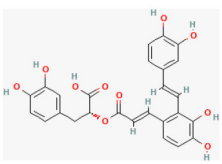
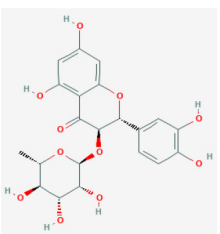
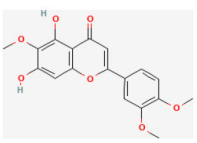
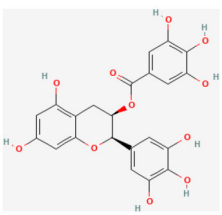
(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Calycosin-7-O-β-D-glucoside		LAD ligation ischemia for 45 min/reperfusion for 180 min in C57BL/6 mice	30 mg/kg oral for 30 min before I/R	Protected against cell apoptosis by activating the JAK2/STAT3 signaling pathway *via* up-regulation of IL-10	(90)
Puerarin		LAD ligation ischemia for 30 min/reperfusion for 180 min in diabetic SD rat	25, 50, 100 mg/kg oral for 28 days before I/R	Suppressed apoptosis, oxidative stress and inflammation (up-regulation of VEGFA/Ang-1 and down-regulation of NF-κB pathways)	(94)
Polydatin		LAC ligation ischemia for 30 min/reperfusion for 120 min in C57BL/6 mice/hypoxia for 3 h/reoxygenation for 3 h in Neonatal rat cardiomyocytes	7.5 mg/kg injection before I/R/1, 10, 100 μmol/L for 1 h before H/R	Reduced ROS and cell death by promoting autophagic flux to clear damaged mitochondria	(76)
Hesperidin		LAD ligation ischemia for 30 min/reperfusion for 240 min in SD rat	200 mg/kg oral for 3 days before I/R	Inhibited excessive autophagy *via* activating the PI3K/Akt/mTOR pathway	(31)
Luteolin		LAD ligation ischemia for 30 min/reperfusion for 24 min in C57BL/6 mice/hypoxia for 2 h/reoxygenation for 2 h in HL-1 cells	25 μg/kg injection for 3 days before I/R/8 μmol/L for 24 h before H/R	Enhanced SERCA2a through SUMOylation at lysine 585 to protect cardiomyocytes	(104)
Honokiol		LAD ligation ischemia for 45 min/reperfusion for 3 h in C57BL/6 mice/hypoxia for 3 h/reoxygenation for 3 h in cardiomyocytes	10 μmol/L injection for 15 min before I/R/5, 10, 20, 40, 80 μmol/L for 3 h before H/R	Promoted autophagic flux (Akt signaling pathway)	(109)
		LAD ligation ischemia for 30 min/reperfusion for 240 min in diabetic SD rat/hypoxia for 1 h/reoxygenation for 4 h in H9C2 cell	5 mg/kg oral for 7 days before I/R/1,2,5 μmol/L for 2 h before H/R	Ameliorated oxidative damage and apoptosis (SIRT1-Nrf2 signaling pathway)	(108)
Tournefolic acid B		Ischemia for 45 min/reperfusion for 60 min in isolating heart	0.5, 1.2 μg/ml perfusate for 20 min before I/R	Suppressed ER stress, oxidative stress, and apoptosis (enhanced the phosphorylation of PI3K and AKT, inhibited the expression of CHOP and Caspase-12, reduced the phosphorylation of JNK, and increased Bcl-2/Bax ratio)	(110)

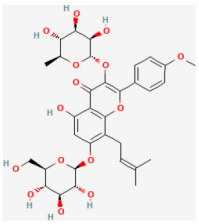
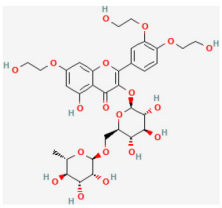
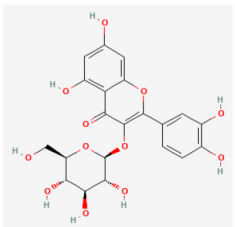
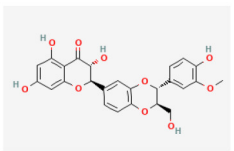
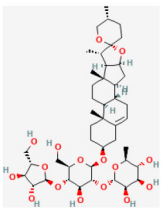
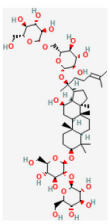
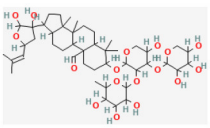
(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Orientin		Hypoxia for 12 h/reoxygenation for 12 h in cardiomyocytes	3, 10, 30 μmol/L for 12 h beforeH/R	Promoted autophagy and enhances cell survival (increasing AMPK-mTORC1 signaling pathway and enhancing the interaction of Beclin 1/Bcl-2)	(113)
Icariin		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	10 mg/kg injection after ischemia	Reduced the apoptosis (PI3K/Akt/eNOS pathway)	(120)
Curcumin		LAD ligation ischemia for 30 min/reperfusion for 180 min in SD rat	10, 20, 30 mg/kg oral for 20 days before I/R	Decreasing oxidative damage and inhibiting myocardium apoptosis (JAK2/STAT3 signal pathway)	(123)
Salvianolic acid A		Ischemia for 30 min/reperfusion for 120 min in isolating heart	20 μmol/L before I/R	Exerted an anti-apoptotic effect and improves cardiac function (JNK/PI3K/Akt signaling pathway)	(126)
Astilbin		LAD ligation ischemia for 30 min/reperfusion for 24 h in diabetic rat/hypoxia for 6 h/reoxygenation in H9C2 cell	12.5, 25, 50, 100 mg/kg injection for 4 h befor I/R/1.5, 5, 15, 50 μmol/L for 24 h before H/R	Blocked inflammatory cascade (HMGB1-dependent NF-κB signaling pathway)	(130)
Eupatilin		Hypoxia for 3 h/reoxygenation 2 h in H9C2 cell	0.1, 1, 10 μmol/L for 24 h before H/R	Suppressed oxidative stress and apoptosis (Akt/GSK-3β signaling pathway)	(27)
Epigallocatechin-3-gallate		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	10 mg/kg injection after ischemia	Mitigated cell death (activating the RISK pathway and attenuating p38 and JNK)	(142)

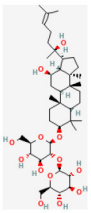
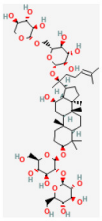
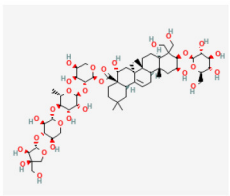
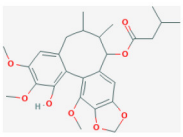
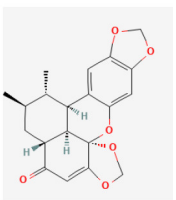
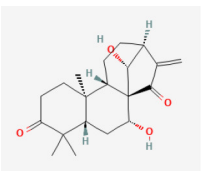
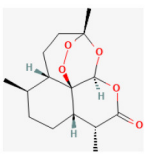
(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Icariin		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	10 mg/kg injection for 5 min before I/R	Decreased inflammatory cytokine TNF-α and IL-10, and inhibited apoptosis (PI3K/Akt signaling pathway)	(144)
Troxerutin		LAD ligation ischemia for 30 min/reperfusion for 60 min in isolating heart	150 mg/kg oral for 1 month before I/R	Exerted significant anti-arrhythmic and anti-inflammatory effects because of the inhibition of inflammatory cytokines activity and reduction of inflammatory reactions	(147)
Isoquercitrin		Hypoxia for 6 h/reoxygenation for 12 h in H9C2 cell	20, 40, 80 mg/ml for 24 h before H/R	Inhibited apoptosis and ROS generation by protecting mitochondrial function and preventing cytochrome c release	(153)
Silibinin		LAD ligation ischemia for 30 min/reperfusion for 24 h in C57BL/6 mice/hypoxia for 6 h/reoxygenation in H9C2 cell	100 mg/kg injection for 7 days before I/R	Inhibited cardiomyocytes apoptosis, reduced ER stress and oxidative stress, and modulating inflammatory response *via* deactivation of NF-κB signaling pathway.	(170)
Saponins					
Polyphyllin I		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	150 mg/kg injection for 2 weeks before I/R	Inhibiting inflammatory response and oxidative stress (NF-κBp65 signaling pathway)	(176)
Ginsenoside Rb1		LAD ligation ischemia for 45 min/reperfusion for 120 min in SD rat	20, 40, 80 mg/kg injection for 3 days before I/R	Decreased the expression of apoptotic related proteins e.g., cleaved-caspase 3 (mTOR signaling pathway)	(25)
Gypenoside A		Hypoxia for 2 h/reoxygenation for 24 h in H9C2 cell	20 μmol/L for 24 h before H/R	Suppressed miR-143-3p *via* the activation of AMPK signaling	(183)

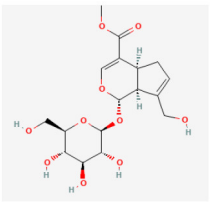
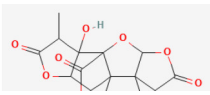
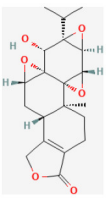
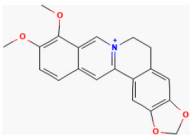
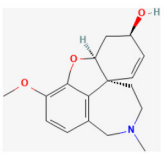
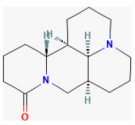
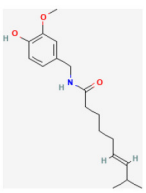
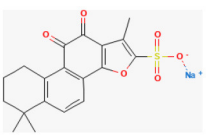
(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Ginsenoside Rg3		LAD ligation ischemia for 30 min/reperfusion for 24 h in SD rat	5, 20 mg/kg oral for 7 days before I/R	Attenuated apoptosis and inflammation	(192)
Ginsenoside Rb3		Hypoxia for 4 h/reoxygenation for 24 h in H9C2 cell	2, 5 μmol/L for 24 h before H/R	Inhibited apoptosis (JNK/NF-κB activation signaling pathway)	(194)
Platycodin D		Hypoxia for 4 h/reoxygenation for 24 h in H9C2 cell	5, 10, 20, 40 μmol/L for 24 h before H/R	Inhibited oxidative stress and apoptosis (Inducing the activation of Akt/Nrf2/HO-1 pathway)	(197)
Lignans					
Isovaleroylbinankac A		LAD ligation ischemia for 45 min/reperfusion for 120 min in C57BL/6 mice/hypoxia for 150 min/reoxygenation for 60 min in neonatal rat ventricle myocytes and H9C2 cell	10, 20, 40 mg/kg injection for 1 h before I/R/0.3, 1, 3 μmol/L for 1 h before H/R	Blocked the apoptosis and inhibiting the ROS generation (activating GR dependent RISK pathway)	(202)
Sauchinone		LAD ligation ischemia for 30 min/reperfusion for 2 h in isolating heart	10 mg/kg injection for 30 min before I/R	Exerted anti-inflammatory and antioxidant effects through inhibition of phosphorylation of p38 and JNK death signaling pathways	(207)
Terpenes					
Glaucocalyxin A		Ischemia for 1 h/reperfusion for 24 h in C57BL/6J mice	10 mg/kg injection after ischemia	Reducted microvascular thrombosis	(212)
		Hypoxia for 24 h/reoxygenation for 2 h in H9c2 cells	5, 10, 20, and 40 μmol/L for 2 h before H/R	Suppressed apoptosis and oxidative stress (Akt/Nrf2/HO-1 signaling pathway)	(213)
Artemisinin		LAD ligation ischemia for 0.5 h/reperfusion for 2 h in SD rat	14 mg/kg oral for 2 weeks before I/R	Suppressed NLRP3 inflammasome activation (decreasing NLRP3, ASC, cleaved caspase-1, IL-1β)	(47)

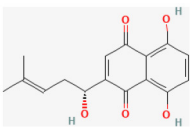
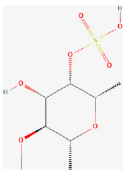
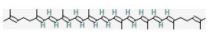
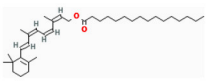
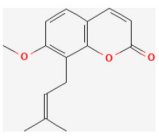
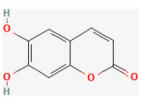
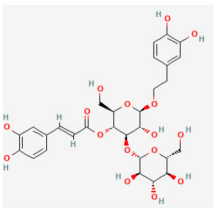
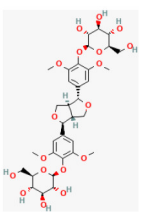
(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Geniposide		LAD ligation ischemia for 0.5 h/reperfusion for 2 h in SD rat/hypoxia for 12 h/reoxygenation for 4 h in H9c2 cells	100 mg/kg oral 30 min before I/R/40 μmol/L for 30 min before H/R	Inhibited the expression of autophagy-related proteins and autophagosome accumulation (activating AKT/mTOR signaling pathways)	(217)
Ginkgolide B		Ischemia for 1 h/reperfusion for 1 h in SD rat	15 mg/kg injection for 10 min before ischemia	Inhibited ER stress-induced apoptosis *via* PI3K/AKT/mTOR signaling pathway	(224)
		Ischemia for 40 min/reperfusion for 120 min in SD rat	8, 16, 32 mg/kg injection for 7 days before ischemia	Alleviated inflammatory response (inhibiting NF-κB p65 subunit translocation, IκB-α phosphorylation, IKK-β activity, as well as the downstream inflammatory cytokines and proteins expressions *via* zinc finger protein *A20*)	(223)
Triptolide		LAD ligation ischemia for 45 min/reperfusion for 3 h in Wistar rat	25, 50, 100 μg/kg injection for 12 h before I/R	Reduced inflammation and oxidative stress (Nrf2/HO-1 defense pathway)	(230)
Alkaloids					
Berberine		LAD ligation ischemia for 30 min/reperfusion for 120 min in Wistar rat/hypoxia for 4 h/reoxygenation for 3 h in H9C2 cell	300 mg/kg oral for 3 days before I/R/50 μmol/L for 3 h before H/R	Promoted mitochondrial autophagy, reduced myocardial enzyme activity, induced cardiomyocytes proliferation, inhibited cardiomyocytes apoptosis (HIF-1α/BNIP3 pathway)	(236)
Galanthamine		LAD ligation ischemia for 30 min/reperfusion for 120 min in SD rat	1, 3 mg/kg injection for 30 min before I/R	Prevented endoplasmic reticulum stress-related apoptosis, and myocardial fibrosis *via* promoting AMPK and Nrf2-related proteins (AMPKα1, Nrf2 and HO-1)	(239)
Matrine		LAD ligation ischemia for 30 min/reperfusion for 24 h in SD rat/hypoxia for 4 h/reoxygenation for 6 h in cardiomyocytes	50, 100 mg/kg injection before I/R/200, 400 μmol/L after hypoxia	Decreased lactate dehydrogenase release, creatine kinase activity, and cardiomyocytes apoptosis (JAK2/STAT3 signaling pathway)	(244)
Capsaicin		Hypoxia for 3 h/reoxygenation for 3 h in H9C2 cell	5, 10, 20, 40, 80 μmol/L for 36 h before H/R	Attenuated generation of ROS, inhibited mPTP opening and caspase-3 activation, downregulated Bax, upregulated 14-3-3η and Bcl-2, and ultimately reduced apoptosis	(250)
Quinones					
Sodium tanshinone IIA sulfonate		LAD ligation ischemia for 30 min/reperfusion for 24 h in SD rat	8 mg/kg injection for 15 min before ischemia and for 0.5, 1, 2, 4, 6 h after ischemia	Protected against oxidative stress and inflammatory responses (NF-κB/HO-1 signaling pathway)	(258)

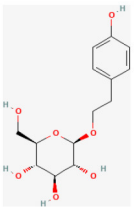
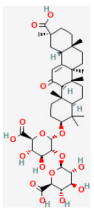
(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Shikonin		Hypoxia for 12 h/reoxygenation for 24 h in H9c2 cells	10, 20, 40 μmol/L for 48 h before H/R	Suppressed apoptosis and increased cell viability, attenuated LDH release (PI3K/Akt signaling pathway)	(261)
Polysaccharides					
Fucoidan		LAD ligation ischemia for 30 min/reperfusion for 0.5–6 h in Wistar rat	27 μg/kg/min injection from 10 min before to 6 h after reperfusion	Blockaded of P-selectin-mediated neutrophil rolling on the vessel wall	(264)
		LAD ligation ischemia for 30 min/reperfusion for 2 h in SD rat	50, 100, 200 mg/kg oral for 7 days before I/R	Regulated the inflammation response *via* HMGB1 and NF-κB inactivation in I/R-induced myocardial damage	(265)
Carotenoids					
Lycopene		LAD ligation ischemia for 20 min/reperfusion for 40 min in C57BL/6 mice/hypoxia for 2 h/reoxygenation for 2 h in HL-1 cells	1 μmol/L injection after ischemia/1, 2, 4 μmol/L for 2 hafter H/R	Inhibited ROS accumulation and inflammation (JNK signaling pathway)	(270)
Retinol palmitate		LAD ligation ischemia for 40 min/reperfusion for 4 h in C57BL/6 mice/hypoxia for 2 h/reoxygenation for 4 h in H9C2 cells	12, 36 mg/kg injection for 3 days before I/R/0.1, 1 μmol/L for 4 h before H/R	Inhibited oxidative stress and apoptosis	(273)
Coumarin					
osthole		LAD ligation ischemia for 30 min/reperfusion for 24 h in SD rat	1, 10, 50 mg/kg injection before I/R	Exerted antioxidant and anti-inflammatory effect (inhibiting the expression of HMGB1 and IκB-α/NF-κB signaling pathway)	(279)
Esculetin		Hypoxia for 3 h/reoxygenation for 6 h in H9c2 cells	5, 10, 20, 40 μmol/L for 24 h before H/R	Suppressed oxidative stress and apoptosis (JAK2/STAT3 signaling pathway)	(282)
Others					
Plantamajoside		Hypoxia for 6 h/reoxygenation for 12 h in H9c2 cells	10, 20, 40, and 80 μmol/L for 24 h before H/R	Suppressed inflammation and oxidative stress (Akt/Nrf2/HO-1 and NF-κB signaling pathways)	(288)
Diallyl trisulfide		Ischemia for 30 min/reperfusion for 1 h in isolating heart	40 mg/kg oral for 3 weeks before I/R	Suppressed oxidative stress and apoptosis with increasing relative gene expression of eNOS, SOD-1 and−2, Bcl-2 and decreasing relative gene expression of NF-κB, IL-17A, Bax, and caspases-3 and−9	(290)
Eleutheroside E		Hypoxia for 4 h/reoxygenation for 24 h in H9c2 cells	30, 60, and 100 μmol/L for 3 h before H/R	Reduced oxidative stress (NF-κB signaling pathway)	(292)

(Continued)

TABLE 1 | Continued

Phytochemical name	Chemical structure depiction	Study model	Dose, route, and duration of administration	Mechanism	References
Salidroside		Ischemia for 30 min/reperfusion for 24 h in SD rat	20, 40 mg/kg oral for 7 days before I/R	Suppressed inflammation and apoptosis (TLR4/NF-κB signaling pathway)	(295)
Glycyrrhizin		Ischemia for 30 min/reperfusion for 24 h in SD rat	0, 2, 4, 10 mg/kg injection for 30 min before I/R	Reduced oxidative stress, iNOS and inflammatory reactions (blocked p38 and JNK signaling pathway)	(299)

anti-sepsis, antiinflammatory, anti-diabetic nephropathy, and hepatoprotection effects (300, 301). It was reported that in rats suffering from myocardial I/R injury, cornuside decreased infarct volume, improved hemodynamics parameters, and alleviated myocardial injury *via* inhibiting PMN infiltration and MPO activity, decreased pro-inflammatory factors, and reduced phosphorylated IB- and NF-B proteins (302).

Phytochemicals and Signal Transduction Pathways

We systematically summarized the phytochemicals' characteristics for their cardioprotective mechanisms in preventing myocardial I/R injury from experimental studies (**Table 1**). Various internal mechanisms related to myocardial I/R injury that control the fate of cardiomyocytes by phytochemicals interventions are systematically summarized (**Figure 2**). Among several signal transduction pathways, NF-κB, PI3K/Akt, Nrf2/HO-1, JAK2/STAT, mTOR, and AMPK signaling pathways take an important position in the modulation of myocardial I/R injury by phytochemicals.

NF-κB Signaling Pathway

NF-κB (Nuclear Factor-kappa B) is composed of different transcription factors—the Rel family. The Rel/NF-κB family regulates immune and inflammatory responses. Activated NF-κB prevents ischemic injury and inhibits both inflammation and apoptosis (304). Paeonol significantly alleviates hypoxia and attenuates I/R injury in H9C2 cells through the BRCA1/ROS-regulated NF-κB/TNF-α/IL-6 pathways and NLRP3 inflammasome (44). Puerarin exerts a similar effect by suppressing NF-κB and upregulating VEGFA/Ang-1 in diabetic rats with myocardial I/R injury (94). Similarly, fisetin reduces ischemic injury and oxidative damage by inhibiting cytokines, such as IL-1β and TNF-α (305). Polyphenols modulate the immune system by inhibiting NF-κB (304).

PI3K/Akt Signaling Pathway

PI3K and the downstream target serine/threonine kinase Akt are crucial in various physiological processes. Activated PI3K/Akt signaling pathways is protective in myocardial I/R injury (306, 307). A study by Wang et al. suggests it is associated with H/R-induced cardiomyocyte apoptosis in Shikonin pretreated cells (261). Another study shows resveratrol inhibits I/R injury-induced cardiomyocyte apoptosis by regulating phosphorylation levels of PI3K/Akt/e-NOS pathway-related proteins (72). The PI3K/Akt signaling pathway regulates the life cycle of cardiomyocytes by regulating the morphology and function (308). 6-Gingerol possesses similar potent *via* this pathway (85).

Nrf2/HO-1 Signaling Pathway

Normally, Nrf2is a transcription factor that regulates the expression of several factors involved in the cellular defense against oxidative stress and inflammation, including heme oxygenase-1 (HO-1) (309). Once activated, it is stabilized and translocates to the nucleus, and binds antioxidant response element (ARE), which activates HO-1 (310). Numerous studies have shown the potential role of the Nrf2/HO-1 pathway in myocardial I/R injury (311). A study by Yu et al. found Nrf2 accumulated more in the nuclear due to triptolide in reperfused myocardium (230). Also, triptolide promoted the activity and expression of HO-1. This study proved triptolide was cardioprotective by activating the Nrf2/HO-1 defense pathway in treatments in I/R injuries (312). In addition, Zhou et al. demonstrated 160-nM triptolide pretreatment for a short period (< 6 h) raised the levels of nuclear Nrf2 and HO-1 in H9c2 cardiomyocytes, but they are downregulated if pretreatment lasted for a longer period (> 9 h) (313). Glaucocalyxin A is also reported to increase cell viability and decrease oxidative stress in H9c2 cells, resulting in fewer cell death from H/R-stimulated oxidative damage. The protective effect of GLA is proved to be associated with the activation of the Akt/Nrf2/HO-1 signaling pathway (213).

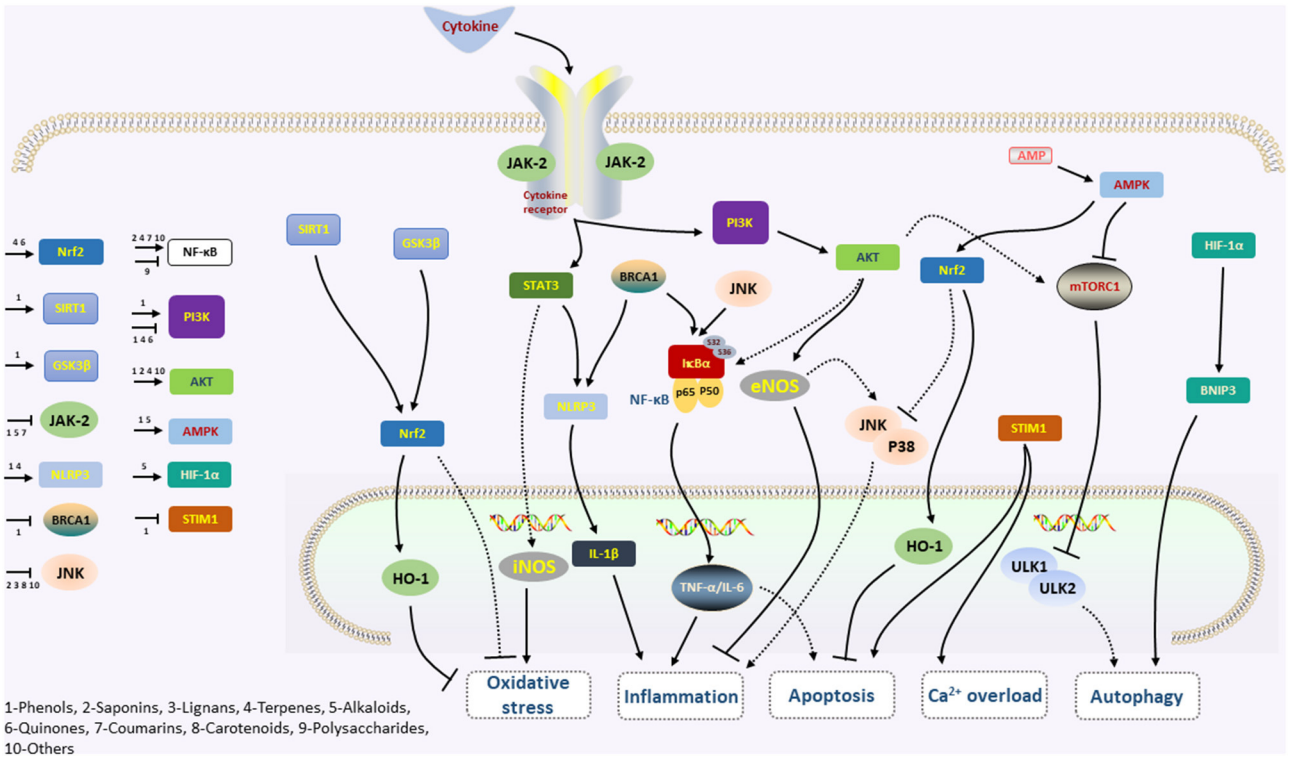


FIGURE 2 | The simplified mechanism scheme of phytochemicals in cardiovascular disease. Phytochemicals reduce the phosphorylation of STAT3 by inhibiting JAK2, which is activated following the binding of cytokines and cognate receptors. Inhibition of the JAK/STAT pathway leads to decreasing of iNOS and NLRP3/IL-1β levels, and thus protects against oxidative stress and inflammation. Activation of the AMPK signaling pathway may also play a key role in the anti-inflammation, further acting on the mTOR and Nrf2 factors and participating in the actions of phytochemicals on oxidative stress, apoptosis, and autophagy. Moreover, the NF-κB signaling pathway, activated by the BRCA1, JNK, and AKT, promotes the expression of TNF-α and IL-6, which regulate inflammation and apoptosis. The phytochemicals are also against apoptosis and inhibit Ca2+ accumulation *via* the STIM1 pathway. The PI3K-AKT signaling pathway is activated by many types of cellular stimuli or toxic insults, activates downstream mTOR, eNOS, and NF-κB, and sequentially regulates the inflammation and apoptosis.

JAK2/STAT Signaling Pathway

Several reports proposed that JAK/STAT signaling is associated with cardiac dysfunction in myocardial I/R injury (314). JAKs are rapidly recruited to the receptor and activated after the upstream receptor molecule, and then catalyze its tyrosine phosphorylation. This process supplies binding sites for the SH2 domain of STATs, ultimately leading to specific gene transcription. In particular, myocardial I/R injury activated JAK1, and JAK2, in turn, activates STAT1 and STAT3. STAT1 promotes apoptosis, while STAT3 protects cardiomyocyte (315). Ming Xu reported baicalin alleviated post-I/R myocardial injury and reduced inflammation *via* JAK/STAT pathway (64). CG pretreatment protected the myocardium against I/R injury by upregulating IL-10 expression (90). Matrine can attenuate myocardial I/R injury by upregulating HSP70, which can be activated by the JAK/STAT pathway (244).

MTOR Signaling Pathway

mTOR is a mammalian target of rapamycin (RAPA) and downregulates autophagy (316). Luo et al. found that GP upregulated p-mTOR^{Ser2448} expression and inhibited autophagy, but these effects were counteracted by RAPA. They also observed that RAPA enhanced p-AKT^{Ser473} expression, which might be associated with the activation of upstream AKT by mTOR inhibition (217). However, RAPA's effects on activating autophagy were inconsistent in myocardial I/R injury. In myocardial I/R injury, GRb1's effects are also controversial. Some studies have shown that mTOR switched on I/R (317), whereas others tend to hold the opposite view. Li et al. proved p-mTOR to be in an inhibitory state in I/R injury. Remarkably, GRb1 treatment reversed the inhibitory state and activated it (25). P-mTOR changes are dynamic after myocardial cell injury, and this may account for the difference in the performance of mTOR in I/R across studies.

AMPK Signaling Pathway

AMPK regulates cell homeostasis and reprograms metabolism. Hou et al. reported Gal alleviated I/R-induced cardiac dysfunction, reduced ERS-related apoptosis, and inhibited myocardial fibrosis by suppressing AMPK/Nrf2 pathways (239). The relationship between the cardio-protective effect of GP depends on suppressing miR-143-3p *via* activating AMPK, which furthered the understanding by connecting their function with miRs (183).

CONCLUSION AND PERSPECTIVES

To date, this review provides the most comprehensive overview of the current knowledge of phytochemicals that interfere with the myocardial I/R injury. Among the phytochemicals with potential anti-I/R injury ability, phenolic compounds take up the largest proportion (45.1%). Saponins, lignans, terpenes, alkaloids, quinones, coumarin, carotenoids, and other compounds make up the remainder, respectively. In addition, phytochemicals extensively modulated autophagy, oxidative stress, Ca^{2+} overload, apoptosis, inflammation, and key regulatory targets and proteases activities. From this point of view, phytochemicals may be a potential panacea for myocardial I/R injury treatment, and studies on their mechanisms rule out the possibility of applying a single molecule as a pathophysiological cause of myocardial I/R injury, while most natural products have more than one "target" and may affect multiple pathways.

Although phytochemicals found in natural products have made great progress in alleviating myocardial I/R injury, future studies focusing on human clinical trials of several potent phytochemicals and their combinations should be carried out. Theoretically, animal models help to explore the probable mechanism; however, there is still a huge anatomic and/or physiological gap between the different species, which may possibly be responsible for the inconsistency between preclinical studies and clinical studies currently (70). Therefore, more appropriate experimental models and precise pharmaceutical intervention studies are needed to simulate human heart physiology. Furthermore, phytochemicals must be investigated for the risk assessment and safety evaluation to observe any undesirable effects, which may hinder further use of phytochemicals as a cardioprotective adjuvant in the human body, as well as the enthusiasm for further pharmaceutical development. In addition, there may be a paradox that the cardio protection of phytochemicals is associated with inhibition of cell death, but it is an antineoplastic activity with the promotion of cell death (318). Cancer cells express different levels of apoptosis-promoting or inhibiting proteases compared to cardiomyocytes, which might partly explain these differences (318). Overall, phytochemicals may be a potential panacea for myocardial I/R injury treatment, but more research is needed to support this promising means of enhancing prognosis and, possibly, prevention.

AUTHOR CONTRIBUTIONS

QL and JW contributed to study conception and design, contributed to final approval, and overall responsibility for this published work. CC, L-TY, and B-RC contributed to acquisition, analysis, and interpretation of data. J-LX, YC, J-LJ, R-LF, LX, X-YQ, DL, JL, YL, X-YC, J-JL, and KZ contributed to article revision. All the authors contributed to the article and approved the submitted version.

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