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Drug-induced cardiotoxicity: Identification, assessment, prevention and management

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Drug-induced cardiotoxicity: Identification, assessment, prevention and management

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CASE REPORT
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Case Report: Oxaliplatin-Induced Third-Degree Atrioventricular Block: First Discovery of an Important Side-Effect

Xi Chen[1], *Hui Wang*[1], *Zijin Zhang*[1], *Yan Xu*[1], *Xuanqi An*[2], *Xin Ai*[3] *and Lin Li*[1]*

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Background: The adverse effects of anticancer therapy in patients with malignancies and cardiovascular diseases are complicated. Oxaliplatin is one of the most commonly used chemotherapy drugs for gastric and colorectal cancers, and oxaliplatin-induced cardiotoxicity has rarely been reported.

Case Summary: We report a 76-year-old man with adenocarcinoma of the esophagogastric junction and a 40-day history of non-ST-elevation myocardial infarction who exhibited a new third-degree atrioventricular block after oxaliplatin administration. We immediately withdrew oxaliplatin treatment and, to avoid future episodes, we implanted a permanent pacemaker for safety and added diltiazem hydrochloride. The third-degree atrioventricular block disappeared after oxaliplatin withdrawal. We detected no recurrence of the third-degree atrioventricular block in future chemotherapies.

Conclusions: This is the first reported oxaliplatin-induced third-degree atrioventricular block, likely mediated by coronary artery spasm. Cancer patients with acute coronary syndrome are a unique and vulnerable population, whom physicians should carefully evaluate and monitor during anticancer treatment. Remarkably, even the most common chemotherapy drugs can cause life-threatening cardiac adverse events.

Keywords: oxaliplatin, adverse event, cardiotoxicity, third-degree atrioventricular block, coronary artery spasm

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INTRODUCTION

Malignancies and cardiovascular diseases have the highest mortality (1). With treatment development, the prognosis of patients with malignancies and cardiovascular diseases has dramatically improved. However, the adverse effects of anticancer therapy in such patients are complicated, due to their comorbidities.

Oxaliplatin is widely used in treating gastric and colorectal cancers, and effectively prevent DNA replication by forming platinum-DNA adducts, stopping the cell cycle, and ultimately leading to mitotic cell apoptosis. Neurotoxicity is its most unique adverse reaction (2), while cardiotoxicity has rarely been reported.

CASE PRESENTATION

A 76-year-old man visited our outpatient clinic complaining of abdominal discomfort with no history of drug allergy. Gastroscopy detected an ulcerated 2-cm diameter mass in the cardia, lying within 1.5 cm of the dentate line. The pathology revealed moderately to poorly differentiated adenocarcinoma, partly mucinous adenocarcinoma, with a mixed type in Laurén's classification. Chest and abdominal enhanced computed tomography suggested possible lymph node metastasis of the hepatogastric ligament without distant metastasis. According to the Union for International Cancer Control/American Joint Committee on Cancer tumor–node–metastasis staging system (8th Edition, 2017), the clinical stage was stage IIIB (T3N2M0).

Hospitalized in the surgical department, the patient suddenly developed precordial pain with heavy sweat. The electrocardiogram (ECG) suggested dynamic lead II, III, aVF ST-segment depression of 0.1 mv with T-wave inversion (**Figure 1**). The cardiac troponin I peaked at 0.8299 ng/ml (0–0.0175) the next day. Echocardiography detected moderate (grade II) left ventricular diastolic dysfunction and preserved left ventricular systolic function without regional wall motion abnormalities. The diagnosis of non-ST-elevation myocardial infarction was suspected. Physicians administered anticoagulants and dual antiplatelet and lipid-lowering therapies. Coronary angiography detected 25% stenosis in the mid-left anterior descending artery, 50% stenosis in the first diagonal artery, 75% stenosis in the second diagonal artery, 50% stenosis in the left circumflex artery, 25% stenosis in the middle right coronary artery, 25–50% stenosis in the distal right coronary artery, and 50% stenosis of the post lateral artery. No stent was placed. He had previously received medications for secondary prevention of coronary heart disease, including nitrates. Postoperative ECG and cardiac enzymes returned to normal. The surgeons did not consider the patient a candidate for surgery or radiotherapy due to the higher risks, and they referred him to our medical oncology department for further anti-tumor treatments.

Upon admission to the medical oncology department, the patient reported no chest discomfort with a negative troponin level. He had a temperature of 36.4°C, pulse rate of 72 bpm, breathing rate of 17 breaths per minute, and blood pressure of 125/71 mmHg. Physical examination was unremarkable. We implemented a chemotherapy course consisting of oxaliplatin and tegafur/gimeracil/oteracil and he received oxaliplatin (150 mg) infusion over 1 h. After 1 h of infusion, the patient suddenly complained of shortness of breath, palpitation, and diaphoresis. The patient was alert and oriented. Physical examination revealed a heart rate of 37 bpm, breathing rate of 26 breaths per minute, and blood pressure of 110/50 mmHg. The patient was afebrile. His face was pale and no rash was seen. Cardiovascular examination was notable for a bradycardic rhythm. The ECG identified a new third-degree atrioventricular block (**Figure 2**). At that time, the patient did not receive tegafur/gimeracil/oteracil. We immediately withdrew oxaliplatin, and the symptoms resolved spontaneously after 20 mins, with the ECG restoring to baseline. His cardiac troponin I cardiac enzymes peaked at 0.0643 ng/ml, 9 h after the onset. Repeated echocardiography revealed no significant changes from baseline. We suspected coronary spasm triggered by oxaliplatin as the etiology, and we administered diltiazem to prevent future onsets. A permanent pacemaker was implanted for safety. The patient complained of no discomfort during the subsequent courses of oxaliplatin and tegafur/gimeracil/oteracil. The pacemaker had not been activated by a low heart rate, and we did not find a third-degree atrioventricular block when we turned down the pacemaker rate during follow-up. A detailed timeline for the case and a graphical abstract image are provided (**Table 1**, **Figure 3**).

DISCUSSION

In the present case, a 76-year-old patient with adenocarcinoma of the esophagogastric junction underwent cancer therapy and suffered from acute non-ST segment elevation myocardial infarction before surgery. Because he was unable to undergo surgery or radiotherapy, we implemented tailored chemotherapy with oxaliplatin and tegafur/gimeracil/oteracil. Unexpectedly, he developed a third-degree atrioventricular block during oxaliplatin infusion, with newly elevated troponin levels. Having suspected coronary spasms triggered by oxaliplatin, we administered diltiazem to prevent future onsets and implanted a permanent pacemaker. The patient reported no discomfort during future chemotherapies, and the pacemaker programming detected no third-degree atrioventricular blocks.

Anti-cancer therapies, particularly chemotherapy, can account for increased risks of cardiovascular disease and mortalities (3). However, in cases of sudden arrhythmia, it is difficult to determine whether arrhythmias are secondary to chemotherapy or the baseline cardiovascular disease, because baseline clinical data are often lacking (4).

We used the Naranjo adverse drug reaction probability scale (5) to evaluate the relationship between chemotherapeutic drugs and arrhythmia in this patient. We calculated the sum of scores, ranging from − 4 to + 13, to evaluate whether a causal relationship existed. Our patient received his first-time 1-h intravenous infusion of oxaliplatin and developed his first third-degree atrioventricular block at the end of the infusion, which suggested that the arrhythmia was temporally related to the use of oxaliplatin. Additionally, the third-degree atrioventricular block could not be explained by concomitant medications, including dexamethasone and tropisetron hydrochloride. Therefore, we calculated the sum of scores as 5, which meant that oxaliplatin likely caused the arrhythmia.

Oxaliplatin is a third-generation platinum-based drug, and it is one of the most commonly used chemotherapy drugs for gastric and colorectal cancers (6). The adverse reactions of oxaliplatin mainly include gastrointestinal, blood and peripheral nervous system disorders, hypersensitivity reactions, and even anaphylactic shock (7). However, there are few clinical reports of oxaliplatin-induced cardiotoxicity. Oxaliplatin is usually provided without cardiac monitoring, which may lead to the underreporting of the cardiotoxicity caused by it, especially when patients are asymptomatic. Furthermore, there have

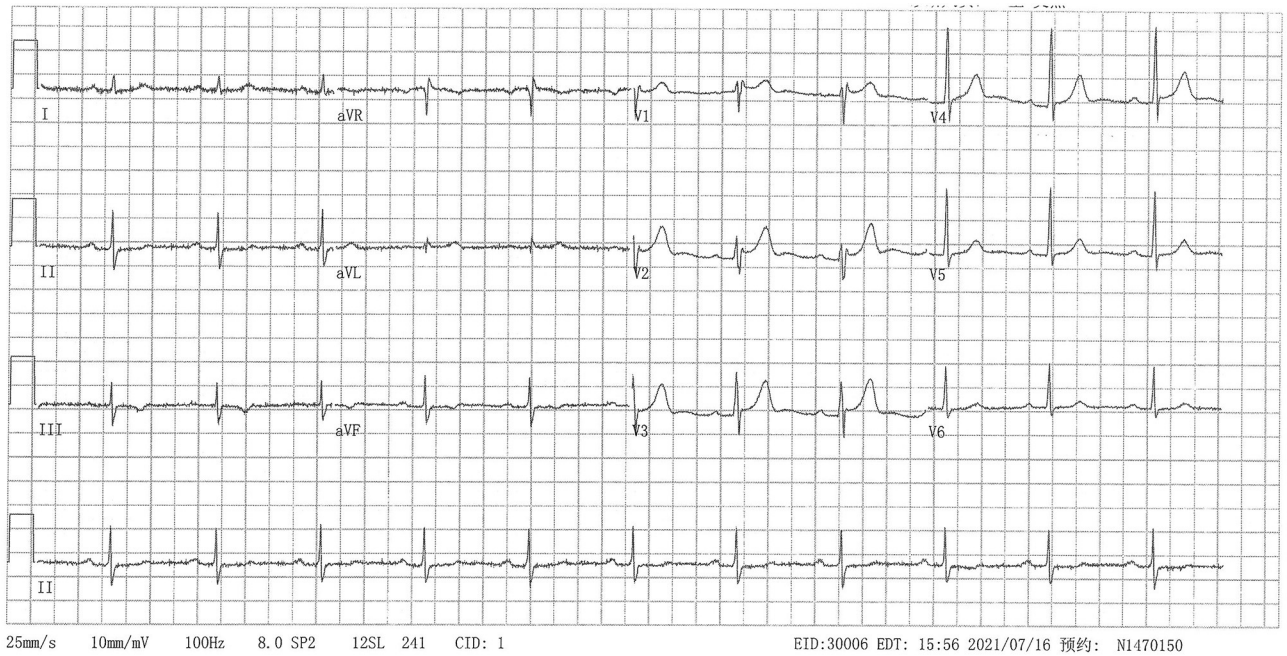

FIGURE 1 | Twelve-lead electrocardiogram showed lead II, III, aVF ST-segment depression of 0.1 mv with T-wave inversion.

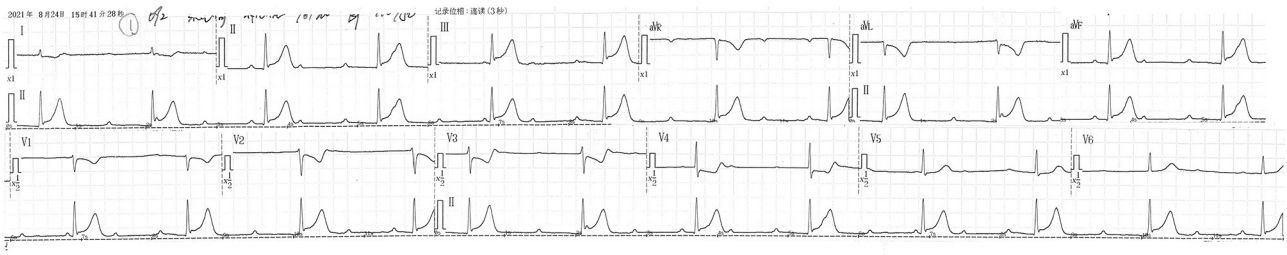

FIGURE 2 | The electrocardiogram identified a third-degree atrioventricular block.

been no literature reports on oxaliplatin-induced third-degree atrioventricular blocks.

Regarding the possible pathophysiology of our case, because prior coronary angiography ruled out the possibility of acute coronary occlusion, the most likely etiology of the spontaneously resolved third-degree atrioventricular block and mildly elevated troponin was coronary artery spasm. Coronary artery spasm, also known as Primzmetal angina or variant angina, was first described in 1959 (8). Its mechanism is assumed to be an increase in coronary artery's vascular smooth muscle tone. rather than blockage caused by plaque or thrombus. Complications of coronary artery spasm include myocardial necrosis, syncope, arrhythmia (such as ventricular tachycardia and third-degree atrioventricular block), and even cardiac arrest. Certain chemotherapeutics, especially 5-fluorouracil (9), have been documented to cause myocardial ischemia by inducing coronary arterial spasms. We found a total of three cases of oxaliplatin-induced coronary spasms. One patient with colorectal cancer metastasizing to the liver received an oxaliplatin infusion (170 mg) and developed T inversion of lead I, II, aVF, and V3 to V6 on the ECG (10). His coronary angiogram was unremarkable. Two other patients with normal coronary angiograms mimicked ST-elevation myocardial infarction in the leads II, III, and aVF (11, 12). In our patient, the third-degree atrioventricular block was accompanied by a narrow QRS complex, suggesting an atrioventricular nodal block or His bundle block.

A possible pathophysiology of oxaliplatin-induced coronary spasms may be through hyperexcitable voltage-gated Na+ channels (10, 13); another may be via inflammatory mediators such as histamine or leukotriene released during allergic injury, triggered by oxaliplatin-induced degranulation of mast cells and basophils after IgE binding (14–16). One article depicted a case of oxaliplatin-induced type 1 Kounis syndrome (11), a simultaneous occurrence of acute coronary events and hypersensitivity allergic reactions, manifesting as coronary artery spasms. One study found that oxaliplatin-free intervals and premedication with

TABLE 1 | Timeline for the case.

Timeline	
7 July 2021	Diagnosis of non-ST-segment elevation myocardial infarction
17 July 2021	Asymptomatic with negative troponin level
23 Aug 2021, 14:30	Administration of oxaliplatin over 1 h
23 Aug 2021, 15:38	Developed shortness of breath, palpitation, and diaphoresis.
23 Aug 2021, 15:41	Electrocardiogram detected third-degree atrioventricular block. Decided to withhold tegafur/gimeracil/oteracil
23 Aug 2021, 17:22	Transferred to the cardiology department of our hospital
25 Aug 2021, 02:09	Troponin level peaked
26 Aug 2021	Administration of diltiazem. Pacemaker implantation

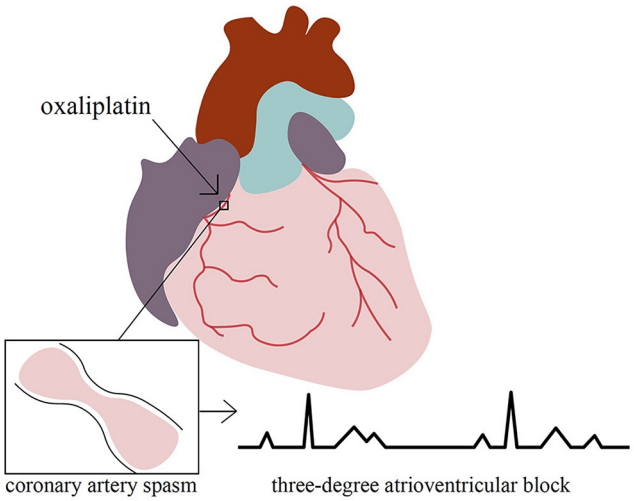

FIGURE 3 | We present a unique case of oxaliplatin-induced third-degree atrioventricular block mediated by coronary artery spasms.

dexamethasone and antihistamines could affect the occurrence of oxaliplatin-related allergic reactions (17). Our patient was treated with dexamethasone before chemotherapy, and he exhibited no signs of allergies, such as skin rash, chest tightness, breathlessness, or anaphylactic shock during the infusion. Therefore, we excluded the diagnosis of Kounis syndrome.

Treatment of coronary artery spasms includes avoiding predisposing factors (such as smoking or vasoactive drugs) and long-acting calcium channel blockers or nitrates. However, their interventions, such as balloon angioplasty, coronary artery bypass surgery, or drug-eluting coronary stent implantation, are ineffective (18). Therefore, we prescribed the patient diltiazem and continued the original chemotherapy at the patient's request. He remained asymptomatic during the subsequent three-course chemotherapy and was satisfied with the overall treatment. At follow-up, the pacemaker had not been activated by a low heart rate, and we did not find a third-degree atrioventricular block when we turned down its rate.

CONCLUSIONS

To the best of our knowledge, this is the first reported case of an oxaliplatin-induced third-degree atrioventricular block. Our patient provided us the opportunity to understand that cancer patients with acute coronary syndrome comprise a unique and vulnerable population who often receive suboptimal anti-cancer and cardiovascular treatment. Therefore, the baseline cardiac status of these patients must be thoroughly evaluated before chemotherapy to avoid unnecessary iatrogenic harm. Moreover, monitoring during treatment and close follow-ups are necessary to ensure immediate and long-term safety. Finally, the management experience for this population was limited to observational studies, as patients with acute coronary syndrome are often excluded from randomized controlled trials. Therefore, we urgently need more clinical experience and research data to assist when formulating treatment plans. However, there are some limitations to this article. The patient refused a cardiac magnetic resonance imaging and further electrophysiological examination or coronary angiography to further confirm the cause, due to financial reasons. This oxaliplatin adverse event needs to be further explored in future work.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: XC, HW, ZZ, and XAn. Data curation: XC, YX, and XAi. Writing—original draft: XC. Writing—review and editing: LL. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Can Dietary Nutrients Prevent Cancer Chemotherapy-Induced Cardiotoxicity? An Evidence Mapping of Human Studies and Animal Models

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Introduction: Chemotherapy has significantly improved cancer survival rates at the cost of irreversible and frequent cardiovascular toxicity. As the main dose-dependent adverse effect, cardiotoxic effects not only limit the usage of chemotherapeutic agents, but also cause the high risk of severe poor prognoses for cancer survivors. Therefore, it is of great significance to seek more effective cardioprotective strategies. Some nutrients have been reported to diminish cardiac oxidative damage associated with chemotherapy. However, the currently available evidence is unclear, which requires a rigorous summary. As such, we conducted a systematic review of all available evidence and demonstrated whether nutrients derived from food could prevent cardiotoxicity caused by chemotherapy.

Methods: We searched Medline (via PubMed), Embase and the Cochrane Library from inception to Nov 9, 2021 to identify studies reporting dietary nutrients against cancer chemotherapy-related cardiotoxicity. We performed descriptive summaries on the included studies, and used forest plots to demonstrate the effects of various dietary nutrients.

Results: Fifty-seven eligible studies were identified, involving 53 animal studies carried on rats or mice and four human studies in cancer patients. Seven types of dietary nutrients were recognized including polyphenols (mainly extracted from grapes, grape seeds, and tea), allicin (mainly extracted form garlic), lycopene (mainly extracted from tomatoes), polyunsaturated fatty acids, amino acids (mainly referring to glutamine), coenzyme Q10, and trace elements (mainly referring to zinc and selenium). Dietary nutrients ameliorated left ventricular dysfunctions and myocardial oxidative stress at varying degrees, which were caused by chemotherapy. The overall risk of bias of included studies was at moderate to high risk.

Conclusion: The results indicated that dietary nutrients might be a potential strategy to protect cardiovascular system exposed to the chemotherapeutic agents, but more human studies are urged in this field.

Systematic Review Registration: https://inplasy.com/inplasy-2022-3-0015/.

Keywords: chemotherapy, cardiotoxicity, heart diseases, oral nutrition, diet therapy, systematic review

INTRODUCTION

Advances in chemotherapy and comprehensive supportive care have contributed to the steadily declined cancer mortality rates over the past decades (1–3). As a result, the survivors have been an increasingly large population (e.g., more than 16.9 million in the USA in 2019) with longer life expectancy (4, 5). However, the great success of chemotherapy has been accompanied by severe cardiovascular toxicity, which is caused by the direct damage to the myocardium through production of oxygen free radicals (5–7). Cardiac toxicity could manifest as subclinical cardiomyopathies at the early stage, such as asymptomatic changes along with left ventricular dysfunction and abnormal cardiac markers. Around 12% (123/1022) pediatric patients with acute myeloid leukemia were reported to suffer cardiotoxicity during and after the chemotherapy regimens over a five-year follow-up (8). In addition, cardiotoxicity would progress to congestive heart failure (CHF) and even cardiac death (5, 9) and these complications have been the leading cause of long-term morbidity and mortality (10–13). The incidence of CHF reported in patients treated with doxorubicin (DOX) was 2.2% (88/4018) (14) and the two-year mortality rate associated with anthracyclines-induced cardiovascular diseases (CVD) was up to 60% (15). Therefore, appropriate early prevention and management for cancer survivors should be implemented to prevent and avoid chemotherapy-induced cardiotoxic progression (16, 17).

Early detection and treatment of chemotherapy-induced cardiac damage have been gradually studied. The common used monitoring methods are echocardiography and cardiac biomarkers. Several drugs were previously investigated as cardioprotective agents for preventing cardiotoxicity (6, 7, 18, 19), but only dexrazoxane was approved by Food and Drug Administration (FDA) to protect the chemotherapy-exposed heart (7, 20). However, dexrazoxane has not been routinely applied in the clinic at present along with debate about its long-term safety. This is largely due to the concerns over its impact on anticancer treatments (21, 22). In addition, the cost and accessibility have also been quite essential impediments for cancer survivors who have already borne considerable treatment overheads in the long-term survivals (5). So, it is of great significance to explore alternative effective, safe, economical, and consistent cardiac protection strategies for long-term cancer survivors.

Dietary nutrients (defined as various nutrients derived from food) are increasingly playing an important role in medicine. Due to the restriction of conventional medicine treatments for cancer, complementary and alternative medicine (CAM) has been playing a broader and more active role in cancer patients (23). Currently, several studies indicated that some fruit and vegetables have been considered as natural antioxidants that could reduce oxidative stress and inhibit chemotherapy-related cardiotoxicity (24–26). Furthermore, dietary factors such as polyunsaturated fatty acids (PUFA) and coenzyme Q10 (CoQ10) have also been reported to be able to protect the chemotherapy-exposed heart on animal models (27, 28). Although there are some narrative reviews (27, 29–32), it seems that the evidence on whether dietary nutrients could alleviate cardiotoxicity induced by chemotherapy has not been systematically summarized.

As such, we hypothesized that dietary nutrients could serve as a novel cardioprotective strategy to prevent cancer chemotherapy-induced cardiotoxicity and conducted a systematic review of the current evidence.

METHODS

This systematic review was conducted based on the guidelines of Systematic Review Protocol for Animal Intervention Studies (33) and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (34), and was registered at https://inplasy.com as INPLASY202230015.

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: (1) subjects: cancer patients or healthy/tumor-bearing animal models, treated with chemotherapeutic agents, with no restrictions on cancer types, animal species and chemotherapeutic agents; (2) intervention: oral intake of dietary nutrients; If the source of the nutrient was reported in the article, we only included cases which the nutrient source was food rather than non-food like drugs. If it was not reported, then we included articles that the nutrient can be obtained from food; (3) comparison: placebo or no intervention (without dietary nutrients mentioned above); (4) outcomes: imaging or biological measures of cardiotoxicity, including echocardiography, serum cardiac markers, oxidative stress markers, and histopathological examinations. Echocardiography is the most common and noninvasive method which measures left ventricular systolic functions like left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS). It is also the most widely used screening method for monitoring cardiotoxicity both during and years after anticancer treatment (16). Cardiac markers, such as cardiac troponin (cTn), N-terminal pro-brain natriuretic peptide (NT-proBNP), creatine kinase (CK), creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH), can indicate abnormal left ventricular structure and increased cardiac stress (17). Measurements of antioxidant defense can reflect the cardiac oxidative stress status in cancer patients, including malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH). The details of detection indicators are represented in **Supplementary Table 1**. Conference abstracts, case reports, reviews, trial protocols, duplicate publications, *in vitro* experiments, and non-controlled studies were excluded.

Search Strategy and Study Selection

A comprehensive search was performed through three separate electronic databases, including Medline (via PubMed), Embase, and the Cochrane Library, from the inception to Nov 9, 2021. In addition, a manual search was also conducted by screening the reference lists from relevant reviews. The search strategies used are provided in **Supplementary Table 2**.

Two reviewers (X-YZ and K-LY) screened the titles and abstracts of records retrieved from the databases and independently screened the full text for eligible studies. Any

disagreements between the two reviewers were resolved through discussion by achieving a consensus.

Data Extraction

Two reviewers (X-YZ and K-LY) independently used a data extraction sheet to extract data from the included studies. The following information was extracted: first author, year of publication, characteristics of subjects, study design, intervention characteristics, and outcome measures. The primary outcomes included LVEF and cTn, and the secondary outcomes were LVFS, CK, CK-MB, LDH, MDA, SOD, and GSH.

Risk of Bias Assessment

Two reviewers (X-YZ and YL) independently assessed the risk of bias for the included studies. For animal studies, we used the risk of bias tool of Systematic Review Center for Laboratory Animal Experimentation (SYRCLE). This tool is designed based on the Cochrane Risk of Bias (RoB) tool for animal experiments. It consists of 10 items, including selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases (35). Each item is rated as "Y" (low risk of bias), "N" (high risk of bias), and "U" (unclear risk of bias). For randomized controlled trials (RCT), we used the Cochrane risk of bias tool (36). It covers 6 domains of bias, namely sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other biases. The items are also assessed as "Y" (low risk of bias), "N" (high risk of bias), and "U" (unclear risk of bias). For non-randomized clinical trials and observational studies, we used the Newcastle-Ottawa Scale (NOS) which contains 8 items in three dimensions of selection, comparability, and outcome (37). It scores from 0 to 9 and higher scores show the lower risk of bias. Any disagreements were resolved by consulting a third reviewer (QW).

Data Analysis

The primary and secondary outcomes of the review were treated as continuous variables represented by mean ± standard deviation. Number of cases and percentages were used to indicate the number of included studies. Effectiveness of dietary nutrients against cardiotoxicity was presented by the comparison between chemotherapy with dietary nutrients groups and chemotherapy groups. Statistical analyses of all outcomes were performed in forest plots using RevMan Software (Version 5.3). When there were more than two arms in the included studies, we presented all the results separately. Due to high heterogeneity from the variations in the baseline of included studies, we used a random-effects model and didn't provide a pooled result as well.

The work flowchart describing the process of the study is shown in **Figure 1**.

RESULTS

Literature Search and Study Selection

A total of 4025 potentially relevant records were initially identified. However, 341 of those were excluded due to duplication, 3,590 studies were excluded by reading titles and abstracts based on the inclusion and exclusion criteria, and 94 potential studies were eligible for full-text screening. We finally included 57 studies, including 53 animal studies and four human studies. The PRISMA flowchart of the literature search and study selection process is shown in **Figure 2**. The reasons for excluding reviews are listed in **Supplementary Table 3**.

Animal Studies

Study Characteristics

The 53 animal studies included were conducted in 14 countries, with most in Egypt (n = 15), India (n = 8), Saudi Arabia (n = 6), Turkey (n = 6), and China (n = 5). The publication years ranged from 1996 to 2021, with 42 before 2010. DOX (n = 48) comprised a significant majority of the included studies, and the other chemotherapeutic agents were cisplatin (n = 3), mitoxantrone (n = 1), and fluorouracil (n = 1). The covered dietary nutrients contained polyphenols (n = 29) (38–66), allicin (n = 3) (67–69), lycopene (n = 2) (70, 71), PUFA (n = 5) (72–76), amino acids (n = 4) (77–80), CoQ10 (n = 5) (81–85), trace elements (n = 3) (86–88), and others (n = 2) (89, 90). 79.24% of the included studies were investigated in Asia and Africa and most studies commonly used allicin from garlic (67–69) and polyphenols from local fruit such as grape (38–43), date palm (55, 56), cranberry (58), cardamom (59), pomegranate (60) and hawthorn (61) as the nutritional interventions. However, American and European studies tended to use amino acid like glycine and glutamine which were rich in animal food or special supplements (77–80). The characteristics of the included animal studies are summarized in **Table 1** and **Figure 3**.

Risk of Bias Assessment

The overall risk of bias of included animal studies was at moderate to high risk and most of the items of SYRCLE depicted unclear risk (**Figure 4** and **Supplementary Table 4**). All animal studies failed to report the sequence generation methods (item 1), allocation concealment (item 3), and random outcome assessment (item 6). Other items also revealed poor outcomes. Four studies (73, 77–79) reported baseline characteristics (item 2), one (84) mentioned the methods of performance blinding (item 5), and another (77) documented the blinded outcome assessment (item 7). In comparison, all the animal studies were free from selective outcome reporting (item 9), and 52 studies did not involve any other sources of bias (item 10).

Effectiveness of Dietary Nutrients

The protective effects of dietary nutrients above on myocardium against cardiac toxicity can be observed by the comparison between chemotherapy with nutrients groups and chemotherapy groups in **Supplementary Figures 1**–**7**.

Polyphenols

In animal studies of our review, polyphenols were defined as a broad class of compounds with multiple phenolic hydroxyls (PhOH) and were reported in 29 studies (38–66). These covered proanthocyanidin (n = 6, all derived from grape seed extract) (38–43), anthocyanin (n = 1, derived from purple corn) (44), resveratrol (n = 2) (45, 46), curcumin (n = 5) (47–51), catechins

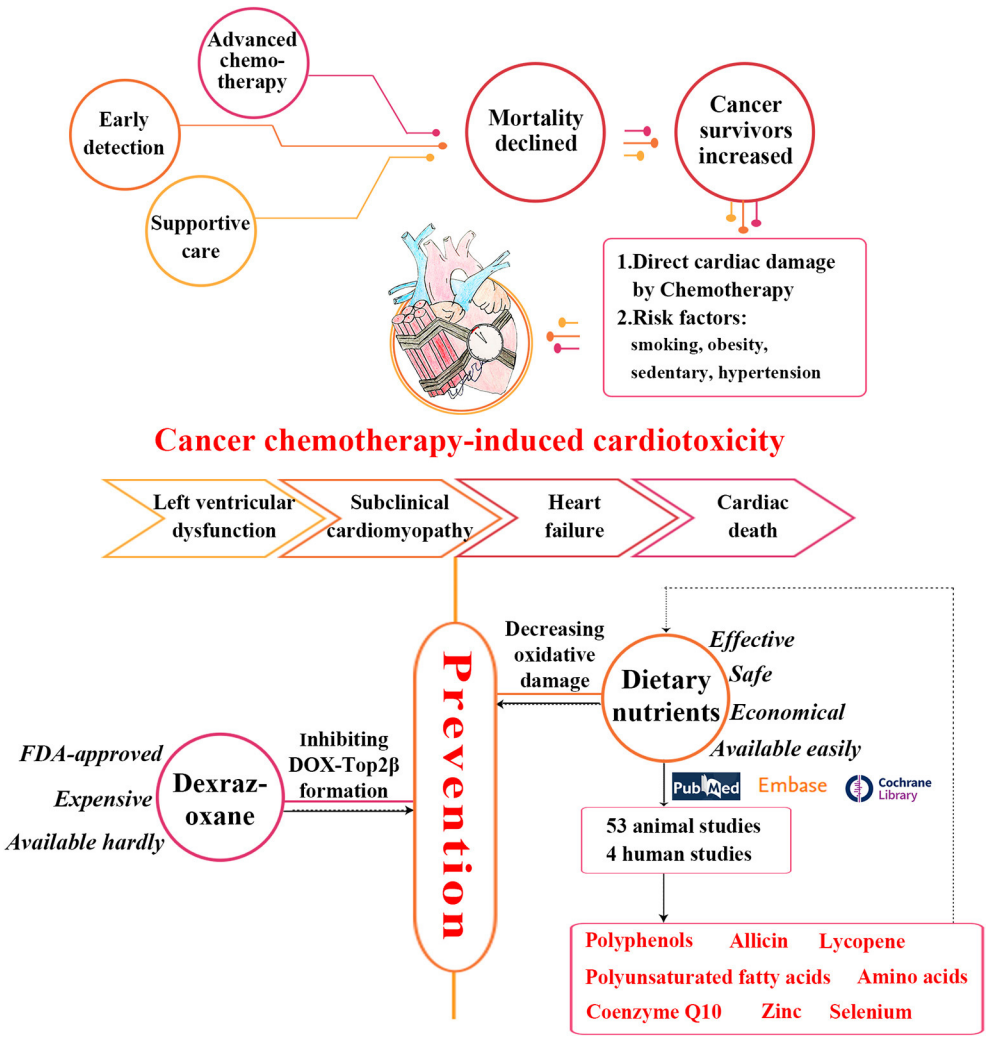

FIGURE 1 | The work flowchart of the study process. Epidemiology, clinical symptoms and current cardioprotective strategies of cancer chemotherapy-induced cardiotoxicity are shown. Our search strategy and main results are also presented. DOX, doxorubicin; FDA, Food and Drug Administration; Top2β, topoisomerase 2β.

(n = 3, derived from green tea and black tea) (52–54), and mixed phenolic compounds extracted from local products [n = 12, derived from date (55, 56), orange (57), cranberry (58), cardamom (59), pomegranate (60), hawthorn (61), naringenin (62), p-coumaric acid (63), honey (64), yogurt (65) and yellow wine (66)]. Three studies demonstrated that supplementation of polyphenols could improve DOX-induced cardiac dysfunctions evaluated by echocardiography (45, 57, 66). LVEF significantly reduced in DOX groups, but was similar in DOX+Nutrients groups and control groups. Cardiac morphological and systolic changes led by DOX were attenuated by polyphenolic nutrients through scavenging free radicals and blocking lipid peroxidation. Biochemical analyses were estimated by serum cardiac markers and antioxidant parameters in 28 studies (38–43, 45–66). LDH, MDA and SOD were reported most. The concentrations of myocardial enzymes in animals received chemotherapy and nutrients were significantly lower compared with those treated with chemotherapeutic agents alone. Oral administration of polyphenols improved the cardiac oxidative changes led by chemotherapy and enhanced the antioxidant enzymatic activities. Histopathological analyses of cardiac tissue captured under the microscope were reported in 16 studies (38, 40, 41, 45, 46, 48–50, 53, 56, 59–62, 65, 66). The incidences of myocardial atrophy, cytoplasmic vacuoles, nuclear pyknosis, and cytoplasmic eosinophilia were significantly higher in heart exposed to DOX, while the polyphenolic substance protected or even restored cardiac disrupted histological structure induced by DOX (**Supplementary Figure 1**).

Allicin and Lycopene

Three studies showed that allicin (all derived from garlic extract) effectively decreased the expression of myocardial tumor necrosis factor-alpha (TNF-α) and mitigated cardiac oxidative damage (67–69) (**Supplementary Figure 2**). Abdel-Daim et al.

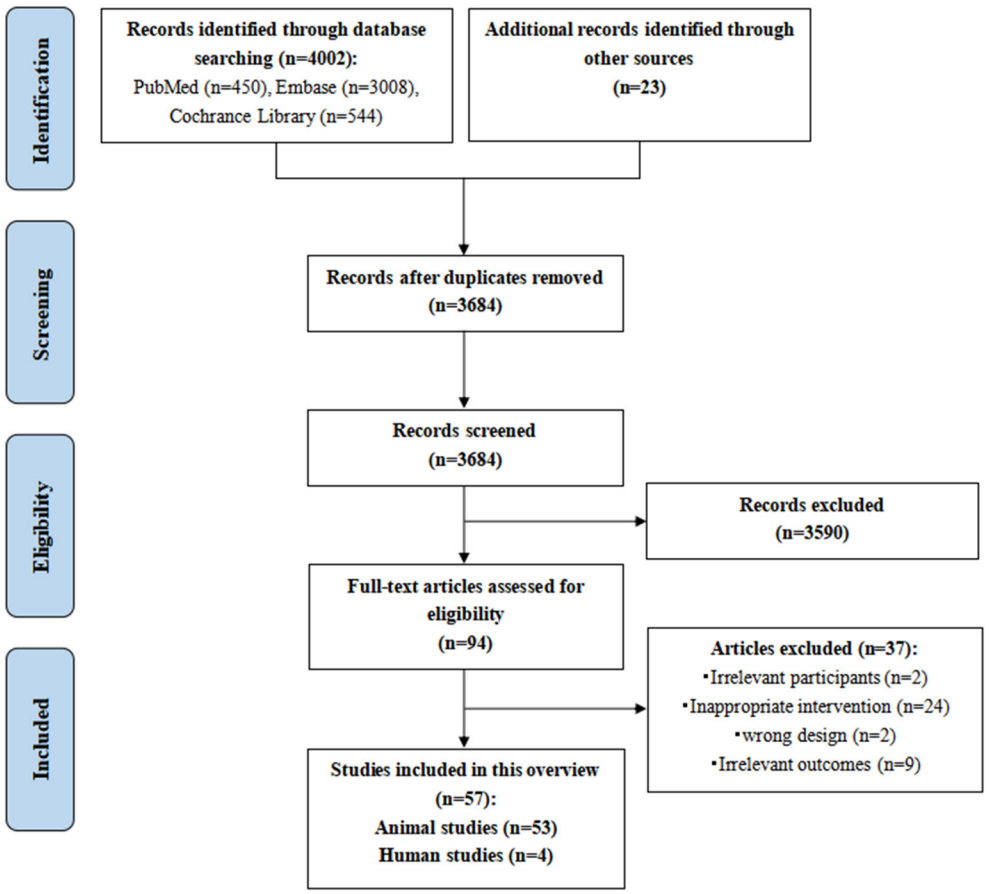

FIGURE 2 | The PRISMA flowchart of the literature search and study selection.

(67) referred that allicin could be a promising cytoprotective agent against DOX-related cardiotoxicity. Two studies revealed that lycopene (all derived from tomatoes) reduced the levels of cardiac oxidative markers and made the histopathological changes maintain nearly normal after the injection of DOX (70, 71) (**Supplementary Figure 3**).

Polyunsaturated Fatty Acids

PUFA were reported in five studies, derived from black chia seed (72), flaxseed (73), fish oil (74, 76), and sesame oil (75). All these studies proved that PUFA attenuated the myocardial necrosis and overall myocardium enlargement and alleviated histopathological alteration in rats/mice treated with DOX (**Supplementary Figure 4**). PUFA were considered as a potential chemoprotectant nutraceutical in combination with chemotherapy to limit the cardiotoxic side effects (72).

Amino Acids, Coenzyme Q10, and Trace Elements

Four studies reported that amino acids [derived from glycine (77) and glutamine (78–80)] could diminish chemotherapy-induced cardiac oxidative damage (**Supplementary Figure 5**). As a vital role in maintaining the cellular redox state, dietary glutamine remained normal cardiac GSH levels in animal models treated with chemotherapeutic drugs and prevented cardiac lipid peroxidation (78–80). Coenzyme Q10 ($n = 5$) (81–85) was proven to be prophylactic in prevention of cardiovascular toxicity through participating with redox function directly in the mitochondrial respiratory chain (**Supplementary Figure 6**), and trace elements [$n = 3$, derived from zinc (Zn) (86, 87) and selenium (Se) (88)] were also exhibited to protect myocardium by preventing mitochondrial dysfunctions and acting in concert with SOD and catalase (**Supplementary Figure 7**).

The details of outcomes are summarized in **Supplementary Table 5**.

Human Studies

Study Characteristics

Four human studies were conducted in Egypt [in 2021 (91) and 2020 (92)], Italy [in 1994 (93)] and the USA [in 1978 (94)]. Three studies (91–93) recruited pediatric patients diagnosed with acute lymphoblastic leukemia (ALL) aged 1 to 16 years and one (94) recruited adults with bronchogenic carcinoma. Two studies (91, 92) were RCTs using DOX as the chemotherapy agent, and the other two studies (93, 94) were non-randomized controlled trials using anthracyclines. The covered dietary nutrients contained PUFA [$n = 2$, derived from omega 3 fatty acids (91) and black

TABLE 1 | The characteristics of the included animal studies.

Dietary nutrients	Studies	Country	Randomization	Animals			Intervention			Comparison				Outcomes
				Species	Tumor-bearing	Chemotherapeutic agents	Food intake	Main ingredients	Duration	Sample size	Grouping	Control groups	Treated groups	
Polyphenols	Adiyaman et al. (38)	Turkey	Not reported	Rats, sprague dawley	Healthy	DOX	Grape seed extract	Proanthocyanidin	35 days	28	4	(1) CON (2) grape seed extract (3) DOX	(4) DOX+grape seed extract	b, c, d
	Ammar et al. (39)	Egypt	Not reported	Rats, sprague dawley	Healthy	DOX	Proanthocyanidin	Proanthocyanidin	10 days	24	4	(1) CON (2) proanthocyanidin (3) DOX	(4) DOX+proanthocyanidin	b, c, e
	Boghdady (40)	Egypt	Yes	Rats, wistar albino	Healthy	DOX	Grape seed extract	Proanthocyanidin	15 days	32	4	(1) CON (2) DOX	(3) DOX+grape seed extract (4) DOX+ginkgo biloba extract	b, c, d
	Yalcin et al. (41)	Turkey	Yes	Mice, albino	Healthy	DOX	Grape seed extract	Proanthocyanidin	21 days	36	6	(1) CON (2) DOX (3) grape seed extract 50 (4) grape seed extract 150	(5) DOX+grape seed extract 50 (6) DOX+grape seed extract 150	c, d
	Yousef et al. (42)	Egypt	Not reported	Rats, sprague dawley	Healthy	Cisplatin	Grape seed extract	Proanthocyanidin	15 days	32	4	(1) CON (2) grape seed extract (3) cisplatin	(4) cisplatin+grape seed extract	b, c, f
	Zhang et al. (43)	China	Yes	Mice, balb/c	Sarcoma	DOX	Proanthocyanidin	Proanthocyanidin	10 days	56	4	(1) CON (2) DOX (3) proanthocyanidin	(4) DOX+proanthocyanidin	b, c
	Petroni et al. (44)	Italy	Not reported	Mice, c57bl/6j	Healthy	DOX	Cyanidin 3-glucoside	Anthocyanin	74 days	24	2	(1) DOX+yellow diet	(2) DOX+red diet	f
	Shoukry et al. (45)	Egypt	Yes	Rats, wister	Healthy	DOX	Resveratrol	Resveratrol	42 days	32	4	(1) CON (2) DOX	(3) DOX+resveratrol(pre) (4) DOX+resveratrol(post)	a, b, d, f
	Arafa et al. (46)	Egypt	Not reported	Rats, wistar albino	Healthy	DOX	Resveratrol	Resveratrol	28 days	40	4	(1) CON (2) resveratrol (3) DOX	(4) DOX+resveratrol	b, c, d, f
	Ibrahim Fouad and Ahmed. (47)	Egypt	Yes	Rats, wistar albino	Healthy	DOX	Curcumin	Curcumin	/	24	4	(1) CON (2) DOX (3) curcumin	(4) DOX+curcumin	b, c
	Bahadir et al. (48)	Turkey	Yes	Rats, wistar albino	Healthy	Cisplatin	Curcumin	Curcumin	14 days	49	7	(1) CON (2) placebo (3) cisplatin (4) beta-carotene (6) curcumin	(5) cisplatin+beta-carotene (7) cisplatin+curcumin	b, c, d
	Benzer et al. (49)	Turkey	Yes	Rats, wistar albino	Healthy	DOX	Curcumin	Curcumin	7 days	35	5	(1) CON (2) curcumin 200 (3) DOX	(4) DOX+curcumin 100 (5) DOX+curcumin 200	b, c, d

(Continued)

TABLE 1 | Continued

Dietary nutrients	Studies	Country	Randomization	Animals			Intervention			Comparison				Outcomes
				Species	Tumor-bearing	Chemotherapeutic agents	Food intake	Main ingredients	Duration	Sample size	Grouping	Control groups	Treated groups	
	Swamy et al. (50)	India	Yes	Rats, albino	Healthy	DOX	Curcumin	Curcumin	14 days	24	4	(1) CON (2) DOX (3) curcumin	(4) DOX+curcumin	b, c, d, f
	Venkatesan (51)	India	Not reported	Rats, wistar	Healthy	DOX	Curcumin	Curcumin	7 days	24	4	(1) CON (2) curcumin (3) DOX	(4) DOX+curcumin	b, c, e
	Ibrahim et al. (52)	Saudi Arabia	Yes	Mice, balb/c	Healthy	Cisplatin	Green tea extract, vitamin E	Catechins, vitamin E	30 days	48	6	(1) CON (2) green tea extract (3) vitamin E (4) cisplatin	(5) cisplatin+green tea extract (6) cisplatin+vitamin E	b, c, f
	Saeed et al. (53)	Egypt	Yes	Rats, wistar	Healthy	DOX	Epigallocatechin-3-gallate	Catechins	12 days	40	5	(1) CON (2) DOX	(3) DOX+epigallocatechin-3-gallate 10 (4) DOX+epigallocatechin-3-gallate 20 (5) DOX+epigallocatechin-3-gallate 40	b, c, d, e
	Amanullah et al. (54)	India	Not reported	Rats, wistar albino	Healthy	DOX	Black tea extract, resveratrol	Catechins, polyphenols	30 days	30	6	(1) CON (2) DOX (3) black tea extract+ resveratrol	(4) DOX+black tea extract (5) DOX+resveratrol (6) DOX+black tea extract +resveratrol	b, c, f
	Mubarak et al. (55)	Egypt	Not reported	Rats, albino	Healthy	DOX	Date palm fruit extract	Anthocyanins, quercetin, procyanidins	30 days	40	4	(1) CON (2) date (3) DOX	(4) DOX+date	b, c
	Sabbah et al. (56)	Saudi Arabia	Yes	Rats, wistar albino	Healthy	DOX	Ajwa date aqueous extract	Polyphenols, flavonoids, Mn	28 days	60	6	(1) CON (2) date 0.75 (3) date 1.5 (4) DOX	(5) DOX+date 0.75 (6) DOX+date 1.5	b, c, d
	Ribeiro et al. (57)	Brazil	Not reported	Rats, wistar	Healthy	DOX	Pera orange juice, Moro orange juice	Hesperidin, anthocyanins	28 days	120	6	(1) CON (2) Pera juice (3) Moro juice (4) DOX	(5) DOX+Pera juice (6) DOX+Moro juice	a, c, f
	Elberry et al. (58)	Saudi Arabia	Yes	Rats, wister	Healthy	DOX	Cranberry extract	Flavonols, flavonoids	10 days	30	4	(1) CON (2) cranberry extract (4) DOX	(4) DOX+cranberry extract	b, c, e
	Abu Gazia and El-Magd (59)	Egypt	Yes	Rats, albino	Healthy	DOX	Cardamom extract	Flavonoids	21 days	30	3	(1) CON (2) DOX	(3) DOX+cardamom extract	b, c, d
	Hassanpour Fard et al. (60)	India	Not reported	Rats, wistar albino	Healthy	DOX	Whole fruit extract of pomegranate	Gallic acid, quercetin	18 days	24	3	(1) CON (2) DOX	(3) DOX+pomegranate extract	b, c, d, e, f

(Continued)

TABLE 1 | Continued

Dietary nutrients	Studies	Country	Randomization	Animals			Intervention			Comparison				Outcomes
				Species	Tumor-bearing	Chemotherapeutic agents	Food intake	Main ingredients	Duration	Sample size	Grouping	Control groups	Treated groups	
	Shatoor and Said Ahmed, (61)	Saudi Arabia	Yes	Rats, wistar albino	Healthy	DOX	Hawthorn extrat	Flavonoids, polyphenols	28 days	36	6	(1) CON (2) hawthorn (3) DOX	(4) DOX+hawthorn(st) (5) DOX+hawthorn(post) (6) hawthorn+DOX(pre)	b, c, d, f
	Subburaman et al. (62)	India	Not reported	Rats, albino	Healthy	DOX	Naringenin	Flavonoids	70 days	18	3	(1) CON (2) DOX	(3) DOX+naringenin	b, c, d, f
	Abdel-Wahab et al. (63)	Egypt	Not reported	Rats, swiss albino	Healthy	DOX	P-coumaric acid	p-coumaric acid (pca)	5 days	24	4	(1) CON (2) P-coumaric acid (3) DOX	(4) DOX+p-coumaric acid	b, c
	Alhumaydhi (64)	Saudi Arabia	Yes	Mice, balb/c	Healthy	DOX	Honey	Polyphenols, fructose, glucose	10 days	40	4	(1) CON (2) honey (3) DOX	(4) DOX+honey	b
	Abu-Elsaad et al. (65)	Egypt	Not reported	Rats, sprague dawley	Healthy	DOX	Tested food: yogurt, green tea extract, carrot	Lactobacillus acidophilus, polyphenols, carrot	154 days	60	5	(1) CON (2) DOX (3) DOX+carvedilol	(4) DOX+tested food (5) DOX+tested food+carvedilol	b, c, d, e
	Lin et al. (66)	China	Yes	Rats, sprague dawley	Healthy	DOX	Yellow wine polyphenolic compounds	Polyphenolic compounds	28 days	50	5	(1) CON (2) yellow wine (3) DOX	(4) DOX+yellow wine	a, c, d, e, f
Allicin	Abdel-Daim et al. (67)	Egypt	Yes	Mice, swiss albino	Healthy	DOX	Allicin	Allicin	14 days	40	5	(1) CON (2) allicin 20 (3) DOX	(4) DOX+allicin 10 (5) DOX+allicin 20	b, c
	Demirkaya et al. (68)	Turkey	Yes	Rats,wistar albino	Healthy	DOX	Aged garlic extract, grape seed extract, hazelnut	Allicin, proanthocyanidin	42 days	135	9	(1) CON (2) DOX 15 (3) DOX 7.5	(4) DOX 15+aged garlic extract (5) DOX 7.5+aged garlic extract (6) DOX 15+grape seed extract (7) DOX 7.5+grape seed extract (8) DOX 15+ hazelnut (9) DOX 7.5+hazelnut	b, c, d
	Mukherjee et al. (69)	India	Not reported	Rats, wistar albino	Healthy	DOX	Garlic homogenate	Allicin	30 days	40	5	(1) CON (2) DOX (3) DOX+PRO	(4) DOX+garlic 250 (5) DOX+garlic 500	c, f
Lycopene	Ferreira et al. (70)	Brazil	Not reported	Rats, wistar	Healthy	DOX	Tomato-oleoresin supplement	Lycopene	49 days	34	4	(1) CON (2) lycopene (3) DOX	(4) DOX+lycopene	d
	Yilmaz et al. (71)	Turkey	Not reported	Rats, sprague dawley	Healthy	DOX	Lycopene	Lycopene	10 days	24	4	(1) CON (2) DOX	(3) DOX+lycopene(pre) (4) DOX+lycopene(post)	c, d
PUFA	Ahmed et al. (72)	India	Yes	Rats, wistar	Healthy	DOX	Chia seed oil	PUFA	7 days	24	4	(1) CON (2) DOX	(3) DOX+chia seed oil 2.5 (4) DOX+chia seed oil 5	b, c, d, e

(Continued)

TABLE 1 | Continued

Dietary nutrients	Studies	Country	Randomization	Animals			Intervention			Comparison				Outcomes
				Species	Tumor-bearing	Chemotherapeutic agents	Food intake	Main ingredients	Duration	Sample size	Grouping	Control groups	Treated groups	
	Asselin et al. (73)	Canada	Yes	Mice, c57bl/6	Healthy	DOX+TRZ	Flaxseed, α-linolenic acid, secoisolariciresinol diglucoside	α-Linolenic acid, secoisolariciresinol diglucoside	42 days	84	5	(1) CON (2) DOX+TRZ	(3) DOX+TRZ+flaxseed (4) DOX+TRZ+α-linolenic acid (5) DOX+TRZ+secoisolariciresinol diglucoside	a, d, e
	Saleh et al. (74)	Egypt	Not reported	Rats, wistar albino	Healthy	DOX	N-3 PUFA	n-3 PUFA	28 days	35-40	5	(1) CON (2) DOX	(3) DOX+n-3 PUFA 25 (4) DOX+n-3 PUFA 50 (5) DOX+n-3 PUFA100	b, c, d, e, f
	Saleem et al. (75)	India	Not reported	Rats, wistar albino	Healthy	DOX	Sesame oil	Linoleic acid, α-linolenic acid, sesamin	30 days	30	5	(1) CON (2) DOX (5) DOX+probucol	(3) DOX+sesame oil 1 (4) DOX+sesame oil 2	b, c, d
	Teng et al. (76)	China	Yes	Rats, sprague dawley	Healthy	DOX	N-3 PUFA	Timnodonic acid, docosahexaenoic acid	112 days	32	3	(1) CON (2) DOX	(3) DOX+n-3 PUFA	a, d
Amino acids	Maneikyte et al. (77)	Austria	Yes	Rats, wag/rij	Colorectal cancer liver metastasis	FOLFOX	Glycine	Glycine	21 days	44	6	(1) casein+sham (2) glycine+sham (3) casein+CON (5) casein+FOLFOX	(4) glycine+CON (6) glycine+FOLFOX	a, b, d
	Todorova et al. (78)	USA	Yes	Rats, fisher344	Mammary carcinoma	DOX	Glutamine	Glutamine	/	50	3	(1) CON (2) DOX+water	(3) DOX+glutamine	a, b
	Todorova et al. (79)	USA	Yes	Rats, fisher344	Mammary carcinoma	DOX	Glutamine	Glutamine	7 days	20	2	(1) DOX+CON	(2) DOX+glutamine	a, c
	Cao et al. (80)	USA	Yes	Rats, fisher 344	Healthy	DOX	Glutamine	Glutamine	28 days	42	6	(1) H_2O+saline (2) H_2O+DOX	(3) glutamine+saline (4) glutamine+DOX	c
CoQ10	Rahmanifard et al. (81)	Iran	Yes	Rats, sprague dawley	Healthy	DOX	CoQ10	CoQ10	21 days	42	6	(1) CON (2) lisinopril (3) CoQ10 (4) DOX	(5) DOX+lisinopril (6) DOX+CoQ10	c, d, e, f
	Shabaan et al. (82)	Egypt	Yes	Rats, wistar	Healthy	DOX	CoQ10	CoQ10	7 days	28	4	(1) CON (2) CoQ10 (3) DOX	(4) DOX+CoQ10	c, d
	Botelho et al. (83)	Brazil	Yes	Rats, wistar albino	Healthy	DOX	CoQ10	CoQ10	14 days	20	4	(1) CON (2) CoQ10 (3) DOX	(4) DOX+CoQ10	b, c, d, e
	Chen et al. (84)	China	Yes	Rats, sprague dawley	Healthy	DOX	CoQ10	CoQ10	21 days	24	4	(1) CON (2) DOX (4) CoQ10	(3) DOX+CoQ10	d, f
	Mustafa et al. (85)	Saudi Arabia	Not reported	Rats, wistar albino	Healthy	DOX	CoQ10	CoQ10	15 days	72	6	(1) CON (2) DOX (3) CoQ10	(4) DOX+CoQ10	b, c, e, f

(Continued)

TABLE 1 | Continued

Dietary nutrients	Studies	Country	Randomization	Animals			Intervention			Comparison				Outcomes
				Species	Tumor-bearing	Chemotherapeutic agents	Food intake	Main ingredients	Duration	Sample size	Grouping	Control groups	Treated groups	
Trace elements	Maryoosh et al. (86)	Iraq	Yes	Rats, wistar albino	Healthy	mitoxantrone	Zinc sulfate	Zinc	20 days	48	6	(1) CON (2) Zinc 15 (3) Zinc 30 (4) mitoxantrone	(5) mitoxantrone+Zinc 15 (6) mitoxantrone+Zinc 30	b, c
	Wu et al. (87)	China	Yes	Rats, sprague dawley	Healthy	DOX	ZnCM	Zinc, curcumin	28 days	42	6	(1) CON (2) DOX	(3) DOX+curcumin 100 (4) DOX+ZnCM 25 (5) DOX+ZnCM 50 (6) DOX+ZnCM 100	a, b, e
	Coudray et al. (88)	France	Not reported	Rats, wistar	Healthy	DOX	Selenium	Selenium	49 days	60	5	(1) CON (2) saline (3) DOX (4) selenium	(5) DOX+selenium	c, f
Others	Radeva-Ilieva et al. (89)	Bulgaria	Yes	Rats, wistar	Healthy	DOX	Methylxanthine from bancha	Methylxanthine	17 days	36	6	(1) CON (2) DOX (3) methylxanthine 5 (4) methylxanthine 1	(5) DOX+methylxanthine 5 (6) DOX+methylxanthine 1	b
	Wahab et al. (90)	Egypt	Yes	Mice, swiss albino	Ehrlich ascites carcinoma	DOX	Vitamin E	Vitamin E	30 days	140	4	(1) DOX	(2) DOX+vitamin E	c

CON, control; CoQ10, coenzyme Q10; DOX, doxorubicin; FOLFOX, 5-fluorouracil+leucovorin+oxaliplatin; PUFA, polyunsaturated fatty acids; TRZ, trastuzumab; ZnCM, zinc+curcumin.
a, echocardiography; b, serum cardiac markers; c, oxidative stress markers; d, histopathological examinations; e, electrocardiogram; f, survival, body weight, heart weight.

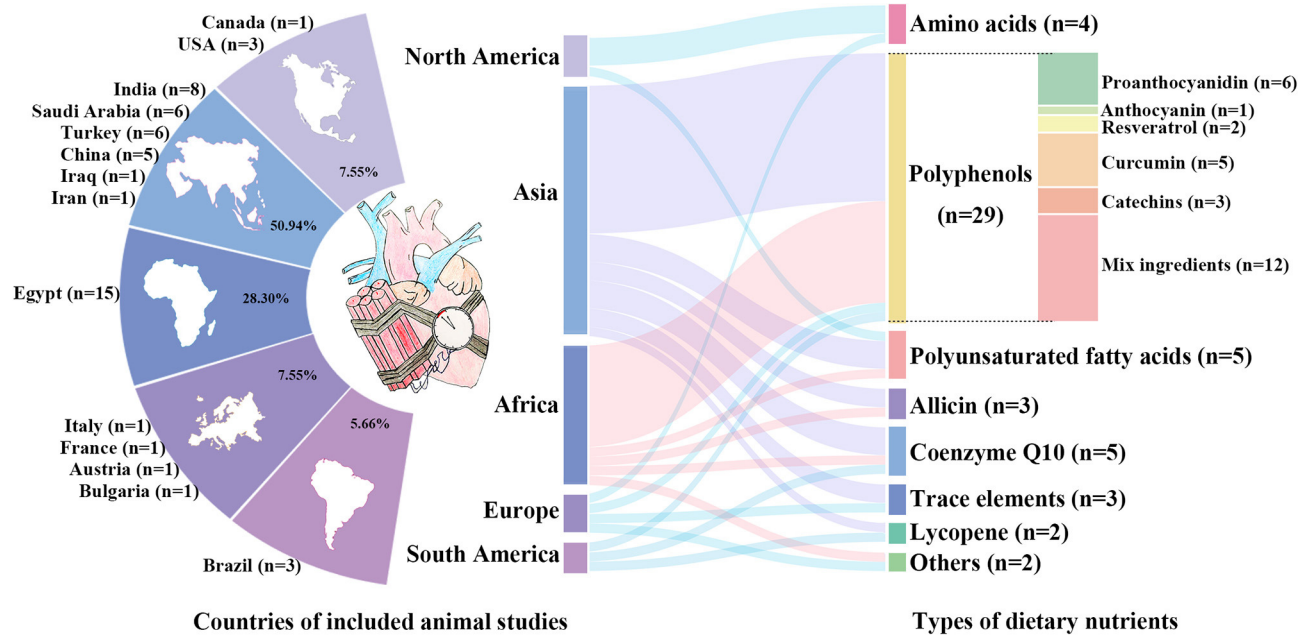

FIGURE 3 | The characteristics of the included animal studies. Distribution of countries of included animal studies are shown in the left. The percentages represent the proportion of included studies in each continent, and the numbers are the number of studies in each country. Among them, African and Asian countries account for the largest share with most studies in Egypt ($n = 15$), India ($n = 8$), Saudi Arabia ($n = 6$), Turkey ($n = 6$), and China ($n = 5$). Types of dietary nutrients are listed in the right, containing polyphenols, allicin, lycopene, polyunsaturated fatty acids, amino acids, coenzyme Q10, and trace elements.

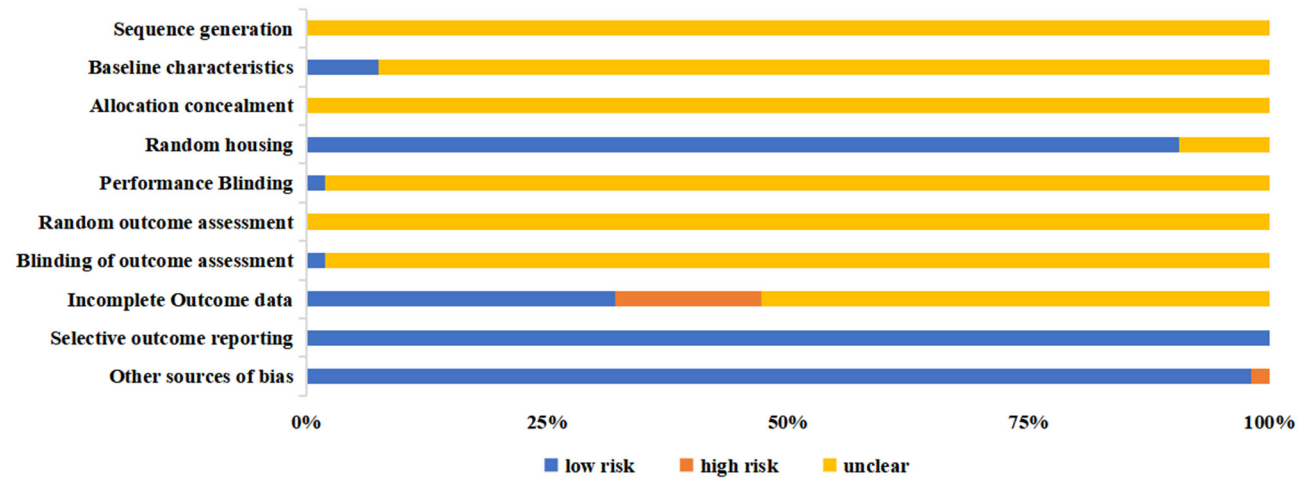

FIGURE 4 | The risk of bias assessment of the included animal studies.

seed oil (92)] and CoQ10 ($n = 2$) (93, 94). The characteristics of the included human studies are summarized in **Table 2.**

Risk of Bias Assessment

The overall risk of bias of included human studies was at moderate to high risk. All items of Cochrane risk of bias tool were rated as low risk in one RCT (91), and three items (blinding of participants and personnel, blinding of outcome assessment, and selective outcome reporting) were rated as unclear risk in another RCT (92). The NOS's scores of the non-randomized trials were 5(93) and 6 (94) respectively, due to the lack of blind evaluation, follow-up and loss to follow up, and the inadequate reports of confounders adjustment. The details of the assessment are presented in **Supplementary Tables 6, 7.**

Effectiveness of Dietary Nutrients

Four studies all showed the cardioprotective effects of the dietary nutrient used in the trials. El Amrousy et al. (91) randomly

TABLE 2 | The characteristics of the included human studies.

Dietary nutrients	Studies	Country	Study design	Participants			Intervention				Comparison		Outcomes
				Cancer type	Gender	Age	Chemotherapeutic agents	Food intake	Main ingredients	Duration	Sample size	Grouping	Control groups	Treated groups	
PUFA	El Amrousy et al. (91)	Egypt	RCT	Acute lymphoblastic leukemia	Male, 36 Female, 24	8.7 ± 1.9 years old	DOX	Omega 3 fatty acids	Omega 3 fatty acids	180 days	60	2	(1) DOX	(2) DOX+omega 3 fatty acids	a, b, c
	Hagag et al. (92)	Egypt	RCT	Acute lymphoblastic leukemia	Male, 25 Female, 15	2-16 years old	DOX	Black seed oil	PUFA (linoleic acid, oleic acid, palmitic acid)	7 days	40	2	(1) DOX+placebo	(2) DOX+black seed oil	a
CoQ10	Iarussi et al. (93)	Italy	Non-randomized controlled trial	Acute lymphoblastic leukemia, non-hodgkin lymphoma	/	1-15 years old	Anthracyclines	CoQ10	CoQ10	/	20	2	(1) anthracyclines	(2) anthracyclines+CoQ10	a
	Cortes et al. (94)	USA	Non-randomized controlled trial	Bronchogenic carcinoma, other carcinoma	Male, 11 Female, 7	56.87 years old	DOX	CoQ10	CoQ10	150 days	18	2	(1) DOX	(2) DOX+CoQ10	a, d

CON, control; CoQ10, coenzyme Q10; DOX, doxorubicin; PUFA, polyunsaturated fatty acids; RCT, randomized controlled trial.
a: echocardiography; b: serum cardiac markers; c: oxidative stress markers; d: electrocardiograph.

divided the children with newly diagnosed ALL into two groups of 30 each. Children in intervention group received 1,000 mg omega 3 fatty acids capsule per day after the administration of DOX for 6 months, and children in control group received the DOX alone. The left ventricular systolic function was preserved in children who took omega 3 for 6 months, while the children in control group experienced significant impairments of cardiac function. Similarly, significantly lower MDA level and higher GSH and SOD levels of children in intervention group revealed that omega 3 fatty acids could decrease the early cardiac damage induced by DOX. Hagag et al. (92) recruited 40 ALL pediatric patients under DOX therapy, including 20 patients treated with black seed oil campus for 1 week and 20 patients treated with equivalent dose of placebo for the same amount of time. A larger reduction in parameters of systolic function arose in children with placebo compared to those with black seed oil. Iarussi et al. (93) carried a controlled trial on 20 children with ALL treated with anthracyclines, consisting of 10 patients with CoQ10 oral therapy and 10 without. Septum wall motion abnormalities were only detected in patients without CoQ10, which demonstrated prophylactic effects of CoQ10 on myocardial function from chemotherapeutic cardiotoxicity. Cortes et al. (94) enrolled 93 consecutive patients with advanced carcinoma to detect DOX-induced cardiotoxicity and the protective effects of CoQ10. Only 10 patients treated with DOX alone for more than 5 months and 8 patients treated with DOX and CoQ10 for more than 5 months were evaluated by systolic time intervals (STI). The mean of serial STIs in ten patients with DOX alone gradually increased during the course of DOX therapy and two patients had CHF. However, STIs were improved in eight patients with DOX and CoQ10 and only one patient had CHF. The effectiveness of dietary nutrients and the details of outcomes are summarized in **Supplementary Figure 8** and **Supplementary Table 8**.

DISCUSSION

Our systematic review included 57 studies published in 14 countries from 1978 to 2021 consisting of 53 animal studies and four human studies, and summarized the cardioprotective effects of dietary nutrients derived from food on target subjects treated with chemotherapy. The descriptive synthetic evidence demonstrated that seven types of dietary nutrients (polyphenols, allicin, lycopene, PUFA, amino acids, CoQ10, and trace elements) might alleviate cardiovascular toxicity induced by chemotherapeutic agents.

As post-mitotic cells, cardiomyocytes are more sensitive to free radical damage due to their high oxidative metabolism and low antioxidant defense level (24). As a result, clinical and subclinical cardiac injuries related with chemotherapy have been a notorious issue. The incidence rates of CHF caused by anthracyclines and cyclophosphamides range 0.14–48% and 7–28%, respectively (6, 95). The childhood cancer survivors are 15 and 10 times more likely to suffer CHF and coronary artery disease, respectively than their siblings (96, 97). As early as in 1967, Tan et al. (98) first described the anthracycline-induced cardiotoxicity and reported that the development of

tachycardia, arrhythmia and CHF in daunomycin patients could be associated with daunomycin. Simultaneously, it was found that cardiovascular toxicity was dose-dependent with a 5% incidence of cardiomyopathy at a cumulative dose of 400 mg/m^2 of anthracyclines, 26% at a cumulative dose of 550 mg/m^2 and up to 48% at 700 mg/m^2 (99). That is the reason why the recommended cumulative dose is limited to 450–500 mg/m^2 (100). Currently, cardiotoxic effects led by anthracyclines, especially DOX, have been most thoroughly studied (7, 19). And this is consistent with our review, in which 90.6% of the included animal studies generated cardiac dysfunctions by the injection of DOX in rats/mice. The most widely proposed mechanism is the anthracyclines' inhibition of topoisomerase 2β, which leads to promote cell apoptosis and generate oxidative damage in cardiomyocytes (13). At present, cardiotoxicity is a broader term without a formal definition (7, 101). The American Society of Echocardiography defines it as a ≥10% drop of LVEF from baseline or the absolute value <53% (101). 2016 European Society of Cardiology Position Paper considers the lower limit of normal LVEF as 50% (102). A clinical trial conducted on pediatric patients with acute myeloid leukemia also defined cardiotoxicity as LVEF <50% on the basis of the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3) definitions (8). However, significantly abnormal cardiac parameters were considered as cardiotoxicity in most of the studies included in our review. Similarly, improved measurements or even back to normal was recognized as the signs of positive cardioprotective effects of nutritional intervention.

The relevant guideline recommended that oncologists considered prevention against chemotherapy-induced cardiotoxicity through long-term management during the early stage of anticancer treatment with support from cardiologists (6). Compared with dexrazoxane, dietary nutrition is more accessible at ordinary times and easier to comply with in long-term survivals. In other words, it can meet the two main advantages of daily and long-term usage. Consequently, it is an additional prevention measure that cancer survivors cannot miss. While the nutritional support has been depicted to improve the adverse effects of chemotherapy (103–105), the current evidence against cardiotoxicity was limited due to the lack of enough clinical studies (29, 30). In addition, the guidelines did not report in detail the aspect of nutrition against cardiomyopathy associated with cancer chemotherapy (6, 20, 96). Several published reviews introduced the application of nutritional intervention in the prevention of cardiac toxicity and covered CoQ10, grape seed extract and ω-3 PUFA (27, 28, 106). But none of them systematically summarized the effectiveness of overall dietary nutrients in this respect. Koss-Mikołajczyk et al. (29) showed that natural products (including fruit, vegetables, herbs, mushrooms, and phytochemicals) could counteract cardiac injury caused by DOX. Despite the comprehensive list of products included in this study, these were all edible plant extracts and foodborne phytochemicals. Nutrients derived from animal food as cardioprotective agents have not been explored. Therefore, our review summarized the current available evidence and filled in the corresponding gaps.

Seven types of dietary nutrients were represented in our review. Among them, polyphenols were in more than half of the included studies possibly due to more than 8,000 species in nature (including flavonoids and non-flavonoids) (107, 108). Polyphenols can eliminate oxygen free radicals by owning multiple PhOH (107) and the oxidation resistance has also made itself as a toxicity-related preventive strategy in some reviews (24, 26, 109). Thus, the extract of fresh fruit (rich in flavonols and flavonoids), grape seeds (rich in proanthocyanidin), and green tea (rich in catechins) were commonly used to ameliorate the chemotherapy-related cardiac damage in the included studies. Besides vegetable food, animal food was also made clear to protect the heart exposed to chemotherapy. PUFA are dietary factors with multiple beneficial effects and could likely protect cardiovascular tissues by adjusting cellular processes and molecular pathways (27, 110). The amino acids can preserve myocardial high-energy phosphate levels and prevent lactate accumulation. Our study refers to glutamine and glycine, which involve GSH synthesis (a vital intracellular antioxidant) (111). CoQ10 is a free-radical scavenger primarily present in metabolically active organs, such as the heart, liver, and kidney (82). Zn has a critical role in maintaining health, primarily through antioxidative stress and anti-inflammation, by catalyzing more than 300 enzymes and binding with over 2,500 proteins. Se prevents oxidative stress and maintains antioxidant enzymes such as the four glutathione peroxidases (GPx) (112, 113).

There were several limitations in our systematic review. First, 93.0% of the included records (53/57) were animal studies along with a relatively moderate to high risk of bias, so the interpretation of results should be more cautious. Second, our findings may still remain a certain distance approaching the clinical application due to the majority of the included studies being animal research. Third, due to the lack of standardization in definition of cardiotoxicity and the high heterogeneity from the variations of included studies, it was a pity that we couldn't provide pooled results in our review.

CONCLUSION

Early prevention and management of cancer chemotherapy-induced cardiotoxicity have been increasingly focused due to the attention to event-free survival during and after cancer therapy. The existing studies have indicated that cardiotoxicity not only puts the patients under high risk of suffering cardiac deterioration but also develops as a social issue concerning the increase of Health System spending (114). The evidence of dietary nutrients against cardiovascular toxicity was still lacking. Our systematic review demonstrated that dietary nutrients (comprising polyphenols, allicin, lycopene, PUFA, amino acids, CoQ10, Zn, and Se) may be a potential strategy to protect cardiovascular system exposed to the chemotherapeutic agents, but more human studies are needed in future. On this basis, the development of cardioprotective strategies for special population, like children,

the pregnant, and the elderly, is now essential for the reason that their vulnerable physical conditions demand much more cardiac protection.

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.

AUTHOR CONTRIBUTIONS

K-WJ, QW, and X-NL contributed to the conception and design of the study. X-YZ and K-LY carried out the search strategy independently and wrote the draft manuscript. YL and YZ contributed to the analysis of the included studies. All authors are responsible for the final content of the manuscript 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.2022.921609/full#supplementary-material

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Association Between the Use of Pre- and Post-thrombolysis Anticoagulation With All-Cause Mortality and Major Bleeding in Patients With Pulmonary Embolism

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Objective: To explore the comparative clinical efficacy and safety outcomes of anticoagulation before (pre-) or following (post-) thrombolytic therapy in systemic thrombolytic therapy for pulmonary embolism (PE).

Methods: PubMed, the Cochrane Library, EMBASE, EBSCO, Web of Science, and CINAHL databases were searched from inception through 1 May 2021. All randomized clinical trials comparing systemic thrombolytic therapy vs. anticoagulation alone in patients with PE and those that were written in English were eligible. The primary efficacy and safety outcomes were all-cause mortality and major bleeding, respectively. Odds ratios (OR) estimates and associated 95% confidence intervals (CIs) were calculated. A Bayesian network analysis was performed using R studio software, and then the efficacy and safety rankings were derived.

Results: This network meta-analysis enrolled 15 trials randomizing 2,076 patients. According to the plot rankings, the anticoagulant therapy was the best in terms of major bleeding, and the post-thrombolysis anticoagulation was the best in terms of all-cause mortality. Taking major bleeding and all-cause mortality into consideration, the most safe–effective treatment was the post-thrombolysis anticoagulation in patients who needed thrombolytic therapy. The net clinical benefit analysis comparing associated ICH benefits vs. mortality risks of post-thrombolysis anticoagulation demonstrated a net clinical benefit of 1.74%.

Conclusion: The systemic thrombolysis followed by anticoagulation had a better advantage in all-cause mortality and major bleeding than the systemic thrombolysis before anticoagulation. The adjuvant anticoagulation treatment of systemic thrombolytic therapy should be optimized.

Keywords: anticoagulation (AC), thrombolysis/thrombolytic agents, all-cause mortality, major bleeding, pulmonary embolism

INTRODUCTION

Pulmonary embolism (PE) commonly occurs in the general community, often resulting in high morbidity and mortality (1–3). Systemic thrombolytic therapy has become an established procedure (4), which can recirculate occluded pulmonary arteries, salvage pulmonary circulation, and reduce mortality. However, a high level of vigilance is needed due to the high frequency of major bleeding complications in patients with systemic thrombolytic treatment. Therefore, the role of systemic thrombolytic therapy remains controversial in non-high-risk/fatal PE. Bleeding can be not only induced by the thrombolytic agent itself but also results from adjunctive therapy with anticoagulation or other risk factors, such as advanced age and hypertension (5). Efforts have been made to adjust the thrombolytic agent or thrombolytic approach to reduce the risk of major bleeding associated with systemic thrombolytic therapy, such as reducing the dose of thrombolytic drugs (6, 7) or catheter-directed thrombolysis (8).

The dynamic balance between thrombosis and thrombolysis is influenced by both optimization of the thrombolysis and the adjunctive antithrombotic therapy. There are two administrations of anticoagulation agents including unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH), which can be started before thrombolysis (pre-thrombolysis anticoagulation) and continuing (9) or started after thrombolysis (post-thrombolysis anticoagulation) according activated partial thromboplastin time (aPTT) (10). The aggressive adjunctive therapy with heparin has been identified as that which increases the risk of major bleeding associated with thrombolytic therapy (11). However, we neglected the effect of the sequence between anticoagulation and thrombolytic therapy on major bleeding. Several randomized, controlled trials have compared the safety and efficiency between heparin and thrombolytic agents in patients with an acute PE (1), but a beneficial effect of pre- and post-thrombolysis anticoagulation on important clinical outcomes is difficult to demonstrate. Therefore, the efficacy and safety of these two anticoagulation strategies of systemic thrombolytic therapy are unclear in patients with acute PE.

To determine whether the treatment effect of thrombolysis with different adjunctive anticoagulation truly exists, we performed this network analysis in the hope of obtaining the optimized anticoagulant therapy of systemic thrombolysis by pooling the results of the available randomized, controlled trials.

METHODS

Search strategy, study selection, data extraction, and analysis of our study were all performed based on a pre-defined protocol (**Supplementary Material 1**).

Search Strategy

PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) statement (12) was referred for a systematic literature review. Two authors (J.S. Tan and N.N. Liu) systematically performed an electronic literature search in PubMed, the Cochrane Library, EMBASE, EBSCO, Web of Science, and CINAHL databases (reported the outcomes within 30 days or in hospital, written in English and published from inception through 1 May 2021; **Supplementary Methods 1**). All the randomized controlled trials were included, which compared a thrombolytic agent [desmoteplase, recombinant tissue plasminogen activator (alteplase), reteplase, streptokinase, tenecteplase, or urokinase] administered systemically by the i.v. route and heparin (low-molecular-weight heparin, unfractionated, fondaparinux, or vitamin K antagonist) with heparin alone in patients with PE. To get a literature search as comprehensive as possible, reference lists from retrieved articles and reference literature (including systematic reviews and guidelines) were examined (**Figure 1**).

Study Selection and Data Extraction

All randomized controlled trials comparing thrombolytic therapy with anticoagulation alone (**Supplementary Methods 2**) in patients with PE were included. We excluded the studies using mechanical thrombectomy along with local catheter-delivered thrombolysis or thrombolytic treatment or those just comparing two regimens of thrombolytic therapy. The possible trials were independently evaluated by two authors (J.S. Tan and N.N. Liu). We excluded the non-relevant studies by screening the title and abstract. The full text was independently screened by two authors (J.S. Tan and N.N. Liu) to assess the study eligibility and they extracted related data (study design and patient characteristics) according to the pre-designed protocol. Once a disagreement about study inclusion or data extraction occurred, it would be resolved by consensus or by a discussion with another author (Dr. Hua).

Outcomes and Measurements

All the outcomes that occurred within 30 days or in the hospital were recorded in the present study. The primary efficacy and safety outcomes were all-cause mortality and major bleeding events, respectively. The secondary safety outcomes were intracranial hemorrhage (ICH). Recurrent PE (confirmed by a validated diagnostic examination) and composite outcomes (including major bleeding, recurrent PE, and all-cause mortality; **Supplementary Methods 3**) were considered the secondary efficacy outcomes.

The definition of major bleeding refers to the International Society of Thrombosis and Hemostasis (ISTH) if sufficient values were available. In other cases, major bleeding was defined according to the original studies. Both trial and patient characteristics, and outcomes were independently extracted from included studies by two authors (J.S. Tan and N.N. Liu).

As is shown in **Figure 2**, all-cause mortality was evaluated in 15 studies (7, 9, 10, 13–24) that satisfy the inclusion criteria. Reporting of ICH, major bleeding events, recurrence, and comprised outcomes were completed by variable studies, and not every study presented all data.

Study Quality and Risk of Bias Assessment

According to the Cochrane Handbook of Systematic Reviews (25), study quality and the risk of bias were evaluated and

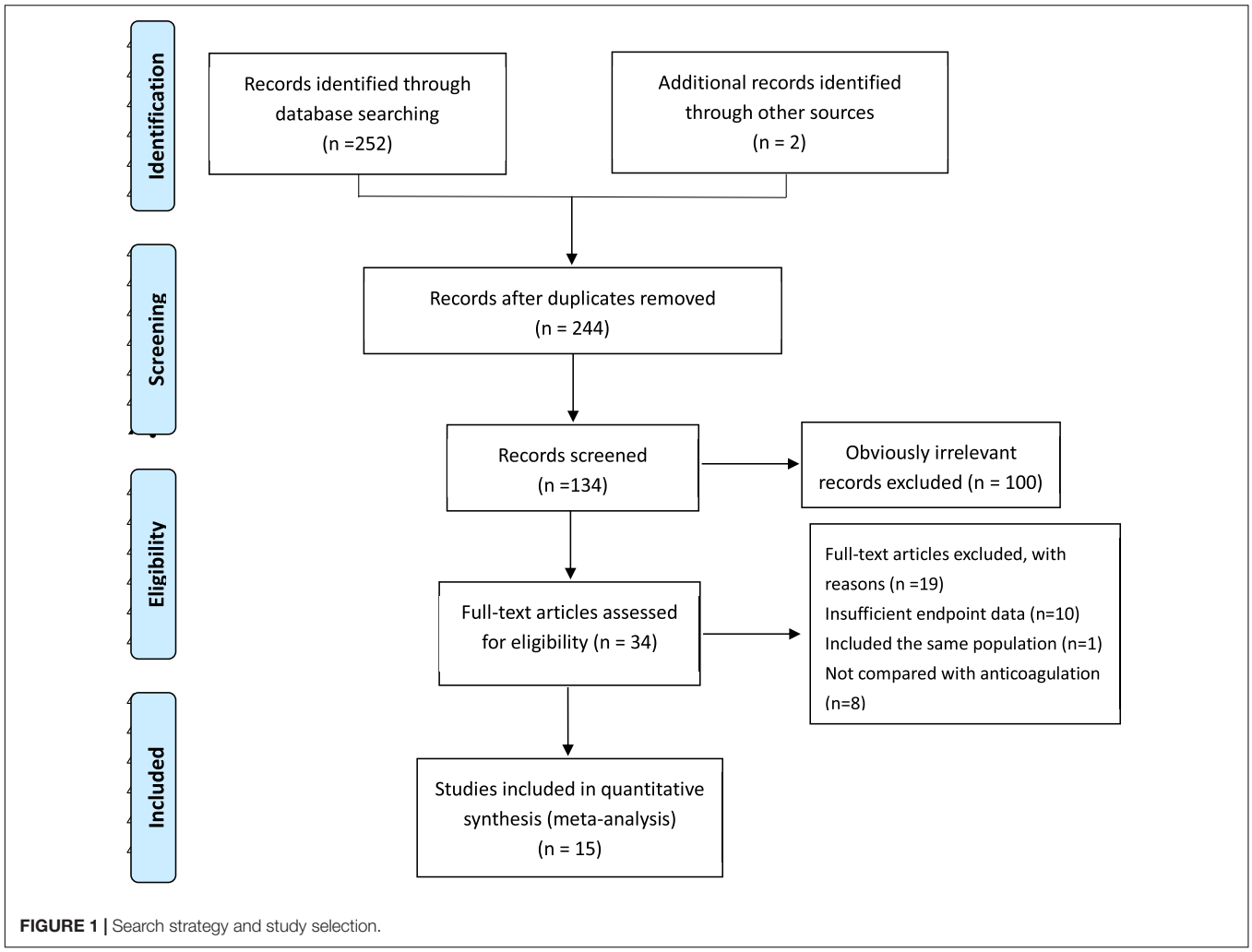

FIGURE 1 | Search strategy and study selection.

they specifically concentrated on the following criteria: (1) proper sequence generation, (2) proper allocation concealment, (3) blinding of the investigator assessing clinical outcomes and the patients, (4) proper outcomes assessment, and (5) short time clinical events recorded during the hospitalization or within 1 month.

Statistical Analysis

Data for further statistical analysis was the intention to treat. The model-used (fixed vs. random-effects) was determined according to the lowest deviance information criterion (DIC) for individual outcomes. Odds ratios (OR) estimates and associated 95% confidence intervals (CIs) were calculated for meta-analysis. We excluded the studies which have 0 events in both arms because they do not contribute to the overall effect. The Bayesian network meta-analysis was performed using R studio software, and the effective and safe treatment rankings were derived.

Sensitivity Analysis

The included trials have been strictly screened by the including criteria. Sensitivity analysis did not repeat for outcomes.

Statistical Heterogeneity and Convergence Assessment

Visual inspection of the forest plots was used to investigate the possibility of statistical heterogeneity, and the I^2 was used to measure heterogeneity (26) [I^2 < 25% was considered mild, I^2 < 75% was moderate, and I^2 > 75% was severe (26)]. Brooks–Gelman–Rubin diagnosis plot and Trace plot were used to diagnose the convergence of the model. Ranking histograms were used to show the ranking possibility for each anticoagulation strategy. In this analysis, a 2-sided $P < 0.05$ was statistically significant. All analyses were performed using R i386 (version 3.2.2, 3 chains were used, including 1,50,000 burn-in iterations followed by 2,00,000 iterations) and SPSS V 24.0 (SPSS Statistics v. 24.0, SPSS Inc.) (**Supplementary Methods 4**).

Net Clinical Benefit

Besides, a net clinical benefit analysis was performed for choosing pre- or post-thrombolysis anticoagulation in systemic thrombolytic therapy for PE. We calculated the short-term risk of ICH (Ti) prevented by post-thrombolysis anticoagulation minus the short-term mortality (Tm) induced by post-thrombolysis

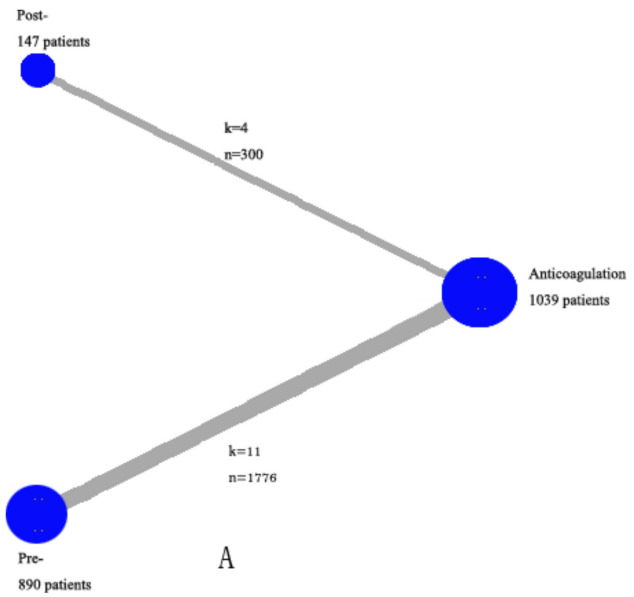

FIGURE 2 | Network of the comparisons for the Bayesian network analysis. The size of the nodes is proportional to the number of patients (in parentheses) randomized to receive the treatment. The width of the lines is proportional to the number of trials (beside the line) comparing the connected treatments. However, we excluded the trials in which both events in the experimental and control groups were 0 in specific analysis. k—number of trials per comparison; n—number of patients per comparison.

anticoagulation. Then, the former was multiplied by a weighting factor of 0.75, suggesting that a single ICH event amounted to 75% of the effect of single mortality. The weighting factor was referred to the related data which demonstrated the serious disability or probability of death owing to ICH (27). The weighting factor was used to provide an accurately and comprehensively conservative estimate of potential benefits associated with post-thrombolysis anticoagulation. The following equation illustrates this definition: net clinical benefit = weighing factor × (Ti_{pre-} − Ti_{post-}) − (Tm_{post-} − Tm_{pre-}) (1).

RESULTS

Study Search and Study Characteristics

Overall, 367 records were identified through database searching and 34 were considered eligible through title and abstract with 15 Randomized controlled trials (RCTs) in the final meta analysis.

As is shown in **Figure 2**, all-cause mortality was evaluated in 15 studies (7, 9, 10, 13–24) that met our inclusion criteria. The detailed selection process is shown in **Figure 1**. Totally, 2,076 patients were enrolled in our analysis. Eleven trials were defined as the pre-thrombolysis anticoagulation group, anticoagulation before thrombolytic therapy in PE. The other trials, anticoagulation following thrombolytic therapy, were defined as post- group (**Figure 2**). The baseline characteristics for every single trial are shown in **Table 1**.

Risk of Bias and Publication Bias

Of the 15 included trials, 7 (46.67%) were assessed as low risk of bias in all the domains. Two (13.33%) were at a high risk of bias for blinding and one (6.67%) for allocation concealment. No studies were at high risk of bias for sequence generation, detection bias, and attrition (**Supplementary Figure 1** and **Supplementary Table 2**). No evidence was proved of publication bias (**Supplementary Figure 2**).

Primary Efficiency Outcome: All-Cause Mortality

For all-cause mortality, 15 studies reported at least 1 event in any group and 2,076 enrolled patients. There were 64 deaths: 17 (1.91%) of 890 patients in the pre-group, 7 (4.76%) of 147 patients in the post- group, and 43 (4.14%) of 1,039 in the anticoagulation group. Compared with the anticoagulation group, both pre-group [OR, 0.490 (0.080, 2.300)] and post- group [OR, 0.120 (0.002, 1.100)] were not associated a difference in all-cause mortality (**Figure 3**).

Primary Safety Outcome: Major Bleeding Events

For major bleeding events, 11 studies reported at least 1 event in any group and 1,814 enrolled patients. There were 130 major bleeding events: 69 (9.11%) of 757 patients in the pre-group, 25 (17.48%) of 143 patients in the post- group, and 36 (3.94%) of 914 in the anticoagulation group. Both pre- [OR, 2.400 (0.810, 6.600)] and post-group [OR, 1.500 (0.290, 5.900)] were not associated with a significant difference (**Figure 3**) when compared with anticoagulation alone.

Secondary Outcomes

Secondary outcomes were not reported in all trials. The detailed analysis results are shown in **Figures 3**, **4**.

Second Efficiency Outcome: Recurrence and Comprised Outcomes

For recurrence, the pre-group [OR, 0.013 (0.025, 0.420)] significantly decreased the risk, but no statistical significance was observed in the post-group [OR, 0.310 (0.017, 1.300); **Figure 3**] when compared with anticoagulation alone. Compared with anticoagulation alone, both pre-group [OR, 0.630 (0.160, 2.000)] and post-group [OR, 0.270 (0.023, 1.500)] were not associated with a difference in comprised outcomes (**Figure 3**).

Secondary Safety Outcome: Intracranial Hemorrhage

Five studies reported at least 1 event in any group and 1,283 patients were enrolled in the ICH analysis. In all, 36 patients were with ICH: 29 (4.88%) of 594 patients in the pre-group, 0 (0%) of 46 patients in the post- group, and 7 (1.09%) of 643 in the anticoagulation group. Due to the small sample size and 0 events in the post-group, we were limited in any further statistical analysis.

TABLE 1 | Baseline characteristics of trials.

Source	No. of patients	Age, mean (Range or SD)	Male, No. (%)	Type of PE	Thrombolysis	Comparator	Pre- or Post-anticoagulation	Major bleeding criteria	Follow-up[1] (d)	Outcomes[3]
A Cooperative Study (38)	160	45.0 (<50), 55.0 (>50)	92 (57.3)	All	Urokinase (2,000 U/lb, then 2,000 U/lb/h for 12 h)	Heparin	Post-	Hematocrit	14	ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Ly et al. (24)	25	53.2 (23–70)	11 (44.0)	All	Streptokinase 72 h	Heparin	Pre-	Not pre-specified	10	ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Becattini et al. (15)	58	68.2 (4.3)	23 (39.7)	Stable	Tenecteplase (30–50 mg) plus heparin	Heparin	Pre-	Bleeding need transfusion, surgical control or fatal or ICH	30	ICH, ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Dotter et al. (23)	31	18–85[a]	12 (38.7)	All	Streptokinase (250,000 IU in 5% dextrose/20–30 min, followed 100,000 IU/hour for 18–72 h)	Heparin	Post-	Not pre-specified	DH[2]	ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Dalla-Volta et al. (18)	36	64.7 (12.5)	12 (33.3)	Stable	Alteplase (100 mg/2 h) plus	Heparin	Pre-	ICH or ≥ 1(units PRBCs transfusion	30	ICH, ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Fasullo et al. (16)	72	56.0 (16.1)	41 (56.9)	Stable	Alteplase (100 mg)	Heparin	Pre-	Bleeding need transfusion, surgical control or fatal or ICH	10	ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Goldhaber et al. (10)	101	58.5 (16.9)	44 (44)	Stable	Alteplse (100 mg)		Post-	ICH, need for surgery	14	ICH, ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Jerjes-Sanchez et al. (13)	8	51.0 (22.9)	5 (63)	All	Streptokinase (1,500,000 IU)	Heparin	Post-	Not pre-specified	30	All-cause Mortality, Comprised outcome
Kline et al. (17)	83	55.4 (14.0)	49 (59.0)	Stable	Tenecteplase (30–50 mg/2 h)	LMWH	Pre-	Not pre-specified	5	ICH, ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Konstantinides et al. (19)	256	62.1 (10.5)	122 (47.6)	Stable	Alteplase (100 mg/2 h)	Heparin	Pre-	Fatal, hemorrhagic stroke, hemoglobin drop ≥ 4 g per deciliter.	DH	ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
Levine et al. (20)	58	60.7 (3.2)	29 (50.0)	Stable	Alteplase (0.6 mg/kg/2 min of ideal body weight)	Heparin	Pre-	Hemoglobin drop > 20 g/L [≥ 2(units PRBCs, retroperitoneal or ICH)]	10	All-cause Mortality, Comprised outcome
Meyer et al. (9)	1,005	66.2 (15.3)	473 (47.1)	Stable	Tenecteplase (30–50 mg)	Heparin	Pre-	Bleeding need transfusion, surgical control and fluid replacement or fatal.	7	ICH, ECH, Major, All-cause Mortality, Recurrence, Comprised outcome
PIOPED Investigators (21)	13	59.3 (16.2)	9 (75.0)	Stable	Alteplase (40–80 mg)	Heparin	Pre-	Not pre-specified	30	ECH, Major, All-cause Mortality
Sharifi et al. (7)	121	58.5 (9.5)	55 (45.5)	Stable	Alteplase (50 mg/2 h)	Heparin or LMWH	Pre-	Not pre-specified	DH	All-cause Mortality, Comprised outcome
Taherkhani et al. (14)	50	55.7 (12.4)	20 (40.0)	Stable	Alteplase (100 mg/90 min) or Streptokinase (1,500,000 u/2 h)	Enoxaparin	Pre-	Fatal, hemorrhagic stroke, hemoglobin drop ≥ 4 g per deciliter	DH	All-cause Mortality, Comprised outcome

[1]The follow-up days did not mean the whole follow-up time in the articles, it just meant the shortest recording events' time in their articles. It often means during the hospital stay or the recorded events' time, which is no more than 1 month.

[2]"DH" means the follow-up was finished during hospitalization.

[3]Outcomes means those events of this trial were counted in the calculation. ICH, intracranial hemorrhage; ECH, extracranial hemorrhage; Mod, intermediate risk (hemodynamically stable with objective evidence of right ventricular dysfunction). [a]Only age range is available but without mean age.

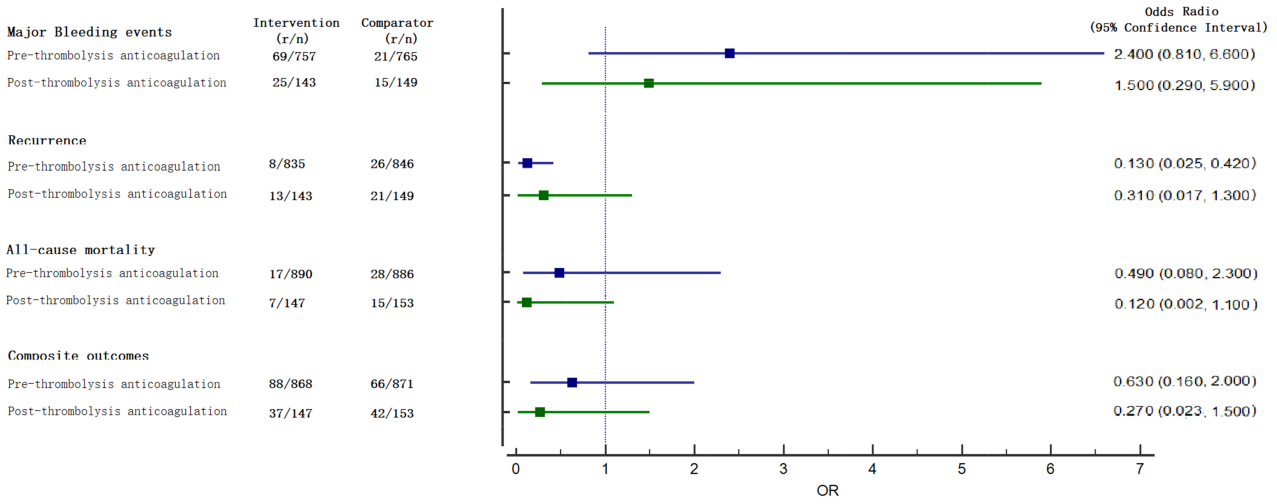

FIGURE 3 | Forest plots for relative effect as compared with anticoagulation.

Strategy Class Rankings

Figure 4 shows the ranking probabilities of each treatment in the 3 possible positions. As is shown in **Figure 4**, recommended ranking in all-cause mortality: post-thrombolysis anticoagulation > pre-thrombolysis anticoagulation > anticoagulation alone; major bleeding: anticoagulation alone > post-thrombolysis anticoagulation > pre-thrombolysis anticoagulation; recurrent PE: pre-thrombolysis anticoagulation > post-thrombolysis anticoagulation > anticoagulation alone; composite outcome: post-thrombolysis anticoagulation > pre-thrombolysis anticoagulation > anticoagulation alone. Post-thrombolysis anticoagulation was the most beneficial treatment just in consideration of all-cause death (0.81) and combined end-point events (0.79). Based on the ranks of effectiveness and safety, the post-thrombolysis anticoagulation was the best in terms of both major bleeding and all-cause mortality (**Figure 5**).

Heterogeneity and Convergence Assessment

Brooks-Gelman-Rubin diagnosis plot (**Supplementary Figure 4**) and Trace plot (**Supplementary Figure 5**) showed that the convergence of the model was good. Heterogeneity test results showed that heterogeneity was low or acceptable, except for combined outcomes.

Net Clinical Benefits

The net clinical benefit analysis comparing associated ICH benefits vs. mortality risks of post-thrombolysis anticoagulation demonstrated a net clinical benefit of 0.0174 (0.0001, 0.0365). This means the net clinical benefit analysis comparing associated ICH benefits vs. mortality risks of post-thrombolysis anticoagulation demonstrated a net clinical benefit of 17.4‰.

DISCUSSION

To our knowledge, there are no RCTs focused on this topic up to now, and this is the first study to explore the efficiency and safety of systemic thrombolysis with pre-thrombolysis anticoagulation or post-thrombolysis in unselected patients with acute PE. In the recommended ranking, systemic thrombolysis followed by anticoagulation was the most beneficial treatment in consideration of all-cause death and combined end-point events, demonstrating a net clinical benefit of 17 fewer deaths per 1,000 people when compared with systemic thrombolysis before anticoagulation.

Major bleeding is an important and apprehensive conundrum for clinicians when choosing thrombolytic therapy in patients with PE. Several meta-analyses have assessed the risk of major bleeding associated with thrombolysis in patients with PE (1, 28, 29). Thabut et al. showed that thrombolytic therapy did lead to a near doubling in the rate of major hemorrhage with a significant reduction in mortality or the recurrence of PE as compared with heparin when administered to unselected patients with acute PE (30). Chatterjee et al. also showed that thrombolytic therapy was associated with lower rates of all-cause mortality but increased risks of major bleeding and ICH among patients with PE (1). Thrombolytic therapy may help reduce mortality but may cause major hemorrhagic events and stroke (31). It should be pointed out that all these previous meta-analyses included the clinical trials of systemic thrombolysis with pre-thrombolysis and post-thrombolysis anticoagulation.

The thrombolytic therapy of PE has followed a similar path to that of myocardial infarction (MI), including adjunctive anticoagulation therapy (9). Heparin should not be infused concurrently with streptokinase or urokinase. For tPA or reteplase, concurrent use of heparin is optional (32). In clinical practice, systemic thrombolysis with pre-thrombolysis anticoagulation was the favored thrombolytics treatment. Eleven clinical trials of the total fifteen trials of our study

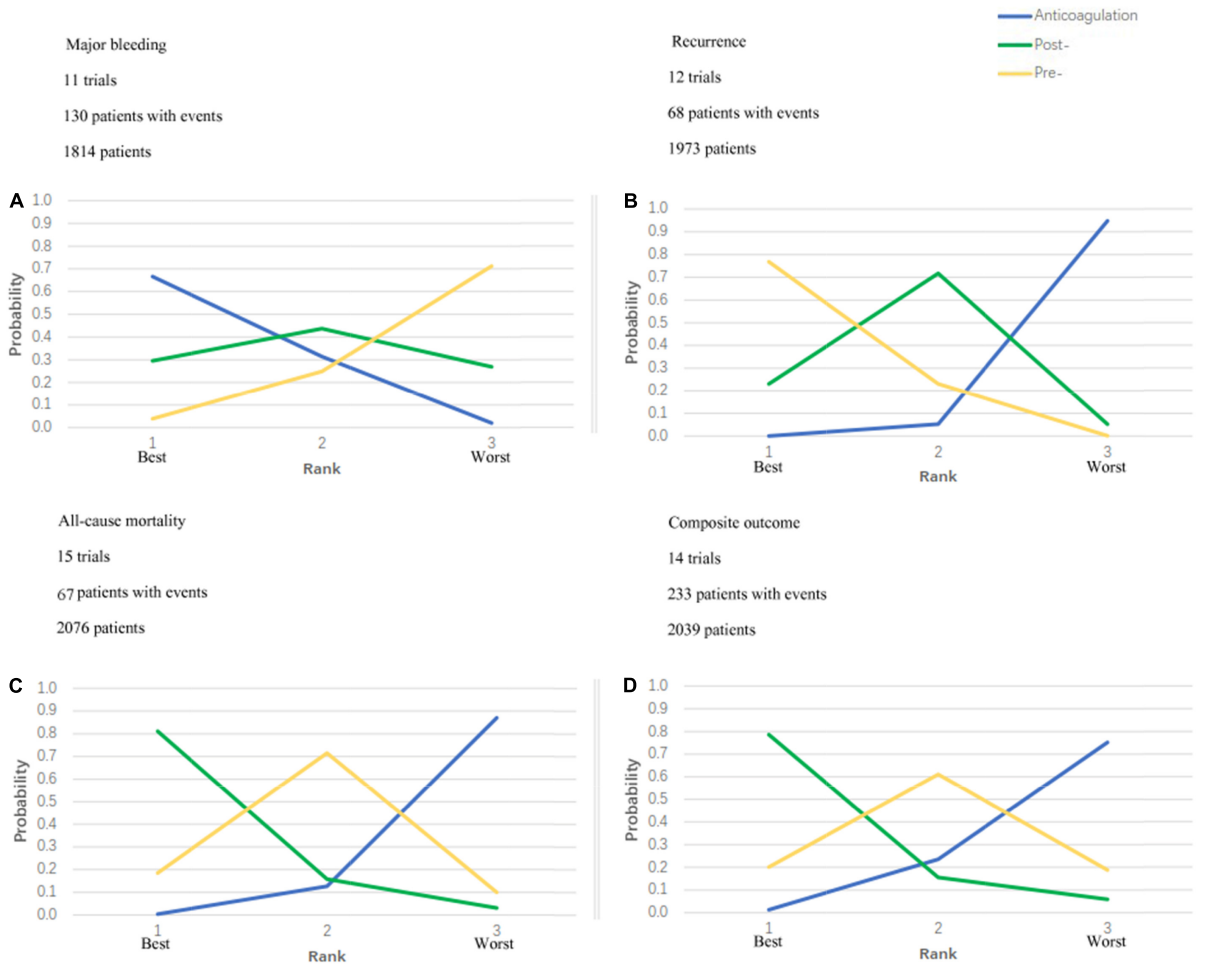

FIGURE 4 | Ranking plots. Strategy ranking plots for primary and secondary outcomes are stratified by treatment. **(A)** Is the ranking plot for major bleeding; **(B)** is the plot for recurrence; **(C)** is the plot for all-cause mortality and **(D)** is the plot for composite outcome. Each line represents 1 strategy and shows the probability of its ranking from best to worst. The peak of the line represents the rank that the strategy is most likely to be for each given outcome. For example, for all-cause mortality, post- thrombolytic anticoagulation is most likely to rank best; pre- thrombolytic anticoagulation, second best; and anticoagulation, worst.

selected pre-thrombolysis anticoagulation while only four trials selected post-thrombolysis anticoagulation. But the hemorrhagic complications of thrombolytic therapy were higher in PE than that in MI (11). One hypothesis to explain the higher rate of hemorrhagic complications following thrombolytic therapy in patients with PE was that venous congestion and an increase in central venous pressure could increase the bleeding risk when PE induces acute cor pulmonale with hemodynamic compromise (33). However, this is just a hypothesis, and there is no strong evidence to support it. Besides, in a patient with ST-elevation infarction, due to the use of heparin, antiplatelet agents, and thrombolytic therapy, the trend of physicians is to avoid punctures in major veins. However, this will not happen in PE where patients are taken for punctures to place a central line and for arterial blood gases, which sometimes includes punctures in the femoral arteries. Therefore, the involvement of arterial and venous punctures may be another possible mechanism of hemorrhagic complications. Furthermore, the well-known risk factor for hemorrhagic complications, liver dysfunction, which induces clotting disorders (34) caused by liver injury due to a combination of arterial hypoxemia, low cardiac output, and liver congestion could be a major factor for the risk of bleeding in patients with acute cor pulmonale and circulatory failure (35). Therefore, we should reexamine the adjunctive anticoagulation therapy of systemic thrombolysis in PE to decrease bleeding events.

We have made the first try to analyze whether pre- and post-thrombolysis anticoagulation could make a difference for patients with PE. Our results revealed that the systemic thrombolysis with post-thrombolysis anticoagulation reduced both all-cause mortality and combined endpoints (**Figure 4**) when compared with anticoagulation alone and systemic thrombolysis with pre-thrombolysis anticoagulation in the ranking plots. Although systemic thrombolysis with post-thrombolysis anticoagulation increased the risk of major bleeding when compared with anticoagulation alone, it is noteworthy that post-thrombolysis anticoagulation reduced the risk when compared with pre-thrombolysis anticoagulation. The

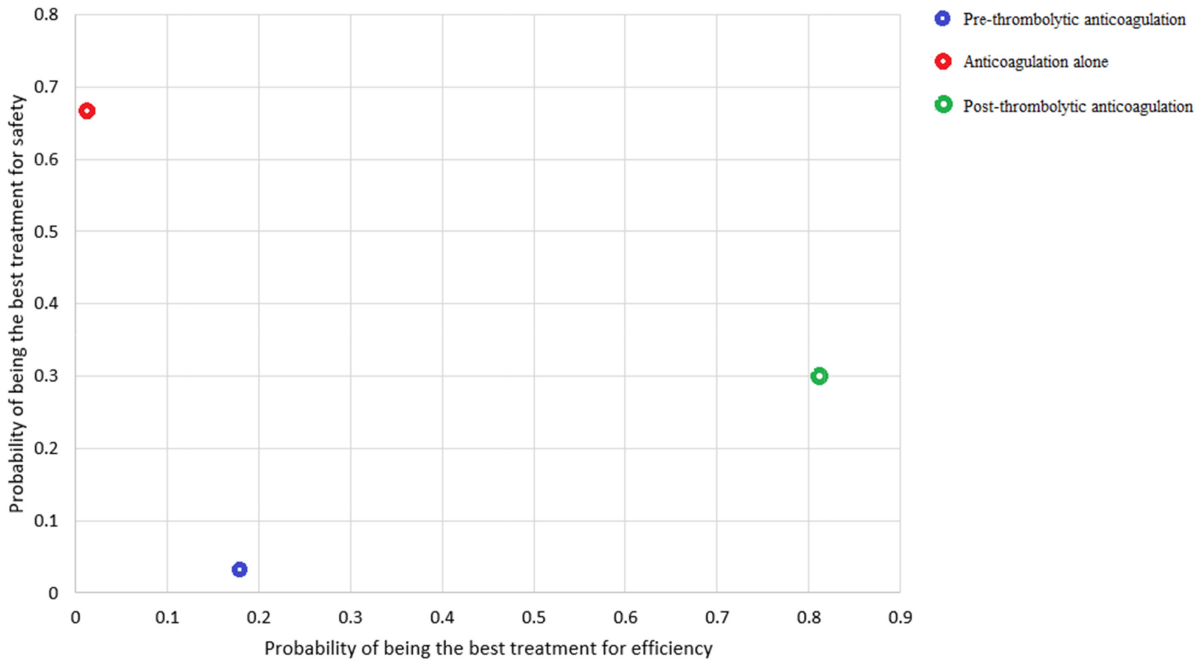

FIGURE 5 | Ranking plot in consideration of efficiency and safety.

international PEITHO (Pulmonary Embolism Thrombolysis) trial (9) enrolled 1,006 patients (506 patients in the tenecteplase group and 499 in the placebo group) with confirmed PE and concluded that in patients with intermediate-risk PE, fibrinolytic therapy could reduce the risk of hemodynamic decompensation, but great caution should be warranted given an increased risk of major hemorrhage and stroke. However, it is worth noting that the anticoagulant administration was started immediately after randomization (also referred to as pre-thrombolysis anticoagulation in our study) in the PEITHO study. In the present meta-analysis, ICH occurred in 29 (4.88%) of the 594 patients in the pre-thrombolysis anticoagulation group, but none occurred in the post-thrombolysis anticoagulation group. In combination with the ranking plots of major bleeding and all-cause mortality, post-thrombolysis anticoagulation seems more favorable than pre-thrombolysis anticoagulation.

In brief, two anticoagulation strategies had differences in safety and effectiveness. If the results of our meta-analysis are confirmed by future randomized clinical trials, there may be a shift in the adjuvant anticoagulation treatment of patients with PE using thrombolytics. Besides, it is also a challenge for researchers to explore other concomitant anticoagulants with thrombolytics, such as the "direct oral anticoagulants (DOAC)," in hemodynamically stable PE (36). Furthermore, previous studies have revealed that fibrinolytic therapy (FT) in patients with PE could accelerate the reversal of right ventricular dysfunction if the patients were properly selected (10) and the weight-adjusted unfractionated heparin regimen was also regarded as a strategy to reduce bleeding complications (37). Future research should concentrate on the probability to accrue maximal clinical benefits by minimizing the risk of bleeding for intermediate-risk PE.

Our study has several limitations which must be taken into consideration for accurate interpretation of the reported efficiency and safety. Firstly, there are no RCTs to compare efficiency and safety between pre- and post-thrombolysis anticoagulation, which means there may be an unequal distribution of potentially confusing factors, and most importantly, the potential imbalance of risk for bleeding between pre- and post-thrombolysis anticoagulation groups. Although the characteristics of the enrolled patients were seemingly matched with each other, the matching degree of basic data was not strict and accurate as RCTs. However, our data were all collected from the RCTs which concentrated on anticoagulants in conjunction with thrombolytics. There were similarly explicit inclusion and exclusion criteria in different RCTs. Secondly, the bias in sample size among different groups included in the present study exists, and the sample size of post-thrombolysis anticoagulation is small than the other two groups. Thirdly, the anticoagulants (heparin or low molecular weight heparin) and thrombolytic agent (such as urokinase, streptokinase, or rtPA) included in the study were inconsistent. Strict criteria for study selection and proper management for pooled data according to QUORUM guidelines and recognized recommendations were employed to emphasize this issue, and the heterogeneity was tested by summary.anohe plot. The heterogeneity was low or acceptable, except for the I^2 in combined end-point events. The presumptive reason is that the combined end-point event was a collection of heterogeneities, though the heterogeneity in combined end-point events would be very high. Thus, bias is unlikely to occur in patient selection and publication. Fourthly, no solicitude was shown for differences in study quality, as all included studies were considered as moderate

to good methodological quality. Lastly, the protocol was not prospectively registered in PROSPERO.

CONCLUSION

The systemic thrombolysis following anticoagulation had a better advantage in all-cause mortality and major bleeding than the systemic thrombolysis before anticoagulation. Therefore, this meta-analysis suggested that early institution of thrombolysis, whenever indicated (without waiting and hesitating for long periods giving anticoagulation alone), maybe a safer approach to reduce the all-cause mortality and major bleeding. However, this study is hypothesis-generating, and a controlled study is required to know the true participation of pre- and post-thrombolysis anticoagulation in the incidence of hemorrhagic complications.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LH and J-ST: full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis, and study concept and design. J-ST, NL, YW, XG, T-TG, X-XY, F-HP, and SH: drafting of the manuscript. J-ST, NL, YW, and XG: critical revision of the manuscript for important intellectual content. J-ST and NL: statistical analysis. LH, NL, X-XY, F-HP, and SH: administrative, technical, or material support. LH: study 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.2022.880189/full#supplementary-material

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Analysis of Clinical Features of Non-steroidal Anti-inflammatory Drugs Induced Kounis Syndrome

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Background: Current knowledge of Kounis syndrome induced by non-steroidal anti-inflammatory drugs (NSAIDs) is based on case reports. This study aimed to investigate the clinical features of Kounis syndrome.

Methods: Case reports of the NSAIDs-induced Kounis syndrome were analyzed by searching Chinese and English databases from 1 January 1950 to 31 January 2022.

Results: The median age of the 45 included patients (28 women) was 51 years (20–80 years). NSAIDs that were the most frequently involved were diclofenac (26.7%, 12/45), metamizole (15.6%, 7/45), and aspirin (13.3%, 6/45). Kounis syndrome occurred mainly within 30 min after administration, with a maximum latency of 1 month. Chest pain (75.6%, 34/45), dyspnea (33.3%, 15/45), and allergic reactions (44.4%, 20/45) were the most common clinical manifestations. Thirty patients (66.7%) had an ST-segment elevation on the electrocardiogram. Echocardiogram and coronary angiography showed abnormalities in 21 patients (75%, 21/28) and 15 patients (37.5%, 15/40). Forty-four patients (97.8%) had a good prognosis after treatment with steroids, antihistamines, and vasodilators.

Conclusion: The possibility of Kounis syndrome should be considered in the presence of coronary artery disease symptoms when taking NSAIDs. Kounis syndrome can be life-threatening. It is essential to identify and treat Kounis syndrome correctly.

Keywords: Kounis syndrome, anaphylaxis, coronary vasospasm, allergic reaction, non-steroidal anti-inflammatory drugs

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INTRODUCTION

Kounis syndrome is a rare acute coronary syndrome (ACS) caused by an allergic reaction that was first described as "anaphylactic angina syndrome" in 1991 (1). The main clinical signs and symptoms of Kounis syndrome are associated with allergic reactions accompanied by cardiac symptoms. Kounis syndrome can be induced by drugs, food, environmental exposures, and other conditions (2). Furthermore, Kounis syndrome causes coronary spasms and affects the cerebral and mesenteric arteries (3, 4).

The true incidence of Kounis syndrome is unknown. Many cases may be missed or underdiagnosed due to their variable presentation and the ignorance of the physician. Epidemiological studies are lacking to determine its prevalence. The unique clinical manifestations and treatment of Kounis syndrome have attracted clinical attention.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs with antipyretic, analgesic, and anti-inflammatory effects. NSAIDs are associated with allergic reactions (5), and the incidence of hypersensitivity reactions is second only to antibiotics (6). Despite the widespread use of NSAIDs, NSAIDs-induced Kounis syndrome is uncommon.

Current knowledge of NSAIDs-induced Kounis syndrome is based on case reports. Furthermore, the syndrome is rarely recognized or reported in clinical practice due to incorrect diagnosis. This study aimed to investigate the clinical characteristics of NSAIDs-induced Kounis syndrome and provide evidence for clinical diagnosis and treatment.

MATERIALS AND METHODS

Retrieval Strategy

We searched the Chinese and English databases from 1 January 1950, through 31 January 2022, including Wanfang, CNKI, VIP, PubMed, Embase, the Cochrane Library, and Web of science. The combination of subject headings and free texts was used for searching. English search terms included: NSAIDs, anaphylaxis, hypersensitivity, coronary vasospasm, cardiac arrest, allergy, Kounis syndrome, non-steroidal anti-inflammatory drugs, various approved non-steroidal anti-inflammatory drugs, myocardial infarction, acute coronary syndrome, and chest pain. We performed an initial evaluation of the titles and abstracts of the articles and read the full texts of all potentially eligible articles. References from studies were checked to identify additional eligible studies.

Inclusion and Exclusion Criteria

Case reports and case series were included. Reviews, animal studies, mechanistic studies, and duplicate cases were excluded.

Data Collection

Two authors independently extracted relevant clinical data according to self-designed tables, including patient country, gender, age, allergy history, past disease history, type and route of administration of NSAIDs, clinical symptoms, laboratory tests, imaging examination, treatment, and prognosis.

Subtypes of Kounis Syndrome

Three variants of Kounis syndrome have been identified (7). The type I variant occurs in patients with structurally normal coronary arteries without cardiovascular risk factors. The acute release of inflammatory mediators induces coronary vasospasm, which may or may not result in acute myocardial infarction. The type II variant occurs in patients with preexisting coronary artery disease. The acute release of inflammatory mediators induces coronary vasospasm, leading to plaque rupture and myocardial infarction. The type III variant occurs in patients with a coronary artery stent. The release of inflammatory mediators can result in stent thrombosis. In this analysis, the type of Kounis syndrome was classified.

Statistical Analysis

Statistical analysis was performed using SPSS 22.0. Descriptive analysis was performed on the extracted data. The measurement data are represented by the median value (minimum, maximum). Enumeration data are expressed as percentages.

RESULTS

Identified Studies

According to the inclusion and exclusion criteria, after independent screening by two authors, 41 articles were included, all of which were case reports. The literature screening is shown in **Figure 1**. The bias assessment of the case reports was evaluated using the National Institute for Clinical Excellence (NICE) quality scale. The quality of the included reports was low, with an overall score of 3–5.

Basic Information

A total of 45 patients (28 women) were included, with a median age of 51 years (range 20–80) (**Table 1**). These patients were mainly from Asia (17.80%, 8/45) and Europe (68.9%, 31/45). The NSAIDs most commonly implicated were diclofenac (26.7%, 12/45), metamizole (15.6%, 7/45), aspirin (13.3%, 6/45), and ibuprofen (11.1%, 5/45). NSAIDs were used mainly to treat pain in 29 patients (82.9%). The most administration routes of NSAIDs were oral (52.5%, 21/40), intravenous (20.0%, 8/40), intramuscular (17.5%, 7/40), suppositories (5.0%, 2/40), intra-articular (2.5%, 1/40), and eye drops (2.5%, 1/40). Twenty patients (44.4%) had previous allergic diseases or reactions. Seventeen patients (37.8%) had cardiovascular diseases or cardiovascular risk factors.

Clinical Symptoms

The clinical symptoms of these patients are summarized in **Table 2**. Kounis syndrome occurred mainly within 30 min after administration, with a maximum latency of 1 month. Chest pain (75.6%, 34/45) and dyspnea (33.3%, 15/45) were the most common symptoms of the acute coronary syndrome. Other common symptoms were digestive symptoms (nausea, vomiting) in 15 patients (33.3%) and neurological symptoms (lightheadedness, syncope, unconsciousness) in 10 patients (22.2%). Thirty-three patients (73.3%) had allergic reactions manifested as itching (42.2%, 19/45), rash (40.0%, 18/45), erythema (20.0%, 9/45), and skin flushing (13.3%, 6/45). Hypotension occurred in 25 patients (69.4%, 25/36). Four patients (8.9%) had a cardiac arrest. Three patients (6.7%) had cardiogenic shock.

Laboratory Test

Laboratory tests are summarized in **Table 2**. Troponin was elevated in 32 patients (84.2%, 32/38), creatine kinase was elevated in 15 patients (60.0%, 15/25), serum tryptase was elevated in 7 patients (70.0%, 7/10), and immunoglobulin E (IgE) was elevated in 10 patients (83.3%, 10/12). Two patients had positive skin prick tests.

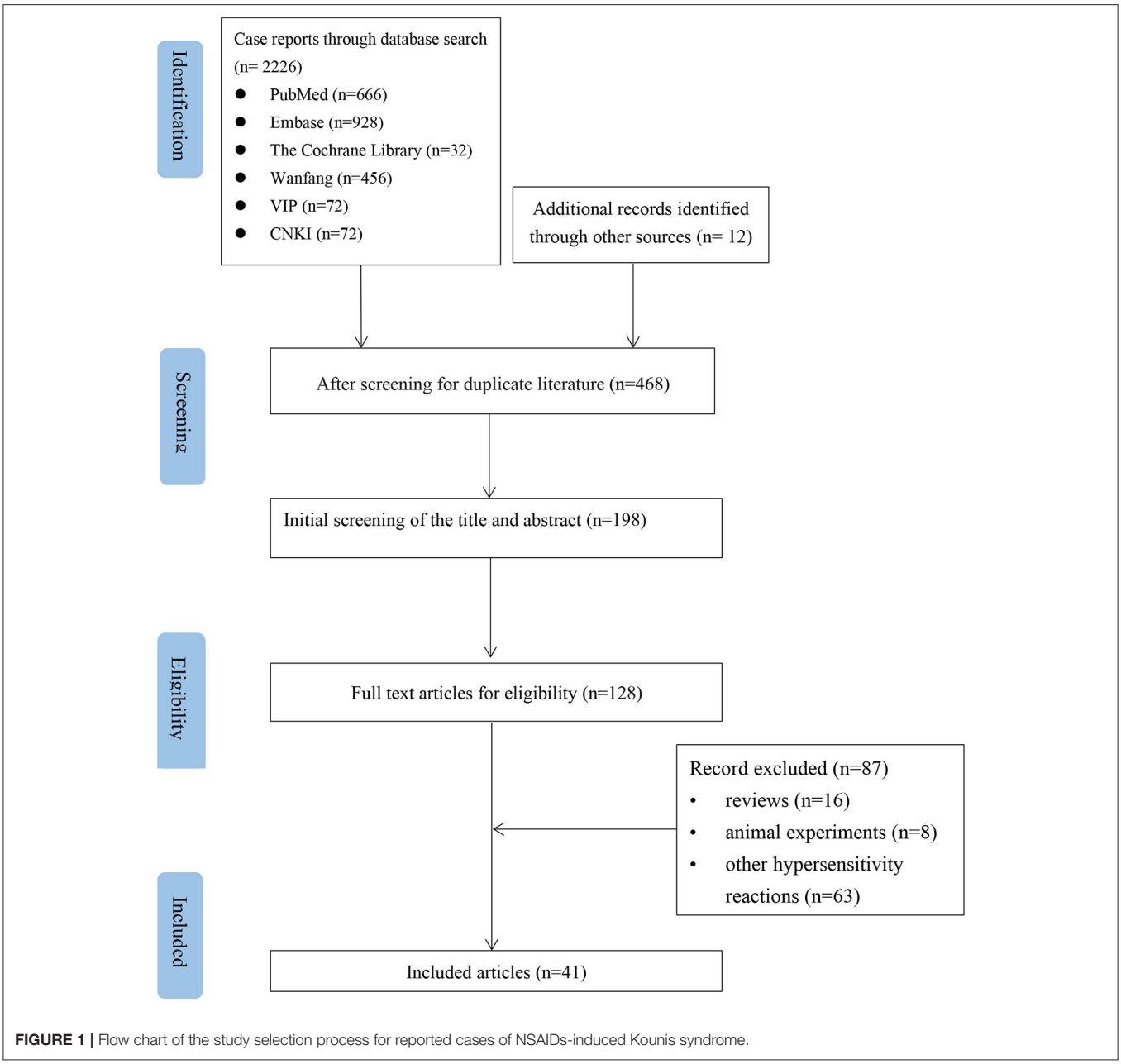

FIGURE 1 | Flow chart of the study selection process for reported cases of NSAIDs-induced Kounis syndrome.

Imaging Examination

The imaging results are summarized in **Table 3**. The most common presentations of electrocardiogram (ECG) were ST-elevation (66.7%, 30/45) and ST-elevation and ST-depression (20.0%, 9/45). Echocardiography in 28 patients showed hypokinesia (39.3%, 11/28), normal (35.7%, 10/28), and reduced ejection fraction (21.4%, 6/28). Coronary angiography showed that 13 patients (32.5%, 13/40) had occlusion, stenosis, and plaques, and two patients (5%, 2/40) had vasospasm. The main coronary arteries involved were the right coronary artery (RCA) and the left anterior descending artery (LAD). The categories of Kounis syndrome were type I variant (73.3%, 33/45), type II variant (24.4%, 11/45), and type III variant (2.2%, 1/45).

Treatment and Prognosis

NSAIDs were discontinued in all patients after developing Kounis syndrome (**Table 4**). Twenty-seven patients (60.0%) received corticosteroids, 26 (57.8%) received antihistamines, and 13 (28.9%) received antiplatelet drugs. Twelve patients (26.7%) received vasodilators including calcium channel blockers (17.8%, 8/45), nitrate (15.6%, 7/45), beta-blockers (4.4%, 2/45), nicorandil (4.4%, 2/45), and ranolazine (2.2%, 1/45). Five patients (11.1%) received intracoronary nitroglycerin and six (13.3%) received heparin. Eight patients (17.8%) underwent percutaneous coronary intervention (PCI). Ultimately, 44 patients (97.8%) had improvement in symptoms or recovered, and one patient (2.2%) died.

TABLE 1 | Basic information on NSAIDs-induced Kounis syndrome ($n = 45$).

Parameter		Value
Age	Years	51 (20,80)[b]
Sex	Male:female	17:28
Country	Turkey	20 (44.4%)
	Spain	6 (13.3%)
	USA	4 (8.9%)
	Japan	4 (8.9%)
	India	3 (6.7%)
	Portugal	2 (4.4%)
	Austria	1 (2.2%)
	Morocco	1 (2.2%)
	Netherlands	1 (2.2%)
	Saudi Arabia	1 (2.2%)
	UK	1 (2.2%)
	Vietnam	1 (2.2%)
Types of NSAIDs	Diclofenac	12 (26.7%)
	Metamizole	7 (15.6%)
	Aspirin	6 (13.3%)
	Ibuprofen	5 (11.1%)
	Dexketoprofen	3 (6.7%)
	Naproxen	3 (6.7%)
	Paracetamol	3 (6.7%)
	Nimesulide	1 (2.2%)
	Acemetacine	1 (2.2%)
	Propyphenazone	1 (2.2%)
	Celecoxib	1 (2.2%)
	Etofenamate	1 (2.2%)
	Indomethacin	1 (2.2%)
Indication (35)[a]	pain	29 (82.9%)
	coronary	2 (5.7%)
	artery disease	3 (8.6%)
	upper respiratory	1 (2.9%)
	tract infection	
	fever	
Route of	oral	21 (52.5%)
administration	intravenous	8 (20.0%)
(40)[a]	intramuscularly	7 (17.5%)
	suppository	2 (5.0%)
	intraarticular	1 (2.5%)
	eye drops	1 (2.5%)
Delay (31)[a]	Within 30 min	22 (71.0%)
	1h–24h	8 (25.8%)
	1 month	1 (3.2%)
Allergic conditions	drug allergy, asthma,	20 (44.4%)
or history	seasonal allergic rhinitis,	
	shaving foam, fruits	
Cardiovascular	smoking	13 (28.9%)
disease or risk	hypertension	9 (20.0%)
factors (17)[a]	diabetes	4 (8.9%)
	coronary artery disease	4 (8.9%)
	hyperlipidemia	3 (6.7%)

NSAIDs, Nonsteroidal anti-inflammatory drugs.
[a]Represents the number of patients out of 21 in whom information regarding this particular parameter was provided.
[b]Median (minimum-maximum).

TABLE 2 | Clinical symptoms and laboratory findings of NSAIDs-induced Kounis syndrome.

Parameter		Value
Clinical symptoms	Chest pain	34 (75.6%)
	Dyspnea	15 (33.3%)
	Nausea	8 (17.8%)
	Vomiting	7 (15.6%)
	Shortness	7 (15.6%)
	Of Breath	6 (13.3%)
	Sweating	5 (11.1%)
	Lightheadedness	5 (11.1%)
	Palpitations	3 (6.7%)
	Syncope	2 (4.4%)
	Unconscious	1 (2.2%)
	Cyanosis	1 (2.2%)
	Abdominal	1 (2.2%)
	Discomfort	33 (73.3%)
	Arm Pain	19 (42.2%)
	Allergy	18 (40.0%)
	Itching	9 (20.0%)
	Rash	6 (13.3%)
	Erythema	3 (6.7%)
	Flushing	
	Swelling	
Cardiac Arrest	Yes	4 (8.9%)
	No	41(91.1%)
Blood pressure (36)[a]	hypotension	25 (69.4%)
	normal pressure	11 (30.6%)
Troponin (38)[a]	elevated	32 (84.2%)
	normal	6 (15.8%)
Creatine kinase (25)[a]	elevated	15 (60.0%)
	normal	10 (40.0%)
Serum tryptase (10)[a]	elevated	7 (70.0%)
	normal	3 (30.0%)
IgE (12)[a]	elevated	10 (83.3%)
	normal	2 (16.7%)

NSAIDs, Nonsteroidal anti-inflammatory drugs; IgE, Immunoglobulin E.
[a]Represents the number of patients out of 21 in whom information regarding this particular parameter was provided.

DISCUSSION

NSAIDs are reversible inhibitors of cyclooxygenase (COX)-1 and COX-2 with varying degrees of selectivity (8) and can be divided into five categories according to the degree of inhibition of COX-1 and COX-2 (9). NSAIDs have long been associated with an increased risk of vascular events (10). NSAIDs can alter the balance of thromboxane-prostacyclin, leading to vasospasm and the formation of a platelet thrombus. The cardiovascular risk may be drug-specific, and further studies are needed to define cardiovascular risk related to NSAIDs (11).

However, the cardiovascular hazard of NSAIDs was driven primarily by the increase in the risk for non-ST-segment elevation. In contrast, NSAIDs did not increase ST-segment elevation myocardial infarction (12). Among the 51 cases of Kounis syndrome reported to the International Agency for Pharmacovigilance (VigiBase™) between 2010 and 2014, most of the cases occurred in the United States. NSAIDs were the most common trigger drugs (13).

In our analysis, NSAIDs-induced Kounis syndrome was more common in southern Europe, especially Turkey and Spain. Type I Kounis syndrome was the predominant type and usually occurred within 30 min. One case of Kounis syndrome

TABLE 3 | Imaging of NSAIDs-induced Kounis syndrome.

Parameter		Value
Electrocardiograph (45)[a]	ST elevation	30 (66.7%)
	ST elevation and ST depression	9 (20.0%)
	Atrioventricular block	5 (11.1%)
	ST depression	3 (6.7%)
	T-wave inversion	3 (6.7%)
	Atrial fibrillation	2 (4.4%)
	Negative T wave	1 (2.2%)
	Prolonged QT interval	1 (2.2%)
Echocardiography (28)[a]	Hypokinesia	11(39.3%)
	Normal	10 (35.7%)
	Systolic dysfunction	2 (7.1%)
	RWMA	2 (7.1%)
	Preserved ejection fraction	3 (10.7%)
	Decreased ejection fraction	6 (21.4%)
Coronary angiography (40)[a]	Normal	26 (65.0%)
	Occlusion	9 (22.5%)
	Stenosis	2 (5.0%)
	Plaques	2 (5.0%)
	Vasospasm	2 (5.0%)
Involved arteries (14)[a]	LCX	1 (7.1%)
	LCA	2 (14.3%)
	RCA	6 (42.9%)
	LAD	9 (64.3%)
Kounis syndrome type[a]	Type I variants	33 (73.3%)
	Type II variants	11 (24.4%)
	Type III variants	1 (2.2%)

RWMA, regional wall motion abnormalities; RCA, right coronary artery; LCX, left circumflex artery; LAD, left anterior descending artery.

[a]*Represents the number of patients out of 21 in whom information regarding this particular parameter was provided.*

TABLE 4 | Treatment and prognosis of NSAIDs-induced Kounis syndrome.

Parameter		Value
Treatment	Discontinued	45 (100%)
	Corticosteroid	27 (60.0%)
	Antihistamine	26 (57.8%)
	Antiplatelet	13 (28.9%)
	Adrenaline	8 (17.8%)
	Calcium channel blocker	8 (17.8%)
	Intracoronary nitroglycerin	5 (11.1%)
	Nitrate	7 (15.6%)
	Heparin	6 (13.3%)
	Morphine	3 (6.7%)
	Beta blocker	2 (4.4%)
	Ranolazine	1 (2.2%)
	Statin nicorandil	2 (4.4%)
	Percutaneous	2 (4.4%)
	coronary intervention	8 (17.8%)
Prognosis	Improve or recover	44 (97.8%)
	Die	1 (2.2%)

occurred 1 month after taking NSAIDs. The probabilities of Kounis syndrome caused by NSAIDs are different, which can be explained by the immune mechanisms of the NSAIDs. Non-allergic NSAIDs reactions are allergy-like reactions that are not immunologically mediated. These reactions are thought to occur mainly due to the inhibition of COX-1 enzymes. Non-allergic NSAIDs reactions are known to be cross-reactive. Immunologically mediated NSAIDs reactions are based on immunoglobulin E (IgE) or T cell response. These reactions do not depend on COX-1 inhibition and can be induced by a single NSAIDs or by a class of NSAIDs with similar chemical structures (14).

The diagnosis of Kounis syndrome is based on clinical signs and symptoms, laboratory tests, ECG, echocardiogram, and coronary angiography (2). Risk factors for Kounis syndrome include previous allergies, hypertension, smoking, diabetes, and hyperlipidemia (7). For patients with suspected Kounis syndrome, a careful review of the clinical history is warranted, including medications and allergy history. In our analysis, patients with NSAIDs-induced Kounis syndrome were predominantly female (62%), contrary to Abdelghany et al. (15). In our study, 44.4% of the patients had a history of allergies or allergic conditions, higher than previously reported (25.1%) (15). ECG usually shows ST-T changes suggesting ischemia, with ST-elevation being the most common finding. Cardiac catheterization may show coronary vasospasm or stenosis. Our study showed that LAD was the culprit artery in >64% of cases, followed by RCA.

Kounis syndrome is not only a single organ arterial disease but also a complex multiorgan disease that can affect the skin, respiratory, and vascular systems (16). Signs and symptoms can be variable, depending on the organ systems. In addition to cardiac manifestations, Kounis syndrome involves the skin or mucosal surfaces (e.g., pruritus, rash, erythema), gastrointestinal system (e.g., diarrhea, vomiting), respiratory system (e.g., shortness of breath, dyspnea), cardiovascular system (e.g., hypotension, palpitations), and nervous system (e.g., unconsciousness, syncope). The severity of Kounis syndrome can range from mild angina with urticaria and pruritus to cardiogenic shock.

Cardiac tissue contains abundant mast cells (17). Infiltration of activated mast cells into plaque erosion or rupture areas is a common pathway between allergic and non-allergic coronary events (18). The burden of cardiac mast cells in coronary plaques in patients with heart disease is 200 times greater than in the coronary arteries in healthy individuals (18). Kounis syndrome is caused by the action of pro-inflammatory mediators released in abundance by mast cells in cardiac tissue, coronary arteries, and plaques. These inflammatory mediators (for example, histamine, neutral proteases, arachidonic acid products, platelet-activating factor, and heparin) lead to peripheral vasodilation, decreased blood pressure and coronary blood flow, coronary spasm, atherosclerotic plaque erosion rupture, and coronary stent thrombosis (19–22).

The treatment of Kounis syndrome is highly challenging. At present, treatment is empirical with no professional guidelines. It is necessary to treat the symptoms of the heart and allergies simultaneously. Symptoms can be eliminated in patients with type I variants after antiallergic treatment. Antiallergic treatment can be performed with intravenous corticosteroids (e.g., hydrocortisone) and H1 and H2 receptor antagonists (e.g.,

diphenhydramine and ranitidine) (23, 24). Administration of vasodilators, such as calcium channel blockers and nitrates, can eliminate vasospasms caused by hypersensitivity reactions (25). For patients with type II variants, acute coronary events must be managed along with anti-allergic therapy with corticosteroids and antihistamines (2). For patients with type III variants, a critical myocardial infarction protocol and emergency thrombus aspiration should be performed, followed by histological examination of the aspirated material and staining for eosinophils and mast cells (26). Epinephrine should be used with caution in Kounis syndrome because it aggravates ischemia, prolongs the QT interval, and causes coronary spasms or arrhythmias (27). Beta-blockers can exacerbate coronary spasms due to the lack of antagonism of α-adrenergic receptors (27).

Stabilizing mast cells and preventing the release of inflammatory mediators may represent a novel therapeutic strategy for Kounis syndrome (23, 28–30). Agents that target stem cell factors are essential for mast cell development, proliferation, survival, adhesion, and homing. These agents include mediator antagonists, inhibitors of mediator biosynthesis, leukotriene antagonists, mediator receptor blockers (sodium nedocromil, sodium cromoglycate, ketotifen, lodoxamide), humanized IgG1 monoclonal antibodies, and other natural molecules that interfere with mast cell stabilization and prevent the release of mast cell contents (31).

Various factors affect the prognosis of Kounis syndrome, including comorbidity, sensitivity, the site of the antibody-antigen reaction, allergen entrance, the allergen concentration, number of allergens the patient is exposed to, and the magnitude of the initial allergic response (21, 32). Type I Kounis syndrome has a more favorable prognosis than the other two variants (15). In our analysis, serious complications of NSAIDs-induced Kounis syndrome were rare, with cardiogenic shock at 6.7% and cardiac arrest at 8.9%. Only one patient with dexketoprofen-induced type I Kounis syndrome died from cardiac arrest (33). The remaining 97.8% of the patients recovered or had symptoms improved after appropriate treatment without any associated long-term sequelae.

CONCLUSION

Kounis syndrome is a rare adverse effect of NSAIDs. The risk of myocardial infarction must be considered when prescribing NSAIDs. Physicians should promptly recognize Kounis syndrome and treat patients with antihistamines, steroids, and calcium channel blockers. Patients may have a good prognosis when appropriate and timely treatment is administered.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LSu and CW conceived of the presented idea. CW, WF, LSo, ZD, ZL, and LSu wrote the manuscript. All authors discussed the results and contributed to the final manuscript. All authors contributed to the article and approved the submitted version.

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Cardiovascular adverse events induced by immune checkpoint inhibitors: A real world study from 2018 to 2022

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Background: The reported rate of cardiovascular adverse events (CAE) caused by immune checkpoint inhibitors (ICI) is low but potentially fatal. Assess the risk of CAE in cancer patients and compare the incidence of CAE between Chinese developed ICIs and imported ICIs.

Methods: A retrospective analysis was performed on cancer patients treated with ICI for at least four cycles in the Second Affiliated Hospital of Dalian Medical University from January 2018 to March 2022. Baseline characteristics, physiological and biochemical values, electrocardiographic and echocardiographic findings were compared between patients with and without CAE.

Results: Among 495 patients treated with ICIs, CAEs occurred in 64 patients (12.93%). The median time to the event was 105 days (61–202). The patients with low neutrophil-to-lymphocyte ratio (L-NLR) were significantly associated with the risk of developing CAE (hazard ratio HR 3.64, 95% confidence ratio CI 1.86–7.15, P = 0.000). Patients with higher comorbidity burden significantly increased the risk of developing CAE (HR 1.30, 95% CI 1.05–1.61, P = 0.014). Those who received a combination of ICI and vascular endothelial growth factor receptor (VEGFR) inhibitors (HR 2.57, 95% CI 1.37–4.84, P = 0.003) or thoracic radiation therapy (HR 32.93, 95% CI 8.81–123.14, P = 0.000) were at a significantly increased risk of developing CAE. Compared to baseline values, creatine kinase is -oenzymes (CK-MB) (95% CI -9.73 to -2.20, P = 0.003) and cardiac troponin I (cTnI) (95% CI -1.06 to -0.06, P = 0.028) were elevated, and the QTc interval prolonged (95% CI -27.07 to -6.49, P = 0.002). Using nivolumab as a control, there was no difference in CAE risk among the eight ICIs investigated. However, the results of the propensity matching showed that programmed death-ligand 1 (PD-L1) inhibitors had lower CAE occurrence compared with programmed cell death protein 1 (PD-1) inhibitors (adjusted HR = 0.38, P = 0.045).

Conclusion: Patients who received concurrent VEGFR inhibitors and ICIs had a history of thoracic radiation therapy, L-NLR, and higher comorbidity burden had an increased risk of CAEs. Elevated cTnI, CK-MB, and QTc, can

be used to monitor CAEs. There was no significant difference in CAE risks between Chinese domestic and imported ICIs. PD-L1 inhibitors had lower CAE occurrence than PD-1 inhibitors.

KEYWORDS

immune checkpoint inhibitors, cardiovascular adverse events, immunotherapy, predictors, surveillance factors

Introduction

Immunotherapy, such as immune checkpoint inhibitors (ICIs), has revolutionized cancer treatment in recent years. ICIs significantly improve the survival rate of patients with advanced cancer. ICIs are monoclonal antibodies against cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1). These agents function by blocking an immune checkpoint at the coreceptor and ligand interface of the T-cell and the antigen-presenting cell (anti-CTLA-4) or by inhibiting the interaction between the T cell and the tumor cell (anti-PD-1 and anti-PD-L1), allowing the increased destruction of cancer cells (1, 2). They have unusual adverse reactions that are different from traditional chemotherapy drugs. With the widespread application of immunotherapy, ICIs can lead to a wide range of immune-related adverse events (irAE). ICIs can induce irAE in multiple organs due to non-specific immune system activation. Most irAEs are manageable in the early stage, but about 10–17% lead to fatal consequences (3). Among the adverse reactions caused by ICIs, the reported rate of cardiovascular adverse events (CAEs) is low, but the mortality rate is high, which may lead to irreversible consequences (4).

CAEs may pose potential physical and economic threats to patients. Most studies on ICI-related cardiotoxicity are case reports, and the incidence of CAEs can be underestimated. Furthermore, the relationship between CAEs and ICIs and the potential associated factors is unclear. Currently, there are eight ICIs being used in China: nivolumab (PD-1), pembrolizumab (PD-1), atezolizumab (PD-L1), sintilimab (PD-1), camrelizumab (PD-1), toripalimab (PD-1), tislelizumab (PD-1), and durvalumab (PD-L1). Among these ICIs, sintilimab, camrelizumab, toripalimab, and tislelizumab were developed in China. There is no comparative study of the effects of domestic ICIs and imported ICIs on CAEs. This study was designed to: (1) provide estimates of the incidence of ICI-related CAEs, (2) determine the clinical characteristics of patients associated with the risk of developing ICI-related CAEs, and (3) compare the differences in CAE between domestic and imported ICIs. This study did not include anti-CTLA-4 drugs due to their limited availability in China.

Materials and methods

Study population and data collection

The study was carried out at the Second Affiliated Hospital of Dalian Medical University. Inclusion criteria were patients 18 years or older who received at least four cycles of ICI treatment from January 2018 to March 2022. The patients were stratified according to whether CAE occurred within 1 year of ICI treatment. The exclusion criteria were patients with a history of severe cardiac disease or patients with incomplete clinical data. The study was approved by the institutional review board of the Second Affiliated Hospital of Dalian Medical University (approval number 2020 NO.044, approval date 2020-11-27).

The following data were collected: age, gender, comorbidities, tumor type, chemotherapies, radiation therapy, vascular endothelial growth factor receptor (VEGFR) inhibitors, human epidermal growth factor receptor 2 (HER-2) inhibitors, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI), PD-1 and PD L-1 inhibitors, PR interval, QTc interval, ejection fraction (EF), creatine kinase (CK), CK-MB, cardiac troponin I (cTnI), B-type natriuretic peptide (BNP), and the occurrence time and type of CAEs. The Charlson Comorbidity Index (CCI) score and the neutrophil-to-lymphocyte ratio (NLR) were calculated for all patients. NLR < 3 can be specified as L-NLR.

Immune checkpoint inhibitors-related cardiovascular adverse events

ICI-related CAEs were defined as CAEs diagnosed within 1 year after the first use of ICIs. The severity of CAEs was classified into grades 1–5 using the Common Terminology Criteria for Adverse Events (CTCAE).

Statistical analysis

The Fine-Gray competing risk model analysis assessed associations between baseline demographic and clinical

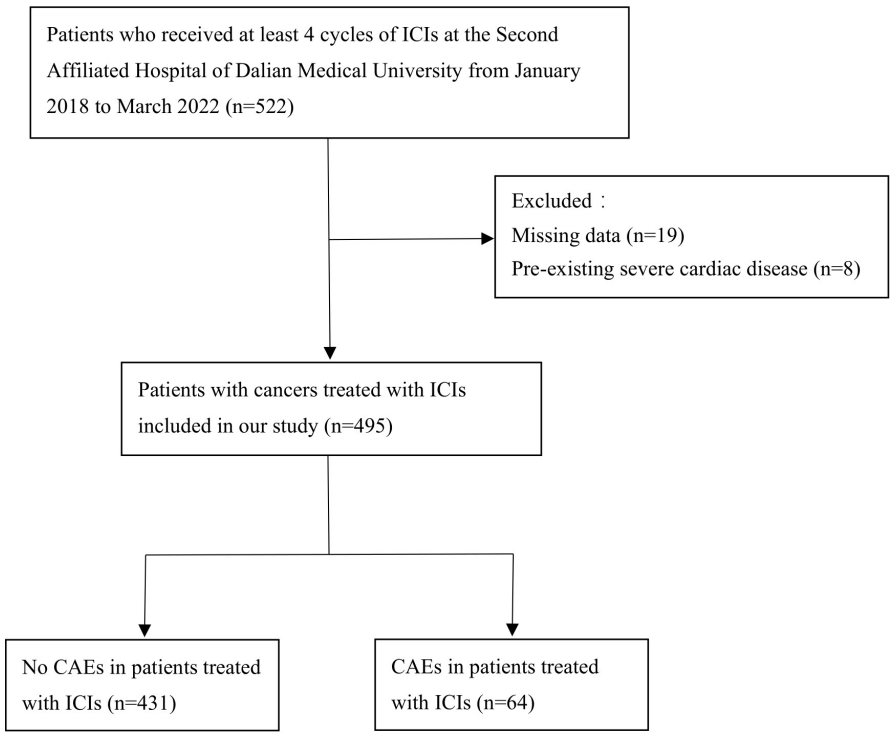

FIGURE 1
The study design for retrospective evaluation of CAEs in patients with ICIs. CAE, cardiovascular adverse events; ICI, immune check inhibitor.

variables and CAEs. Based on the clinical data of the patients in descriptive statistics, the results were presented as mean ± SD. Other data were evaluated for significance using the Mann-Whitney *U*-test (non-parametric), paired *t*-tests, and independent *t*-tests. The comparison data between PD-1 and PD-L1 inhibitors were processed by propensity matching with a ratio of 1:1. All statistical tests were two-sided and $p < 0.05$ was considered statistically significant. All statistics were analyzed using Stata 17 and SPSS 22.

Results

Predisposing factors for immune checkpoint inhibitors-related cardiovascular adverse events

After applying the inclusion and exclusion criteria, 495 patients were included in the analysis (**Figure 1**). Sixty-four (12.93%) patients developed CAEs within 1 year of starting treatment with ICIs. **Table 1** shows the demographic and clinical characteristics of the patients. The increase in CCI score significantly increased the risk of CAEs (hazard ratio HR 1.30, 95% confidence interval CI 1.05–1.61, $P = 0.014$). A total of 218 patients had NLR < 3 (L-NLR), and 42 (19.27%) of these patients had CAEs. L-NLR was significantly associated with the risk of CAEs (HR 3.64, 95% CI 1.86–7.15, $P = 0.000$). CAEs occurred in two of ten cervical cancer patients (20.00%) after using ICIs (HR 17.61, 95% CI 1.85–167.62, $P = 0.013$). When analyzed for ICI combination therapies, VEGFR inhibitors (HR 2.57, 95% CI 1.37–4.84, $P = 0.003$) significantly increased the risk of CAEs. Twenty-four patients with a history of radiation therapy within 90 days were treated with ICIs, and three patients who received thoracic radiation therapy developed CAEs (HR 32.93, 95% CI 8.81–123.14, $P = 0.000$). Using nivolumab as a control, there were no statistically significant differences in the risk of CAEs between the eight ICIs.

Classification of cardiovascular adverse events caused by immune checkpoint inhibitors

The most common CAE were: arrhythmia, 53.13% (34/64); acute non-ST segment elevation myocardial infarction, 17.19% (11/64); pericarditis, 10.94% (7/64); myocarditis, 7.81% (5/64); and others, 10.94% (7/64). CAE of grade 1 (30%), and grade 2 (47%) accounted for the largest proportion of total adverse events (**Figure 2**).

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TABLE 1 The baseline characteristics of cardiac adverse events (n = 495).

	No CAE (N = 431, 87.07%)	CAE (N = 64, 12.93%)	HR	95% CI	P
Age (years)	62.28 ± 10.18	61.78 ± 9.97	0.99	0.96–1.02	0.378
<50	42 (9.74)	8 (12.50)			
[50, 59]	104 (24.13)	14 (21.88)			
[60, 69]	201 (46.64)	29 (45.31)			
[70, 79]	67 (15.55)	13 (20.31)			
[80, 89]	17 (3.94)	0			
Sex (n, %)			1.97	0.96–4.05	0.065
Male	317 (73.55)	52 (81.25)			
Female	114 (26.45)	12 (18.75)			
CCI score	5.16 ± 1.88	6.03 ± 1.86	1.30	1.05–1.61	0.014
L-NLR	176 (40.84)	42 (65.63)	3.64	1.86–7.15	0.000
Tumor type (n, %)					
Lung cancer	256 (59.4)	37 (57.81)	3.80	0.71–20.41	0.119
Stomach cancer	32 (7.42)	6 (9.38)	4.57	0.77–27.28	0.096
Esophageal cancer	27 (6.26)	3(4.69)	3.41	0.45–25.99	0.237
Liver cancer	19 (4.41)	6 (9.38)	4.95	0.93–26.40	0.061
Colorectal cancer	19 (4.41)	5 (7.81)	4.42	0.95–20.53	0.058
Cholangiocarcinoma	6 (1.39)	1 (1.56)	6.43	0.46–90.35	0.167
Pancreatic cancer	6 (1.39)	1 (1.56)	4.49	0.29–69.91	0.284
Cervical cancer	8 (1.86)	2 (3.13)	17.61	1.85–167.62	0.013
Lymphoma	16 (3.71)	1 (1.56)	1.14	0.17–7.83	0.891
Other	45 (10.44)	5 (7.81)	4.35	0.62–30.43	0.138
Chemotherapy (n, %)					
Antimetabolite	137 (31.79)	25 (39.06)	0.75	0.32–1.76	0.503
Anti-tubulin	163 (37.82)	19 (29.69)	0.82	0.34–2.00	0.669
Topoisomerase	63 (14.62)	11 (17.19)	1.38	0.45–4.24	0.572
Platinum	111 (25.75)	11 (17.19)	0.73	0.36–1.48	0.382
Alkylating agent	5 (1.16)	1 (1.56)	2.76	0.51–14.99	0.240
Radiation therapy (n,%)					
Thoracic radiotherapy	1 (0.23)	3 (4.69)	32.93	8.81–123.14	0.000
Radiation therapy to other sites	17 (3.94)	3 (4.69)	0.69	0.23–2.12	0.521
Other therapies (n, %)					
Anti-VEGFR	68 (15.78)	23 (35.94)	2.57	1.37–4.84	0.003
Anti-HER-2	3 (0.70)	2 (3.13)	1.82	0.24–13.68	0.561
EGFR-TKI	59 (13.69)	15 (23.44)	1.14	0.52–2.50	0.745
ICI (n,%)					
(ref = Nivolumab)					
Pembrolizumab	50 (11.60)	10 (15.63)	2.43	0.47–12.64	0.292
Atezolizumab	17 (3.94)	2 (3.13)	0.63	0.09–4.37	0.635
Sintilimab	159 (36.89)	28 (43.75)	1.82	0.37–9.01	0.466
Camrelizumab	94 (21.81)	13 (20.31)	1.75	0.31–9.74	0.524
Toripalimab	17 (3.94)	1 (1.56)	0.37	0.02–5.91	0.480
Tislelizumab	55 (12.76)	4 (6.25)	1.21	0.18–8.04	0.843
durvalumab	23 (5.34)	4 (6.25)	1.30	0.17–10.14	0.802

L-NLR, low-neutrophil to lymphocyte ratio < 3; CCI, Charlson Comorbidity Index; VEGFR, vascular endothelial growth factor receptor; HER-2, human epidermal growth factor receptor 2; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; HR, hazard ratio; CI, confidence interval.

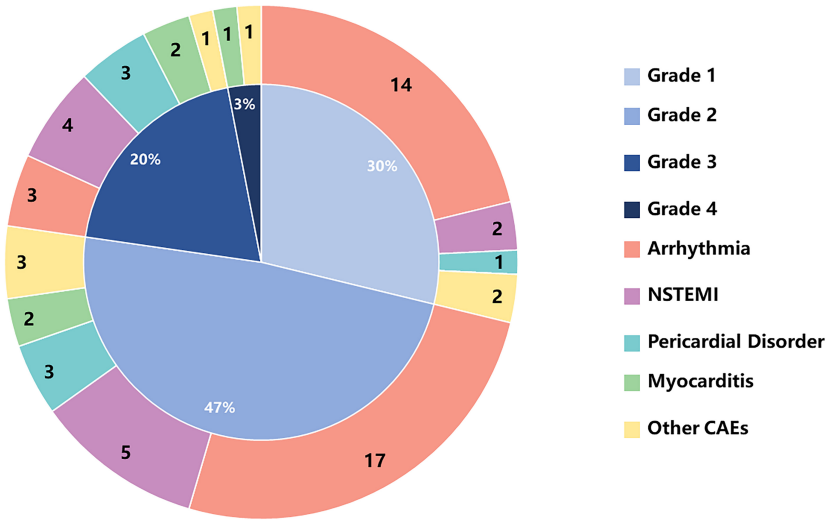

FIGURE 2
The numbers of CAEs and percentages of CAE toxicities based on CTCAE grading in cancer patients receiving ICIs. CTCAE, Common Terminology Criteria for Adverse Event; NSTEMI, non-ST-segment elevation myocardial infarction.

Time of occurrence of cardiovascular adverse events

Among the 64 patients who developed CAE within the first year, the median time to cardiac adverse events was 105 days (interquartile range IQR: 61–202 days): 14.06% within 30 days and 68.75% within 6 months after the beginning of ICI treatment. Details are shown in **Figure 3**.

Clinical and laboratory parameters of cardiovascular adverse events

Cardiac color Doppler ultrasound, electrocardiogram, and myocardial enzymes were obtained in patients with CAE. Most of the patients had normal sinus rhythm (58 ± 5%). Compared to baseline, there were significant differences in the QT interval corrected for heart rate (QTc), 449.55 ms vs. 432.79 ms (95% CI -27.02 to -6.49, $P = 0.002$), creatine kinase-MB (CK-MB), 20.29 U/L vs. 14.33 U/L (95% CI -9.73 to -2.2, $P = 0.003$), and cardiac troponin I (cTnI), 1.02 pg/mL vs. 0.46 pg/mL (95% CI -1.06 to -0.06, $P = 0.028$). The details are shown in **Table 2**.

The cumulative incidence of cardiovascular adverse events of programmed cell death protein 1 and programmed death-ligand 1 inhibitors

Among the 495 patients analyzed, 449 (90.7%) received PD-1 inhibitors, and 46 (9.3%) received PD-L1 inhibitors. The risks of PD-1 and PD-L1 for causing CAEs were analyzed by propensity matching. **Table 3** shows the characteristics of the propensity match cohort. The results of PD-1/PD-L1 before matching show that the CCI score ($P = 0.004$), L-NLR ($P = 0.016$), lung cancer ($P = 0.000$), stomach cancer ($P = 0.04$), antimetabolite ($P = 0.003$), anti-tubulin ($P = 0.000$), and topoisomerase ($P = 0.000$) had statistical differences. However, the covariates between PD-1 and PD-L1 were balanced after matching without significant differences. Results showed that PD-L1 inhibitors had a lower incidence of CAE compared with PD-1 inhibitors (adjusted hazard ratio aHR 0.38, $P = 0.045$). The adjusted cumulative incidence rates of CAE are shown in **Figure 4**.

Discussion

As a new method of cancer treatment, ICIs act on the immune system of cancer patients, restore the immune system's ability to fight tumors. However, in addition to providing excellent survival benefits, ICIs can induce specific hyperactivation of immune responses, leading to non-cancer tissue damage and inevitable drug toxicity (5). Due to the rareness of cardiotoxicity, most studies present CAEs as case reports, and the incidence is often underestimated in clinical trials. Myocarditis can be more common after ICI therapies. It can develop early after initiation of ICI therapy and has a malignant course (6).

Data from a large network of healthcare organizations showed that of 5,518 cancer patients treated with at least one ICI cycle, 691 (12.5%) developed cardiotoxicity. The most common

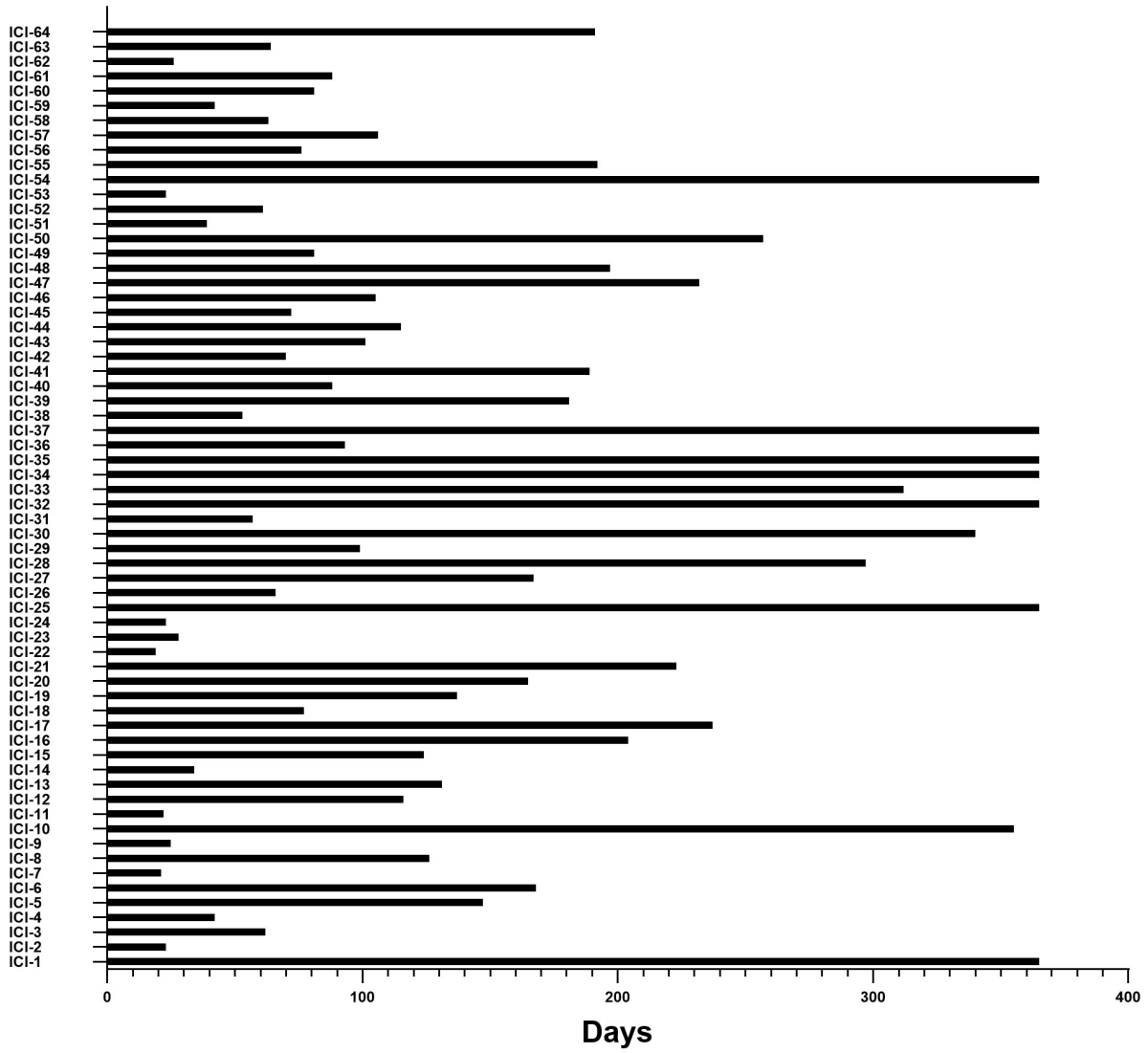

FIGURE 3
Time from initiation of ICI therapies to the occurrence of CAEs. X-axis, time (days) from cancer treatment with ICIs to the onset of CAE; Y-axis, the number of individual subjects with CAE in 64 of the 495 ICI-treated cohort patients in the study.

TABLE 2 The characteristics of biomarkers in patients with CAE (*n* = 64).

Parameter, *n* (%) N		Baseline	CAEs	95% CI	*P*
WMA		0	1		
EF, %	25	59.24 ± 6.35	58.28 ± 5.25	−0.59 to −2.51	0.212
PR interval, ms	52	151.37 ± 19.18	151.73 ± 25.52	−7.77 to 7.04	0.921
QTc interval, ms	58	432.79 ± 27.06	449.55 ± 37.17	−27.02 to−6.49	0.002
CK, U/L	38	60.32 ± 31.66	394.36 ± 1148.58	−711.25 to 43.18	0.081
CK-MB, U/L	41	14.33 ± 5.72	20.29 ± 11.86	−9.73 to −2.20	0.003
cTnI, ng/ml	41	0.46 ± 0.40	1.02 ± 1.50	−1.06 to −0.06	0.028
BNP, pg/ml	12	50.95 ± 66.42	89.93 ± 118.55	−85.41 to 7.86	0.094

WMA, wall motion abnormalities; EF, ejection fraction; CK, creatine kinase; cTnI, cardiac troponin I; BNP, B-type natriuretic peptide.

TABLE 3 Propensity matching of patients.

Variable	Whole cohort			Propensity score matched cohort		
	PD-1 inhibitor *n* = 449	PD-L1 inhibitor *n* = 46	*P*	PD-1 inhibitor *n* = 46	PD-L1 inhibitor *n* = 46	*P*
Age (years)	61.93 ± 10.18	64.91 ± 9.50	0.058	64.17 ± 9.44	64.91 ± 9.50	0.709
<50	47 (10.47)	3 (6.52)		8 (17.39)	3 (6.52)	
[50,59]	111 (24.72)	7 (15.22)		9 (19.57)	7 (15.22)	
[60,69]	205 (45.66)	25 (54.35)		20 (43.48)	25 (54.35)	
[70,79]	71(15.81)	9 (19.56)		8 (17.39)	9 (19.56)	
[80,89]	15 (3.34)	2 (4.35)		1 (2.17)	2 (4.35)	
Sex (*n*, %)			0.336			0.615
Male	332 (73.94)	37 (80.43)		35 (76.09)	37 (80.43)	
Female	117 (26.06)	9 (19.57)		11 (23.91)	9 (19.57)	
CCI score	5.20 ± 1.91	6.02 ± 1.42	0.004	6.33 ± 1.21	6.02 ± 1.42	0.272
L-NLR	189 (42.09)	28 (60.87)	0.016	21 (45.65)	28 (60.87)	0.146
Tumor type (*n*, %)						
Lung cancer	252 (56.12)	40 (86.96)	0.000	42 (91.3)	40 (86.96)	0.505
Stomach cancer	38 (8.46)	0	0.04	1 (2.17)	0	0.317
Esophageal cancer	30 (6.68)	0	0.071	0	0	–
Liver cancer	23 (5.12)	2 (4.35)	0.819	0	2 (4.35)	0.155
Colorectal cancer	24 (5.35)	0	0.180	0	0	–
Cholangiocarcinoma	5 (1.11)	2 (4.35)	0.077	0	2 (4.35)	0.155
Pancreatic cancer	7 (1.56)	0	0.394	1 (2.17)	0	0.317
Cervical cancer	12 (2.67)	0	0.262	0	0	–
Lymphoma	17 (3.79)	0	0.180	1 (2.17)	0	0.317
Other	48 (10.69)	2 (4.35)	0.174	1 (2.17)	2 (4.35)	0.559
Chemotherapy (*n*, %)						
Antimetabolite	156 (34.74)	6 (13.04)	0.003	11 (23.91)	6 (13.04)	0.182
Anti-tubulin	176 (39.2)	6 (13.04)	0.000	8 (17.39)	6 (13.04)	0.564
Topoisomerase	44 (9.80)	30 (65.22)	0.000	27 (58.7)	30 (65.22)	0.522
Platinum	111 (24.72)	11 (23.91)	0.904	7 (15.22)	11 (23.91)	0.296
Alkylating agent	6 (1.34)	0	0.431	0	0	–
Radiation therapy (*n*, %)						
Thoracic radiotherapy	4 (0.89)	0	0.521	2 (4.35)	0	0.155
Radiation therapy to other sites	18 (4.01)	2 (4.35)	0.912	4 (8.7)	2 (4.35)	0.401
Other therapies (*n*, %)						
Anti-VEGFR	84 (18.71)	7 (15.22)	0.561	14 (30.43)	7 (15.22)	0.084
Anti-HER-2	69 (15.37)	0	0.472	0	0	–
EGFR-TKI	5 (1.11)	5 (10.87)	0.416	10 (21.74)	5 (10.87)	0.160

PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1; L-NLR, low-neutrophil to lymphocyte ratio < 3; CCI, Charlson Comorbidity Index; VEGFR, vascular endothelial growth factor receptor; HER-2, human epidermal growth factor receptor 2; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor.

cardiotoxicity was arrhythmia (9.3%), and 2.1% of the patients developed myocarditis at 12 months (7). In this real-world study, we found that the incidence rate of CAE in patients who received at least four cycles of ICI within 1 year was 12.93%. The most common CAE was arrhythmia, accounting for 53.13% of the total CAEs. Among the 64 patients who developed CAE in the first year, the median time to diagnose CAE was 105 days, which was longer than a retrospective study of 46 days (IQR: 17–83 days) conducted from 2015 to 2018 (4). The longer time in our study could be affected by COVID-19 as it could impact hospital admissions and delay the diagnosis of patients with CAE.

One study reported that ICI-induced cardiotoxicity might be predominant in men. However, this does not necessarily represent susceptibility to men compared to women because men are overrepresented at baseline in both ICI use and clinical trial registries (8). This study showed that ICI-induced cardiotoxicity was not related

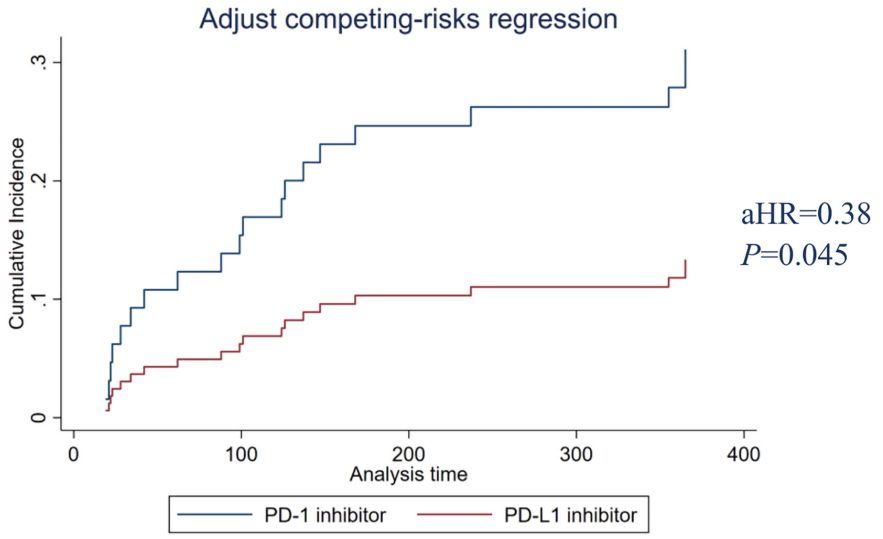

FIGURE 4
PD-1 and PD-L1 inhibitors adjusted cumulative incidence rates. PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1.

to gender. Several studies have shown that CAE caused by ICI do not appear to be age-susceptible and occur in patients with a wide age range (20–90 years) (6, 9). In our study, the patients were generally older, and age had no significant effect on the occurrence of CAE. A large population-based study from multiple medical institutions showed that patients with CAEs had a higher burden of comorbidities than patients who did not develop CAEs within 1 year of ICIs initiation (P = 0.0042). Patients who developed CAEs were more likely to have cerebrovascular disease (12.9% vs. 10.3%, P = 0.0375), congestive heart failure (5.9% vs. 2.6%, P < 0.001), myocardial infarction (4.8% vs. 2.3%, P = 0.0002), peripheral vascular disease (15.6% vs. 12.3%, P = 0.0129), hypertension (50.8% vs. 45.1%, P = 0.0046), or renal disease (13.7% vs. 10.8%, P = 0.0221) (7). In addition, an international registry identified combination therapy, diabetes, obesity, and anti-CTLA-4 therapy were independent risk factors for cardiotoxicity (10). Pre-existing autoimmune disease may also be an independent risk factor (11). Our study also confirmed that patients who developed CAEs had a higher comorbidity burden (HR 1.30, P = 0.014).

NLR is the ratio of absolute neutrophil count to absolute lymphocyte count in peripheral blood, which has been shown to correlate with the prognosis of various malignancies (12). This ratio appears to reflect a balance between non-specific inflammatory and immune responses that may influence response to ICI therapies (13). Data from a retrospective analysis showed that L-NLR was significantly associated with the appearance of irAEs (OR, 2.2, P = 0.018) (14). Our study found that L-NLR was significantly related to the risk of CAE (HR 3.64, P = 0.000), which was consistent with the previous research.

With the deepening of research and the progress of clinical trials, to increase anti-tumor response, more and more ICIs are used in combination with each other or with chemotherapy, radiotherapy, and targeted therapy, increasing the risk of the complexity of toxicity (15, 16). We did not find increased risks of CAEs in patients treated with combination ICIs and chemotherapeutic drugs. VEGFR inhibitors are known to increase the risk of cardiotoxicity (17). Patients who received concomitant or previous VEGFR inhibitors combined with ICI had an increased risk of major adverse cardiovascular events (MACE) compared to patients who received ICIs alone (HR 2.15, 95% CI 1.05–4.37, P = 0.04) (18). The results of our study also confirmed that the combined use of VEGFR inhibitors with ICI significantly increased the risk of CAE (HR 2.57, P = 0.003).

The combination of radiation therapy and immunotherapy is also a hot spot in tumor treatment because radiation therapy has the effect of presenting antigens on tumor cells. The synergistic effect of radiation therapy and immunotherapy could trigger an endogenous antigen-specific immune response, thus increasing the incidence of MACE through recognizing shared antigens (19). The results of a meta-analysis showed similar grade 3–4 toxicity in ICI combined with radiation therapy (16.3%) and ICI alone (22.3%). The grade 5 toxicities were 1.1 and 1.9% for ICI alone and ICI with radiation therapy (20). A retrospective analysis found that exposure to cardiac radiation dose increased the risk of MACE. A mouse model with concurrent thoracic

radiation and PD-1 blockade showed increased radiation-induced cardiotoxicity and a decreased left ventricular EF. However, there was no significant difference in cumulative chest radiation dose between the ICI and non-ICI groups (21). We found that thoracic radiation significant increased the risk of CAE (HR 32.93, P = 0.000). On the contrary, radiation to other sites, such as the head and neck, did not increase CAE risk.

Nivolumab was the first PD-1 antibody investigated in patients in 2006 and the first PD-1 antibody approved by the FDA in 2014 (22). On June 15, 2018, China's Drug Administration approved nivolumab, the country's first immuno-oncology, and the first PD-1 therapy. Since 2020, the number of pivotal clinical trials of PD1/PD-L1 drugs developed by Chinese companies has exceeded that of PD1/PD-L1 drugs developed by biopharmaceutical companies in other countries (23). In this study, there was no significant difference in CAE between domestic ICIs and imported ICIs. In addition, the effects of PD-1 and PD-L1 inhibitors on CAE were also studied. Among them, PD-1 inhibitors block the PD-1/PD-L2 pathway, resulting in increased binding of PD-L2 to the repulsive guidance molecules B (RGMb) receptor, which may affect immune system homeostasis. Anti-PD-L1 still allows PD-1 to interact with its other ligand, PD-L2, and may be less toxic as PD-L2 signaling protects immune homeostasis (24). A systematic review investigated differences in the toxicities of PD-1 and PD-L1 inhibitors in patients with non-small cell lung cancer (NSCLC). Patients treated with PD-1 inhibitors were found to have a slightly higher rate of irAEs (16% vs. 11%, P = 0.07) and pneumonitis (4% vs. 2%, P = 0.01) compared to patients who received PD-L1 inhibitors (25). Compared with PD-1 inhibitor use, PD-L1 inhibitor use was significantly associated with lower risks of cardiac complications both before and after propensity score matching (26). We found that PD-L1 inhibitors had a lower incidence of CAE than PD-1 inhibitors.

Our study found that the decrease in EF from baseline was not related to CAE, which was consistent with previous studies (6, 18), suggesting that the dependence on EF alone to detect the occurrence of CAE in patients receiving ICIs is inadequate. Clinicians should not rely on EF as a discriminant indicator of the severity of ICI-related CAE (6). The QTc interval is a standardized measure available routinely from a 12-lead ECG and predominantly represents ventricular repolarization (27). In a retrospective analysis, QTc was more prolonged (26.8 ± 12.0 from baseline; P = 0.036) at the time of MACE (4). Our study also showed that prolonged QTc was significantly associated with an increased risk of CAE (P = 0.002).

The CK value has low specificity, and our study found no significant difference in the CK value before and after the occurrence of CAE. In a retrospective analysis of patients with lung cancer treated with ICIs, mild elevations in cTnI were observed at the time of MACE (4). Abnormal levels of troponin were observed in 94% of patients with ICI-associated myocarditis (6). Elevated troponin usually indicates myocardial cell death. Our study found that elevated cTnI was significantly associated with the appearance of CAE (P = 0.028). Therefore, monitoring troponin in each treatment cycle could allow patients with potential myocarditis to be admitted to the hospital as soon as possible.

The strength of our study is a real-world study. Due to the low incidence of ICI-related myocarditis, pericarditis, and heart failure related to ICI, cancer patients without clinical cardiovascular symptoms treated with ICI have not received enough attention from oncologists to perform regular cardiovascular evaluations (28). Our knowledge of ICI-associated CAE has been significantly enhanced by case series and pharmacovigilance databases. Due to a retrospective design, selection bias remains a concern, as there was no prospective cardiovascular screening protocol in all sites, and screening for cardiac biomarkers and other tests was left to the discretion of the individual providers (18). In addition, due to the invasive nature of endocardial biopsy, there was a lack of biopsy-proven cases.

Conclusion

Using nivolumab as a control, there was no independent association between the eight ICIs and CAE risk. However, PD-L1 inhibitors had a lower rate of CAE than PD-1 inhibitors. Combination therapies of ICI with VEGFR inhibitors significantly increased the risk of CAE. Patients who had a history of previous thoracic radiation therapy taking ICIs also had increased CAE risk. L-NLR and higher comorbidity burden were associated considerably with CAE and could be used as a risk predictor for CAEs. Cardiac biomarkers such as cTnI, CK-MB, and QTc were significantly elevated when CAEs were present and could be used as monitoring factors. Patients will benefit from close monitoring by incorporating clinical assessment, cardiac biomarkers, and cardiac examination into the management recommendations for ICI therapy.

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 the Department of Pharmacy, The Second Affiliated Hospital of Dalian Medical University, Dalian, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

GF and NS: full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis and obtained funding. SW, HB, and LZ: concept and design and drafting of the manuscript. JH, XL, and SYW: acquisition, analysis, and interpretation of data. HB, XL, and GF: critical revision of the manuscript for important intellectual content. HB and LZ: statistical analysis. SW, HB, and GF: supervision. All authors contributed to the article and approved the submitted version.

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.

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Naringenin ameliorates myocardial injury in STZ-induced diabetic mice by reducing oxidative stress, inflammation and apoptosis *via* regulating the Nrf2 and NF-κB signaling pathways

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Diabetes-induced myocardial damage leads to diabetic cardiomyopathy and is closely associated with the generation of oxidative stress and inflammation. Naringenin (NG) exhibits antioxidant and anti-inflammatory effects. However, whether NG has cardioprotective effects against diabetic cardiomyopathy by regulating oxidative stress and inflammation remains unknown. This study investigated the effect of NG on diabetic cardiomyopathy based on an analysis of streptozotocin (STZ)-induced type 1 diabetic mice. The results indicated that NG reduced cardiac fibrosis and cardiomyocyte apoptosis in this diabetic model, accompanied by reduced blood glucose. NG inhibited pro-inflammatory cytokines, the level of reactive oxygen species and the expression of nuclear factor kappa-B (NF-κB), whereas the expression of antioxidant enzymes and nuclear factor erythroid 2-related factor 2 (Nrf2) were greatly enhanced by NG. Furthermore, in high glucose-treated H9C2 myocardial cells, NG effectively reduced cell apoptosis by inhibiting the formation of reactive oxygen species and pro-inflammatory cytokines. NG's antioxidant and anti-inflammatory activities were mechanistically associated with NF-κB inhibition and Nrf2 activation in animal and cell experiments. Data analysis showed that NG could regulate Nrf2 and NF-κB pathways to protect against diabetes-induced myocardial damage by reducing oxidative stress and inhibiting inflammation.

KEYWORDS

naringenin, inflammation, oxidative stress, diabetic cardiomyopathy, Nrf2, NF-κB

Introduction

It is estimated that by 2040, there will be 642 million diabetes patients aged 20 to 79 years globally (1). Studies have indicated that diabetic cardiovascular complications have become the leading cause of mortality among diabetic patients (2). Some patients have a hyperglycemia-induced myocardial injury but do not have high blood pressure or coronary artery disease. This disease, termed diabetic cardiomyopathy (3), is currently of considerable interest among researchers (4). The underlying mechanisms of hyperglycemia-induced myocardial injury include cardiac inflammation, increased reactive oxygen species (ROS), interstitial fibrosis, and cardiac cell apoptosis (2, 5). Such adverse reactions induce structural remodeling and affect heart diastolic function (6). Therefore, pharmacological inhibition of inflammation, ROS, and cell apoptosis has been proven to confer cardioprotection.

Many natural flavonoids have been shown to exhibit potent cardioprotective properties (7, 8). Several lines of evidence have demonstrated that naringenin (NG), a flavonoid abundant in grapefruit, plays a valuable role in the cardiovascular system. NG has been shown to reduce cardiac damage following ischemia-reperfusion injury by inhibiting mitochondrial oxidative stress damage (9). In the mouse sepsis model, NG protected against septic cardiac dysfunction by inhibiting NF-κB-dependent cardiac inflammation (10). Furthermore, NG treatment alleviated ischemia-reperfusion injury-induced myocardial cell death in rat models (11). These studies suggest that anti-inflammatory and antioxidant properties may be involved in the cardioprotective mechanism of NG. However, it remains unknown whether NG improves diabetes-induced myocardial injury.

Therefore, this study investigated the influence of NG on myocardial injury and related signaling pathways in STZ-induced diabetic mice and high glucose-induced H9C2 cells.

Materials and methods

Animals and treatment

Animal experiments followed the protocol previously described with minor changes (12, 13). In this study, C57BL/6 mice (Chengdu Experimental Animals Co., Ltd., Chengdu, China) were kept at room temperature of 23 ± 1°C with a 12:12 h light: dark cycle. For the experimental protocol, the *in vivo* experiment was conducted based on that stated by the National Institutes of Health for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

After 1 week of adaptive feeding, the animals were randomly assigned to five groups (six mice per group): control group (Ctrl), diabetes mellitus group (DM), 25 mg/kg NG + DM group (LNG + DM), 50 mg/kg NG + DM group (MNG+DM), and 75 mg/kg NG + DM group (HNG + DM). Following 12 h of fasting, 80 mg/kg of 1% STZ (Sigma, St. Louis, MO, USA) was injected intraperitoneally in diabetic mice groups (DM with/without treatment). The mice of the Ctrl group received an intraperitoneal injection with an equal volume of sodium citrate buffer. After 72 hours, the tail blood was sampled. The criterion for successfully establishing the model was a blood glucose level >16.7 mmol/L. Following the establishment of the model, different doses of NG (25, 50, and 75 mg/kg; Civi Chemical Technology Co., Ltd., Shanghai, China) were administered by gavage to the respective NG groups once daily. After euthanasia of the mice, the hearts were fixed in 4% paraformaldehyde, prepared in paraffin, and sections of 4 μm thick were cut. Fresh heart tissues were used for Western blot detection after storage at −80°C.

Analysis of myocardial histology

Hematoxylin and eosin (HE) and Masson staining was performed and images of the stained paraffin sections were obtained using a light microscope.

Determination of malondialdehyde (MDA) and superoxide dismutase (SOD) levels

The total protein concentration was determined using the bicinchoninic acid assay (BCA) method. The MDA and SOD levels in the cardiac tissues were detected using test kits from the Jiancheng Bioengineering Institute (Nanjing, China).

Analysis of immunohistochemistry

The paraffin sections were deparaffinized and placed in citrate buffer. The sections were heated to boiling when the power was switched off. After 5 min, the process was repeated. After cooling, the sections were washed twice with phosphate-buffered saline (PBS) for 5 min to retrieve antigens. The sections were blocked with goat serum for 20 min at room temperature and incubated with interleukin (IL)-6 (1:100), tumor necrosis factor (TNF)-α (1:100), or NF-κB p65 (1:100) antibodies at 4°C overnight. A secondary antibody was applied for 30 min at 37°C. The sections were washed three times with PBS for 5 min, and diaminobenzidine (DAB) was used to develop the color. Finally, sections were observed, and images were obtained using a light microscope (OLYMPUS BX53, Japan). For NF-κB p65 and Nrf2 fluorescence staining, fixed cardiomyocytes were incubated with NF-κB p65 (1:100) or Nrf2 (1:100) antibodies overnight at 4°C followed by phycoerythrin (PE)-conjugated secondary antibody (1:200). The analysis of fluorescent images was performed using a fluorescence microscope.

Apoptosis in tissue or cells was quantified using the TUNEL apoptosis test kit from Beyotime Biotechnology (Jiangsu, China) according to the manufacturer's instructions. Fluorescence microscopy was performed to analyze the samples.

Western blot

The tissues and cells were washed twice using ice-cold PBS and lysed in lysis buffer. Lysates were separated by sodium dodecyl sulfate-polyacrylamide (10%−12%) gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were washed with Tris-buffered saline/Tween-20 (TBST) and blocked in 5% skim milk powder dissolved in TBST for three hours, followed by the incubation with the respective primary antibody at dilutions according to the supplier's instructions.

The membranes were examined using an anti-NF-κB p65 antibody (1:1,000, Cell Signaling Technology), anti-heme oxygenase-1 (HO-1) antibody (1:1,000, Abcam), anti-Nrf2 antibody (1:1,000, Cell Signaling Technology), anti-Bax antibody (1:500, Santa Cruz Biotechnology), anti-cleaved caspase-3 antibody (1:500, Santa Cruz Biotechnology), anti-IL-6 antibody (1:500, Santa Cruz Biotechnology), anti-TNFα antibody (1:500, Santa Cruz Biotechnology), anti-nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) antibody (1:500, Santa Cruz Biotechnology), or anti-NQO1 antibody (1:500, Abcam). Following conjugation to horseradish peroxidase, the corresponding immunoglobulin G secondary antibody (1:1,000, Beyotime Biotechnology) was used to detect the primary antibodies. Enhanced chemiluminescence (Pierce, MA, USA) was used to visualize the bands.

Cell culture and treatment

The H9C2 cell line was kindly provided by Dr. Xiaoqiu Tan (Cardiovascular Research Institute, Southwest Medical University, Luzhou, China). H9C2 cells were cultured according to the usual protocol reported previously (14). Additionally, pretreatment of cells with 10 μM NG was performed for 2 h before exposure to 33 mM glucose (HG) for 24 h.

ROS detection

Tissue sections or H9C2 cells were incubated for 30 min at 37°C using dihydroethidium (DHE; KeyGEN Biotech, Nanjing, China) or 2,7-Dichlorodi -hydrofluorescein diacetate (DCFH-DA; Beyotime Biotechnology), respectively. Following three washes with PBS, images of the tissue sections and cells were obtained using a fluorescence microscope. Graphical analysis software was applied to determine the mean fluorescence intensity.

Flow cytometric analysis

Apoptosis was analyzed by flow cytometry (15). Cells were trypsinized, harvested, washed twice with cold PBS, and centrifuged. The supernatant was removed, and the cells were resuspended in 1 ml of binding buffer. Cells were vortexed gently, incubated for 10 min at room temperature in the dark, and stained using 5 μl Annexin V-FITC. Cell staining was performed with a 5 μl propidium iodide (PI) solution for 5 min at room temperature in the dark. Cells were resuspended in 500 μl PBS and gently vortexed. Cell analysis was carried out by flow cytometry within 1 h.

Statistics

Measurements are expressed as means ± standard deviations. Analysis of variance was applied to determine

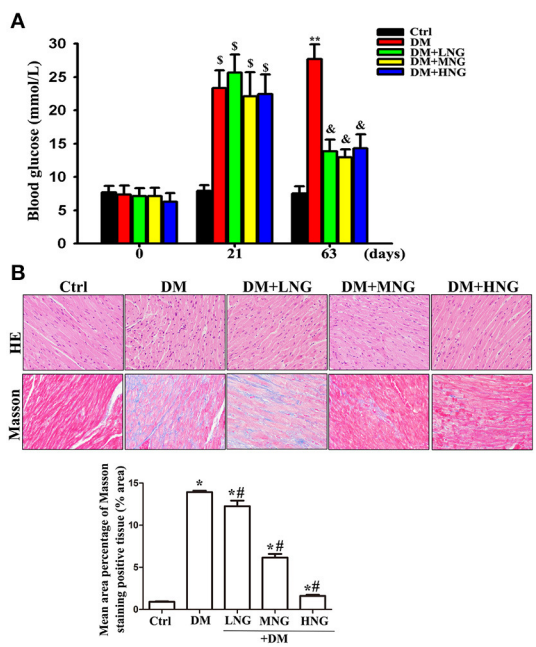

FIGURE 1
NG improves hyperglycemia and pathologic damage to cardiac tissue in diabetic mice. **(A)** Blood glucose of mice from different treatments. Data are presented as the mean ± SD ($n = 6$). $^{\$}P <$ 0.05 vs. corresponding groups in 0 day; $^{**}P < 0.05$ vs. Ctrl group in 63 days, $^{\&}P < 0.05$ vs. DM group in 63 days. **(B)** Representative images of HE or Masson staining for each group, light microscope (400 ×). The data are presented as mean ± SD ($n =$ 3). $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group.

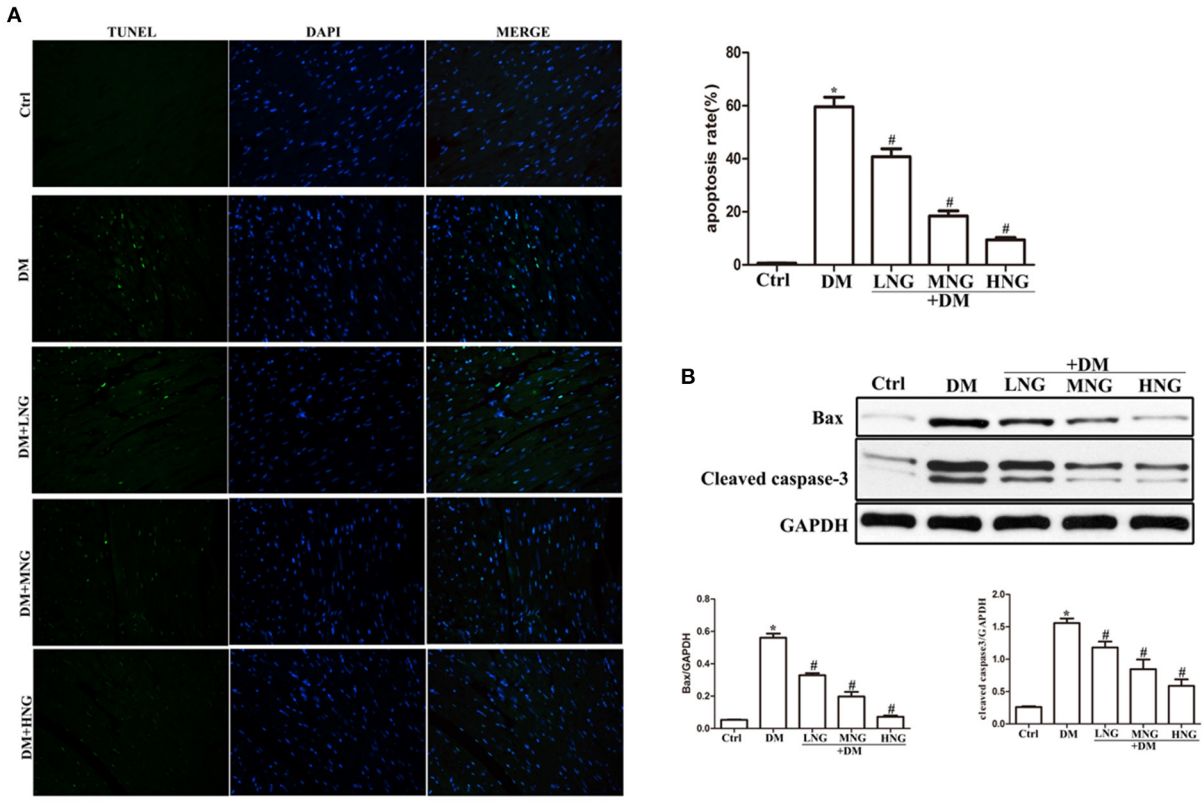

FIGURE 2
NG reduces apoptosis of cardiomyocytes in cardiac tissues of diabetic mice. **(A)** Representative images of TUNEL staining for each group, fluorescence microscope (200×). Data are presented as the mean ± SD ($n = 3$). $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(B)** Quantitative analysis of the protein expression of Bax and cleaved caspase-3 in cardiac tissues. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group.

the statistical significance between groups. Individual points were analyzed for statistical significance using Student's *t*-test. Statistics were conducted using SPSS 17. A value of $P < 0.05$ was considered statistically significant.

Results

NG ameliorated hyperglycemia and pathologic damage to cardiac tissue in diabetic mice

The blood glucose level of the STZ-treated groups at 21 days significantly increased compared to day 0 ($P < 0.05$, Figure 1A). After 63 days, blood glucose in the DM group was also significantly elevated compared to the Ctrl group ($P < 0.05$). In contrast, the differences in blood glucose after NG interventions were significantly reduced compared with the DM group ($P < 0.05$). HE and Masson staining demonstrated that cardiac structures were ordered in the Ctrl group, with a clear outline of tissues and collagen fibers in the interstitium (Figure 2B). On the contrary, degeneration, necrosis of some cardiomyocytes, disordered arrangement of cells, and most of the collagen fibers in the interstitium were observed in the DM group. These morphological injuries were improved in the DM groups treated with the different doses of NG (Figure 2B).

NG reduced cardiomyocyte apoptosis in the cardiac tissues of diabetic mice

TUNEL staining was used to detect cardiomyocyte apoptosis. The apoptosis rate in the DM group was significantly increased compared with the Ctrl group ($P < 0.05$, Figure 2A). Treatment with the different doses of NG significantly reduced the apoptosis rate ($P < 0.05$). Cardiomyocyte apoptosis is a significant factor inducing cardiac dysfunction (16). Therefore, apoptosis-related signal proteins were evaluated. Induction of hyperglycemia prominently increased Bax and cleaved caspase-3 compared with the Ctrl group ($P < 0.05$, Figure 2B). By contrast, the expressions of Bax and cleaved caspase-3

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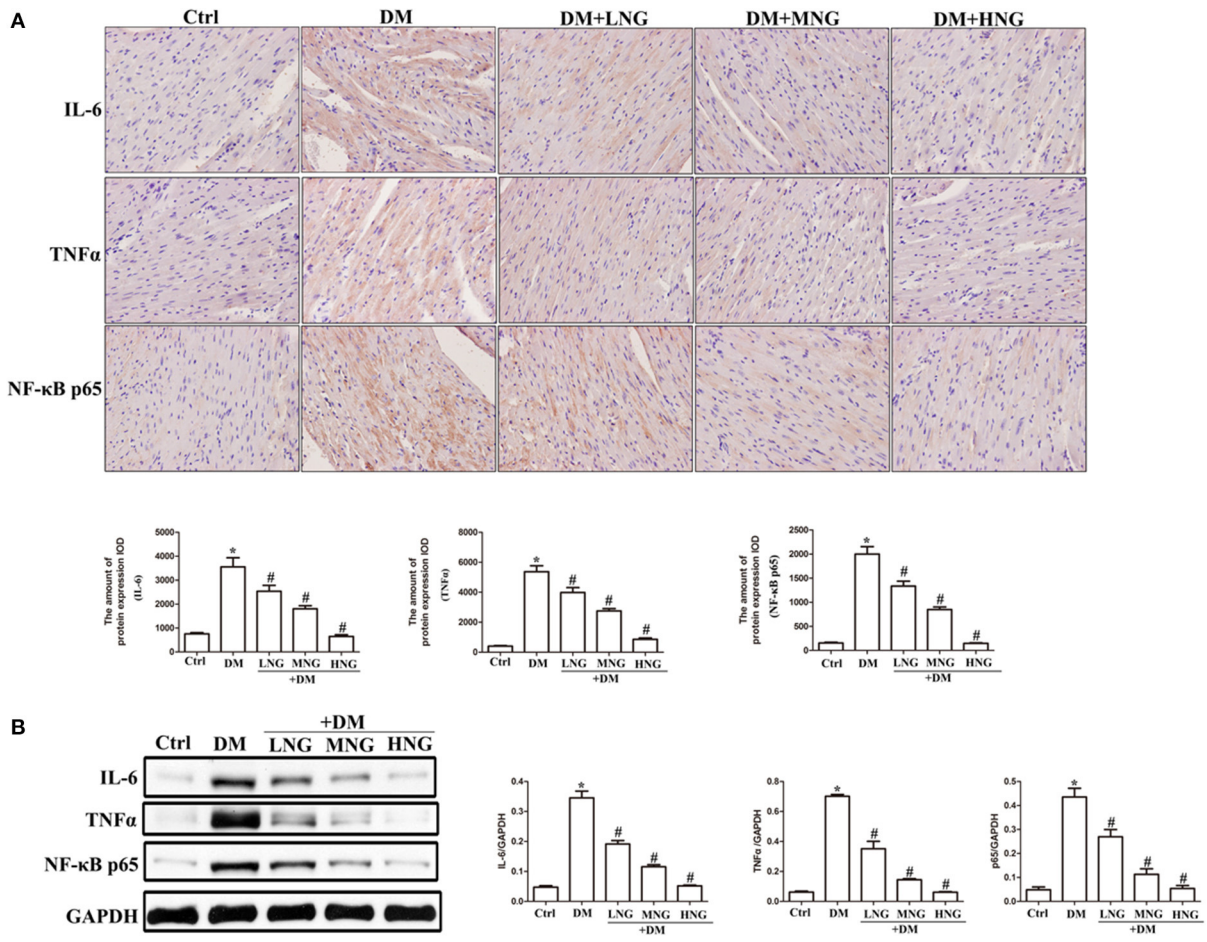

FIGURE 3
NG inhibits the expression of inflammatory cytokines in cardiac tissues of diabetic mice. **(A)** Representative immunohistochemical staining of IL-6, TNF-α, and NF-κB p65 in cardiac tissues. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(B)** Quantitative analysis of the protein expression of IL-6, TNF-α, and NF-κB p65 in cardiac tissues. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group.

proteins were inhibited by treatment with the different NG doses ($P < 0.05$).

NG inhibited the expression of inflammatory cytokines in the cardiac tissues of mice with diabetes

The images of cardiac sections from the groups stained for IL-6, TNF-α, or NF-κB p65 are shown in Figure 3A. As demonstrated by the quantitative analysis results, apparent upregulation of IL-6, TNF-α, and NF-κB p65 was observed in the DM group compared with the Ctrl group ($P < 0.05$, Figure 3A). Additionally, NG at the different doses used to treat diabetic mice reduced the expression of IL-6, TNF-α, and NF-κB p65 in a dose-dependent manner (Figure 3A), as well as decreased the IL-6, TNF-α, and NF-κB p65 protein levels (Figure 3B).

NG reduced diabetes-induced oxidative stress in cardiac tissues of mice with diabetes

The images of the cardiac sections stained with DHE from the different groups are shown in Figure 4A. Quantitative analysis demonstrated a significant ROS upregulation in the DM group compared with the Ctrl group ($P < 0.05$, Figure 4B). In contrast, the ROS levels in the different NG groups were reduced dose-dependent compared to the DM group ($P < 0.05$, Figure 4B). Furthermore, NG treatment resulted in decreased MDA levels (a biomarker of oxidative

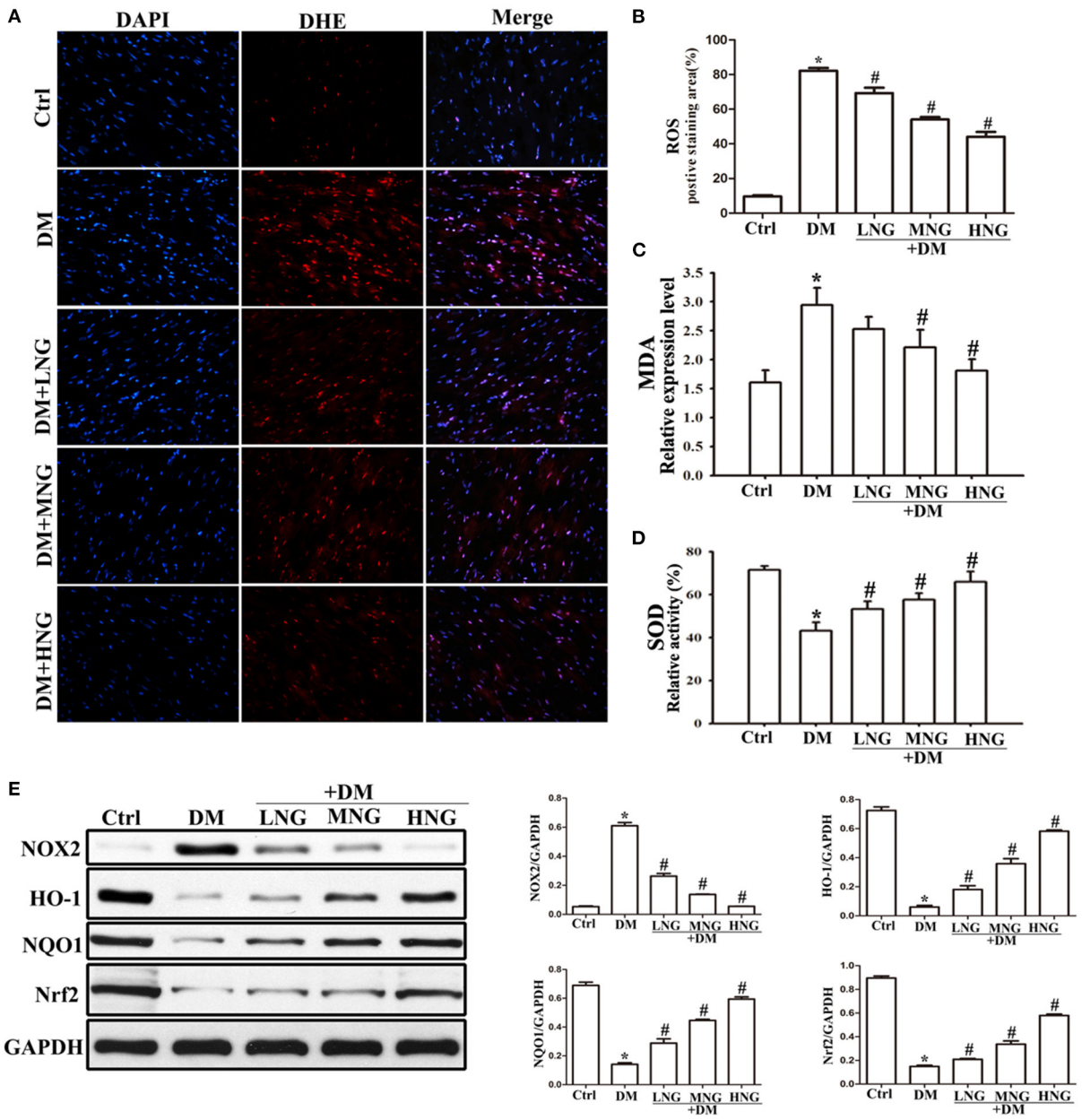

FIGURE 4
NG reduces diabetes-induced oxidative stress in cardiac tissues of diabetic mice. **(A)** Representative images of DHE staining for each group, fluorescence microscope (200×). **(B)** Quantitative analysis of the number of DHE fluorescence cells in cardiac tissue samples. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(C)** The level of malondialdehyde (MDA) from different treatments in cardiac tissue. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(D)** The level of superoxide dismutase (SOD) of different treatments in cardiac tissue. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(E)** Quantitative analysis of the protein expression of NOX2, HO-1, NQO1, and Nrf2 in cardiac tissues. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group.

damage) in a dose-dependent manner (Figure 4C), together with increased antioxidant SOD enzyme activity (Figure 4D). Nrf2 was previously shown to have a significant effect in inducing phase II detoxifying enzymes, for example, HO-1 and NQO1 (17). Therefore, Figure 4E indicates that Nrf2 and its downstream signaling proteins HO-1 and NQO1 are down-regulated in the DM group compared with the Ctrl group ($P < 0.05$), whereas they are elevated by NG treatment. Since NOX2 is the primary source of ROS, these results showed that NOX2 protein expression was up-regulated in the DM group compared

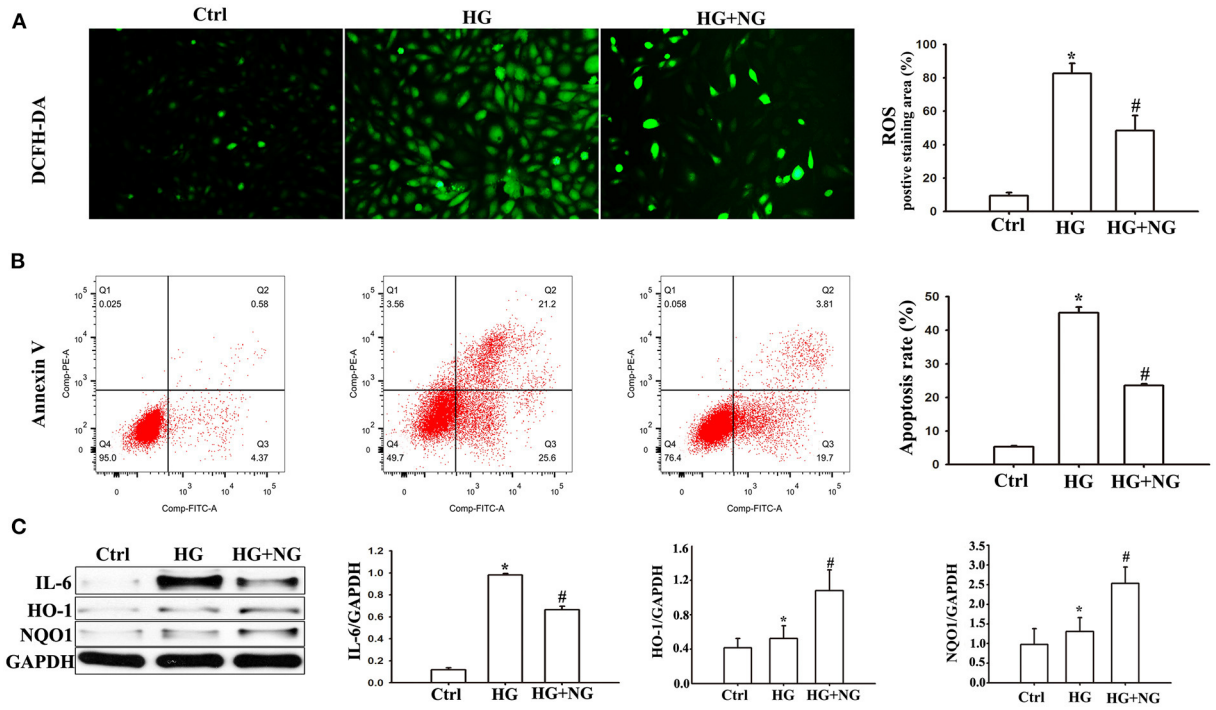

FIGURE 5
NG reduced HG-induced oxidative stress and apoptosis in H9C2 cells. **(A)** Representative images of DCFH-DA staining for each group, fluorescence microscope (200×). Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(B)** Evaluation of apoptosis by flow cytometry using Annexin V staining. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(C)** Quantitative analysis of the protein expression of IL-6, HO-1, and NQO1 in H9C2 cells. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group.

with the Ctrl group ($P < 0.05$, Figure 4E) but was reduced by NG treatment.

NG reduced HG-induced oxidative pressure and apoptosis in H9C2 cells

Reactive oxygen species analysis by DCFH-DA staining demonstrated a much higher percentage of ROS in H9C2 cells cultured under HG conditions compared to the Ctrl group ($P < 0.05$, Figure 5A). However, there was a significantly decreased percentage of ROS cultured in HG after NG treatment ($P < 0.05$, Figure 5A). The apoptosis analyses by flow cytometry showed a considerable increase in the percentage of apoptotic cells among the H9C2 cells cultured under HG conditions compared to the Ctrl group ($P < 0.05$, Figure 5B). For comparison, the percentage of apoptotic cells cultured in HG was markedly reduced after treatment with NG ($P < 0.05$, Figure 5B). In addition, significant changes in pro-inflammatory cytokine and antioxidant proteins were also detected. IL-6 expression increased in the DM group compared with the Ctrl group ($P < 0.05$, Figure 5C), while NG treatment reduced IL-6 expression compared to the DM group ($P < 0.05$, Figure 5C). The expression of HO-1 and NQO1 increased in the DM group compared with the Ctrl group ($P < 0.05$, Figure 5C), while the NG treatment reduced the expression of HO-1 and NQO1 compared to the DM group ($P < 0.05$, Figure 5C). These results indicated that the protective effects of NG were probably related to its antioxidant and anti-inflammatory characteristics.

NG regulated NF-κB and Nrf2 expression in H9C2 cells under HG condition

The influence of NG on NF-κB and Nrf2 activation was analyzed to determine the signaling pathways responsible for inflammatory cytokines and oxidative stress. Analysis of immunostaining data indicated that more positive staining for NF-κB p65 was observed in the nucleus under the HG condition compared to the Ctrl group (Figure 6A). At the same time, NG treatment reduced the positive staining for NF-κB p65 compared to the untreated HG group (Figure 6A). Consistent with this, NF-κB p65 protein expression in the nucleus was significantly suppressed by NG under the HG condition ($P < 0.05$, Figure 6B). Furthermore, analysis of the immunostaining showed less positive staining for Nrf2 in the nucleus under

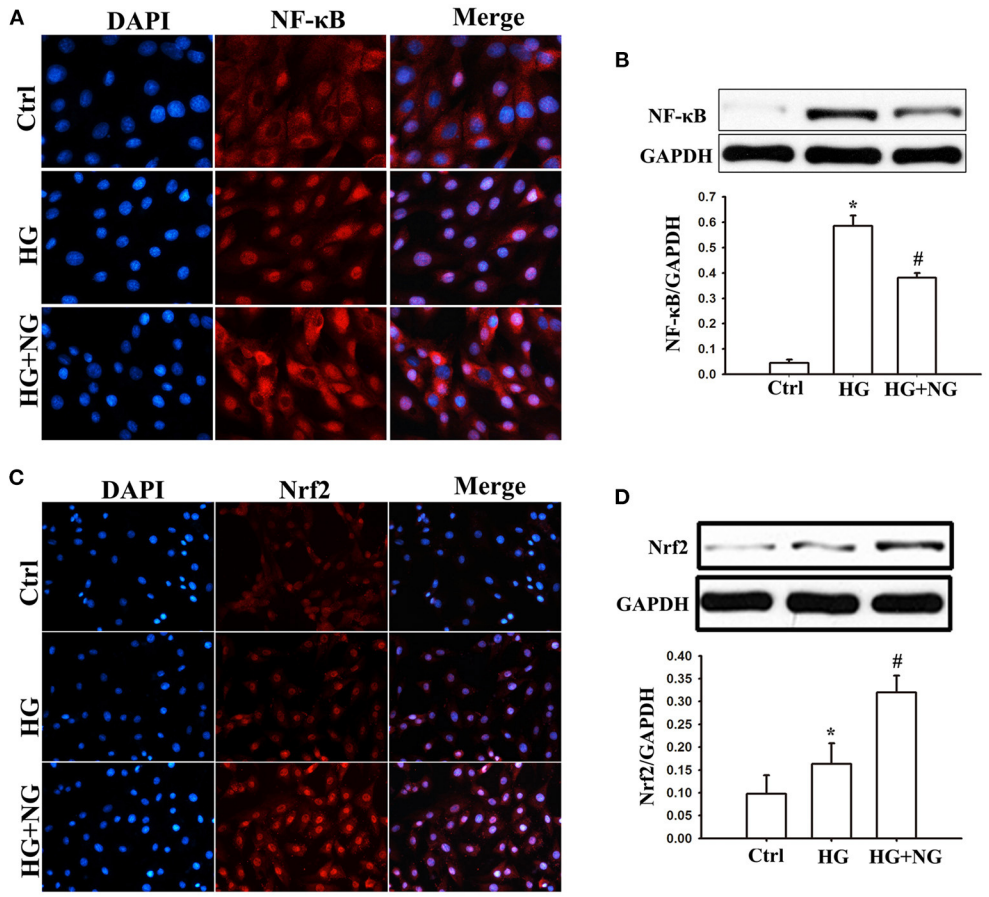

FIGURE 6
NG regulates the expression of NF-κB and Nrf2 in H9C2 cells under the HG condition. **(A)** Representative immunofluorescence staining of NF-κB for each group, fluorescence microscope (200×). **(B)** Quantitative analysis of the NF-κB protein expression in H9C2 cells. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group. **(C)** Representative immunofluorescence staining of Nrf2 for each group, fluorescence microscope (200×). **(D)** Quantitative analysis of the Nrf2protein expression in H9C2 cells. Data are expressed as mean ± SD ($n = 3$), $^{*}P < 0.05$ vs. Ctrl group; $^{\#}P < 0.05$ vs. DM group.

the HG conditions compared to the Ctrl group (Figure 6C). At the same time, NG treatment increased the positive staining for Nrf2 compared with the untreated HG group (Figure 6C). NG significantly upregulated the Nrf2 protein expression in the nucleus under the HG condition ($P < 0.05$, Figure 6D).

Discussion

Naringenin has been reported to have many cardioprotective activities (7, 18, 19). Although NG has been reported to improve high glucose-induced hypertrophy of cardiomyocytes in diabetic models (20, 21), the mechanism by which it exerts these protective effects against diabetic cardiomyopathy is not fully understood. Progressive oxidative stress and inflammation development are prominent markers of diabetic cardiomyopathy (22, 23). Therefore, inhibiting oxidative stress and inflammation during disease progression may be a promising strategy for treating diabetic cardiomyopathy.

Several lines of evidence have shown that NG exerts antioxidant and anti-inflammatory pharmacological activities (24, 25). Our previous study shows that NG ameliorates fibrosis by down-regulating Rho A/Rho-associated protein kinase (ROCK) signaling pathways in diabetic nephropathy mice (26). Furthermore, NG induced the expression of the antioxidant protein HO-1, reducing endothelial cell apoptosis under the HG condition (15). In the current study, NG improved diabetes-induced myocardial injury by reducing oxidative stress and inflammation by regulating the Nrf2 and NF-κB signaling pathways (Figure 7).

Chronic persistent inflammation can be one of the main reasons that hyperglycemia leads to changes in myocardial structure and function (16, 27). NF-κB is a vital transcription regulator for proinflammatory cytokine genes, including IL-6

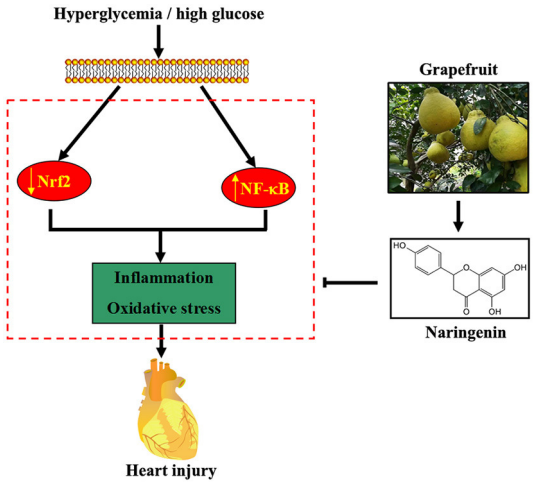

FIGURE 7
Proposed pathway for the cardioprotective effect of NG against diabetes-induced myocardial injury. Hyperglycemia or HG reduces Nrf2 expression and induces NF-κB expression, resulting in oxidative stress and inflammation. NG, a flavonoid abundant in grapefruit, partially attenuates diabetes-induced heart injury by activating the Nrf2 pathway and inhibiting the NF-κB pathway.

and TNF-α (28). The hearts of diabetic rats were characterized by increased NF-κB p65, IL-6, and TNF-α expression (29). Sophocarpine inhibition of NF-κB-mediated inflammation attenuated diabetic cardiomyopathy (30). In the present study, NG reduced the hyperglycemia-induced expression of proinflammatory cytokines and NF-κB. Therefore, the mediation of the protective effect of NG was based on inhibiting the NF-κB pathway.

In addition to cardiac inflammation, hyperglycemia-induced oxidative stress also affects the progression of diabetic cardiomyopathy. Enhancing antioxidant defense may protect against diabetes-induced cardiac dysfunction (31). As one of the most important transcription factors, Nrf2 exerts antioxidant and anti-apoptotic effects by activating multiple antioxidant genes, including HO-1 and NQO1 (6, 32). Our study showed that hyperglycemia increased ROS and MDA levels *in vivo*. Furthermore, NG treatment increased antioxidant enzymes (HO-1, NQO1, and SOD), reduced ROS generation, and activated Nrf2 in cardiac tissues from diabetic mice. Therefore, the results suggested that the Nrf2 pathway might be involved in the cardioprotective effect of NG. In particular, exposure to HG caused slightly higher Nrf2 expression than the control group. The findings differ from our *in vivo* study in which hyperglycemia suppressed Nrf2 expression. The cause of this discrepancy may be attributed to the duration of stimulation. We hypothesize that a short exposure of the cell to HG induces the compensatory protection mechanism. Therefore, Nrf2 is activated to exert its antioxidant stress effect. However, when animals are exposed to longer-term hyperglycemia, the antioxidant system will be weakened. Thus, Nrf2 expression is down-regulated by long-term hyperglycemia.

Oxidative stress and inflammation lead to cardiomyocyte apoptosis (33, 34). Therefore, the effect of NG on cardiomyocyte apoptosis was observed both *in vitro* and *in vivo*. In diabetic mice, NG treatment reduced hyperglycemia-induced cell apoptosis. Subsequently, NG also downregulated the expression of cleaved caspase-3 and the pro-apoptotic protein Bax. These data showed that inhibition of cardiomyocyte apoptosis could be one of the critical mechanisms of NG to improve diabetes-induced cardiac dysfunction. Furthermore, subsequent cardiac interstitial collagen deposition was attenuated by NG treatment.

This study found that NG ameliorated myocardial injury in diabetic mice. NG inhibited pro-inflammatory cytokines, ROS level, and NF-κB expression, while antioxidant enzymes and Nrf2 expression were significantly enhanced. The main limitation of this study was the lack of application of signal pathway inhibitors or gene knockdown in *in vitro* experiments. There is no direct evidence that NG inhibits oxidative stress, inflammation, or apoptosis by regulating Nrf2 or NF-κB. However, in support of our findings, inhibition of Nrf2 has been reported to suppress the NG-induced protective effect induced by NG in cardiac fibroblasts and vascular endothelial cells (15, 35). We will confirm these observations in future studies.

This study demonstrated that NG ameliorated myocardial injury in STZ-induced diabetic mice by improving Nrf2-mediated antioxidant stress and reducing NF-κB-mediated inflammation. Furthermore, targeting Nrf2 and NF-κB may be an important therapeutic strategy for reducing diabetic complications.

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.

Ethics statement

The study was approved by the Ethical Committee of Chongqing University Cancer Hospital.

Author contributions

YH and SW performed the experiment and wrote the paper. HS wrote the paper. YL analyzed the data. JF reviewed and revised the paper. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Preventive use of beta-blockers for anthracycline-induced cardiotoxicity: A network meta-analysis

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Background: Anthracyclines are commonly used chemotherapeutic agents to treat malignant tumors. However, cardiotoxicity is a potentially serious adverse effect of anthracyclines. Beta-blockers may be effective in preventing anthracycline-induced cardiotoxicity (AIC). However, the lack of direct comparisons of various beta-blockers interferes with clinical decision-making. Network meta-analysis (NMA) was performed to assess the effectiveness of beta-blockers for AIC.

Methods: We searched PubMed, Embase, Web of Science, and the Cochrane Central Register of Clinical Trials. The last update was in May 2022. Randomized controlled trials (RCT) of beta-blockers for AIC were included. Four beta-blockers were selected for comparison based on the number of studies. NMA was conducted with STATA 14.0 software.

Results: A total of 10 RCTs (875 patients) met the selection criteria. NMA results showed that carvedilol was superior to bisoprolol [$SMD = -0.50$, 95% CI (-0.91, -0.10)] and nebivolol [$SMD = -1.46$, 95%CI (-2.82, -0.11)] in a delay of LVEF. The results of the cumulative probability ordering are as follows: carvedilol (83.8%) > metoprolol (71.8%) > bisoprolol (43.9%) > placebo (40.9%) > nebivolol (9.5%).

Conclusion: Based on the available evidence, carvedilol is the best beta-blocker for AIC, followed by metoprolol. However, additional studies with large samples should be conducted to confirm our findings.

KEYWORDS

beta-blockers, anthracycline, cardiotoxicity, systematic review, network meta-analysis

Introduction

Anthracyclines are anticancer drugs, including Adriamycin, erythromycin, and epi-amycin. They can be used to treat various types of cancer, including breast cancer, lymphoma, and leukemia (1, 2). Although their anticancer effects are notable, numerous clinical studies have found these drugs have serious adverse effects, with cardiotoxicity particularly prominent (3, 4). Anthracycline-induced cardiotoxicity (AIC)

is a dose-limiting and possibly fatal complication of anthracycline administration that can arise during any period of chemotherapy (5). The main representative features are arrhythmias, pericardial effusion, and myocardial ischemia. AIC can contribute to cardiac failure and decrease survival (6). Mechanisms of AIC are complex and include free radicals, calcium overload, impaired energy metabolism, and apoptosis (7–12). A liposome-encapsulated formulation, doxorubicin liposome, was developed to limit anthracycline exposure in the myocardium. Liposomal doxorubicin improves the therapeutic index of conventional anthracyclines (13). However, AIC is still a pressing issue.

Beta-blockers are effective in treating hypertension, heart disease, and cardiac failure (14–16). Beta-blockers improve ventricular remodeling and reduce arrhythmias mainly by altering the status of adrenergic receptors (17, 18). Beta-blockers can delay the progression to heart failure in patients who develop cardiotoxicity (19, 20). Several beta-blockers are already available for clinical use, such as metoprolol, atenolol, nebivolol, bisoprolol, and carvedilol. Studies have shown that beta-blockers could be effective in preventing AIC (21). However, previous meta-analyses that evaluated the efficacy of beta-blockers to ameliorate AIC in terms of changes in left ventricular ejection function (LVEF) showed inconsistent results (19, 22–26). Furthermore, due to the small sample size of most studies, there is a lack of direct comparison between beta-blockers to determine which beta-blocker is most effective in preventing AIC.

Network meta-analysis (NMA) synthesizes evidence from direct and indirect comparisons to rank treatment interventions and guides drug selection (27). To provide additional evidence on beta-blocker treatment against AIC, a comprehensive systematic evaluation and NMA of relevant randomized controlled trials (RCTs) were conducted to assess which beta-blockers provided the best cardioprotective effect.

Methods

Network meta-analysis was conducted following the guidelines of the Preferred Reporting Items for Systematic

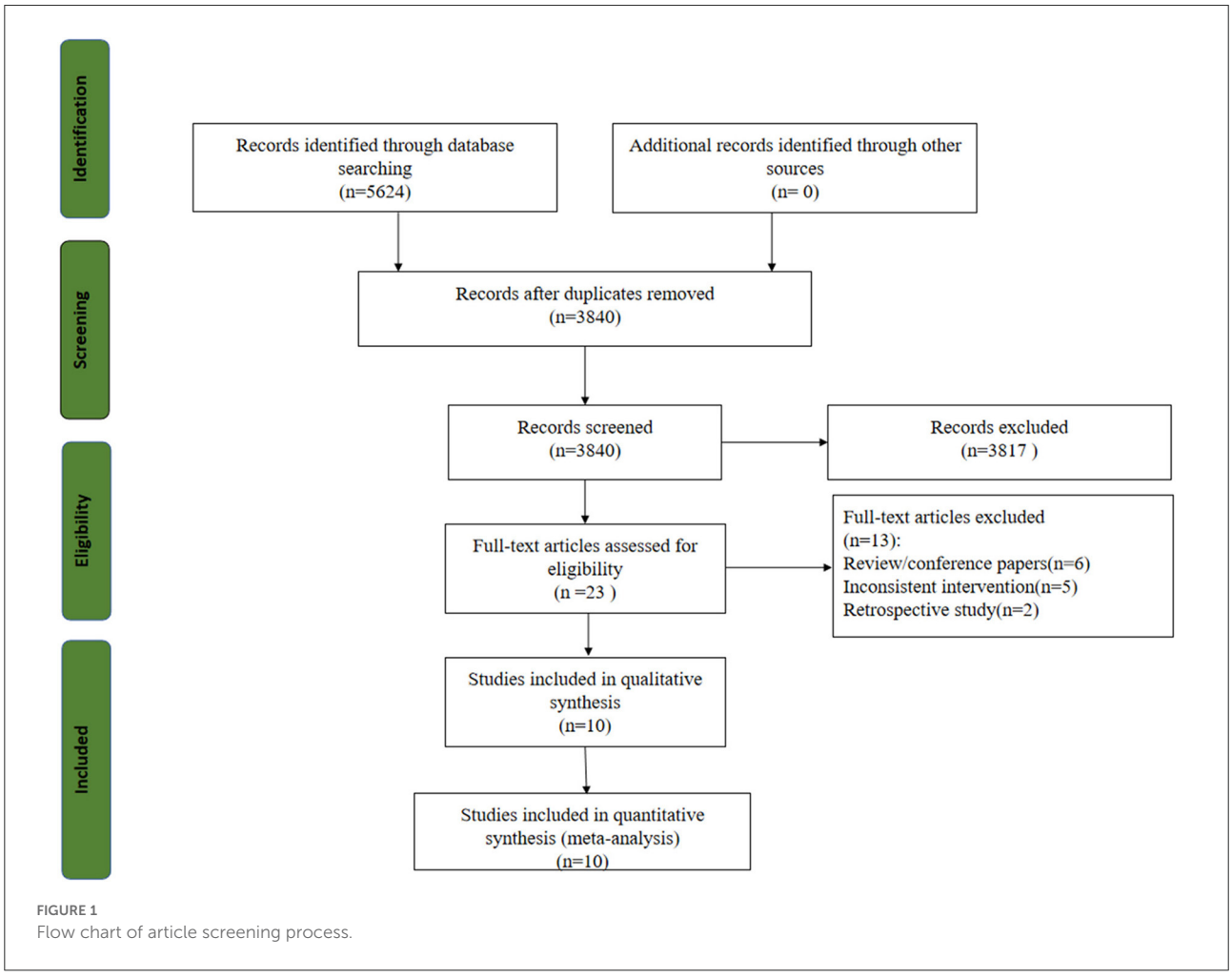

FIGURE 1
Flow chart of article screening process.

Reviews and Meta-Analysis Statements for Network Meta-Analysis (PRISMA-NMA) (28).

Inclusion and exclusion criteria

The inclusion criteria were: (1) participants: patients were diagnosed with tumors by pathology or imaging and were older than 18 years. All patients received anthracyclines, including epirubicin, pirarubicin, or doxorubicin. The dose and duration of drug treatment were unlimited; (2) type of study: randomized clinical trials (RCTs); (3) interventions: the experimental group began using beta-blockers before chemotherapy to counteract the cardiotoxicity of anthracycline-based chemotherapy. In the control group, a placebo was used; and (4) outcomes: LVEF at baseline and after chemotherapy, mortality, and adverse events. The exclusion criteria were: (1) non-RCT; (2) studies with insufficient data, duplicate data, or data that could not be extracted; (3) cardiotoxicity due to non-anthracycline chemotherapy; and (4) reviews, conference abstracts, or meta-analysis.

Search strategy

Randomized clinical trial studies on beta-blockers for the prevention of AIC were searched in PubMed, Embase, Web of Science, and the Cochrane Central Register of Clinical Trials. The last search was in May 2022. References from included studies were checked to identify additional studies. Search terms included anthracycline, chemotherapy, cardiotoxicity, doxorubicin, atenolol, carvedilol, metoprolol, nebivolol, bisoprolol, arotinolol, adrenergic beta-antagonists, randomized controlled trial, and random*. The search was conducted using a combination of medical subject headings and free-text words (Supplementary Table S1).

Data extraction

Data extracted included: (1) basic information: title, source, author, year; (2) baseline characteristics of the study population: number of trial participants, age, disease type; (3) intervention details, follow-up time; (4) key elements of the risk of bias evaluation; and (5) data on outcome indicators and outcome measures: LVEF, adverse events, and mortality.

Quality assessment

Two investigators independently assessed the risk of bias in the included studies and cross-checked the results. A bias

TABLE 1 General characteristics of selected RCTs.

Author year	State	Mean age		Sample size		Cancer type	Drug type	Doses	Follow-up duration	Outcome indicators
		T	C	T	C					
Livi et al., 2021 (36)	Italy	24~75	24~75	45	42	Breast cancer	Bisoprolol	5 mg	24, m	LVEF
Gulati et al., 2016 (33)	Norway	50.5 ± 9.1	50.8 ± 9.2	30	30	Breast cancer	Metoprolol	100 mg	NA	LVEF, side effects
Kaya et al., 2013 (35)	Turkey	51.4 ± 9.4	50.5 ± 11.1	27	18	Breast cancer	Nebivolol	5 mg	NA	LVEF
Abuosa et al., 2018 (30)	Saudi Arabia	46.1 ± 13.0/41.3 ± 18.2/ 42.0 ± 15.0	40.4 ± 14.0	41/38/37	38	Mostly breast cancer	Carvedilol	6.25 mg/12.5 mg/25 mg	6, m	LVEF, mortality
Avila et al., 2018 (31)	Brazil	50.8 ± 10.10	52.9 ± 9.05	96	96	Breast cancer	Carvedilol	25 mg	6, m	LVEF, mortality, side effects
Beheshti et al., 2016 (38)	Iran	29~54	29~54	30	40	Breast cancer	Carvedilol	6.25 mg	NA	LVEF
Elitok et al., 2014 (32)	Turkey	33.4 ± 5.8	34.3 ± 6.1	40	40	Breast cancer	Carvedilol	12.5 mg	6, m	LVEF
Kalay et al., 2006 (34)	Turkey	46.8 ± 14	49.0 ± 9.8	25	25	Mostly breast cancer	Carvedilol	12.5 mg	6, m	LVEF, mortality
Nabati et al., 2017 (37)	Iran	47.57 ± 8.75	47.1 ± 12.17	46	45	Breast cancer	Carvedilol	12.5 mg	6, m	LVEF
Salehi et al., 2011 (21)	Iran	45.70 ± 14.16/ 52.52 ± 11.00	43.50 ± 15.27	15/17	14	Breast cancer and lymphoma	Carvedilol	12.5 mg/25 mg	4, m	LVEF

NA, data not available; T, treatment group; C, control group; m, months; LVEF, left ventricular ejection fraction.

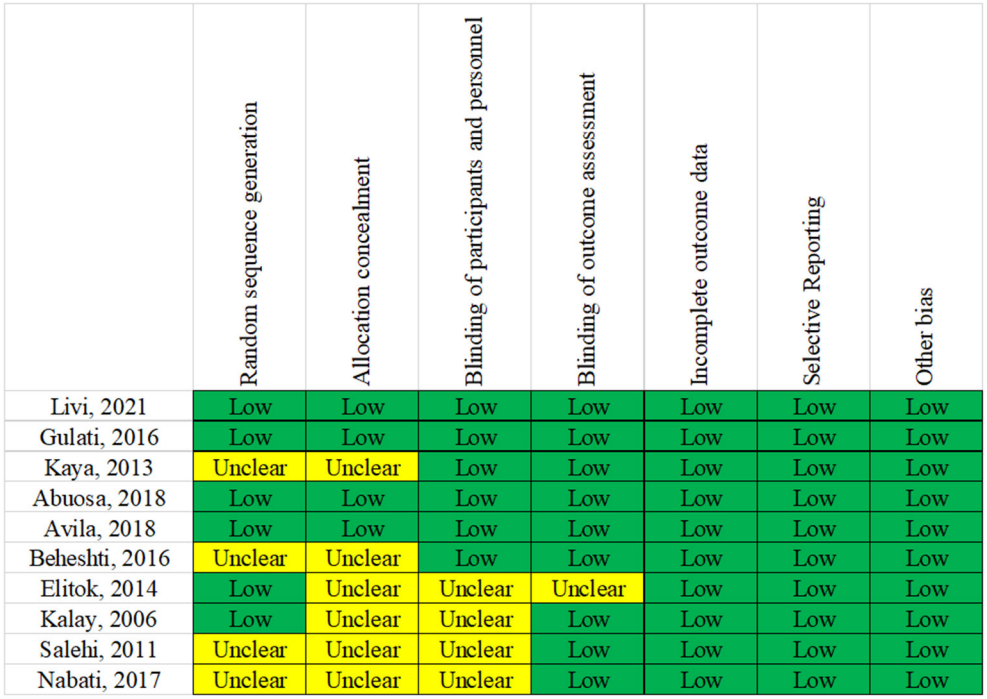

FIGURE 2
Risk of bias in the included studies.

assessment tool recommended in the Cochrane Handbook 5.1.0 was used to evaluate the risk of bias in RCT (29).

Statistical analysis

Stata 14.0 was used to analyze the data. Standardized mean difference (*SMD*) was used as the effect analysis statistic for the measurement data. Dichotomous variables were analyzed using the risk ratio (*RR*) as the effect analysis statistic. Each effect size was provided with its 95% confidence interval (95%*CI*). The χ^2 test was used to assess statistical heterogeneity between the results of the studies, while the magnitude of heterogeneity was determined by combining I^2 quantification. Fixed effects were used if there was no heterogeneity between studies ($I^2 < 50\%$, $P > 0.1$). If there was heterogeneity ($I^2 > 50\%$, $P < 0.1$), the source of heterogeneity was analyzed, and a meta-analysis was performed with random effects after excluding the influence of heterogeneity.

Due to the limited data available in the literature, subgroup analyses were only performed for doses of carvedilol (6.25 mg vs. 12.5 mg vs. 25 mg). Sensitivity analysis was used to test the stability of the meta-analysis results. The mvmeta package was used for data preprocessing in the NMA. When network relationship diagrams were drawn, inconsistency tests should be performed to determine if there were closed loops in the network relationship diagrams. In the present study, no closed loop was formed for each outcome indicator. Therefore, no inconsistency test was performed. The outcome indicators for each intervention were ranked by plotting the surface under the cumulative ranking curve (SUCRA). Comparison-adjusted funnel plots were used to assess publication bias and the effects of the small sample in included studies.

Results

Search results

Five thousand six hundred twenty-four studies were obtained from the initial review, and 10 RCTs were included after screening (21, 30–38). The study selection flow chart is shown in Figure 1.

Study and patient characteristics

There were 875 patients in the 10 RCTs. Most of the tumor types were breast cancer. Four beta-blockers were included: bisoprolol, metoprolol, nebivolol, and carvedilol. Two studies compared the efficacy of different doses of beta-blockers compared to placebo. The beta-blocker doses ranged from 5 to 100 mg. The experimental groups were comparable to the

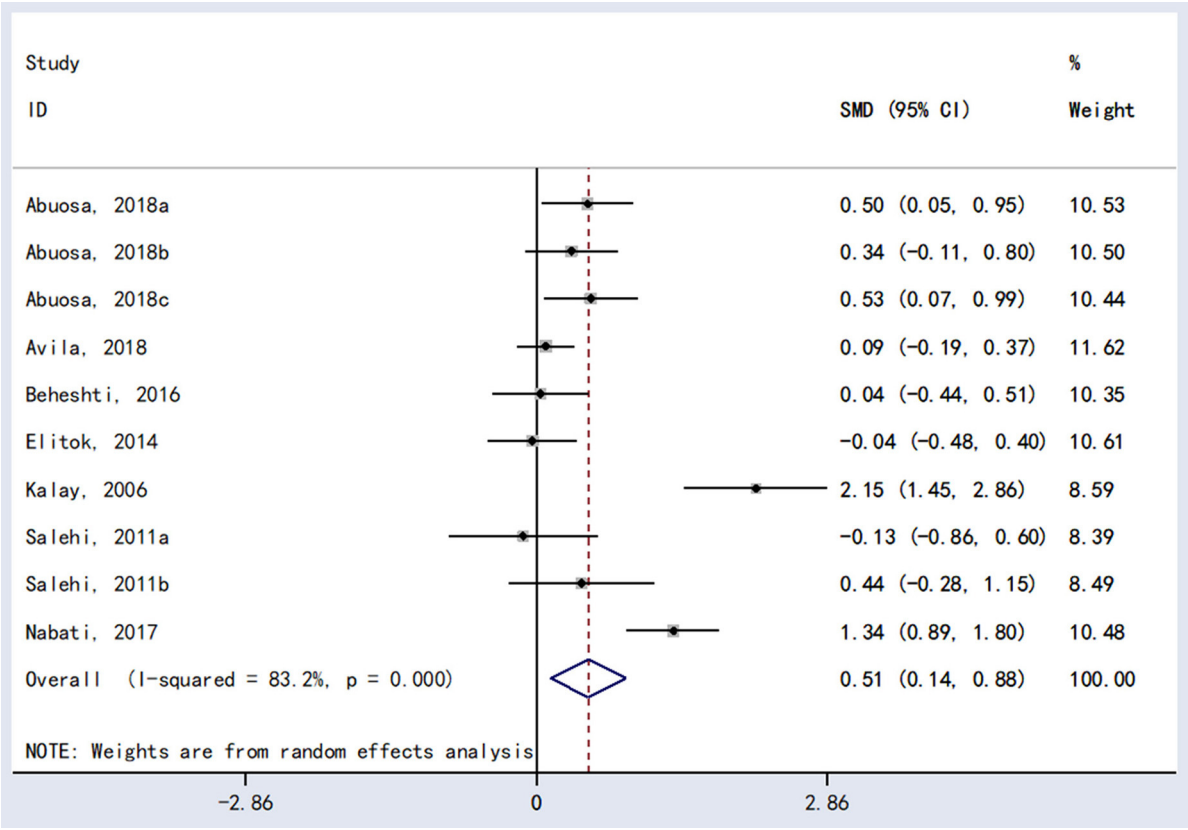

FIGURE 3
The forest plot of the effect of carvedilol on LVEF.

control groups at baseline in these RCTs. The characteristics of the included studies are shown in Table 1.

Quality assessment results

All included RCTs mentioned that the grouping was performed using a random method. Bias risk assessment for randomization showed low-risk bias in six RCTs and unclear in four RCTs. Regarding the concealment of random assignment, four RCTs showed low-risk bias, and six showed unclear results. Placebos were used in all RCTs to implement the blinded method. Regarding data completeness, selective reporting and other aspects showed a low risk of bias. The bias evaluation is shown in Figure 2.

Pairwise comparison of meta-analysis results

The meta-analysis showed a statistically significant difference between carvedilol and placebo in LVEF [$RR = 0.51$, 95%CI (0.14, 0.88), $P = 0.007$; Figure 3].

Compared to placebo, bisoprolol and nebivolol had an advantage in LVEF, with a statistically significant difference [$RR = 0.67$, 95%CI (0.23, 1.10), $P = 0.002$; $RR = 1.49$, 95% CI (0.82, 2.16), $P < 0.0001$]. However, the difference between metoprolol and placebo was not statistically significant [$RR = 0.06$, 95%CI (−0.44, 0.57), $P = 0.803$].

In terms of mortality, the meta-analysis did not show statistically significant differences between carvedilol and placebo ($RR = 1.08$, 95% CI (0.51, 2.27), $P = 0.889$; Figure 4].

Adverse events

There were no significant differences in adverse events between metoprolol and placebo [$RR = 4.00$, 95% CI (0.47, 33.73), $P = 0.203$] or carvedilol and placebo [$RR = 0.50$, 95% CI (0.13, 1.94), $P = 0.317$].

Subgroup analysis

Subgroup analysis was performed for different doses of carvedilol (6.25 mg vs. 12.5 mg vs. 25 mg). There were no

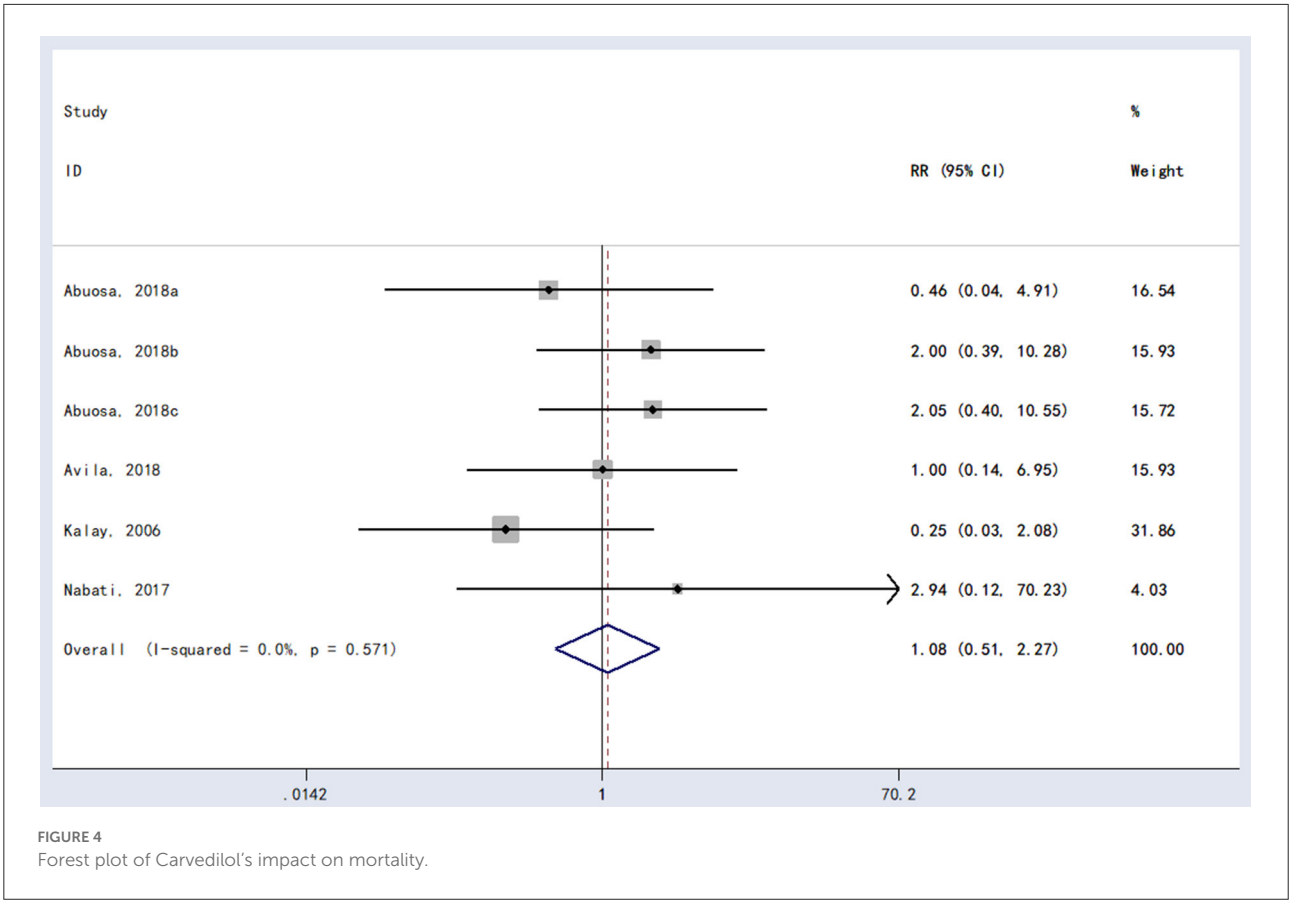

FIGURE 4
Forest plot of Carvedilol's impact on mortality.

statistically significant differences in LVEF performance between 6.25 and 12.5 mg [$RR = 0.28$, 95%CI (−0.18, 0.73), $P = 0.234$] or between carvedilol 6.25 mg carvedilol and 25 mg [$RR = 0.54$, 95%CI (−0.18, 1.25), $P = 0.140$] or between carvedilol 12.5 mg carvedilol and 25 mg [$RR = 0.64$, 95%CI (−0.10, 1.38), $P = 0.091$]. The results are shown in Figure 5.

Sensitivity analysis

The sensitivity analysis of the results of LVEF of carvedilol compared to placebo was performed using the one-by-one elimination method. The results showed that the meta-analysis results were stable (Figure 6).

Results of network meta-analysis

In terms of LVEF, the network relationships for the four beta-blockers are shown in Figure 7. According to the evidence network diagram of NMA comparisons, the width of each edge is proportional to the number of RCTs comparing each pair of treatments, and the size of each treatment node is proportional to the number of randomized participants (sample size).

Four different beta-blockers were subjected to NMA, yielding 10 two-by-two comparisons, two of which were statistically significant (Figure 8). NMA results showed that carvedilol was superior to bisoprolol [$SMD = -0.50$, 95% CI (−0.91, −0.10)] and nebivolol [$SMD = -1.46$, 95% CI (−2.82, −0.11)] in delaying the reduction in LVEF.

Probability ranking result

The four beta-blockers were ranked based on the SUCRA values (Figure 9). The results of the cumulative probability ordering are as follows: carvedilol (83.8%) > metoprolol (71.8%) > bisoprolol (43.9%) > placebo (40.9%) > nebivolol (9.5%).

Publication bias analysis

Comparison-adjusted funnel plots for LVEF as an outcome indicator were plotted for publication bias. These results showed

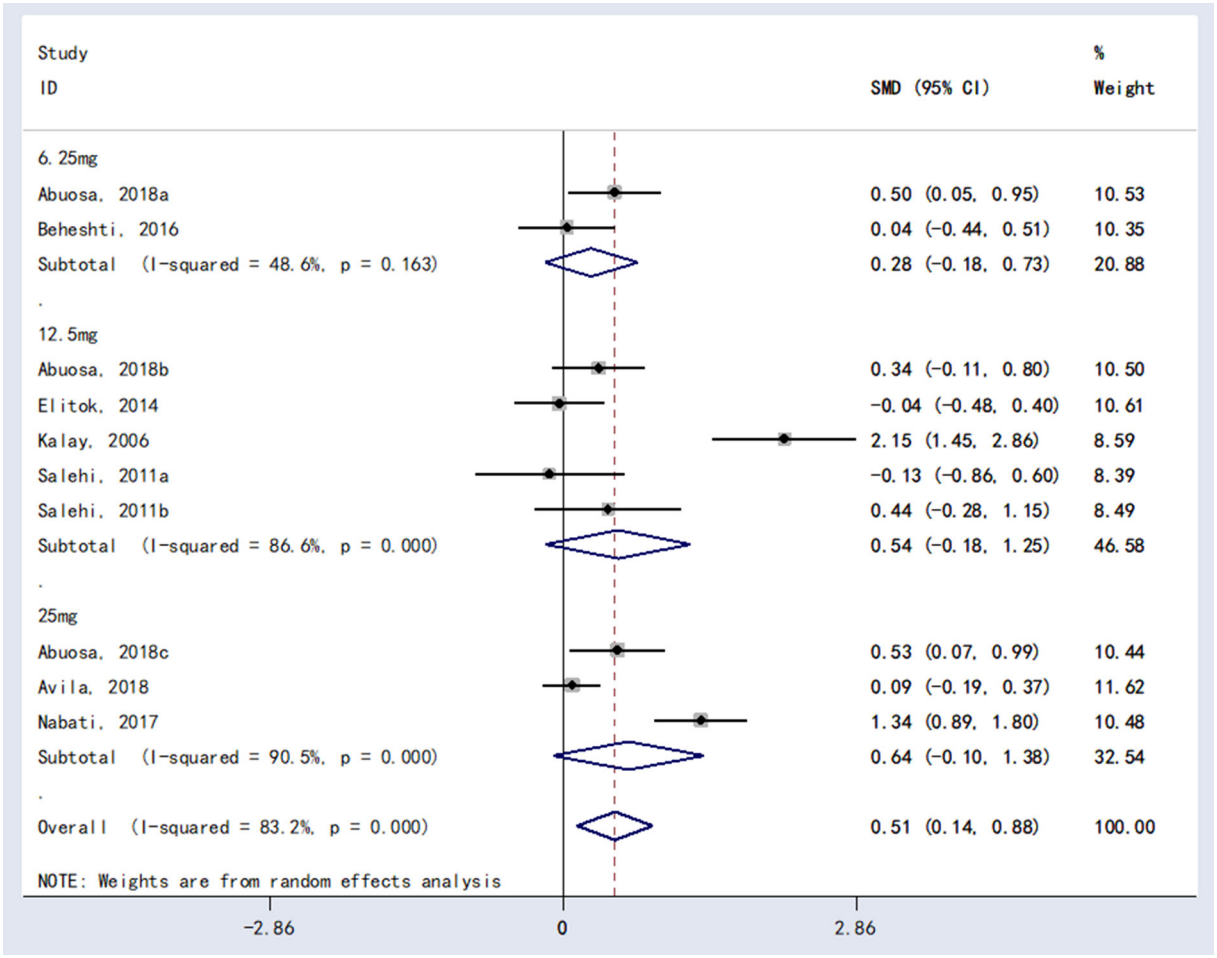

FIGURE 5
Subgroup analysis forest plot of effects of different doses of carvedilol on LVEF.

poor symmetry, suggesting a possible degree of publication bias (Figure 10).

Discussion

This study evaluated the efficacy of different beta-blockers vs. placebo for preventing AIC by NMA. NMA included 10 RCTs, including 875 patients. Beta-blockers included in the NMA were bisoprolol, metoprolol, nebivolol, and carvedilol. A previous meta-analysis confirmed the carvedilol cardioprotective effects in patients treated with anthracyclines, improving the significant decrease in LVEF and reducing the incidence of cardiovascular events (23). Long-term follow-up studies have shown that LVEF at the end of treatment is an independent predictor of cardiotoxicity (39). Lower LVEF is associated with an increased risk of cardiotoxicity (40). Therefore, a decrease in LVEF is recommended to define chemotherapy-related cardiotoxicity (41, 42).

Anthracyclines are among the most popular chemotherapy drugs due to their broad-spectrum and potent anticancer effects (43). While it provides sound anticancer therapeutic effects, the development of cardiotoxicity limits its application. The cardiotoxic effects become more pronounced as the cumulative dose increases. The risk of congestive heart failure is positively correlated with anthracycline doses (44–46). Early monitoring and timely intervention are essential to avoid the progression to irreversible heart damage (47). Beta-blockers can treat heart failure by stimulating the Gs-AC-cAMP-PKA signaling pathway to produce positive inotropic effects in cardiac myocytes (48).

The results of the direct comparative meta-analysis of this study showed an advantage of carvedilol in causing the delay in the reduction of LVEF compared to placebo. The results are consistent with other studies (22, 23, 25). The result stability was also confirmed by sensitivity analysis ruling out the possibility of false-positive results. In addition, bisoprolol and nebivolol were equally advantageous in mitigating the decline in LVEF.

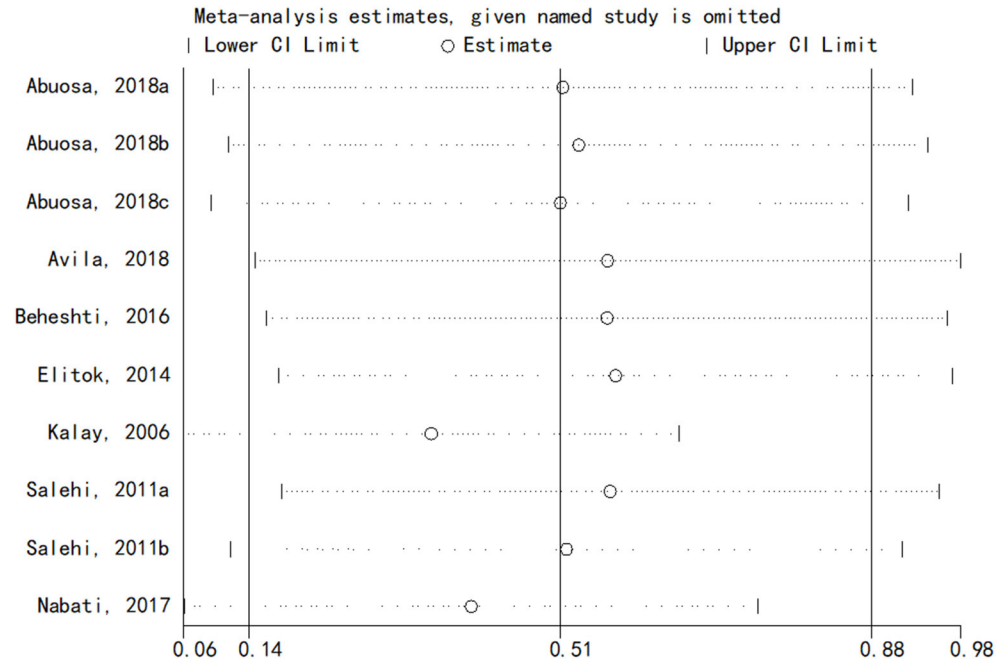

FIGURE 6
Sensitivity analysis results.

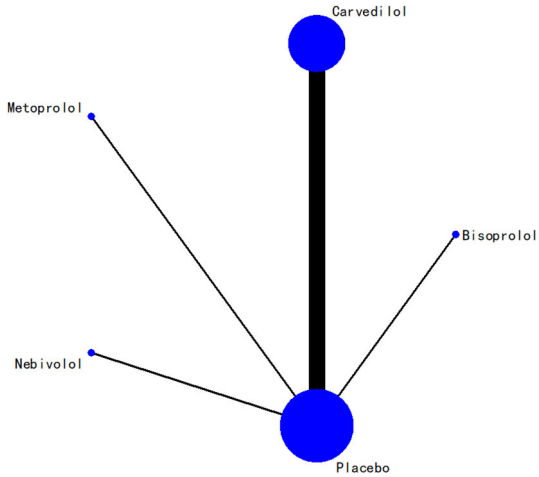

FIGURE 7
Evidence network diagram.

However, more evidence is needed to support the findings due to the size of the included studies. Unlike placebo, metoprolol was not statistically significant in mitigating the LVEF decline. This may be related to the ineffective protective effect against cardiotoxicity due to the absence of antioxidant activity of metoprolol (49).

The NMA results showed that carvedilol was superior to bisoprolol and nebivolol in delaying LVEF reduction. The results of the probability ranking indicated that carvedilol was the best beta-blocker to prevent AIC. Based on direct comparisons, carvedilol and placebo had no statistically significant difference in mortality. Therefore, we recommend carvedilol as the preferred regimen for preventing AIC. Carvedilol is an antioxidant and has more potent antioxidant properties than other types of beta-blockers (34). The metabolites of carvedilol exhibit antioxidant properties. The metabolites are 50 or 100 times more powerful than carvedilol (50). Carvedilol inhibits the lipid peroxidation in cardiac cell membranes and oxygen release from neutrophils. It preserves the body's natural antioxidant system by scavenging peroxides, hypochlorous radicals, and oxygen radicals (51). We attempted to compare the effect of different doses of carvedilol in delaying the reduction of LVEF by subgroup analysis. Unfortunately, no meaningful recommended dose was found. Therefore, future studies with varying doses of carvedilol to prevent AIC should be conducted.

Limitations

First, because of the lack of direct comparisons between different beta-blockers in included studies, the comparisons between other beta-blockers in NMA were obtained by indirect comparisons. Therefore, the results, effectiveness, and safety of the actual drugs may be biased. Second, the included

Carvedilol	0.06 (-1.22,1.34)	**0.50 (0.10,0.91)**	0.66 (-0.59,1.91)	**1.46 (0.11,2.82)**
-0.06 (-1.34,1.22)	Metoprolol	0.44 (-0.90,1.78)	0.60 (-1.19,2.39)	1.40 (-0.46,3.26)
-0.50 (-0.91,-0.10)	-0.44 (-1.78,0.90)	Bisoprolol	0.16 (-1.16,1.47)	0.96 (-0.46,2.38)
-0.66 (-1.91,0.59)	-0.60 (-2.39,1.19)	-0.16 (-1.47,1.16)	Placebo	0.80 (-1.04,2.65)
-1.46 (-2.82,-0.11)	-1.40 (-3.26,0.46)	-0.96 (-2.38,0.46)	-0.80 (-2.65,1.04)	Nebivolol

FIGURE 8
Results of network meta-analysis.

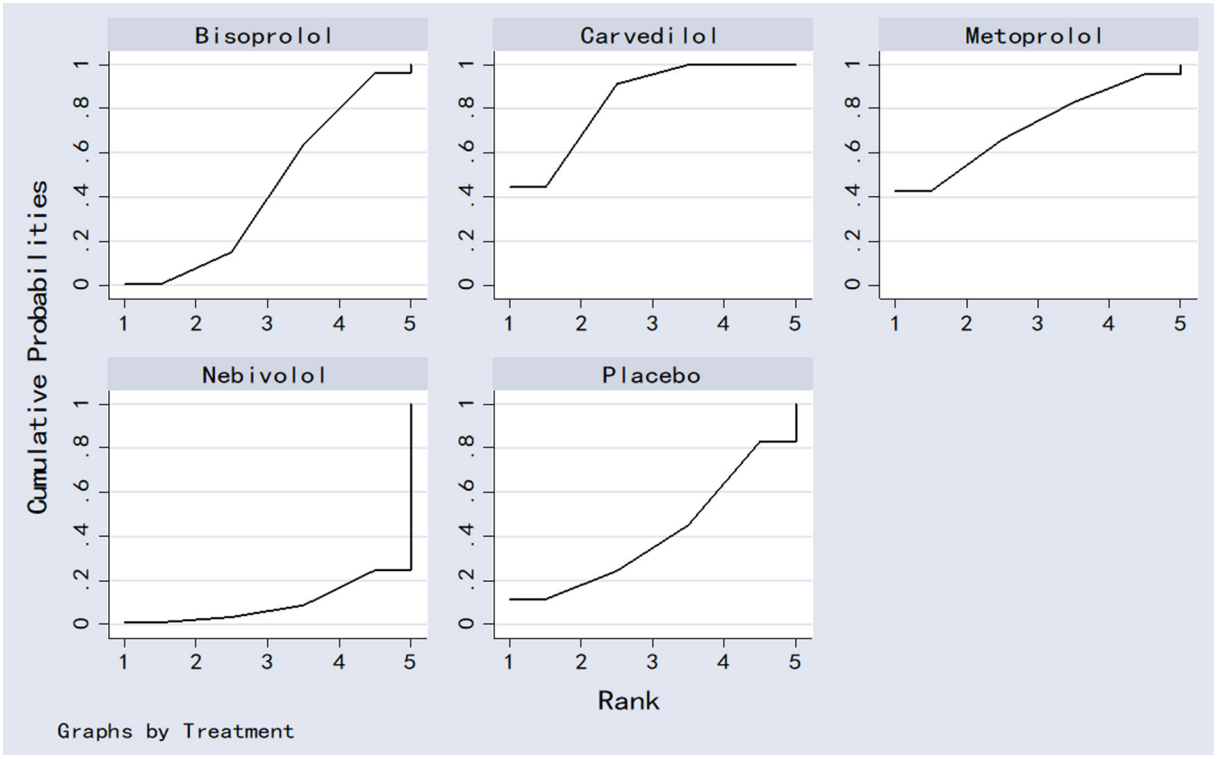

FIGURE 9
The surface under the cumulative ranking curve plots.

studies were mainly focused on carvedilol (seven studies), while there was only one study for bisoprolol, metoprolol, and nebivolol. Therefore, the results of the studies were prone to bias. Finally, the small sample size of patients included in some of the studies may reduce the credibility of the trial results.

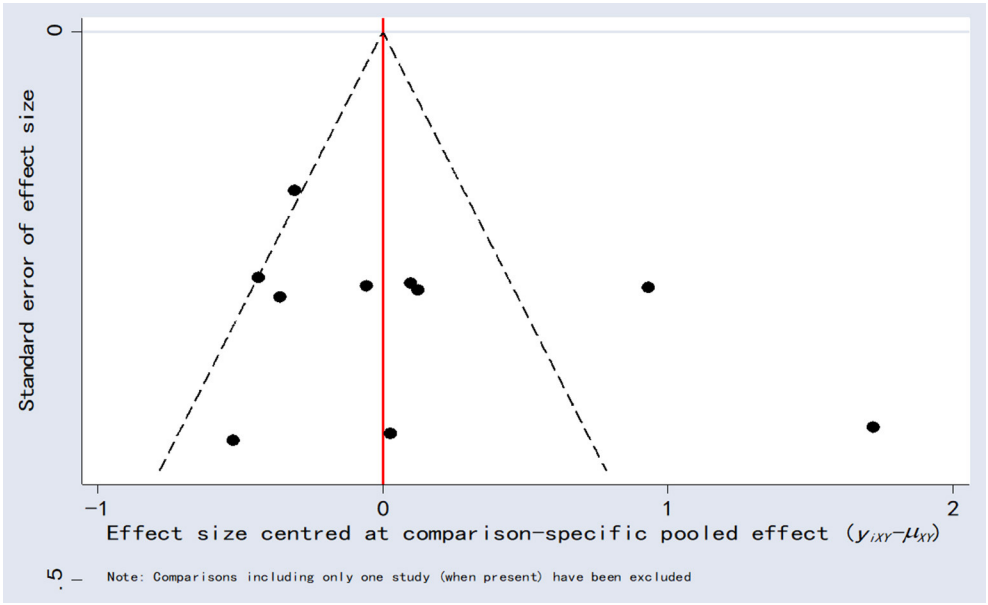

FIGURE 10
Comparison-correction funnel plot.

Conclusions

Carvedilol may be the best beta-blocker for preventing AIC, followed by metoprolol. To confirm and support the findings of this NMA, larger sample sizes and high-quality RCTs are needed.

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 authors.

Author contributions

DH and JH write this paper and analyze the data. YL and XZ design this study, perform the statistical analysis, and review this paper. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.968534/full#supplementary-material

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Safety of chronic high-dose calcium channel blockers exposure in children with pulmonary arterial hypertension

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Background: Chronic calcium channel blockers (CCBs) are indicated in children with idiopathic/heritable pulmonary arterial hypertension (IPAH/HPAH) and positive response to acute vasodilator challenge. However, minimal safety data are available on the long-term high-dose exposure to CCBs in this population.

Methods: Patients aged 3 months to 18 years who were diagnosed with IPAH/HPAH and treated with CCB in the past 15 years were retrospectively reviewed. The maximum tolerated dose and the long-term safety of high-dose CCBs on the cardiovascular and noncardiovascular systems were assessed.

Results: Thirty-two eligible children were enrolled in the study, with a median age of 9 (6–11) years old. Thirty-one patients were treated with diltiazem after diagnosis. The median maximum tolerated dose was 12.9 (9.8–16.8) mg/kg/day. Children younger than 7 years used higher doses than children in the older age group, 16.4 (10.5–28.5) mg/kg/day vs. 12.7 (6.6–14.4) mg/kg/day, $P < 0.05$. Patients were followed up for a median period of 6.2 (2.6–10.8) years. One patient died from a traffic accident, and others showed a stable or improved WHO functional class status. Thirteen (40.6%) and 10 (31.3%) patients developed arrhythmias and hypotension. Nine (28.1%) patients had sinus bradycardia, five (21.9%) had first-degree or second-degree type II atrial-ventricular blocks, and two (6.3%) had second-degree type II atrial-ventricular blocks. Most of these arrhythmias were transient and relieved after CCB dose adjustment. The most reported noncardiovascular adverse effect was gingival hyperplasia (13, 40.6%), accompanied by different degrees of dental dysplasia. No liver or kidney dysfunction was reported.

Conclusion: Diltiazem was used in a very high dose for eligible children with IPAH/HPAH. The toxicity of long-term CCB use on the cardiovascular system is mild and controllable. Clinicians should also monitor the noncardiovascular adverse effects associated with drug therapy.

KEYWORDS

pediatric, pulmonary arterial hypertension, calcium channel blocker, diltiazem, high-dose, toxicity

Introduction

Pediatric idiopathic/heritable pulmonary arterial hypertension (IPAH/HPAH) is a rare disease characterized by increased pulmonary vascular resistance (PVR) and pressure, leading to right ventricular failure and death (1). The annual incidence for IPAH from the Netherlands registry was 0.7 cases per million children (2). If left untreated, the median survival is only 10 months (3). Patients are classified as responders and nonresponders according to the acute pulmonary vascular response to vasodilator challenge during a right heart catheterization. Responders can benefit from high-dose calcium channel blockers (CCBs) with a 5-year survival of 97% (4). At the same time, nonresponders can only be treated with targeted therapies with a much worse 5-year survival (48%) (5).

CCBs such as diltiazem, amlodipine, and nifedipine can be used to treat responders in IPAH (6–10). These drugs should be started at a low dose and progressively titrated to the maximum amount based on each patient, considering patients' cardiac function, heart rate, and blood pressure (4, 5, 7, 11, 12). Traditionally, the dose in this clinical setting is much higher than that typically suggested for other pediatric indications. However, little is known about the maximum tolerated dose of CCB in this patient population and its long-term safety over high-dose exposure.

In this study, we retrospectively reviewed children with IPAH/HPAH who were identified responders in the last 15 years at Fuwai Hospital. The study aimed to determine the maximum CCB dose in these patients and assess the long-term cardiovascular and noncardiovascular safety of CCB therapy.

Methods

Study design and participants

The study population was patients aged 3 months to 18 years, diagnosed with IPAH/HPAH, and who responded to acute vasodilator challenge. All consecutive patients who visited Fuwai Hospital between January 2006 and March 2021 were retrospectively reviewed on the hospital's electronic medical platform. The patients' clinical characteristics and hemodynamic data at the time of diagnosis and the data during follow-up were collected and abstracted by trained study personnel using a standardized electronic case record database. The blood sugar, liver or renal function was monitored by biochemical test every time patients were followed up. The detailed scheme for the CCB dose titration and its adverse effects was obtained from medical records. Follow-up data were recorded annually through outpatient visits, hospitalizations, or by telephone. The last date of follow-up was September 2021.

Ethical approval was obtained from the Fuwai Hospital Research Ethics Committee (No. 2021-1484). Written consent was obtained from the guardian of each patient.

Patients' diagnosis and CCB treatment

The diagnosis of IPAH/HPAH conformed to the Third World Symposium on Pulmonary Hypertension (2003). The hemodynamic criteria were right heart catheterization (RHC), demonstrating a mean pulmonary arterial pressure (mPAP) $\geq$25 mm Hg at rest, mean pulmonary artery wedge pressure (mPAWP) $\leq$15 mm Hg, and pulmonary vascular resistance index (PVRi) $\geq$3 WU·m^2 (13).

Acute vasodilator response (AVR) testing was performed in all patients during RHC. A positive AVR was defined according to the Sitbon criteria: a decrease in mPAP after vasodilator challenge of at least 10 mm Hg to a value of $<$40 mm Hg without change or an increase in cardiac output relative to baseline value (5).

Responders were treated with CCB, with or without a combination of targeted therapy according to the physician's judgment. Drugs administered were diltiazem, amlodipine, or nifedipine. The choice depended on the patient's heart rate (HR), systemic arterial pressure (SAP), and adherence. The diltiazem dose adjustment scheme was as follows: starting with an initial dose of 1.5–2.0 mg/kg per day in three divided doses and increasing the dose every 2–4 weeks to the maximum tolerated dose. Amlodipine and nifedipine were alternatives when the resting HR was $<$ 70 beats/min (bpm).

Statistical analysis

As appropriate, data are presented as mean $\pm$ SD, median (interquartile interval), and number (%) of patients. Wilcoxon's rank-sum test was used to compare differences in CCB dose between different age groups. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA).

Results

Study group

One hundred and fifty children met the IPAH/HPAH criteria, and all had vasoreactivity tested during RHC. Thirty-two children (21%) were identified as responders to acute vasodilator challenge and were included in the study. These patients were treated with CCB immediately after diagnosis. The baseline clinical characteristics of the patients are listed in Table 1.

TABLE 1 Baseline clinical characteristics of patients.

Characteristics	
Demographics	
Age (years)	9 (6–11)
Height (cm)	134 ± 23
Weight (kg)	30 ± 12
BSA	1.16 ± 0.25
Female, *n* (%)	22 (68.8)
HR (beats/min)	95 ± 16
mSAP (mmHg)	75 ± 10
WHO FC, *n* (%)	
FC I	1 (3.1)
FC II	22 (68.8)
FC III	9 (28.1)
Biochemical test	
NT-proBNP (pg/ml)	306 (136–1,495)
ALT (umol/l)	21 ± 12
AST (umol/l)	30 ± 12
UA (umol/l)	347 ± 83
Cr (umol/l)	47.7 ± 12.7
Echocardiogram	
LVEDD (mm)	34 ± 7
RV (mm)	23 ± 7
sPAP (mmHg)	66 ± 19
TAPSE (mm)	17 ± 3
Hemodynamics from RHC	
mPAP (mmHg)	49 ± 10
PVRi (WU/m^2)	11.9 ± 4.3
CI (L/min/m^2)	3.6 ± 1.2
Maximum diltiazem dose (mg/day)	360 (240–420)
Maximum diltiazem dose (mg/kg/day)	12.9 (9.8–16.8)
> 7 years old ($n = 17$)	12.7 (6.6–14.4)
* <=7 years old ($n = 14$)	16.4 (10.5–28.5)

Values are mean ± SD; median (interquartile interval); and n (%).
*P < 0.05 when compared with doses in children older than 7 years.
BSA, body surface area; mSAP, mean systemic arterial pressure; HR, heart rate; FC, functional class; NT-proBNP, N-terminal pro-B-type natriuretic peptide; ALT, Alanine transaminase; AST, aspartate aminotransferase; UA, urine acid; Cr, creatinine; LVEDD, left ventricular end-diastolic dimension; RV, right ventricular; sPAP, systolic pulmonary arterial hypertension; TAPSE, tricuspid annulus plane systolic excursion; mPAP, mean pulmonary artery pressure; PVRi, pulmonary vascular resistance index; CI, cardiac index.

The median age of PAH diagnosis was 9 (6–11) years old, 22 (68.8%) were females, and 23 (71.9%) had the World Health Organization (WHO) Functional Class I-II. The level of N-terminal pro-B-type natriuretic peptide (NT-proBNP) elevated to 306 (136–1,495) pg/ml at diagnosis (14). The mean baseline SAP and HR were 75 ± 10 mmHg and 95 ± 16 bpm. All children had normal liver and renal functions before CCB initiation. The echocardiogram observed a marked elevation in systolic pulmonary arterial pressure (66 ± 19 mmHg) and a low tricuspid annulus plane systolic excursion (17 ± 3 mm). mPAP and PVRi were 49 ± 10 mmHg and 11.9 ± 4.3 WU·m^2 from the diagnostic RHC.

Long-term oral high-dose CCB for patients

All children started oral CCBs after diagnosis. Detailed information for CCB dose is shown in Tables 1, 2. Thirty-one patients were treated with diltiazem and one with amlodipine due to a baseline HR of < 70 bmp. At the end of the follow-up, 28 patients were still taking diltiazem and two with amlodipine (including one patient taking diltiazem and amlodipine combination therapy). One patient did not respond to long-term CCB treatment and was switched to PAH-targeted therapy.

The median maximum tolerated diltiazem dose was 12.9 (9.8–16.8) mg/kg per day, ranging from 3.3 to 34.3 mg/kg per day. Children younger than 7 years used a higher dose than patients in the older age group, 16.4 (10.5–28.5) mg/kg/day *vs.* 12.7 (6.6–14.4) mg/kg/day, $P < 0.05$. The six children younger than 3 years old at diagnosis were all treated with diltiazem during the entire follow-up period. The maximum tolerated diltiazem dose in these six youngest patients ranged from 7.5 mg/kg/day to 34.3 mg/kg/day.

The effects of high-dose CCB treatment

The patients were followed for a median period of 6.2 (2.6–10.8) years. A patient died from a traffic accident, although his symptoms were markedly relieved with diltiazem. All other children had stable or improved WHO functional class and normal NT-proBNP, 14 (45.2%) in functional class I and 17 (54.8%) in functional class II (Table 2). Systolic pulmonary arterial pressure and tricuspid annulus plane systolic excursion were improved to 37 ± 9 mmHg and 21 ± 3 mm.

Adverse effects of high-dose CCB

The adverse effects of CCB are shown in Tables 2, 3. Thirteen patients had arrhythmias: nine (28.1%) sinus bradycardia (HR < 60 bpm), five (21.9%) first-degree or second-degree type I atrial-ventricular block (AV block), and two (6.3%) second-degree type II AV block. No patient had third-degree AV block, atrial flutter, and atrial fibrillation. Hypotension occurred in 10 patients (31.3%): four complained of mild orthostatic dizziness accompanied by nausea, three complained of fatigue when exercising, and the rest were asymptomatic. The symptoms of bradyarrhythmias and hypotension were relieved after optimizing the diltiazem dose, except for the patient with

TABLE 2 Detailed clinical information of each patient.

ID	Age (years)	follow-up* (years)	CCB therapy	Maximum dosage (mg/kg/day)/(mg/d)	Side-effects	mSAP (mmHg)		HR (beat/min)		WHO FC	
						baseline	follow-up	baseline	follow-up	baseline	follow-up
1	0.7	2.6	diltiazem	11.3/135	II-I AV-block	70	60	105	88	2	2
2	2	1.4	diltiazem	17.1/240	gingival hyperplasia, increase in body hair	73	60	102	65	3	2
3	3	13.3	diltiazem	32.0/480	sinus bradycardia, gingival hyperplasia, increase in body hair	85	78	98	65	2	1
4	3	5.1	diltiazem	34.3/480	sinus bradycardia, gingival hyperplasia	84	65	120	94	2	2
5	3	2.7	diltiazem	7.5/120	sinus bradycardia, II-I AV-block, hypotension	66	58	117	75	3	1
6	3	1.7	diltiazem	28.0/420	sinus bradycardia, gingival hyperplasia, increase in body hair	67	55	90	56	3	2
7	5	12.5	diltiazem	30.0/600	hypotension, gingival hyperplasia, constipation	80	65	102	66	3	1
8	5	2.2	diltiazem	10/210	none	61	52	142	114	2	1
9	6	5	diltiazem	12.3/270	none	86	72	71	61	2	2
10	6	6.1	diltiazem	7.8/180	sinus bradycardia, atrial tachycardia, hypotension	77	68	77	112	3	1
11	6	11.1	diltiazem	19.6/450	sinus bradycardia, I degree AV-block	75	57	102	59	2	2
12	7	14.4	diltiazem	17.5/420	constipation	70	58	96	70	2	2
13	7	6.3	diltiazem	15.7/360	none	74	68	87	86	2	2
14	7	3.2	diltiazem	10.6/255	hypotension, gingival hyperplasia	73	60	95	80	2	1
15	8	13.2	diltiazem	16.2/420	II-I AV-block	68	59	110	75	1	1
16	8	14.4	diltiazem	16.8/420	gingival hyperplasia	50	50	100	75	2	1
17	9	2.6	amlodipine	0.38/5	sinus bradycardia, hypotension	81	71	79	70	3	1
18	9	13.5	diltiazem	12.9/360	hypotension, gingival hyperplasia	61	58	88	70	2	1
19	9	6.5	diltiazem	13.3/360	gingival hyperplasia, increase in body hair	102	63	99	74	2	2
20	10	2.6	diltiazem	13.0/390	none	74	66	77	67	2	2
21	10	5.7	diltiazem	15.5/480	none	74	64	63	75	2	2
22	10	9.9	diltiazem	10.9/360	gingival hyperplasia	79	64	110	69	3	1
23	11	3.5	diltiazem	9.8/315	hypotension, rash	72	63	78	63	2	1
24	11	6.9	diltiazem	13.3/465	none	79	69	86	74	2	2
25	11	2.4	diltiazem	16.4/525	gingival hyperplasia, hypotension	68	60	108	77	3	2

(Continued)

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TABLE 2 (Continued)

ID	Age (years)	follow-up* (years)	CCB therapy	Maximum dosage (mg/kg/day)/(mg/d)	Side-effects	mSAP (mmHg)		HR (beat/min)		WHO FC	
						baseline	follow-up	baseline	follow-up	baseline	follow-up
26	12	13.7	diltiazem	12.7/420	gingival hyperplasia, increase in body hair	75	70	84	70	2	1
27	12	8.6	diltiazem and amlodipine	10.6 and 0.16/330/2.5	sinus bradycardia, gingival hyperplasia	72	59	98	60	2	2
28	15	0.6	diltiazem	4.9/180	none	107	death	87	death	3	death
29	15	9.7	diltiazem	3.3/120	II-II AV-block, hypotension	90	88	98	89	2	2
30	15	1.6	diltiazem	7.1/270	Rash	70	58	108	64	2	1
31	17	9.1	diltiazem	6/240	sinus bradycardia, II-I AV-block, hypotension	84	70	89	70	2	2
32	17	9.1	diltiazem	5.5/225	II-II AV-block, atrial tachycardia, fatigue, dizziness	98	83	82	84	2	2

*The median follow-up period is 6.2 (2.6–10.8) years.
&the patient died from traffic accident 7 months after diagnosis.
CCBs, calcium channel blockers; AV-block, atrial-ventricular block; mSAP, mean systemic arterial pressure; HR, heart rate; FC, functional class.

TABLE 3 Long-term adverse effects of calcium channel blockers.

Side effect of calcium channel blocker	*n* (%)
Sinus bradycardia	9 (28.1)
I degree/ II degree I type A-V block	5 (21.9)
II degree II type A-V block	2 (6.3)
Hypotension	10 (31.3)
Gingival hyperplasia	13 (40.6)
Increase in body hair	5 (15.6)
Others	
Rash	2 (6.3)
Constipation	2 (6.3)
Liver dysfunction	none
Renal dysfunction	none

AV-block, atrial-ventricular block.

FIGURE 1
A patient with severe gingival hyperplasia. Female, 3 years old at diagnosis, treated with diltiazem at the maximum dose of 34.3 mg/kg/day, gingival hyperplasia is severe, companied with dental abnormalities.

second-degree type I AV block. Severe hypotension, cardiac arrest, and cardiogenic shock were not documented during the study.

Gingival hyperplasia was reported in 13 (40.6%) patients, accompanied by a different degree of dental dysplasia (Figure 1). The adverse effect mostly occurred 16 months after initiation of CCB and when the dose was more than 10 mg/kg per day. Other adverse effects included were increase in body hair ($n = 5$), constipation ($n = 2$), and rash ($n = 2$). Hyperglycemia and liver or kidney dysfunction were not reported.

Discussion

In pediatric patients with IPAH/HPAH, only a minority can benefit from chronic treatment with CCB. These patients

are defined as vasodilator responders. This subset of the population is very rare. In the past 15 years, we diagnosed 150 pediatric IPAH/HPAH patients in our PAH center; only 32 patients (21%) were positive responders. Similarly, in a global registry of pediatric PAH and pediatric REVEAL cohort from the United States, only 32 and 19 children were responders using the same criteria (8, 10). Most previous studies focused on the long-term effect of CCBs in pediatric patients with IPAH/HPAH. There are minimal data on the actual use of CCBs, their maximum tolerated doses, and whether chronic exposure to high-dose CCB results in severe toxicity to the cardiovascular system or other body organs. In the present study, we attempted to answer these questions by reviewing our eligible patients to characterize the safety of chronic high-dose CCBs in pediatric patients.

Initially, CCBs were studied as vasodilator agents to test pulmonary vasoreactivity (6, 15, 16). However, their use is limited by possible severe adverse clinical events, including profound hypotension and cardiogenic shock. Therefore, vasodilators that are more selective to the pulmonary arteries are used. The guidelines recommend that CCBs only be indicated in PAH patients with significant acute pulmonary vasoreactivity (17, 18). CCBs should start from a low dose and then gradually titrated upward to the maximum tolerated dose over weeks to months.

Long-term CCB responders in adult PAH patients have been treated with diltiazem at a mean daily dose of 482 ± 151 mg (range, 180 to 720 mg), nifedipine 102 ± 27 mg (range, 60 to 120 mg), or amlodipine 20 mg, without reported severe cardiovascular toxicity (5). The following CCB regimens have been recommended for pediatric patients with PAH: diltiazem at the initial dose of 1.5–2 mg/kg/day in three divided doses and the maintenance dose of 3–5 mg/kg/day in three divided doses, or nifedipine started at 0.6–0.9 mg/kg/day with a maintenance dose of 2–5 mg/kg/day (19). However, maximum tolerated doses have not been reported or recommended. On the contrary, the maximum dose of diltiazem for pediatric hypertension was said to be 6 mg/kg per day up to 360 mg per day (20).

Our study found that diltiazem was the most frequently administered CCB in our practice. The median maximum tolerated diltiazem dose was 12.9 mg/kg per day, several times higher than the recommended maintenance dose for pediatric patients with IPAH/HPAH or the maximum dose for pediatric hypertension. Younger patients seemed to tolerate higher doses, calculated by body weight, than older ones. In this study, a 3-year-old patient received the highest diltiazem dose, 34.3 mg/kg/day. Similar to the findings of other pediatric IPAH/HPAH cohorts, CCBs worked well in our patient population to improve cardiac function and survival (4, 8, 10). This may be explained because PAH patients who responded positively to acute vasodilator challenge are supposed to have almost exclusively vasoconstrictive abnormality without severe cellular narrowing of the arterioles (12). Pulmonary vasoconstriction could be relieved or reversed by high-dose CCB.

Several factors might have contributed to the high utilization of diltiazem in our practice. First, most children were documented to have a rapid HR when PAH was diagnosed, and diltiazem has a negative chronotropic effect to counteract the HR. Second, the half-life of diltiazem is shorter than that of amlodipine, which enables physicians to handle complications in case emergencies occur. Acute CCB overdose was ranked as the top sixth overall substance category associated with the most reported fatalities. Pediatric poisonings are mainly unintentional ingestions (< 6 years old) or intentional exposure (13 to 19 years old) (21). However, most literature focused on acute CCB overdose in pediatric patients. Data for chronic high-dose CCB toxicity are minimal. We found that the dose titration strategy managed by the PAH professionals displayed satisfactory safety over a long follow-up period even though most patients received higher doses of CCBs. A small proportion of patients were recorded to have bradycardia, conduction disturbances, or hypotension. However, most of these symptoms were transient and moderate and disappeared after adjusting the diltiazem dose. No emergency treatment of overdose toxicity was reported.

Theoretically, diltiazem has a negative inotropic effect and can decrease cardiac contractility.

In contrast, we observed improvement in cardiac function during the follow-up. This is also attributed to intensive pulmonary artery dilation and decreased right ventricular afterload under high-dose CCB treatment. Throughout our study, hepatic or renal function impairment and the potential adverse effect of hyperglycemia were not recorded.

Gingival hyperplasia was the most common chronic noncardiovascular adverse effect reported during follow-up, characterized by an excessive enlargement of gingival tissue. The disorder is also widely reported in adults. The prevalence rates of nifedipine- or amlodipine-induced gingival hyperplasia were 20 to 50% (22, 23) and 3.3% (24). The median onset of this adverse effect was 262 days, and more men were present with this than women (22–24). However, data in pediatrics are rare. In this study, 40.6% of children with diltiazem presented with gingival hyperplasia, mainly 16 months after starting the drug with a diltiazem dose of more than 10 mg/kg per day. Gingival hyperplasia can interfere with esthetics, chewing, speech, and psychological health, especially in children. However, management is challenging (25). Given the devastating character of PAH, changing CCB therapy is not wise or perhaps not recommended. PAH experts, stomatologists, and psychologists should work together to find the most appropriate alternative therapies for very severe cases.

Limitations

This study suffers from limitations with the retrospective design and single-center data. Pediatric PAH centers are rare in China, especially 10 years ago. As the largest PAH center in China, we treat patients throughout the country, and our data represent wide Chinese patients. Furthermore, we enrolled all eligible children treated at our hospital for a 15-year period with continuous observation. The patients were followed for a median of more than 6 years. As a rare disease, our relatively large cohort of patients and the long duration of the study effectively avoided selective bias and ensured the representativeness of the study.

Conclusion

The appropriate administration strategy of long-term high-dose CCBs in pediatric PAH patients is effective with mild to moderate adverse effects. Close lifelong monitoring is necessary to continuously observe the growth, development, and organ functions in this unique population.

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.

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Committee of Fuwai Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

YW wrote the analysis plan and had the primary responsibility for writing the article. XG and LH supervised the study and data analysis. YW, XG, and LH designed the protocol. YW, F-HP, and X-XY designed the data collection forms. YW, F-HP, X-XY, XG, FZ, LH, J-ST, and SH collected the data. YW, LH, FZ, J-ST, and SH analyzed the data. YW, F-HP, X-XY, XG, FZ, and LH interpreted the data. All authors provided critical reviews of drafts and approved the final submitted version.

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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.

Publisher's note

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The protective effects of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract on oxidative stress induced by 2,2′-azo (2-methylpropamidine) dihydrochloride in HL-1 mouse cardiomyocytes

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To investigate the protective effects of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract (CSTE) on oxidative stress injury in HL-1 mouse cardiomyocytes induced by 2,2′-azo (2-methylpropamidine) dihydrochloride (AAPH, 1 mmol/L), HL-1 cells were co-cultured with different concentrations (10–100 μg/mL) of the CSTE for 24 h. A cell damage model was established by continuously culturing the cells in Dulbecco's Modified Eagle Medium plus AAPH for 4 h. Cell survival rates were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay, and by measuring intracellular malondialdehyde (MDA) content. MDA and total reactive oxygen species (ROS) levels were determined by thiobarbituric acid colorimetry and the 2′,7′-dihydrodichlorofluorescent sodium yellow diacetate probe, respectively. Apoptosis was measured by flow cytometry. The intracellular catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione s-transferase (GST), γ-glutamylcysteine synthetase (γ-GCS), and glutathione (GSH) contents were determined by colorimetric methods. CSTE content was determined by high performance liquid chromatography. The CSTE pretreatment improved survival rates in damaged HL-1 cells, reduced total intracellular ROS and MDA levels, and reduced apoptosis. The CSTE also increased the activities of the antioxidant enzymes (CAT, SOD, GSH-Px, and GST), as well as the γ-GCS and GSH levels in damaged cells. Real-time fluorescence quantitative polymerase chain reaction analysis indicated that the CSTE upregulated CAT, SOD1, and GSH-Px mRNA expression levels. Additionally, the CSTE reduced MDA and ROS levels in HL-1 cells by improving the endogenous antioxidant system; thus, alleviating the oxidative stress damage caused by AAPH. Our compositional analyses revealed that the CSTE contained caffeic acid, isoquercetin, rosmarinic acid, luteolin, and baicalin. The CSTE demonstrates antioxidant and protective effects in myocardial cells.

KEYWORDS

HL-1 mouse, oxidative stress, cardiomyocytes, heart, AAPH

Introduction

Clerodendranthus spicatus (Thunb.) C. Y. Wu is a plant found in Asia and Oceania, it is a perennial herb in the *Lamiaceae* family (1), the aerial part of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu is used as medicine or health tea (Figure 1). Previous studies have reported that a *C. spicatus* (Thunb.) C. Y. Wu extract (CSTE) exerts protective effects on some internal organs, including the kidneys, and protects against nephritis edema, hypertension, cholelithiasis hepatitis, rheumatoid arthritis, and inflammation of the fallopian tubes (2–4), which results in painful and poor urination, kidney atrophy, swollen hands and feet, and acute hepatitis; thus, the CSTE has been used as a traditional Chinese medicine in China (5). In addition, the CSTE may also have antibacterial and anti-inflammatory qualities when used in tea.

Superoxide anions (O_2^-), peroxide ions (O_2^{2-}), hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2), peroxynitrite [$ONOO^-$reactive oxygen species (ROS)], organic peroxyradicals (ROO•), and lipid peroxyradicals (LOO•) are physiological by-products of metabolism. Excessive accumulation of ROS in the body induces oxidative stress and causes disease. Endogenous antioxidant dysfunction in the heart leads to a high degree of oxidative stress in some chronic heart diseases (6, 7). Therefore, excessive ROS accumulation not only induces lipid peroxidation of polyunsaturated fatty acids in cell membranes but also degenerates macromolecules, such as proteins and genetic material in cells, leading to oxidative stress injury in cardiac cells and necrosis (8–10). Therefore, ROS are a significant pathogenic factor leading to chronic heart disease. Some plants used in traditional Chinese medicine have good antioxidant capacities, as they are rich in antioxidant molecules, and are often used to treat chronic diseases or play a preventive role as drinks. Therefore, ingesting this medicine (foods homologous to traditional Chinese medicine) helps improve antioxidant capacity, alleviates the adverse effects of oxidative stress on tissues and vital organs, and prevents the occurrence of related diseases (11). In some specific circumstances, ROS may be protective against ischemia-reperfusion injury during myocardial ischemia-reperfusion injury (12). In this study, only the common ROS-induced oxidative stress myocardial injury was investigated. The complex mechanism of ROS on cardiomyocytes needs further study.

HL-1 is a mouse cardiomyocyte cell line used to study oxidative stress-induced cardiomyocyte injury (13). We evaluated the potential preventative and health effects of the CSTE in combatting oxidative stress injury. Cardiomyocytes were treated with 2,2'-amidine hydrochloride (2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) to construct an oxidative damage cell model. We explored the protective mechanisms of the CSTE against oxidative stress induced by AAPH, and provide a theoretical reference point for the CSTE in preventing heart disease caused by oxidative stress.

Materials and methods

Extraction of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu

C. spicatus (Thunb.) C. Y. Wu is produced in Sipsongpanna, Yunnan Province, China. The *C. spicatus* (Thunb.) C. Y. Wu from Sipsongpanna is the only one approved for medicinal use in China, so this study selects samples of this product. After the *C. spicatus* (Thunb.) C. Y. Wu (Kunming Xuanqing Biotechnology Co., Ltd, Kunming, Yunnan, China) was freeze-dried, it was crushed, ground, and sieved, and a particular amount of the sample powder was accurately weighed into a beaker. Ethanol (70%) was added at a liquid material ratio of 20:1. The solution was placed in a 60°C water bath for 2 h twice, suction filtrated, and the liquid was passed through FL-3 macroporous resin. The water and ethanol were removed from the resinous liquid using a rotary evaporator (evaporated until no liquid flowed into the beaker) and dried at a constant temperature of 60°C for 48 h. The dried sample was ground, weighed, sealed in an EP tube, and stored at 4°C.

Cell culture and groups

HL-1 cardiomyocytes (National Collection of Authenticated Cell Cultures, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Solarbio Life Science, Beijing, China) plus 10% fetal bovine serum and 1% cyanin-streptomycin dual antibody solution at 37°C in 5% CO_2, and the medium was changed every 2 days. The cells (2×10^5) were added to 96- and 6-well cell culture plates and cultured for the experiments. The oxidative damage cell model was prepared by continuously culturing the cells in DMEM plus 1 mmol/L AAPH for 4 h. Damaged cells (1×10^4) were enumerated and added to wells containing 100 μL of the CSTE (10, 50, and 100 μg/mL) in 96-well plates for 24 h. Normal HL-1 cells without AAPH were used as the control group.

3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl-2H-tetrazolium bromide (MTT) cell viability assay

Fifty 4-week-old female BALB/c mice (weight 20–25 g) were purchased from Hunan Slike. The HL-1 cells were treated and cultured for 24 h. Then, the medium was discarded, and 100 μL of MTT solution (0.5 mg/mL, Solarbio Life Science) was added to the cells for 4 h. The supernatant was discarded, 100 μL of dimethyl sulfoxide was added to the wells, the plate was shaken for 30 min, and the $OD_{490\ nm}$ value was measured. The cell survival rate was calculated using the formula: cell survival rate (%) = OD treatment group/OD normal group × 100.

FIGURE 1
Observation diagram of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu, **(A)** Dried *Clerodendranthus spicatus* (Thunb.) C. Y. Wu, commodity condition; **(B)** *Clerodendranthus spicatus* (Thunb.) C. Y. Wu in vegetative state.

Cell malondialdehyde (MDA) production

After the treatments, MDA production was determined by thiobarbituric acid colorimetry. The cells were rinsed in phosphate-buffered saline (PBS), scraped, and lysed in precooled lysis solution. Then, 500 μL of the lysis supernatant, 15% trichloroacetic acid, and 0.67% thiobarbituric acid (400 μL, Solarbio Life Science) were added to a 5 mL tube. After mixing and incubating for 20 min at 95°C, the tubes were cooled, and 3 mL of isopropyl alcohol was added to extract the pigments. The OD_{532nm} value was measured, and total protein content in the cells was determined with a kit. MDA production was calculated according to the formula: MDA production (ng/mg Pro) = MDA content (ng/mL) × 1.5 mL/total protein weight (mg).

Determination of intracellular ROS levels

After the cells were treated and grown in 6-well cell plates, Dulbecco's modified Eagle's medium (DMEM) plus 20 μmol/L 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA, Solarbio Life Science) was added for 20 min at 37°C. The cells were washed twice in cold PBS and absorbance was determined at an excitation wavelength of 485 nm. When the emission wavelength was 530 nm, the FLUOstar OPTIMA was used to measure fluorescence intensity, and relative ROS levels were calculated according to the formula: relative ROS content (%) = fluorescence intensity of the treatment group/fluorescence intensity of the normal group × 100.

Determination of antioxidant enzyme activities, glutathione (GSH) levels, and γ-glutamylcysteine synthetase (γ-GCS) activity in cells

Cells (2×10^5/well) were added to 6-well plates, treated, and cultured. An appropriate amount of SOD, catalase (CAT), GSH-Px, GSH, and γ-GCS (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) were taken from the cell lysis solution. Enzyme activity was expressed in specific enzyme activity units (U/mg Pro), and GSH content and γ-GCS activity were expressed as μmol/mg Pro. The total protein content in the cells was corrected.

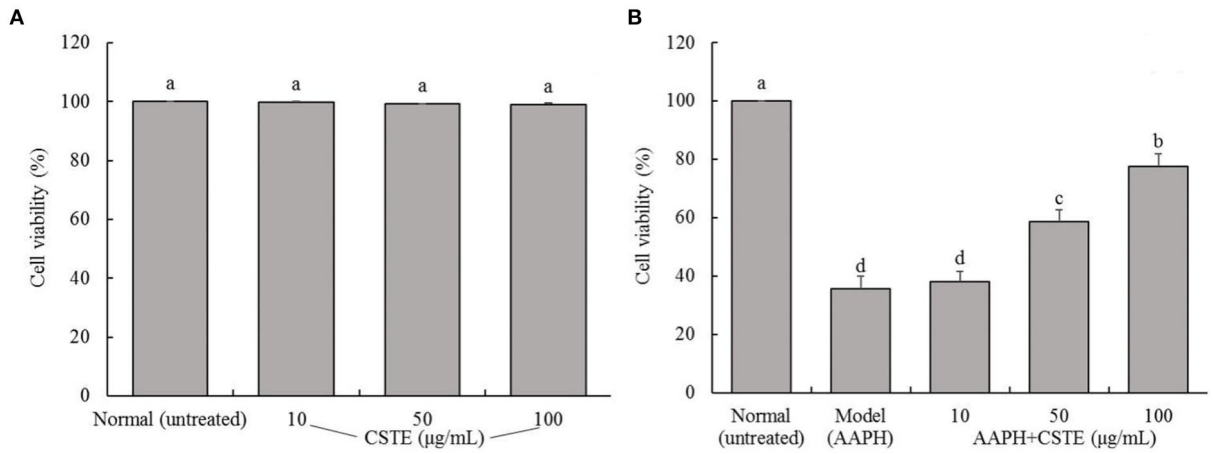

FIGURE 2
Survival rate of HL-1 cardiomyocytes, **(A)** cells not treated with AAPH, **(B)** AAPH induced oxidative damage of cells; CSTE: *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract. $^{a-d}$ according to Duncan's multi range test, the average values of different letters in the same column have significant differences ($P < 0.05$, $n = 6$).

Flow cytometry to detect cell death

After the cells were treated with MDLE, a 1 mL cell suspension (5×10^5 cell/mL) was centrifuged at 4,500 rpm and 4°C, and the supernatant was discarded. The cells were rinsed in pre-chilled PBS and resuspended in 500 μL of PBS. The cells were mixed with 5 μL of Annexin V-FITC (ThermoFisher Scientific, Waltham, MA, USA) and 5 μL of propidium iodide and incubated in the dark at 37°C for 15 min (AccuriC6, BD Biosciences, San Jose, CA, USA). Flow cytometry was used to detect apoptosis (14).

Antioxidant gene expression using real-time fluorescence quantitative polymerase chain reaction

Total RNA was extracted from the cells using Trizol reagent (ThermoFisher Scientific) and RNA concentrations were adjusted to the same levels after UV spectroscopy was performed to assess purity. RNA (2 μg) was added to the OligodT_{18}, RNase, dNTPs, and MLV enzyme (1 μL each), and 5× Buffer (10 μL) in the PCR tubes. Then, cDNA was synthesized at 37°C for 120 min at 99°C for 4 min, and at 56°C for 3 min, and CAT, GSH-Px, and superoxide dismutase 1 (SOD1, Cu/Zn-SOD) mRNA expression levels were detected by RT-fluorescence qPCR. cDNA (2 μL, ThermoFisher Scientific), upstream and downstream primers (10 μmol/L), 10 μL of the SYBR Premix Ex Taq II (2×), and 0.4 μL of the ROX Reference Dye (50×) were added to the total reaction system (20 μL) and 5.6 μL of sterile double-distilled water was added and thoroughly mixed with the reagents and placed in the QuantStudio^{TM} 6 Flex PCR apparatus for the reaction. The amplification parameters were: 95°C for 35 s, 55–59°C for 30 s, 95°C for 15 s, 60°C for 60 s over 40 cycles, and 95°C for 15 s (SteponePlus, ThermoFisher Scientific). Amplifications were performed three times in parallel, and the mean cycle threshold (Ct) value was recorded. The expression level of the target gene (F) was calculated according to the following formula. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference (15).

$$F = \frac{2^{Ct_1 - Ct_2}}{2^{Ct_3 - Ct_4}}$$

Where Ct_{1-4} are the Ct values of the genes in the samples, GAPDH in the test samples, genes in the blank samples, and GAPDH in the blank samples, respectively.

High-performance liquid chromatography

The CSTE and standard samples were extracted with methanol, passed through 0.22 μm organic filter membranes, and transferred to brown liquid vials for testing. The composition of the CSTE was determined by HPLC (UltiMate3000, ThermoFisher Scientific) using an Accucore C18 column (5 μm, 4.6 × 250 mm). Mobile phase A was 0.5% acetic acid in water and mobile phase B was acetonitrile. The flow rate was 0.5 mL/min, and the column temperature was 30°C. The detection wavelength was 359 nm and the injection volume was 5 μL. Pre-equilibrium was performed for 10 min.

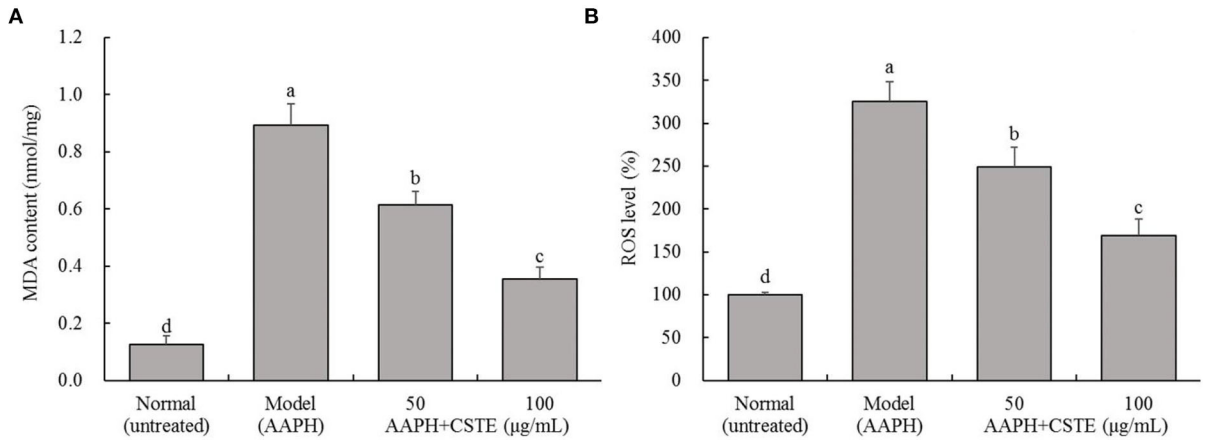

FIGURE 3
MDA content and ROS level of AAPH inducing oxidative damaged HL-1 cardiomyocytes, **(A)** MDA content, **(B)** ROS level; CSTE: *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract. $^{a-d}$ according to Duncan's multi range test, the average values of different letters in the same column have significant differences ($P < 0.05$, $n = 6$).

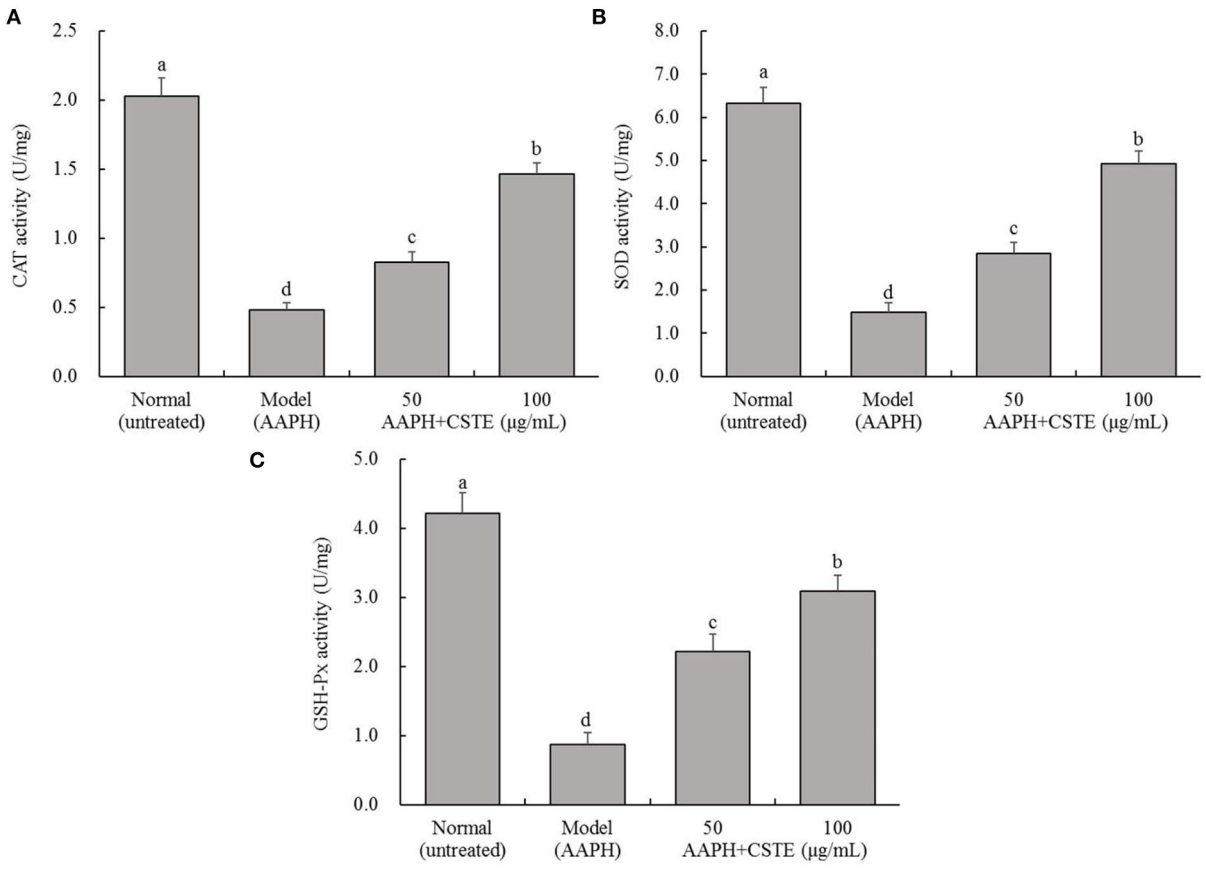

FIGURE 4
CAT, SOD, and GSH-Px activities of AAPH inducing oxidative damaged HL-1 cardiomyocytes, **(A)** CAT activity, **(B)** SOD activity, **(C)** GSH-Px activity; CSTE: *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract. $^{a-d}$ according to Duncan's multi range test, the average values of different letters in the same column have significant differences ($P < 0.05$, $n = 6$).

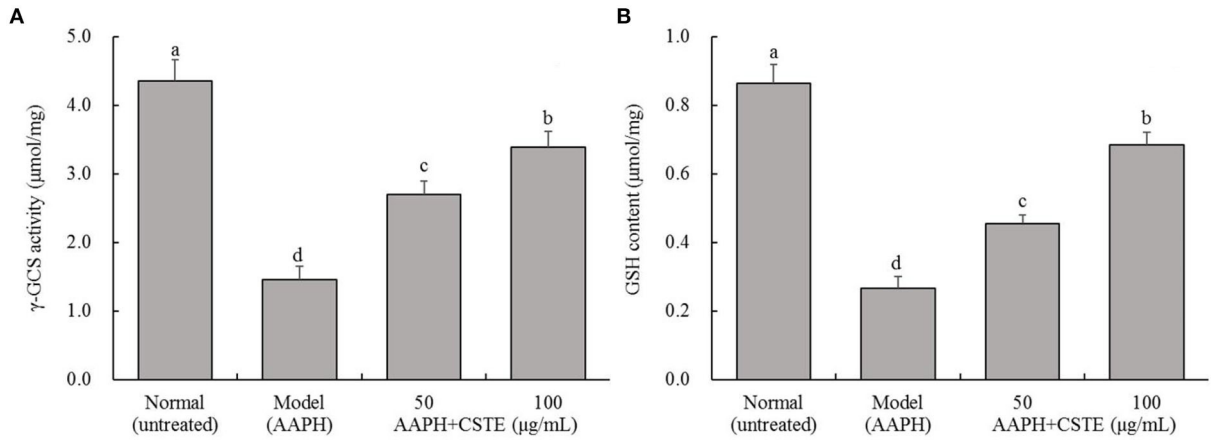

FIGURE 5
γ-GCS activity and GSH content of AAPH inducing oxidative damaged HL-1 cardiomyocytes, **(A)** γ-GCS activity, **(B)** GSH content; CSTE: *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract. $^{a-d}$ according to Duncan's multi range test, the average values of different letters in the same column have significant differences ($P < 0.05$, $n = 6$).

Data analyses

All experiments were repeated three times, and the results are expressed as mean ± standard deviation. All statistical analyses were performed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), and one-way analysis of variance was used to detect differences. A *P*-value < 0.05 was considered significant.

Results

Effects of the CSTE on the survival of HL-1 cells

The survival rates of the HL-1 cells after the CSTE treatments (10, 50, and 100 μg/mL) were > 90%, suggesting that the CSTE had no cytotoxic effect on the cells (Figure 2A). Additionally, direct exposure to AAPH (1 mmol/L) for 4 h significantly decreased the survival rates of the treated HL-1 cells when compared with the untreated group ($P < 0.05$). However, the survival rates of damaged cells increased when compared with untreated cells after treatment with the different CSTE concentrations for 24 h. Additionally, the protective effect was significantly enhanced ($P < 0.05$) as the CSTE concentration was increased to the 50 and 100 μg/mL concentrations (Figure 2B).

Effects of the CSTE on MDA content and ROS levels in damaged HL-1 cells

The MDA content in the HL-1 cells increased significantly after the 4-h AAPH treatment (1 mmol/L) compared with untreated cells ($P < 0.05$; Figure 3). However, MDA content decreased significantly after the CSTE treatments (50 and 100 μg/mL) ($P < 0.05$). The MDA content was lowest in the 100 μg/mL CSTE treatment. Additionally, the AAPH treatment significantly increased ROS levels in HL-1 cells ($P < 0.05$); however, the ROS levels in the damaged cells tended to decrease after the 24-h CSTE treatment (50 and 100 μg/mL). ROS activity was strongly inhibited when the CSTE concertation was 100 μg/mL.

Effects of the CSTE on CAT, SOD, and GSH-Px activities in damaged HL-1 cells

AAPH reduced CAT, SOD, and GSH-Px activities in the HL-1 cells (Figure 4). In contrast, antioxidant enzyme activities increased gradually in the damaged cells after the 24 h treatment with different CSTE concentrations (50 and 100 μg/mL), and a significant difference was observed when the damaged model groups were compared with untreated cells ($P < 0.05$).

Effects of the CSTE on γ-GCS activity and GSH content in damaged HL-1 cells

The AAPH treatment significantly inhibited intracellular γ-GCS activity and significantly reduced intracellular GSH content ($P < 0.05$; Figure 5). The γ-GCS content in damaged HL-1 cells increased gradually after the 24-h CSTE (50 and 100 μg/mL) treatments. Additionally, intracellular GSH content recovered and tended to increase. The higher CSTE dose (100 μg/mL) exerted significant effects on γ-GCS activity and GSH content in damaged HL-1 cells compared with the model group ($P < 0.05$).

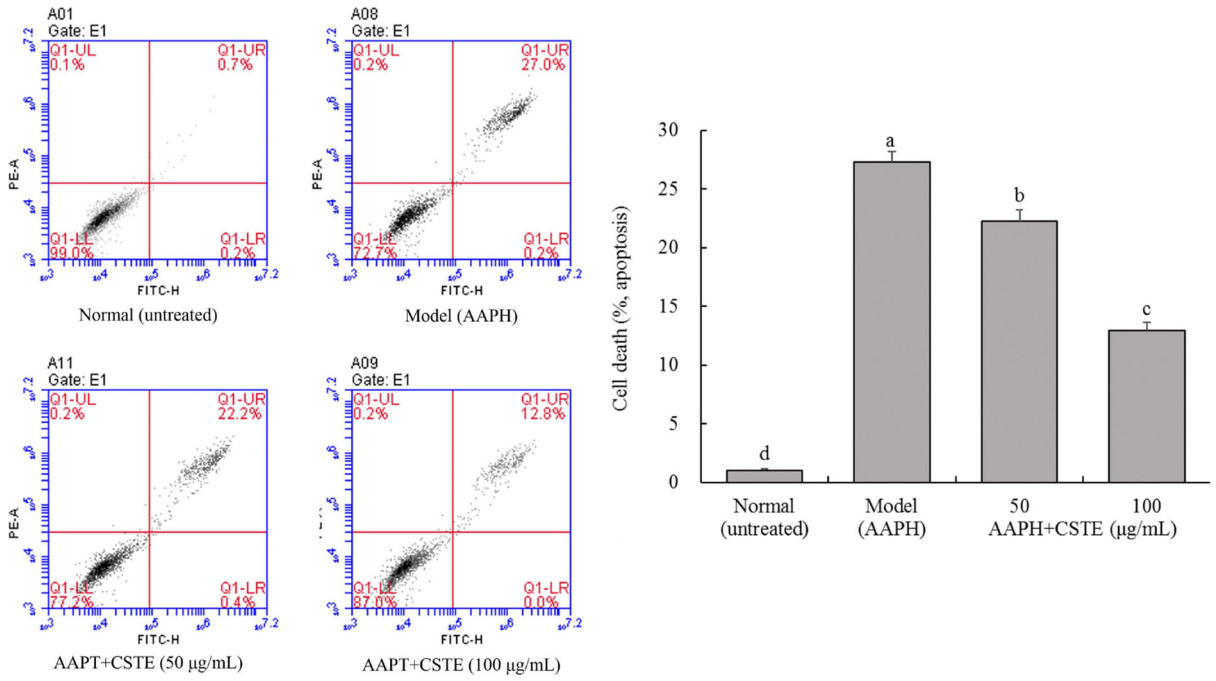

FIGURE 6
Flow cytometry of apoptosis HL-1 cells, CSTE: *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract. $^{a-d}$ according to Duncan's multi range test, the average values of different letters in the same column have significant differences ($P < 0.05$, $n = 6$).

Effects of the CSTE on cell death in the oxidatively injured HL-1 cells

Cell apoptosis is detectable by flow cytometry. The oxidatively damaged HL-1 cardiomyocytes appeared to have undergone apoptosis. About 27.3% of the cells in the model group underwent apoptosis, and these cells were considered dead. The CSTE significantly reduced apoptosis due to oxidative stress, thereby reducing the number of dead cells. The number of dead cells decreased with an increase in the CSTE concentration (Figure 6). Therefore, the CSTE significantly inhibited apoptosis in cardiomyocytes following oxidative damage, thereby reducing cell death.

Effects of the CSTE on SOD1, GSH-Px, CAT, and Nrf2 gene expression levels in HL-1 cells

The RT-fluorescent qPCR results showed that 1 mmol/L AAPH significantly decreased SOD1, GSH-Px, CAT, and Nrf2 mRNA levels in HL-1 cells ($P < 0.05$; Figure 7). However, these levels gradually increased after the CSTE treatment. The higher CSTE doses (50 and 100 μg/mL) exerted significant effects on SOD1, GSH-Px, CAT, and Nrf2 mRNA expression levels in injured cells compared with the model group ($P < 0.05$).

Analysis of the active compounds in the CSTE

The chromatogram of the standards showed that the peak times of caffeic acid, isoquercetin, rosmarinic acid, luteolin, and baicalin were 7.533, 11.817, 14.887, 20.147, and 25.407 min, respectively. The HPLC analyses revealed that the CSTE contained caffeic acid, isoquercetin, rosmarinic acid, luteolin, and baicalin at the peak times of 7.573, 11.827, 14.863, 18.590, and 25.390 min, among which rosmarinic acid was the most important active compound (Figure 8).

Discussion

Cardiomyopathy is a group of disorders that progressively impairs cardiac function due to structural changes in the lower chambers of the heart (i.e., ventricles) and impaired myocardial wall function. Cardiomyopathy is characterized by the aggravation of heart failure and serious arrhythmias, such as chest tightness, shortness of breath, dyspnea, wheezing, coughing foam-like sputum, enlargement of the liver and

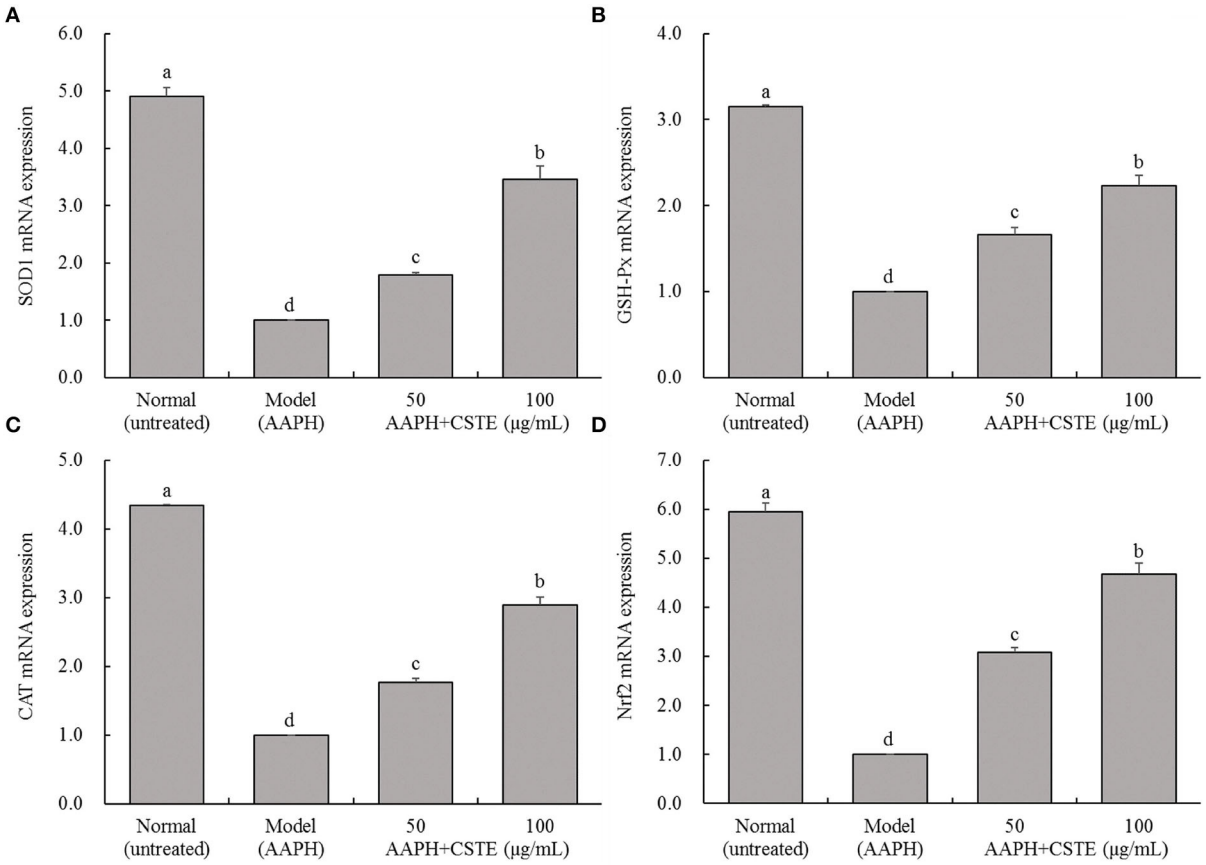

FIGURE 7
SOD1, GSH-Px, CAT and Nrf2 mRNA expression of AAPH inducing oxidative damaged HL-1 cardiomyocytes, **(A)** SOD expression, **(B)** GSH-Px expression, **(C)** CAT expression, (D) Nrf2 expression; CSTE: *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract. $^{a-d}$ according to Duncan's multi range test, the average values of different letters in the same column have significant differences ($P < 0.05$, $n = 6$).

spleen, abdominal water, and edema of the lower limbs. Cardiomyopathy is a common disease in older people (16). Because older people frequently develop cardiomyopathy, the physiological changes in older people are closely related to the pathogenesis of cardiomyopathy. An imbalance of oxidative stress may be an important factor in the pathogenesis of cardiomyopathy. Excessive ROS accumulation is an important pathogenic factor in several chronic diseases. As important free radical donors, azo compounds, such as AAPH, spontaneously generate peroxy-free radicals, which damage DNA, proteins, lipids, and other biological macromolecules, leading to cell death. In this study, the survival rate of the HL-1 cells decreased significantly after exposure to AAPH (1 mmol/L). The survival rate was significantly higher than that of damaged cells treated with the CSTE for 24 h ($P < 0.05$). Additionally, excessive ROS generation induces lipid peroxidation of unsaturated fatty acids in cell membranes, thereby increasing MDA and 4-hydroxynonenal, which are toxic to cells (17, 18). Therefore, MDA is used as a marker of cell damage caused by oxidative stress (19). Treating the damaged HL-1 cells with the CSTE reduced total ROS levels and MDA content. The appropriate administration of antioxidants reduces ROS-induced lipid peroxidation in cardiomyocytes and prevents oxidative stress damage (20). A clinical study reported that eating an antioxidant-rich diet reduces oxidative stress levels in patients with chronic heart disease and eases the progression to end-stage heart disease (21). Furthermore, antioxidant therapy ameliorates the tissue damage caused by excessive oxidative stress in the heart tissue of patients with chronic heart disease (22). The water-soluble azo compound AAPH generates free radicals by thermal decomposition at physiological temperatures, which attack proteins or lipids to initiate peroxidation, ultimately leading to oxidative damage. Cell membranes are rich in unsaturated fatty acids, which are very sensitive to free radical-induced peroxidation, and can cause cell hemolysis (23). As the rate at which free radicals are generated by AAPH can be easily controlled and measured, the use of AAPH to induce hemolysis provides a good way to study

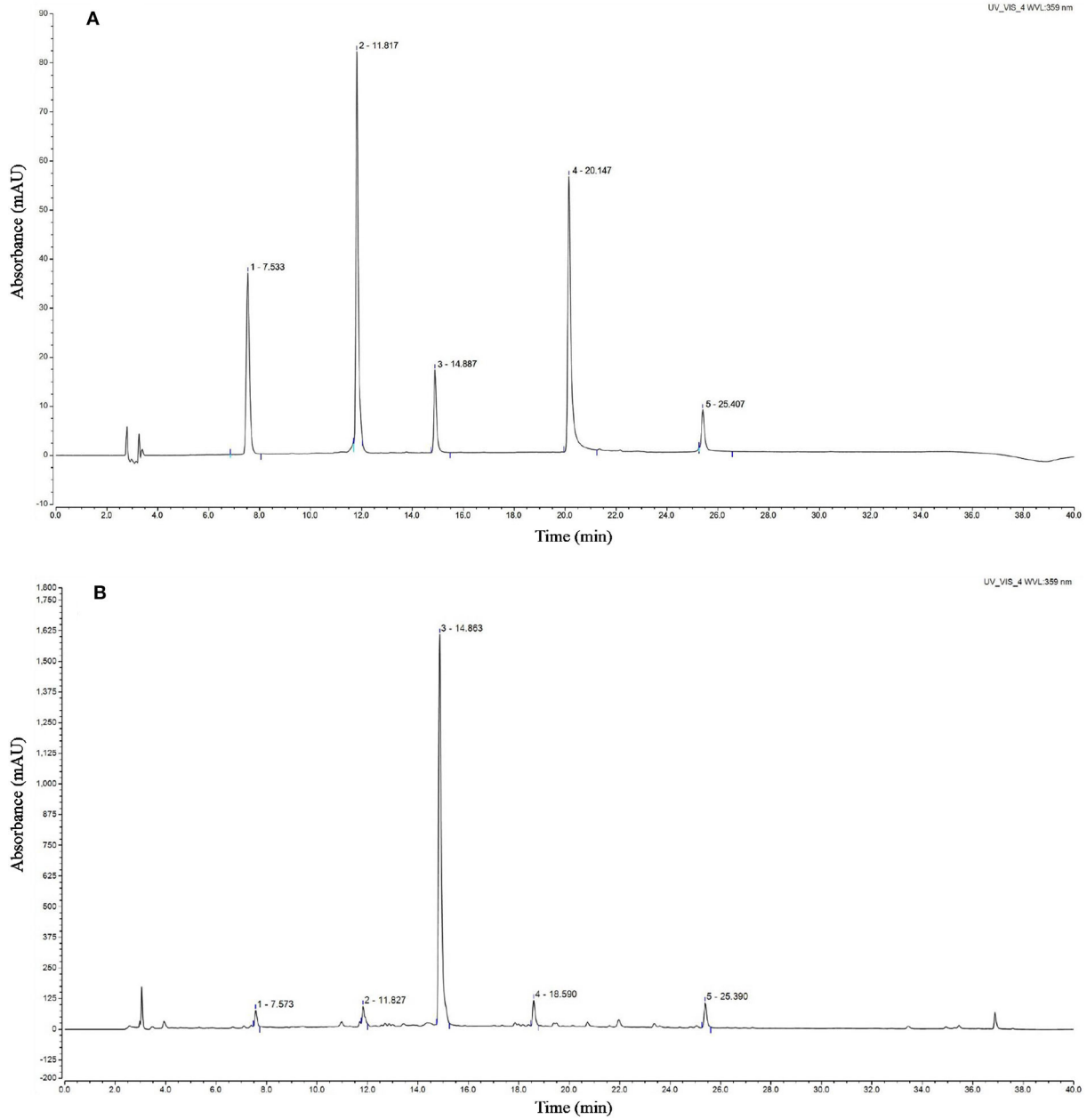

FIGURE 8
HPLC chromatogram of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu extract, **(A)** standard chromatogram, **(B)** *Clerodendranthus spicatus* (Thunb.) C. Y. Wu chromatogram. 1: caffeic acid, 2: isoquercetin, 3: rosmarinic acid, 4; luteolin, 5: baicalin.

free radical-induced membrane damage and detect myocardial cell damage.

The endogenous antioxidant enzymes (CAT, SOD, GSH-Px, and GST) and non-enzymatic GSH effectively fight against oxidative stress injury under normal physiological conditions. For example, SOD converts excess superoxide anions to H_2O_2, which is converted to water by CAT and GSH-Px (24). Additionally, GSH-Px uses GSH as a substrate to reduce H_2O_2 and alkane hydroperoxide levels and also reduces organic hydroperoxides (ROOH) to hydroxyl compounds (ROH) (25). GST helps GSH-Px remove excessive ROOH from the body. In our study, the activity of the antioxidant enzymes CAT, SOD, GSH-Px, and GST as well as γ-GCS content increased in damaged cells after pretreatment with different CSTE concentrations, GSH increased as well. Enhanced CAT and SOD activities prevented the damage caused by oxidative stress,

inhibited lipid peroxidation reactions in cell membranes, and further alleviated oxidative stress damage to the cells (26, 27). As a major non-enzymatic antioxidant, GSH directly reduces toxic lipid peroxides and GSH-Px through H_2O_2 and indirectly inhibits free radical chain reactions, avoiding the cell damage caused by free radicals (28). γ-GCS is the rate-limiting enzyme in GSH biosynthesis and promotes GSH synthesis. After the ROS level increases, the activity of antioxidant enzymes and the expression of related genes in cells increases during the early stage of oxidative stress to resist oxidative damage (25, 26). Studies have shown that Nrf2 is the basis for defense against ROS. Nrf2 reduces the ROS level in the body and regulates the antioxidant enzymes to play a role in balancing oxidative stress. The CSTE also upregulated mRNA transcription levels of the major antioxidant enzymes in damaged HL-1 cells. By enhancing CAT, GSH-Px, and Nrf2 transcription levels, the CSTE improved the activity of intracellular total copper/zinc superoxide dismutase and alleviated oxidative stress injury in cells (29, 30).

Caffeic acid, isoquercetin, rosmarinic acid, luteolin, and baicalin have good antioxidant activities (31–35). Studies have reported that caffeic acid inhibits injury of adriamycin-induced cardiomyocytes (31). Roeosinic acid protects myocardial cells from hypoxia and reoxygenation injury, reduces cardiac fibrosis induced by stress, and inhibits myocardial hypertrophy induced by high glucose levels (36). An *in vitro* study showed that luteolin reduces the oxidative damage in cells caused by H_2O_2 (34). Luteolin significantly inhibits damage to H9c2 cardiomyocytes caused by an N_2 saturated hypoxia chamber and $Na_2S_2O_4$ hypoxic environment, and effectively maintains the balance between antioxidant defense and free radicals (37). Baicalein also protects the heart by inhibiting myocardial damage and infarction (38). Other studies have also shown that caffeic acid and rosmarinic acid components are the main components of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu, but other studies have also shown that *Clerodendranthus spicatus* (Thunb.) C. Y. Wu also contains other components, such as methyl caffeate, ethyl caffeate, and vanillic acid, etc. (3, 39). Therefore, it can be seen that the composition of *Clerodendranthus spicatus* (Thunb.) C. Y. Wu from different origins is quite different, but the *Clerodendranthus spicatus* (Thunb.) C. Y. Wu used in this study are the only origins that can be used as medicines, and are representative.

Conclusions

The combined actions of these five compounds may constitute the protective effects of the CSTE on the myocardium. There are very few studies on the physiological activity of CSTE, especially the research on the mechanism between the active ingredient and the physiological effect. In this study, the association and mechanistic effects of the physiological effects of the CSTE and the active ingredients were investigated. Our study provides a theoretical basis for the CSTE as a health food or traditional Chinese medicine with potent antioxidant capacity. However, as our study only involved *in vitro* analyses of HL-1 cells, specific *in vivo* CSTE-mediated molecular actions must be elucidated if the factors and antioxidant protective mechanisms in the cells are to be identified.

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 the Ethical Committee of Chongqing Hospital of Traditional Chinese Medicine.

Author contributions

YL wrote the first draft of the manuscript. JW collected the data and performed experiments. JJ and XL performed the experiments. MW contributed to conception and design of the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.

Publisher's note

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Protective activity of *Malus doumeri* leaf extract on H_2O_2-induced oxidative injury in H9C2 rat cardiomyocytes

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In this study, *Malus doumeri* leaf extract (MDLE) was used to test its anti-oxidation capacity *in vitro*, it has been preliminarily analyzed for H_2O_2-induced oxidative damage in H9C2 cells and its main active components. The antioxidant capacity through DPPH (1, 1-Diphenyl-2-Picrylhydrazyl), $ABTS^{+}\bullet$ [2,2,2'-azino-BIS-(3-ethylbenzo-thiazoline-6-sulfonic acid)] radical ion, •OH (hydroxyl radical), and $\bullet O_2^-$ (superoxide anion) were determined *in vitro*. The proliferation of H9C2 cells was examined by MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-Tetrazolium bromide]. MDA (malondialdehyde), SOD (superoxide dismutase), CAT (catalase), GSH (glutathione), and GSH-Px (glutathione peroxidase) were determined by colorimetry. Apoptosis induced by oxidative damage was detected by flow cytometry. The mRNA expression of antioxidant related genes of SOD, CAT, GSH, and GSH-Px were checked by qRT-PCR (quantitative real-time polymerase chain reaction). The MDLE main active components were analyzed by HPLC (high-performance liquid chromatography). MDLE had significant scavenging effects on DPPH, $ABTS^{+}\bullet$, •OH, and superoxide anion radicals in a concentration-dependent manner. H_2O_2 treatment could significantly lead to oxidative stress injury of H9C2 cells, and MDLE treatment significantly improved the degree of H9C2 cell damage, and showed a positive correlation with concentration. MDLE can also reduce apoptosis caused by oxidative damage. MDLE treatment could significantly reduce MDA content and increase CAT, SOD, GSH, and GSH-Px contents and expression. In addition, by HPLC analysis, the following six bioactive components were detected from MDLE: chlorogenic acid, isoquercitrin, quercetin, baicalin, and phloretin. Therefore, MDLE has a good protective effect on myocardial cells.

KEYWORDS

***Malus doumeri* leaf, oxidative toxicity stress, hydrogen peroxide, SOD, HPLC**

Introduction

Plant drinks, including tea, originated from the Bayu region in south China and were used as medicinal drink (1). Tea contains many active compounds such as tea polyphenols, so that tea has strong free radical scavenging and reducing activities (1). *Malus doumeri* leaf belongs to another type of tea, that is, a plant that does not belong to the *genus Camellia* in the Theaceae family. It is produced in the Yangtze River Basin, China, and is mainly refined and processed from the young leaves of local wild *Malus doumeri*. Its leaves contain various beneficial amino acids, flavonoids, and so on (2, 3) (Figure 1). Since the leaves grow in an original ecological environment, the picked leaves can be dried directly under appropriate temperature and humidity, active substances are produced through complex chemical changes triggered by a special fermentation process. Studies have found that *Malus doumeri* leaf has a variety of health care effects, such as blood lipids, relieving nerves, anti-aging, strengthening teeth, inhibiting inflammation, preventing cancer, and improving immunity (4, 5).

Reactive oxygen species (ROS) are the main cause of oxidative stress. Under physiological conditions, the concentration of ROS is very low. It is an important substance involved in signal transduction and can regulate the production of immune inflammatory factors in the body. At the same time, peroxides can induce apoptosis (6, 7). ROS can attack proteins, oxidize amino acids, change their structure, and make them lose their physiological functions. It can oxidize lipids on the surface of cell membranes, changing their structure and function. It can also modify bases and interfere with the function of genetic material (8). In the presence of Fe^{2+}, H_2O_2 generates hydroxyl radicals (·OH) with strong oxidizing ability, and triggers more other reactive oxygen species to achieve the degradation of organic matter, thereby causing oxidative damage to the body (9). Therefore, this study examined the protective effect of different concentrations of MDLE on oxidative stress-injured cells through H_2O_2-induced H9C2 cell injury model. Moreover, the preliminary analysis of potentially active components was conducted to provide some reference for the effect of MDLE on human chronic diseases.

Materials and methods

Malus doumeri leaf extract preparation

The *Malus doumeri* leaf was produced in Honghua village, Sanxi Township, Wushan County, Chongqing, China, it was produced in 2021, the freeze-dried *Malus doumeri* leaf (Chongqing Wushan Xiajiang Tea Industry Co., Ltd, Chongqing, China) was crushed, ground, and screened, and then a certain amount of *Malus doumeri* leaf powder was precisely weighed into a beaker. Seventy percent ethanol (v/v) was added to the liquid to the material at a 20:1 ratio (w/w), and incubated at 60°C in a water bath for 2 h. This procedure was performed twice. Remove water and ethanol from the resin liquid by rotary evaporator (steam until no liquid flows in the beaker), and dry at 60°C for 48 h; the dried sample was removed, ground, and weighed, then stored in an EP tube and stored in 4°C for later use.

Hydroxyl radical scavenging experiment

Salicylic acid colorimetric method (10). Take 10 mL corkscrew tube, add 2 mL MDLE aqueous solution of different concentrations (0.2, 0.6, and 1.0 mg/mL) (11), then add 2 mL 9 mmol/L ethyl-salicylic acid solution and 1 mL 9 mmol/L ferrous sulfate solution (prepared with ferrous sulfate seven water, Solarbio Life Science, Beijing, China) successively. Finally, 2 mL 8.8 mmol/L hydrogen peroxide (Solarbio Life Science) was added to initiate the reaction. The samples were incubated at 37°C in a water bath for 30 min, and the OD at 510 nm was measured. Three independent OD measures were performed per group (Themo Genesys10s, ThermoFisher Scientific, Waltham, MA, USA).

DPPH free radical scavenging assay

0.5 mL MDLE aqueous solution with different concentrations (0.2, 0.6, and 1.0 mg/mL) was added to 2 mL DPPH ethanol solution (0.33 mM, Solarbio Life Science) (11), and incubated in the dark at room temperature for 30 min. The 517 nm value of absorbance in the solution was tested (12), three parallel in each group (Themo Genesys10s, ThermoFisher Scientific).

ABST clearance experiment

In a 5 mL stopper tube, 1 mL ABTS (2,2,2′-azino-BIS-(3-ethylbenzo-thiazoline-6-sulfonic acid, Solarbio Life Science) free radical working solution and 0.4 mL MDLE solution with different concentrations (0.2, 0.6, and 1.0 mg/mL) were added (11). The tube was filled with solvent, and left in darkness for 30 min, and the OD value at 734 nm was measured. Samples were prepared in triplicate. For the ABTS free radical working solution preparation, 3 mg ABTS was dissolved into 0.8 mL double steamed water to prepare the Liquid A. For Liquid B, 1 mg potassium persulfate was dissolved in 1.5 mL double steamed water. Then, 0.2 mL of liquid A and liquid B were mixed, oxidized in darkness for 12 h, and diluted to A

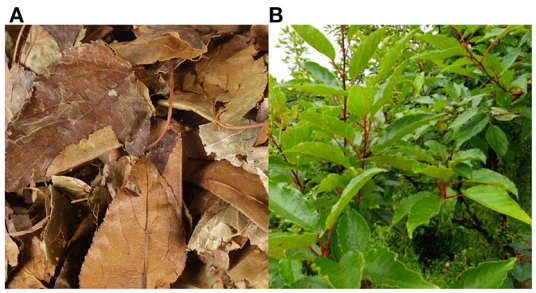

FIGURE 1
Observation diagram of *Malus doumeri* leaf, **(A)** dry leaves, **(B)** plant leaves.

TABLE 1 The sequences of reverse transcription-polymerase chain reaction primers in this study.

Gene name	Sequence
SOD	Forward: 5′- AGATGGTGTGGCCGATGTGT-3′
	Reverse: 5′- TCCAGCGTTTCCTGTCTTTGTA-3′
CAT	Forward: 5′- TGTTGCTGGAGAATCGGGTTC-3′
	Reverse: 5′- TCCCAGTTACCATCTTCTGTGTA-3′
GSH	Forward: 5′- TACGGCTCACCCAATGCTC-3′
	Reverse: 5′- CTATGGCACGCTGGTCAAATA-3′
GSH-Px	Forward: 5′- GTCGGTGTATGCCTTCTCGG-3′
	Reverse: 5′- CTGCAGCTCGTTCATCTGGG-3′
GAPDH	Forward: 5′- TCA AGA AGG TGG TGA AGC AGG-3′
	Reverse: 5′- AGC GTC AAA GGT GGA GGA GTG-3′

with anhydrous ethanol and determined at OD_{734nm} (Themo Genesys10s, ThermoFisher Scientific) (13).

Superoxide anion removal experiment

The pyrogallol autoxidation method was used for superoxide anion removal experiment (14). In a 10 mL corkscrew tube, 1 mL MDLE aqueous solution (0.2, 0.6, and 1.0 mg/mL) was mixed with 4.5 mL 0.1 mol/L TRIS-HCl buffer solution (pH = 8.2, Solarbio Life Science), and reacted at 37°C in a water bath for 20 min, then allowed to cool to 25°C. Then 0.4 mL of 50 mmol/L pyrolenol solution (Solarbio Life Science) was placed in a water bath at 37°C for 5 min, and then 0.1 mL of 8.0 mol/L concentrated HCl was added immediately to stop the reaction. All samples were prepared in duplicate.

H9C2 cell culture

The H9C2 cells (National Collection of Authenticated Cell Cultures, Shanghai, China) were recovered and inoculated in DMEM medium (high glucose, containing 10% fetal bovine serum and 1% penicillin-streptomycin diabody solution, Solarbio Life Science), and cultured at 37°C in a 5% carbon dioxide environment.

Cell viability assay (MTT method)

The concentration of H9C2 was adjusted to 1.0×10^4 cells/mL after culturing, and then 200 μL of cell suspension was added to each well of a 96-well cell culture plate, and then adherent culture was carried out at 37°C for 24 h. After adherent cells are treated in two cases, first, 200 μL of MDLE medium solutions at concentrations of 0, 40, 100, 160 μg/mL were added to the wells. In the second case, before the MDLE treatment, the cells were first treated with 20 μL of hydrogen peroxide with a concentration of 0.3 mmol/L for 4 h, and then the cells were treated with the above concentration of MDLE medium solution. After 48 h, the cells were further treated with 200 μL of MTT reagent (Solarbio Life Science) at a concentration of 5 mg/mL for 4 h. Then, after all the medium was drained, 200 μL of sterile DMSO was added and shaken for 30 min. The final absorbance value was measured at 490 nm (Themo Genesys10s, ThermoFisher Scientific) (15).

MDLE affected the contents of MDA, SOD, GSH, GSH-Px, and CAT in H9C2 cells injured by oxidative stress

H9C2 cells in logarithmic growth phase were digested with 0.25% trypsin and adjusted to a concentration of (1×10^5 cells/mL), then seeded into 6-well cell culture plates, and then 2 mL of DMEM medium was added to the wells, were incubated at 37°C for 24 h in 5% carbon dioxide. After adhesion, 200 μL hydrogen peroxide (0.3 mmol/L) was added, mixed, and cultured for 4 h to obtain the oxidative damage model. For the H9C2 oxidative damage cell model, 200 μL MDLE aqueous extract (0, 40, 100, and 160 μg/mL) was added to each well, and the final volume was adjusted with PBS buffer (0.1 M). Samples were incubated at 37°C, 5% CO_2 in a saturated and humid environment for 24 h. After MDLE treatment, H9C2 cells were washed with pre-cooled PBS, dissociated with 200 μL trypsin, and transferred to a 1.5 mL centrifuge tube for supernatant removal. The cell pellet was washed again with pre-cooled PBS and centrifuged at 4,000 r/min for 15 min to remove the supernatant. The pellet was homogenized in 800 μL normal saline. The MDA (malondialdehyde), SOD (superoxide dismutase), CAT (catalase), GSH (glutathione), and GSH-Px (glutathione peroxidase) contents in cell homogenate were determined according to the instructions of the relevant kit (Solarbio Life Science) (16).

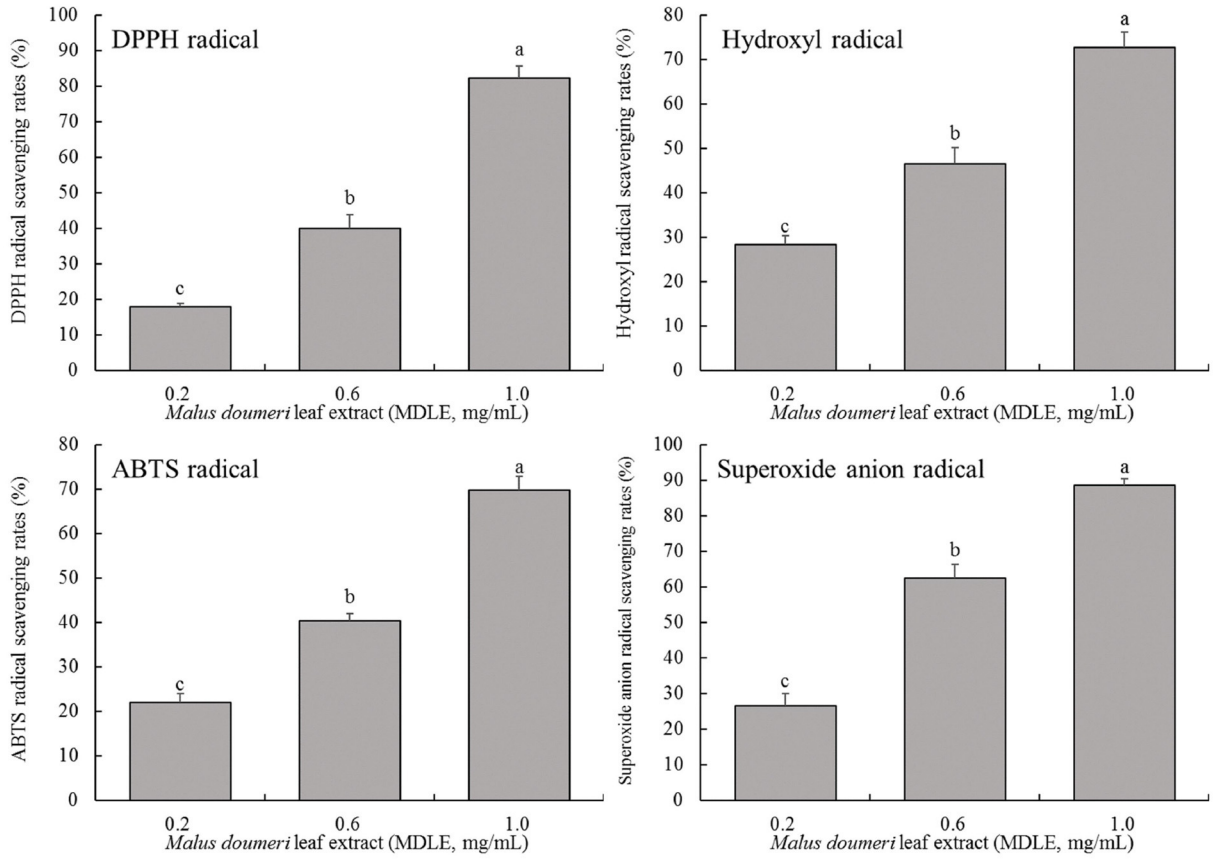

FIGURE 2
Hydroxyl radical, DPPH free radical, ABTS free radical and superoxide anion free radical scavenging rates of *Malus doumeri* leaf extract (MDLE). [a–c]Lowercase letters are used to indicate significant differences at the $P < 0.05$ level. The same letter group indicates no difference, and the letter difference group indicates difference.

TABLE 2 Effect of *Malus doumeri* leaf extract (MDLE) treatment on survival rate of H_2O_2 induced oxidative damage of H9C2 cells.

Group	Normal	Oxidative damage	H_2O_2+MDLE (μg/mL)	
			80	160
OD_{490}	0.508 ± 0.005^a	0.214 ± 0.009^d	0.332 ± 0.008^c	0.427 ± 0.007^b
Survival rate (%)	100.00 ± 0.00^a	42.09 ± 1.91^d	65.30 ± 2.22^c	84.13 ± 1.85^b

[a–d]Lowercase letters are used to indicate significant differences at the $P < 0.05$ level. The same letter group indicates no difference, and the letter difference group indicates difference.

Flow cytometry

After MDLE treatment, 1 mL cell suspension of 5×10^5 CFU/mL was centrifuged at 4,500 RPM and 4°C. The supernatant was discarded, and the cells were washed with pre-cooled PBS and suspended in 500 μL PBS. Cells were mixed with 5 μL annexin V-FITC and 5 μL propidium iodide (ThermoFisher Scientific) at 37°C in the dark for 15 min (AccuriC6, BD Biosciences, San Jose, CA, USA). Finally, flow cytometry was used for detection.

H_2O_2 effect on antioxidative genes expression in H9C2 cells

After the cells were cultured according to the previous experimental method, 1.0 mL RNAzol was added to extract the RNA from the tissue. Then measure the absorbance values of RNA extracts at 260 and 280 nm, and adjust the concentration of RNA to 1 μg/μL after calculating OD260/OD280. After reverse transcription, prepare a cDNA reaction system. The system solution includes cDNA (1 μL), SYBR Green PCR Master Mix (10 μL), upstream primers (1 μL, Table 1, ThermoFisher

Scientific), downstream primers (1 μL) and sterile distilled water (7 μL). After the reaction solution was prepared, it was placed in a real-time quantitative PCR instrument, and under the set conditions (60 s at 95°C and 15 s at 95°C for 40 cycles, then 30 s at 55°C, 35 s at 72°C, 30 s at 95°C, 35 s reaction at 55°C) for mRNA amplification (SteponePlus, ThermoFisher Scientific), using GAPDH as the internal reference, and calculating the relative expression intensity of each gene according to the $2^{-\Delta\Delta CT}$ assay (17).

The main active components of MDLE were analyzed by high-performance liquid chromatography

MDLE extract was dissolved in DMSO to obtain a concentration of 10 mg/mL solution, then diluted with 50% methanol to obtain the final concentration of 2 mg/mL liquid phase sample through a 0.22 m organic filtration membrane test (injection volume: 10 μL). The Chromatographic column was an Accucore C18 column (2.6 μm, 4.6 × 150 mm), the Mobile phase A consisted of 0.5% acetic acid water, and the Mobile phase B consisted of acetonitrile. The flow rate was 0.6 mL/min, the column temperature was 35°C, and the detection wavelength was 254 nm. The Gradient elution conditions were 0–10 min in 12–25% B and 10–30 min in 25–45% B (UltiMate3000, ThermoFisher Scientific).

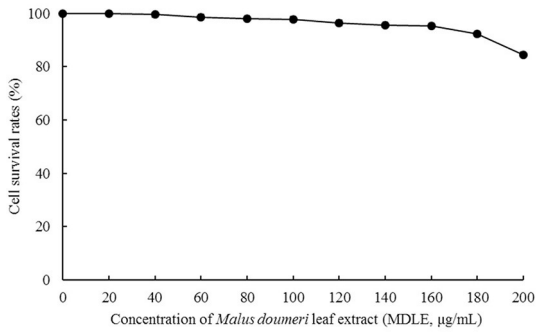

FIGURE 3
Effect of *Malus doumeri* leaf extract (MDLE) treatment on survival rate of H9C2 cells.

Statistical analysis of data

The experimental data were analyzed using SPSS 20.0 statistical software. Experimental results are expressed as mean ± standard deviation. Duncan's multi-range test was used to analyze the results by one-way analysis of variance (ANOVA), and $P < 0.05$ was considered to be statistically significant.

Results

MDLE antioxidant test *in vitro*

As shown in Figure 2, MDLE aqueous extract at concentrations of 0.2, 0.6, and 1.0 mg/mL significantly increased the scavenging rates of hydroxyl radical (28.4, 46.5, and 72.8%), DPPH free radical (31.9, 58.5, and 83.7%), ABTS free radical (22.0, 40.4, and 69.8%), and superoxide anion free radical (34.2, 62.7, and 87.6%). The results showed that MDLE had dose-dependent scavenging ability on four kinds of free radicals, and there were significant differences ($P < 0.05$).

H9C2 cell survival rate test (MTT method)

As shown in Figure 3, the survival rates of H9C2 cells were higher than 95% after MDLE treatments (0–160 μg/mL), indicating that MDLE within the concentration range (0–160 μg/mL) had no obvious lethal effect on H9C2. Therefore, 40, 100, and 160 μg/mL MDLE aqueous extracts were used for subsequent studies.

As shown in Table 2, H_2O_2 greatly reduced the survival rate of H9C2 cells after injury. After treatment with 80 and 160 μg/mL MDLE, the cell survival rate was significantly

TABLE 3 SOD, GSH, GSH-Px, and CAT contents of *Malus doumeri* leaf extract (MDLE) treatment on H_2O_2 induced oxidative damage of H9C2 cells.

Group	Normal	Oxidative damage	H_2O_2+MDLE (μg/mL)	
			80	160
SOD (U/mg)	7.82 ± 0.42^{a}	3.23 ± 0.25^{d}	5.02 ± 0.34^{c}	6.16 ± 0.32^{b}
GSH (μmol/mg)	0.79 ± 0.05^{a}	0.33 ± 0.03^{d}	0.48 ± 0.03^{c}	0.63 ± 0.04^{b}
GSH-Px (U/mg)	5.12 ± 0.26^{a}	2.02 ± 0.21^{d}	3.15 ± 0.26^{c}	3.99 ± 0.16^{b}
CAT (U/mg)	3.19 ± 0.23^{a}	0.79 ± 0.18^{d}	1.45 ± 0.20^{c}	2.32 ± 0.21^{b}

$^{a-d}$Lowercase letters are used to indicate significant differences at the $P < 0.05$ level. The same letter group indicates no difference, and the letter difference group indicates difference.

improved, and the protective effect of high concentration (160 μg/mL) was more pronounced ($P < 0.05$).

MDLE treatment changes the MDA content in oxidative-damaged H9C2 cells

As shown in Figure 4, after H_2O_2 treatment for 4 h, the MDA content of MDLE-treated H9C2 cells (0.76 ± 0.06 nmol/mg) was significantly higher than that of untreated cells (0.18 ± 0.03 nmol/mg). Different concentrations of MDLE aqueous extracts (80 and 160 μg/mL) significantly reduced the MDA content, and 160 μg/mL MDLE treatment had the lowest MDA content (0.36 ± 0.05 nmol/mg).

MDLE treatment changed the SOD, GSH, GSH-Px, and CAT contents in oxidative damaged H9C2 cells

As shown in Table 3, the SOD, GSH, GSH-Px, and CAT contents in H_2O_2-treated H9C2 cells (0.3 mmol/L) for 4 h were significantly lower than in normal cells. The cells' SOD, GSH, GSH-Px, and CAT contents were significantly improved after MDLE treatments (80 and 160 μg/mL), and the effect was the best in the 160 μg/mL MDLE treatment.

Effect of MDLE on apoptosis of oxidative-damaged H9C2 cells

Apoptosis and death were induced in H_2O_2-treated H9C2 cells. MDLE significantly inhibit H_2O_2 induced oxidative H9c2 cell death, indicating that MDLE had an inhibitory effect on cell oxidative damage (Figure 5). And the inhibitory effect was positively correlated with the concentration.

MDLE affected *SOD*, *CAT*, *GSH*, and *GSH-Px* genes expression levels in damaged H9C2 cells

Real-time fluorescence quantitative PCR analysis shows that *SOD*, *CAT*, *GSH*, and *GSH-Px* mRNA expression levels in H9C2 cells were significantly decreased after H_2O_2 induced (0.3 mmol/L) injury, as shown in Figure 6. After MDLE treatment, the expression levels of endogenous antioxidant enzymes *SOD*, *CAT*, *GSH*, and *GSH-Px* in damaged cells were significantly increased ($P < 0.05$).

Liquid chromatography analysis of main components of MDLE

Figure 7 shows the liquid chromatogram of MDLE and related standard substances. HPLC detected six compounds, including chlorogenic acid, isoquercitrin, quercetin, baicalin, and phloretin.

Discussion

Oxygen-free radicals (OFR) can attack polyunsaturated fatty acids in biofilms and cause lipid peroxidation, which leads to oxidative damage to cells (18). There are two types of antioxidant systems in the body, which enable cells to maintain the redox homeostasis of free radicals and ROS. One is an enzymatic antioxidant system, including SOD, CAT, and GSH-Px; the other is a non-enzymatic antioxidant system, including antioxidants such as glutathione, vitamin C, vitamin E, coenzyme Q10, and alpha-lipoic acid. These antioxidants can neutralize free radicals and ROS, reduce the damage of free radicals and ROS to cells, and protect cells and tissues from oxidative damage (19). The body protects itself from damage caused by free radicals and ROS through a range of cell-intrinsic enzymatic and non-enzymatic antioxidants. These potentially harmful free radicals and ROS are regulated by the expression of endogenous antioxidants. The use of antioxidants helps to improve the body's antioxidant capacity, eliminate the harmful effects of free radicals and ROS, and prevent damage caused by a chain reaction of free radicals and ROS. *In vitro* studies confirm that various active components of plants can improve oxidative stress (20). DPPH is a stable free radical that becomes a stable

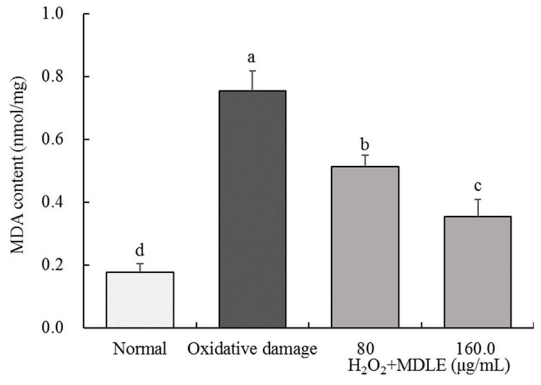

FIGURE 4
MDA content of *Malus doumeri* leaf extract (MDLE) treatment on H_2O_2 induced oxidative damage of H9C2 cells. $^{a-d}$Lowercase letters are used to indicate significant differences at the $P < 0.05$ level. The same letter group indicates no difference, and the letter difference group indicates difference.

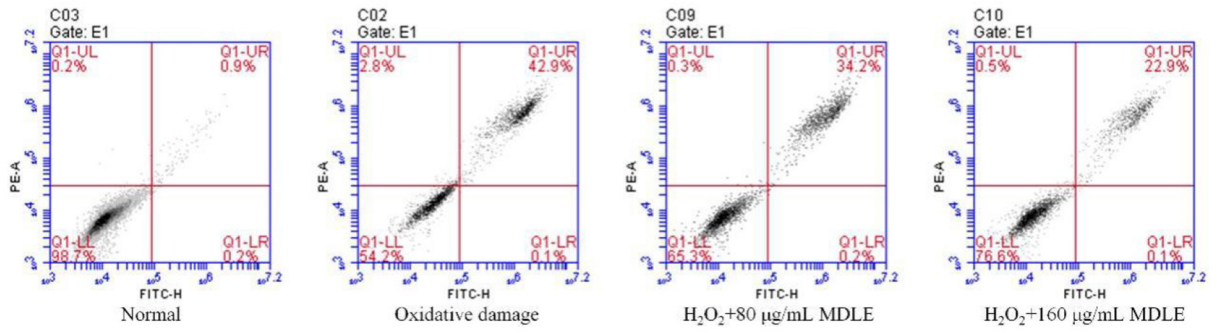

FIGURE 5
Apoptosis and death of H_2O_2 induced oxidative damage of H9C2 cells.

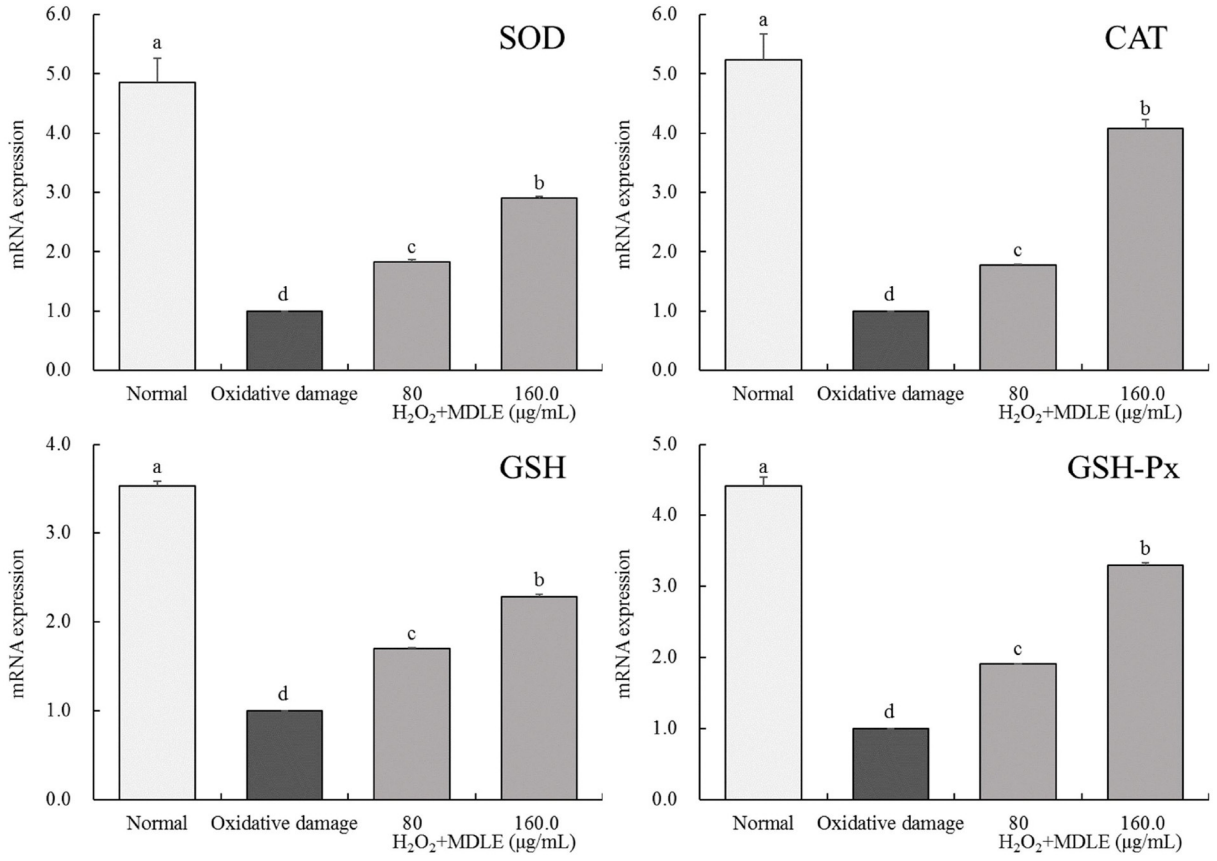

FIGURE 6
SOD, *CAT*, *GSH*, and *GSH-Px* mRNA expression of *Malus doumeri* leaf extract (MDLE) treatment on H_2O_2 induced oxidative damage of H9C2 cells. $^{a-d}$Lowercase letters are used to indicate significant differences at the $P < 0.05$ level. The same letter group indicates no difference, and the letter difference group indicates difference.

yellow molecule after combining with electrons or hydrogen free radicals. It is one of the effective means to detect the antioxidant effect of substance extracts. The *in vitro* evaluation of the antioxidant activity of plant compounds or plant extracts is an important aspect of studying functional factors (21). Unbalanced levels of hydroxyl radicals in the body will cause oxidative damage to DNA, proteins and lipids and damage the body. The effect of antioxidants on oxidative damage can be

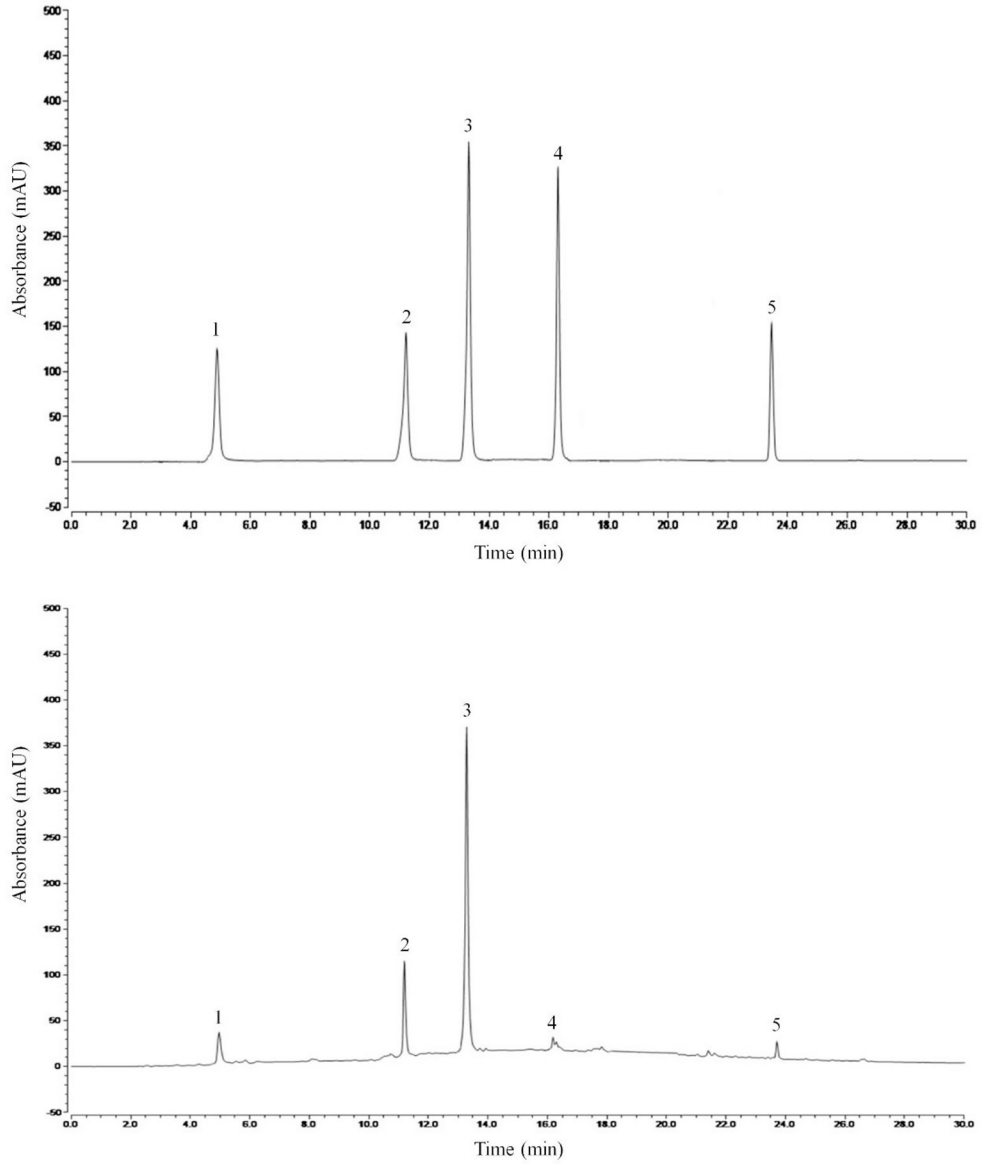

FIGURE 7
HPLC standard and *Malus doumeri* leaf extract chromatogram: 1, chlorogenic acid; 2, isoquercitrin; 3, quercetin; 4, baicalin; 5, phloretin.

detected by capturing •OH in a Fenton reaction system by salicylic acid (22). An oxidant can oxidize ABTS to produce blue-green ABTS•$^{+}$; and the effect of antioxidants can make it fade (23). O_2^- can be produced by the chain reaction of pyrogallol autooxidation system, and the color will be developed after o2- reaction O_2^-. The reaction attenuates its luminous signal (24). This study preliminarily confirmed that MDLE has antioxidant capacity in a dose-dependent manner through free radical scavenging experiments *in vitro*.

MDA is one of the important products of oxidative stress and lipid peroxidation. Its massive production and accumulation can cause serious damage to cell membranes, which in turn leads to cell death and damage to tissues and internal organs (25, 26). Hypoxia can cause myocardial tissue to generate a large number of oxygen free radicals (OFR). OFR acts on the unsaturated fatty acids on the cell membrane to produce peroxidation of membrane lipids, which in turn leads to myocardial cell damage and the formation of lipid peroxides. The important metabolites of OFR in the body can better reflect the degree of tissue peroxidation (27). After H9C2 cells were exposed to H_2O_2, the level of MDA increased, and MDLE could inhibit this occurrence, and the higher the dose, the

better the effect. Superoxide dismutase is an active substance derived from living organisms, which can eliminate harmful substances produced by organisms in the process of metabolism, and has a special anti-aging effect on the human body by continuously supplementing SOD. SOD is one of the most effective antioxidants for scavenging free radicals. Its antioxidant capacity is 20 times that of vitamin C and 50 times that of vitamin E. Its specificity and efficiency in scavenging free radicals are unmatched by other antioxidants (28). Catalase is an antioxidant enzyme that is ubiquitous in almost all organisms, mainly in the liver and red blood cells of animals. It is the marker enzyme of peroxisome and accounts for about 40% of the total amount of peroxisomal enzymes (29). Glutathione peroxidase (GSH-Px) is an important peroxide-decomposing enzyme widely existing in the body, which can reduce toxic peroxides to non-toxic hydroxyl compounds, and at the same time promote the decomposition of H_2O, thereby protecting the structure and function of the cell membrane from interference and damage by peroxides (30). Glutathione reductase can use NADPH to catalyze GSSG to produce GSH, and the activity level of glutathione peroxidase can be calculated by detecting the decrease of NADPH (31). MDLE can regulate the levels of CAT, SOD, GSH-Px and GSH in cells, thereby inhibiting lipid peroxidation and protecting cells.

Chlorogenic acid is a plant polyphenol that is often found in food. Studies have shown that it has a good antioxidant effect, which can also exert its anti-inflammatory effect (32). Isoquercetin is also a compound with antioxidant effects, and its physiological activities include anti-inflammatory, anti-viral, anti-tumor, and hypotensive (33). Quercetin is present in many plants, it is not only a good antioxidant, but also can lower blood sugar, and also has a certain effect on digestive system cancer (34). Baicalin is an active substance that can protect cardiovascular and cardiomyocytes, and can avoid heart damage (35). Phloretin is a kind of plant polyphenol with dihydrochalcone structure, which has antioxidant, anti-tumor, anti-inflammatory, immunosuppressive and other pharmacological effects (36). Some studies showed that *Malus doumeri* leaf from other places in China mainly contained 9 compounds, including the 5 compounds detected in this study (11, 37). In previous studies, the content of these 5 compounds was also high (38). Therefore, it could be considered that these 5 compounds were the main functional ingredients in *Malus doumeri* leaf from different places. Therefore, the presence of these bioactive chemicals can explain many pharmacological effects of MDLE.

Conclusions

In conclusion, we evaluated the antioxidant activity of MDLE through *in vitro* experiments. We preliminarily analyzed the improvement effect and potential active compounds of MDLE under oxidative stress at the cellular level. MDLE had significant effects on DPPH, OH, O_2^-, and ABTS radical had significant scavenging activity. Meanwhile, the contents of CAT, SOD, GSH-Px and non-enzyme antioxidant GSH could be increased. Real-time quantitative PCR showed that MDLE could also up-regulate the transcription level of mRNA of related antioxidant enzymes to reflect the alleviating effect of oxidative stress. In addition, nine compounds, mainly flavonoids, were detected from MDLE by HPLC. These active compounds have been proved to have many biological activities. Finally, this study only carried out a preliminary study on the *in vitro* antioxidant activity and the effect of improving oxidative damage of *Malus doumeri* leaves, and the protective mechanism of its antioxidant effect needs to be further studied.

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 the Affiliated Hospital of Guizhou Medical University.

Author contributions

WL and DW conceived and designed the study. YS, ZS, PL, ZC, and BW conducted most of the experiments and data analysis and wrote the manuscript. DL, XS, and JP participated in collecting data and helped to draft the manuscript. All authors reviewed and approved the manuscript.

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Guizhou Province atherosclerotic disease prevention and treatment technology innovation].

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.

Publisher's note

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Cardiotoxicity is mitigated after a supervised exercise program in HER2-positive breast cancer undergoing adjuvant trastuzumab

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Background: Trastuzumab is used, alone or in conjunction with standard chemotherapy, to treat HER2-positive breast cancer (BC). Although it improves cancer outcomes, trastuzumab. can lead to cardiotoxicity. Physical exercise is a safe and effective supportive therapy in the management of side effects, but the cardioprotective effects of exercise are still unclear.

Objectives: The primary aim of this study was to test whether trastuzumab-induced cardiotoxicity [left ventricular ejection fraction (LVEF) under 50%, or an absolute drop in LVEF of 10%] was reduced after a supervised exercise program of 3 months in patients with HER2-positive breast cancer. Secondary endpoints were to evaluate (i) cardiotoxicity rates using other criteria, (ii) cardiac parameters, (iii) cardiorespiratory fitness and (iv) whether a change in LVEF influences the cardiorespiratory fitness.

Methods: 89 women were randomized to receive adjuvant trastuzumab in combination with a training program (training group: TG; $n = 46$) or trastuzumab alone (control group: CG; $n = 43$). The primary and secondary endpoints were evaluated at the end of the supervised exercise program of 3 months (T3).

Results: After exercise program, 90.5 % of TG patients and 81.8% of CG patients did not exhibit cardiotoxicity. Furthermore, whatever the used criterion, percentage of patients without cardiotoxicity were greater in TG (97.6 and 100% respectively) than in CG (90.9 and 93.9% respectively). LVEF and GLS

values remained stable in both groups without any difference between the groups. In contrast, at T3, peak VO_2 (+2.6 mL.min^{-1}.kg^{-1}; 95%CI, 1.8 to 3.4) and maximal power (+21.3 W; 95%CI, 17.3 to 25.3) increased significantly in TG, whereas they were unchanged in CG (peak VO_2: +0.2 mL.min^{-1}.kg^{-1}; 95%CI, −0.5 to 0.9 and maximal power: +0.7 W, 95%CI, −3.6 to 5.1) compared to values measured at T0. No correlation between LVEF changes and peak VO_2 or maximal power was observed.

Conclusion: A 12-week supervised exercise regimen was safe and improved the cardiopulmonary fitness in particular peak VO_2, in HER2-positive BC patients treated with adjuvant trastuzumab therapy. The study is under powered to come to any conclusion regarding the effect on cardiotoxicity.

Clinical trial registration: www.ClinicalTrials.gov, identifier: NCT02433067.

KEYWORDS

breast cancer, HER2 overexpression, cardiotoxicity, supervised exercise program, prevention, supportive care

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer death among women (1). The human epidermal growth receptor-2 (HER2) is overexpressed and/or amplified in approximately 20–25% of BC patients (2). Trastuzumab, a humanized monoclonal antibody against the extracellular domain of HER2, improves disease-free and overall survival in patients with BC overexpressing HER2 (3–6). Although well tolerated, trastuzumab may have adverse cardiac effects, ranging from an asymptomatic drop in left ventricular ejection fraction (LVEF) to symptoms of heart failure (7–9).

There is no universal consensus on the definition of cardiotoxicity. In clinical trials, and according to several professional societies, different thresholds of change in LVEF are used to define cardiotoxicity (2, 10–13). Cardiac dysfunction is considered either as (i) LVEF below 50% or an absolute decrease of 10% from baseline, or (ii) LVEF below 50%, or (iii) an absolute decrease in LVEF of more than 15% from baseline, with LVEF remaining above 50% (11), or (iv) 10% subclinical and asymptomatic reduction in LVEF from baseline (2). Thus, baseline evaluation of cardiac function is recommended prior to initiation of trastuzumab-based therapy and regularly during trastuzumab treatment.

Currently, to assess LVEF before, during and after chemotherapy or trastuzumab treatment, echocardiography is the main non-invasive method for detecting myocardial dysfunction (14). The main advantages of this method are its wide availability, lack of radiation, and its ability to assess hemodynamics and other cardiac structures. Furthermore, echocardiography makes it possible to assess not only 3D-based LVEF, but also left ventricular (LV) global longitudinal strain (GLS). Indeed, GLS was proposed as an early marker of imminent cardiotoxicity (14, 15). A reduction of 15% during chemotherapy is associated with a higher probability of significant left ventricular systolic dysfunction (14, 15).

Adjuvant therapies for BC may induce a cascade of cardiac dysfunction, starting with LV alterations, resulting in LVEF decrease and abnormal LV contractility, a stroke volume reduction and cardiac output, and ultimately, a decrease in nutrient and oxygen supply (16, 17). These alterations may lead to dyspnea, lower gas diffusion capacity, thereby compromising the oxygen supply and elimination of carbon dioxide (18, 19). In addition, cardiotoxicity is accompanied by a decrease in cardiorespiratory capacities, which in turn exacerbate exercise intolerance and deconditioning (20–22). It is therefore essential to prevent cardiotoxicity as early as possible, from the initiation through to the end of treatment, and beyond (23–25).

Physical exercise is increasingly recognized as an effective non-pharmacological approach to counteracting the adverse effects of cancer therapy (26–35). Nevertheless, data are sparse regarding the effects of physical exercise on cardiac toxicity induced by adjuvant treatment with trastuzumab. Besides, Murray et al., reported recently that the role of

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate transaminase; BC, Breast Cancer; BMI, body mass index; CG, Control group; ER, Estrogen Receptor; GLS, left ventricular global longitudinal strain; HER2, human epidermal growth factor receptor 2; HR, Heart rate; LV, left ventricular; LVEF, left ventricular ejection fraction; Peak VO_2, Peak oxygen uptake; PR, Progesterone Receptor; SaO_2, oxygen arterial saturation; TG, Training group; TIC trastuzumab-induced cardiotoxicity; VO_2/HR, Oxygen pulse; WHO, world health organization.

exercise on cancer treatment-related cardiac dysfunction is still unclear (36).

To date, considering the small number of available data and the heterogeneity between studies, there is inconclusive evidence of a cardioprotective effect of physical activity in patients receiving adjuvant trastuzumab. Therefore, the primary aim of this study was to ascertain whether a supervised exercise training program lasting 3 months would reduce trastuzumab-induced cardiotoxicity (TIC) in HER2-positive breast cancer undergoing adjuvant trastuzumab. Secondary objectives were to assess (i) cardiotoxicity defined using various different criteria; (ii) cardiac parameters (LVEF and GLS); and (iii) cardiorespiratory fitness (peak VO_2 and maximal power). We hypothesized that a supervised exercise program would be effective in preventing TIC and improving cardiorespiratory fitness.

Materials and methods

Ethics approval

The CARDAPAC study was conducted in compliance with the Declaration of Helsinki. It received approval by the Ethics Committee (Comité de Protection des Personnes Est-II), Besançon, France under the number P/2014/241 and by the National Health Products Safety Agency (N° ID RCB 2014-A01911-46). The trial was registered on ClinicalTrials.gov under the number NCT02433067. Financial support was provided by the Ligue Contre le Cancer association (CCIR-GE).

Study design

We performed the present phase II, randomized, prospective, multicentre, non-comparative trial, and included patients from 5 sites in Eastern France (one university teaching hospital, three non-academic public hospitals and one private clinic). Detailed methods have been published elsewhere (37).

Briefly, women were recruited based on the eligibility criteria summarized in Table 1. To be included, patients had to be aged 18 to 85 years, had a first HER2-positive breast cancer, histologically confirmed with a WHO performance status $\leq$ 1, had to have completed chemo-radiotherapy, had to have a normal cardiac function with LVEF $\geq$ 50% (less than 3 months) and had to present a certificate of non-contraindication to the practice of physical activity. All participants provided written informed consent prior to enrolment. Patients were randomly assigned in a 1:1 ratio to receive adjuvant trastuzumab in combination with a supervised training exercise program (training group, TG) or trastuzumab alone without exercise program (control group, CG). Randomization was performed according to the minimization technique with stratification (eRandomisation software Tenalea®) by age (18–30 vs. 30–50 vs. 50–85 years) and global health score defined from a quality of life questionnaire [QLQ-C30 (0–30 vs. 30–50 vs. 50–70 vs. >70)].

TABLE 1 Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
- Patients aged 18 to 85 years - First HER2 positive breast cancer, confirmed histologically - WHO Performance status ≤1 - Completed chemo-radiotherapy - Normal renal function (creatinine clearance ≥ 60 ml.min^{-1}) - Normal heart function with LVEF ≥ 50% (As assessed by echocardiography dating from less than 3 months previously) - Normal liver function (normal ASAT and ALAT) - Certificate of non-contraindication to the practice of physical activity - Active contraception or menopaused	- HER2 negative breast cancer - Patients with metastases - Heart failure (LVEF ≤ 50%) - Resting oxygen saturation (SaO_2) ≤ 92% - Autoimmune disease (systemic lupus erythematosus, rheumatoid arthritis) - Symptomatic osteoarthritis, cardiovascular disease (angina or uncontrolled hypertension) or lung disease (chronic obstructive pulmonary disease) - Patients suffering from malnutrition (BMI <18 kg.m^{-2}) or weight loss of >10% during the previous 3 months - Patients with psychiatric or cognitive disorders deemed unsuitable for physical activity - Pregnant or breastfeeding patients

ALAT, alanine aminotransferase; ASAT, aspartate transaminase; BMI, body mass index; HER2, human epidermal growth factor receptor 2; LVEF, left ventricular ejection fraction; SaO_2, oxygen arterial saturation; WHO, world health organization.

Study protocol

All evaluations were carried out at enrolment (T0), and at three (T3) and 6 months (T6). Between T0 and T3, both groups received standard oncological care either with (TG) or without (CG) supervised exercise program. Between T3 and T6, both groups had standard oncological care without supervised physical activity.

Cardiorespiratory exercise testing

Cardiorespiratory exercise testing was conducted using a cyclo-ergometer (Ergoselect 200; Ergoline; Bitz, Germany) under the supervision of a respiratory medicine specialist. The assessor provided standardized encouragement until maximal power was reached. After a 3-min warm-up period at a power of 30 watts, intensity was gradually increased by 10 watts every min until the patient was exhausted or limited by

factors such as fatigue, refusal to continue the exercise, and/or appearance of cardiac symptoms (i.e., ECG abnormalities or arterial hypertension). The cadence was maintained between 50 and 70 revolutions per min until the tolerated power. The highest oxygen achieved during the final 60 seconds of the test was considered as peak (peak VO_2). Active recovery was pursued for 10 min, at the same power as that employed during the warm-up.

Heart rate (HR) was continuously monitored, from rest to the end of recovery, with a 12-lead electrocardiogram (CASE P2, GE Healthcare, Buckinghamshire, UK).

The ventilatory parameters [ventilation per (VE), respiratory rate (RT), tidal volume (VT)], oxygen consumption (VO_2) and carbon dioxide release (VCO_2)] were recorded continuously, using a gas exchange analyzer system (MGC-CPX System; MGC Diagnostics Corporation, Saint Paul, MN, USA), which was calibrated using gases of known concentration. Ventilatory variables were averaged every 30 seconds.

The first and second ventilatory thresholds (VT_1 and VT_2) were assessed from the relation between the respiratory exchange ratios (VCO_2/VO_2, VE/ VO_2, VE/ VCO_2) by two experts, in a blinded fashion, using the V-slope method according the Wasserman method (38). The mean of the two closest values was taken as the VT and the corresponding power (watts) was recorded. Power corresponding at VT_1 and VT_2 was used to guide intensity for the supervised exercise program.

Supervised exercise program

Patients allocated to the TG performed a supervised exercise training, which was carried out on a cycloergometer, with electromagnetic braking, and comprised three sessions of 55 min per week for 12 weeks, giving a total of 36 sessions. Each session began with 5 min of warm-up at an intensity equal to half the power of VT_1 (½ VT_1), followed by 9 work bouts of 5 min each, for a maximum total exercise time of 45 min. Each 5-min work bout consisted of 4 min of moderate intensity power, denoted "base", followed by a 1-min-high intensity work bout denoted "peak". Initially, the "base" exercise level was chosen as the VT_1 power and the "peak" level as the VT_2 power. At the end of the last "peak", an active recovery period of 5 min was performed at the same power as that of the warm-up (½ VT_1).

The exercise program was supervised by an adapted physical activity specialist to ensure the safety and quality of the program. During each session, HR was continuously measured with a fingertip pulse oximeter (Onyx® Vantage 9590, Nonin Medical, Inc., USA), at the end of the warm-up, at the end of each "base" and each "peak", as well as every min during active recovery. The peak and base loads were alternately readjusted by 10 watts when values of HR at the end of the session were 10 to 12 beats/min below the target heart rate.

It should be noted that the proposed exercise intervention was never intended to replace or interfere with the standard of care.

Primary endpoint

Cardiotoxicity was defined as either a decrease of the LVEF under 50% at T3 (independently of the baseline value) or an absolute drop in LVEF of at least 10% from T0 to T3 (criterion 1).

All echocardiographic measurements were performed in the supine position, on 3 representative beats, and data were averaged. Echocardiographic acquisitions were performed by a single experienced cardiologist blinded to the patient assignment group, to avoid measurement bias.

LVEF was assessed by transthoracic Doppler echocardiography (Philips EPIQ7, Philips Healthcare, Andover, MA, USA) in the apical 4-chamber (4C) view using Simpson's biplane rule, according to the American Society of Echocardiography recommendations (35, 36).

Secondary endpoints

Cardiotoxicity defined using other criteria

Due to the different criteria used in numerous clinical trials (2, 10–13), cardiotoxicity was also assessed using other criteria, namely: (i) LVEF less than or equal to 50% at T3 or an absolute decrease in LVEF of at least 15% from T0 to T3 (criterion 2) and (ii) LVEF less than 50% at T3 (criterion 3).

Additionally, an absolute decrease in GLS of 15% from T0 to T3 was used as the 4th criterion for cardiotoxicity (39–41). GLS was assessed in the basal, mid-ventricular and apical segments in the 4-chamber view. Since the recommendations of the European Society of Cardiology were only published in 2016 (42), GLS was measured only from the second year of inclusion onwards.

Other outcomes

Cardiac parameters were evaluated by studying baseline LVEF and GLS and cardiopulmonary functions were assessed by maximal power (watts), peak VO_2 ($ml.min^{-1}.kg^{-1}$), heart rate ($beats.min^{-1}$) and VO_2/HR ($mL.\ beats^{-1}$) as described above.

Furthermore, two subgroups were set-up: TOX and NoTOX according to the presence or absence of cardiotoxicity as defined by criterion 1.

Sample size

Since the beginning of the study and the publication of the design (37), the sample size was modified, in agreement

with methodologists and authors for two reasons: (i) a low rate of inclusion (1.5 patients/month) despite 5 years of inclusion, (ii) the recent introduction of targeted therapies, in particular trastuzumab emtansine (T-DM1), an antibody drug combining the anticancer properties of trastuzumab and the antineoplastic cytotoxic drug DM1 (43–45). Patients receiving this treatment could not be included, reducing the active supply of patients eligible for our study. The power initially was set at 90% but was decreased to 80% to guarantee the feasibility of the research.

In the experimental arm, according to Fleming's one-stage design with a one-sided alpha risk of 5% and power of 80%, 45 patients were required in the TG to test the following hypotheses:

- H0 (Null): A cardiotoxicity free rate at 3 months of 75% (uninteresting).
- H1 (Alternative): A cardiotoxicity free rate at 3 months of 90% (warranting further investigation in phase III trial).

These hypotheses were based on cardiotoxicity rates observed in randomized clinical trials, which were approximately 13 to 27%, depending on the molecules used during chemotherapy (10).

The decision rule involves 45 evaluable patients in the TG (a patient was considered evaluable when LVEF data was available at T0 and T3), with a 3-month follow-up from randomization:

- If 38 or fewer patients were free of cardiotoxicity at 3 months (84.4%), the supervised exercise program was declared to be of limited interest,
- If 39 or more patients were free of cardiotoxicity at 3 months (86.7%), the supervised exercise program was declared to warrant further phase III evaluation.

CG group served as calibration to validate the H0 hypothesis.

Statistical analysis

Qualitative variables were described as number and percentage, and quantitative variables as median with interquartile range (IQR) or median with range (Min-Max) for age. Echocardiography variables and cardiorespiratory parameters at maximal exercise were described as mean ± standard deviation (SD) at T0, T3 and T6. Differences over time were described as the difference in means, with 95% confidence interval (CI). Violin plots were used to obtain a longitudinal representation of data during follow up. The Wilcoxon-Mann-Whitney test was used to compare median values of LVEF (according to criterion 1) and cardiorespiratory parameters of patients according to the presence or absence of cardiotoxicity at T0 and T3. Spearman's correlation coefficient was used to assess the association between changes in LVEF, peak VO_2 and maximal power.

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and R software version 4.0.3 (R Development Core Team, Vienna, Austria; http://www.r-project.org).

Results

Population

At enrolment, all patients had completed chemotherapy and radiotherapy. They were treated only with trastuzumab in adjuvant for 12 months with a total of 18 cycles, as already described in detail in Jacquinot et al. (37).

Between April 2015 and February 2020, among of 205 patients screened, 89 were randomized, 46 patients (51.7%) to the TG and 43 patients (48.3%) to the CG (Figure 1). The baseline clinical characteristics of all participants are presented in Table 2. The median age was 51.0 years (IQR: 43.4–55.8) and median body mass index was 25 $kg.m^{-2}$ (IQR: 22.5–28.9). The average score obtained from the QLQ-C30 questionnaire, which evaluates various dimensions of health-related quality of life and global health, was 66.7/100 (IQR: 54.2–75.0).

Among the overall population of 89 patients, 58 (65.2%) were estrogen positive, 64 (71.9%) had a conservative surgery and 78 (88.6%) had received radiotherapy. Chemotherapy regimens were similarly distributed between the two groups. Indeed, 71 patients (79.8%) were treated sequentially with chemotherapy based on anthracyclines and taxanes, 17 patients (19.1%) with anthracyclines without taxanes and one patient (1.1%) without anthracyclines. All patients also received trastuzumab concomitantly to taxanes. Among the 89 patients, 57 (64%) were non-smokers, 45 (51.1%) did not consume alcohol and 66 (74.2%) did not take analgesic medication. Furthermore, 84 patients (94.4%) regularly performed physical activity, 33 of them (39.8%) practiced less than 1 h a week, 39 (47%) at least 1 to 3 h a week and 11 (13.3%) more than 3 h per week.

Cardiotoxicity

For the primary endpoint, 42 (91.3%) patients were evaluable in TG (4 patients excluded) and 33 (76.3%) in CG (10 patients excluded; Figure 1). In TG, although we did not reach the target of 45 evaluable patients randomized, to observe at least 39 patients free of cardiotoxicity, we nevertheless observed 38 patients free of cardiotoxicity at T3, among the 42 evaluable patients randomized [90.5%, 90%CI (79.5–96.7)]. Patients, who presented a cardiotoxicity, had an absolute decrease of LVEF at least 10% but none of them had a LVEF $< 50\%$ (Table 3). The

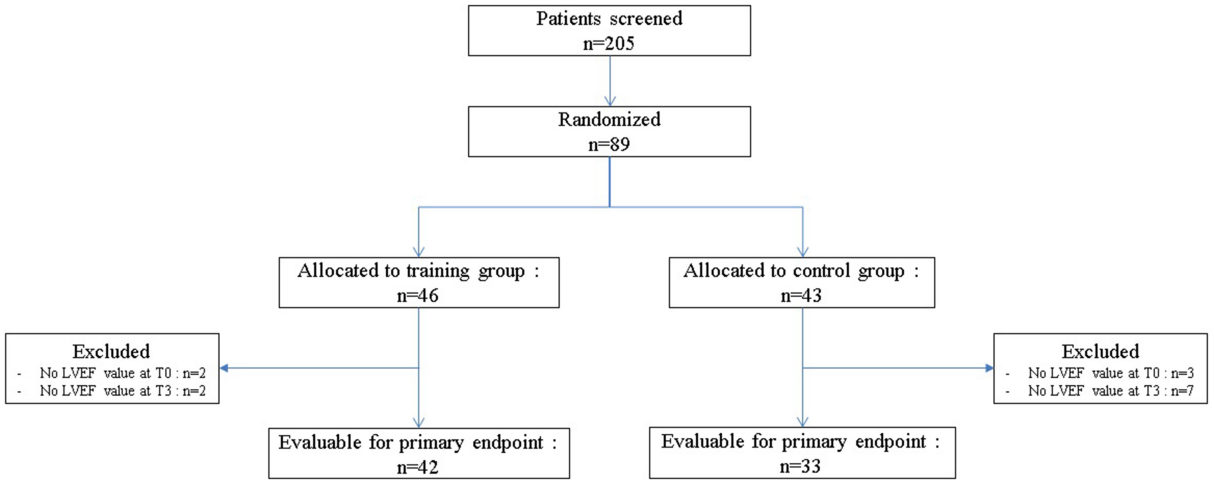

FIGURE 1
The study flow chart.

lower limit of the binomial 90% confidence interval for the rate of patients free of cardiotoxicity at T3 in TG is higher than the H0 hypothesis rate (75%) demonstrating that this estimation is clearly above 75% and that the intervention can therefore be considered to warrant further evaluation.

In the control group, at T3, 27 patients were free of cardiotoxicity among the 33 evaluable patients randomized [81.8%, 90%CI (67.2–91.8)]. Among of 6 patients who presented a cardiotoxicity (18.2%), an absolute decrease of LVEF at least 10% in 4 patients and a LVEF < 50% in 2 patients were observed. The non-comparative context of the study did not provide sufficient statistical power to demonstrate a significant difference in the rate of cardiotoxicity at T3 between the two groups.

Regarding the secondary endpoints, based on criterion 2, 97.6% (n = 41) in TG and 90.9% (n = 30) in CG did not experience cardiotoxicity. Indeed, according to this criterion, one patient from each group presented an absolute decrease of LVEF by at least 15% and 2 patients in CG a LVEF <50%. Furthermore, using criterion 3, 100% (n = 42) of patients in TG and 93.9% (n = 31) in CG were free of cardiotoxicity (Table 3), only 2 patients from CG having presented a LVEF <50%. Whatever the criterion (1, 2 or 3), there were consistently more patients without cardiotoxicity in TG compared to those in CG. According to criterion 4 (absolute drop in GLS of 15% from baseline), 83.3% (n = 20) of patients in TG did not have cardiotoxicity vs. 70 % (n = 14) in CG (Table 3).

Cardiac parameters

At inclusion (T0), median values of LVEF [TG: 61.6 % (6.9); CG: 59.6 % (6.7)] and GLS [TG: −20.8 % (2.6); CG: −19.3 (2.0)] were within normal range, greater than or equal to 50 or −18% respectively (Table 4).

At T3 and T6, values remained stable both in TG or CG, with similar medians (Table 4). The violin plots in Figure 2 show the changes in LVEF between T0, T3, and T6 with heterogeneous individual trajectories in both groups.

Cardiorespiratory variables

The training program was well tolerated overall, and that no adverse effects were observed in the patients in TG.

At T3, peak VO_2 increased significantly in TG (mean difference, 2.6 mL.min^{-1}.kg^{-1}; 95% CI, 1.8 to 3.4) whereas it was unchanged in CG (mean difference, 0.2 mL.min^{-1}.kg^{-1}; 95% CI, −0.5 to 0.9; Table 5). Similarly, maximal power increased in TG (mean difference 21.3 W; 95% CI, 17.3 to 25.3), whereas it did not change in CG (mean difference 0.7 W; 95% CI,−3.6 to 5.1; Table 5). VO_2/HR ratio increased significantly in TG (mean difference 0.7 mL.beats^{-1}; 95% CI, 0.3, 1.1) whereas it remained unchanged in CG (mean difference 0.1 mL.beats^{-1}; 95% CI, −0.2, 0.4). Furthermore, in both groups, HR reached the maximal predicted value and failed to show any difference (TG: mean difference 2.5 beats.min^{-1}; 95% CI, −2.8 to 7.7 and CG: mean difference −1.4 beats.min^{-1}; 95% CI, −5.7 to 3.0).

At T6, peak VO_2, maximal power, and VO_2/HR values decreased in TG but were still higher than those measured at T0. Conversely, in CG, these variables were unchanged in comparison with baseline values.

When considering only TG patients who presented cardiotoxicity at T3 (shown in red in Figure 3), peak VO_2 increased in 2 out of 4 patients, remained stable in one patient

TABLE 2 Patient's characteristics at inclusion.

	Overall population $n = 89$		Training group $n = 46$		Control group $n = 43$	
	n	%	n	%	n	%
STRATIFICATION CRITERIA						
Age (years)						
Median (IQR)	51.0 (43.4–55.8)		51.1 (43.1–55.8)		51.0 (43.4–56.4)	
Median [range]	51.0 [28.7–74.5]		51.1 [28.7–66.8]		51.0 [32.6–74.5]	
Age (years)						
18–30	1	1.1	1	2.2	0	0
30–50	40	44.9	21	45.7	19	44.2
50–85	48	53.9	24	52.2	24	55.8
Global health score - QLQ-C30						
Median [range]	66.7 [25.0–100.0]		66.7 [25.0–100.0]		66.7 [41.7–100.0]	
Missing	9		4		5	
Global health score - QLQ-C30						
0–30	2	2.5	2	4.8	0	0
31–50	18	22.5	10	23.8	8	21.1
51–70	31	38.8	16	38.1	15	39.5
71–100	29	36.2	14	33.3	15	39.5
Missing	9	-	4	-	5	-
DISEASE AND TREATMENT CHARACTERISTICS						
Scarff-Bloom-Richardson grade						
I	4	4.6	3	6.7	1	2.4
II	32	36.8	17	37.8	15	35.7
III	51	58.6	25	55.6	26	61.9
Missing	2	-	1	-	1	-
ER						
+	58	65.2	33	71.7	25	58.1
–	31	34.8	13	28.3	18	41.9
PR						
+	41	46.1	24	52.2	17	39.5
–	48	53.9	22	47.8	26	60.5
HER2						
+	89	100	46	100	43	100
Type of surgery						
Mastectomy	25	28.1	13	28.3	12	27.9
Conservative surgery	64	71.9	33	71.7	31	72.1
Chemotherapy cycles						
Anthracyclines + taxanes	71	79.8	36	78.3	35	81.4
Anthracyclines without taxanes	1	1.1	0	-	1	2.3
Without anthracyclines	17	19.1	10	21.7	7	16.3
Radiotherapy						
Yes	78	88.6	39	86.7	39	90.7
No	10	11.4	6	13.3	4	9.3
Missing	1	-	1	-	0	-
Trastuzumab cycles						
18	89	100	46	100	43	100
LIFE HABITS						
Tobacco consumption						
Smoker	16	18.0	11	23.9	5	11.6
Non smoker	57	64.0	33	71.7	24	55.8
Ex-smoker	16	18.0	2	4.4	14	32.6
Alcohol consumption						
Yes	43	48.9	22	48.9	21	48.9
No	45	51.1	23	51.1	22	51.1
Missing	1	-	1	-	0	-

(Continued)

TABLE 2 (Continued)

	Overall population $n = 89$		Training group $n = 46$		Control group $n = 43$	
	n	%	n	%	n	%
Analgesic medication						
Yes	23	25.8	13	28.3	10	23.3
No	66	74.2	33	71.7	33	76.7
Physical activity						
Yes	84	94.4	43	93.5	41	95.4
No	5	5.6	3	6.5	2	4.6
Physical activity level						
Less than 1h/week/year	33	39.8	12	28.6	21	51.2
1h to 3h/week/year	39	47.0	21	50.0	18	43.9
More than 3h/week/year	11	13.3	9	21.4	2	4.9
Missing	1	-	1	-	0	-
ANTHROPOMETRIC VARIABLES						
Height (cm)						
Median (IQR)	163 (159–169)		163.0 (159.0–166.0)		166.0 (160.0–173.0)	
Missing	1		1		0	
Body mass (kg)						
Median (IQR)	67.5 (60.0–78.0)		65.2 (60.0–71.0)		72.2 (60.0–84.3)	
Body Mass Index (kg.m^{-2})						
Median (IQR)	25.0 (22.5–28.9)		24.7 (22.3–28.4)		26.0 (22.7–30.1)	
Missing	1		1		0	

ER, Estrogen Receptor; HER2, human epidermal growth factor receptor 2; PR, Progesterone Receptor; QLQ-C30, Quality of Life Questionnaire – Core 30.

TABLE 3 Study of cardiotoxicity at T3 according to the different criteria, in patients of both groups (TG and CG).

	Overall population $n = 75$		Training group $n = 42$		Control group $n = 33$	
	n	%(90%CI)	n	%(90%CI)	n	%(90%CI)
Cardiotoxicity (Criterion 1)						
Yes	10	13.3 (7.4–21.6)	4	9.5 (3.3–20.5)	6	18.2 (8.2–32.8)
No	65	86.7 (78.4–92.6)	38	90.5 (79.5–96.7)	27	81.8 (67.2–91.8)
If yes, reason ?						
Absolute decrease of at least 10%	8	80.0	4	100	4	66.7
LVEF at T3 < 50%	2	20.0	0	0.0	2	33.3
Cardiotoxicity (Criterion 2)						
Yes	4	5.3 (1.8–11.8)	1	2.4 (0.0–10.8)	3	9.1 (2.5–21.8)
No	71	94.7 (88.2–98.2)	41	97.6 (89.2–100)	30	90.9 (78.2–97.5)
If yes, reason?						
Absolute decrease of at least 15%	2	50.0	1	100	1	33.3
LVEF at T3 < 50%	2	50.0	0	0.0	2	66.7
Cardiotoxicity (Criterion 3)						
Yes	2	2.7 (0.5–8.2)	0	0.0 (0.0–7.0)	2	6.1 (1.1–17.9)
No	73	97.3 (91.8–99.5)	42	100 (93.0–100)	31	93.9 (82.1–98.9)
Cardiotoxicity (Criterion 4)						
Yes	10	22.7	4	16.7	6	30.0
No	34	77.3	20	83.3	14	70.0
Missing	31	-	18	-	13	-

LVEF, left ventricular ejection fraction.

TABLE 4 Evaluation of cardiac parameters.

	Training group ($n = 42$)								Control group ($n = 33$)							
	T0		T3		T6		T3-T0	T6-T3	T0		T3		T6		T3-T0	T6-T3
	n	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n* Mean (95% CI)	*n* Mean (95% CI)	*n*	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)	*n* Mean (95% CI)	*n* Mean (95% CI)
LVEF (%)	42	61.6 (6.9)	42	61.7 (6.5)	30	61.5 (6.3)	42 0.1 (−2.6, 2.8)	30 −0.9 (−4.3, 2.6)	33	59.6 (6.7)	33	59.0 (5.5)	31	61.2 (6.9)	33 −0.7 (−3.0, 1.6)	31 2.1 (−0.3, 4.5)
GLS (%)	24	−20.8 (2.6)	29	−20.1 (1.8)	25	−20.2 (1.9)	24 0.5 (−0.8, 1.8)	20 0.1 (−0.9, 0.8)	21	−19.3 (2.0)	21	−21.3 (2.9)	26	−19.8 (2.6)	20 −1.6 (−2.7, −0.4)	20 0.7 (−0.8, 2.2)

LVEF, left ventricular ejection fraction; GLS, left ventricular global longitudinal strain.

and declined slightly in one patient (Figure 3A). As for the CG, peak VO_2 decreased in 3 out of 5 patients and increased in 2 patients (Figure 3B). Unlike LVEF, homogeneous individual trajectories were observed (Figure 3). Furthermore, the same pattern of individual trajectories was observed for the maximal power (Figure 4).

No correlation between LVEF changes and either peak VO_2 or maximal power was observed ($R = 0.035$; $p = 0.78$; and $R = 0.004$; $p = 0.97$ respectively).

TOX and NoTOX groups

As shown in Table 6, LVEF was significantly higher at T0 in the TOX group [68.0 % (67.0–75.0)] compared to the NoTOX group [60.0% (56.0–63.0); $p < 0.01$]. At T3, it was significantly lower in TOX group [TOX: 56.0 (54.0–62.0)] than in NoTOX group [60.0 (57.0–64.0); ($p < 0.04$)] with a significant relative difference (TOX: −12.0 (−14.0, −11.0) vs NoTOX: 1.0 (−3.0, 5.0); $p < 0.01$).

In contrast, at T0 and T3, no difference was observed between groups with regard to peak VO_2 and maximal power, although values were numerically slightly lower in the TOX group than in the NoTOX group (Table 6).

Discussion

Although trastuzumab improves outcomes in patients with HER2-positive breast cancer (BC) (46), trastuzumab-induced cardiotoxicity (TIC) compromises the health-related quality of life and overall survival of cancer patients (47, 48). A progressive LVEF decline and potentially overt heart failure may occur. However, recent findings demonstrated that this form of cardiomyopathy is mostly reversible with early detection and prompt introduction of an appropriate therapeutic strategy (49, 50).

To our knowledge, few studies have investigated the effects of an exercise training program on TIC in BC patients treated by trastuzumab. The CARDAPAC study is one of the first randomized, clinical studies to offer a supervised exercise training with HER2-positive BC patients under adjuvant trastuzumab, to prevent TIC.

The major findings are that an exercise program, combining high and moderate intensities, minimized TIC. Indeed, the rate of patients free of cardiotoxicity observed after a supervised exercise training of 3 months (T3) was 90.5% [90%CI (79.5–96.7)] vs. 81.8% [90%CI (67.2–91.8)] in TG and CG patients respectively. The lower limit of the binomial 90% confidence interval for the rate of patients free of cardiotoxicity at T3 in the TG was higher than the H0 hypothesis rate (75%), demonstrating that this estimation is above 75% and therefore, that the results must be considered worthy of further evaluation.

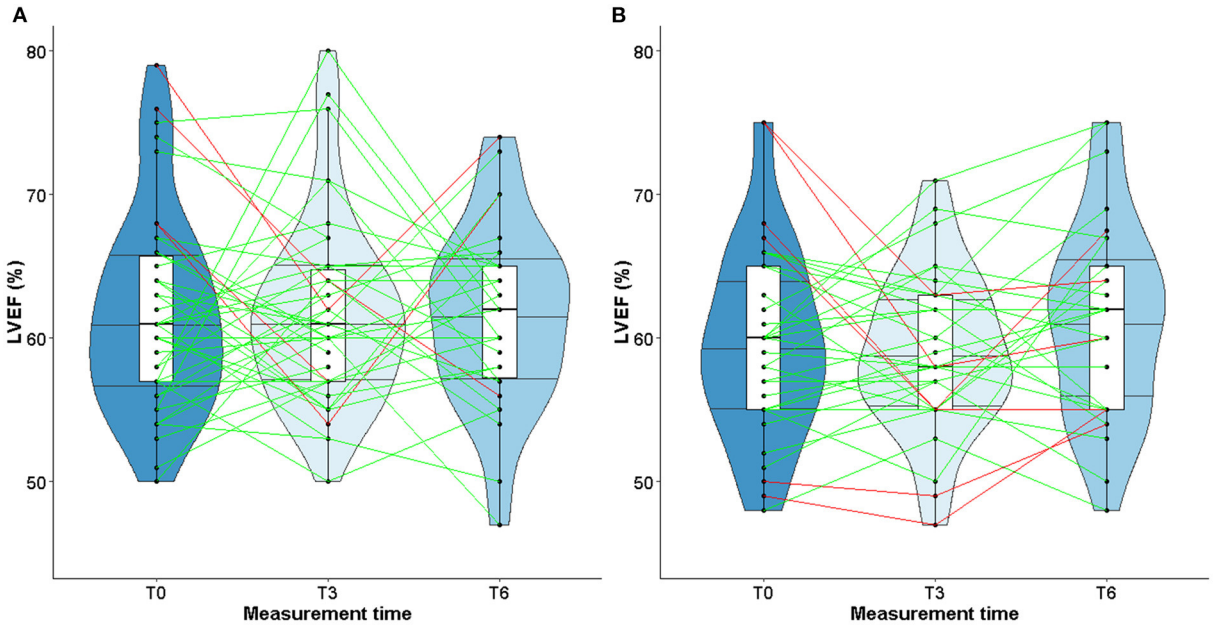

FIGURE 2
Violin plot for evolution of LVEF in TG **(A)** and CG **(B)**.

Regardless of the criterion used to defined cardiotoxicity (1, 2, 3 or 4), the number of patients without cardiotoxicity was higher in the TG than in the CG, although the non-comparative nature of the study did not allow sufficient statistical power to demonstrate a significant difference in the rate of cardiotoxicity at T3 between the two groups.

In the literature, Foulkes et al. did not identify cardiotoxicity in trained patients during anthracycline chemotherapy, whereas 25% of non-trained patients were independently diagnosed with cardiotoxicity (51). Their study took place during an anthracycline-based chemotherapy regimen, and only 7 out of 17 patients received treatment with trastuzumab whereas in our study, all patients were treated by adjuvant trastuzumab only at enrolment. In our study, the rate of cardiotoxicity as defined by criterion 1, reached 13.3% in the whole population (9.5% in TG and 18.2% CG). This percentage is consistent with the results of Naumann et al. (52) and Lemieux et al. (53) who observed cardiotoxicity rates of respectively 15.7 and 13.5% in women treated with trastuzumab for primary BC. Those percentages were higher than those reported by Smith et al. (4) and Pivot et al. (11) in BC patients having received also only trastuzumab (5.7 and 3.4% respectively).

These discrepancies might be due either to the lack of consensus regarding the definition of cardiotoxicity (54) or to patient eligibility criteria, with some studies excluding patients with LVEF <55%, patients aged over 65 years, patients with cardiac risk factors, or those who had undergone mediastinal radiotherapy or hormone therapy. Thus, the question of whether cardiotoxicity is underestimated or not likely depends on the criterion chosen. It should be noted that in our study, the average decline in LVEF was 12 points (with criterion 1). Therefore, an absolute decrease of 10% in LVEF from baseline makes it possible to identify patients with an early decline in LVEF compared to other criteria. Although the evaluation of LVEF remains the reference for the measurement of cardiac dysfunction, the European Society of Cardiology (42) has recommended, since 2016, the assessment of GLS to identify subclinical lesions (55). Nevertheless, to date, as for LVEF, a consensual definition of GLS is still lacking, making it difficult to compare results between studies.

Currently, there is a paucity of data regarding the effects of exercise on the incidence of cardiotoxicity. Recent research reported the possible cardioprotective effects of exercise and its potential role in maintaining LVEF and GLS in BC patients undergoing chemotherapy or anti-HER2 antibodies (26, 51, 56). In our study, at inclusion, mean LVEF was 61.6% (SD: 6.9) in the TG and 59.6% (SD: 6.7) in the CG patients. These latter had completed surgery, chemotherapy and radiotherapy and were approximately 6 months from the beginning of treatment with trastuzumab. Similar data were found in the PHARE study (57) in which baseline LVEF was 66%. However, in that study, LVEF gradually decreased by 3.6% to reach a nadir at 12 months, in the absence of a physical exercise program (57). Thus, we can confirm that physical exercise, such as the program proposed in our study, may have tempered the decline in LVEF at T6. Besides, as suggested by Fei et al. (58), the maintenance of LVEF

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TABLE 5 Cardiorespiratory variables at maximal exercise.

	Training group (n = 42)								Control group (n = 33)							
	T0		T3		T6		T3-T0	T6-T3	T0		T3		T6		T3-T0	T6-T3
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n Mean (95% CI)	n Mean (95% CI)	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n Mean (95% CI)	n Mean (95% CI)
Peak VO_2 ($mL.min^{-1}.kg^{-1}$)	41	25.7 (6.1)	35	28.4 (6.1)	27	26.4 (6.2)	35 2.6 (1.8, 3.4)	26 −1.0 (−1.8, −0.2)	33	24.3 (8.3)	28	24.2 (6.6)	26	23.8 (6.9)	28 0.2 (−0.5, 0.9)	24 0.4 (−0.5, 1.2)
Maximal power (watts)	41	119.2 (23.7)	34	138.7 (27.7)	26	134.7 (32.3)	34 21.3 (17.3, 25.3)	24 −5.0 (−9.3, −0.8)	32	118.4 (25.5)	29	120.1 (30.2)	26	121.8 (27.2)	29 0.7 (−3.6, 5.1)	24 4.1 (−0.2, 8.5)
HR ($beats.min^{-1}$)	40	169.1 (17.8)	35	170.5 (21.5)	27	170.4 (17.5)	34 2.5 (−2.8, 7.7)	26 1.8 (−4.5, 8.1)	33	164.0 (15.0)	29	163.7 (13.6)	26	167.9 (13.8)	29 −1.4 (−5.7, 3.0)	24 5.7 (0.2, 11.2)
VO_2/HR ($mL.beats^{-1}$)	40	10.1 (1.7)	35	10.8 (1.6)	27	10.5 (1.7)	34 0.7 (0.3, 1.1)	26 −0.4 (−0.7, −0.0)	33	10.3 (2.4)	28	10.5 (2.4)	26	10.1 (2.4)	28 0.1 (−0.2, 0.4)	24 −0.1 (−0.5, 0.2)

Peak VO_2, Peak oxygen uptake; HR, Heart Rate; VO_2/HR, Oxygen pulse.

during follow-up might result either from the young age of our patients, who did not present heart failure and in whom cardiovascular risk was low because patients had completed their treatment with taxanes and/or anthracyclines, known to worsen cardiotoxicity. Here, the drop in LVEF was more important in patients who presented cardiotoxicity at T3 (−12 points [IQR: −14.0, −11.0]) than in patients without cardiotoxicity (+1.0 point [IQR: −3.0, 5.0]). These observations have previously been reported by Sendur et al. who found significant LVEF loss and higher cardiac biomarkers in patients having developed TIC (59).

Recently, a non-randomized study, conducted in women with early-stage BC who received usual care with or without physical training, showed a significant reduction in LVEF, but no difference after training in peak VO_2 (26). Conversely, a randomized controlled study by Hornsby et al. reported that supervised aerobic training did not change LVEF, cardiac output, stroke volume, or diastolic and systolic volumes, but increased peak VO_2, maximal power and pulse oxygen (60). Our results agree with this latter study since we found higher peak VO_2 and maximal power after training without modification of LVEF and GLS.

To the best of our knowledge, available data exclusively in women with HER2 positive BC are scarce. Haykowsky et al. reported left ventricular dilation and a reduction in LVEF in HER2 positive BC patients treated by adjuvant trastuzumab, despite aerobic exercise training during the first 4 months of trastuzumab therapy (61). Furthermore, they did not observe any improvement in maximal power, peak VO_2, heart rate, or perceived effort (61). Conversely in our study, the supervised exercise program was intermittent, with personalized target intensities (vs. continuous between 60–90% peak VO_2) and the duration of sessions was longer in the study by Haykowsky et al. (55 vs. 30–60 min). Moreover, our program began at a distance from chemotherapy, while theirs was concomitant with chemo-radiotherapy.

More recently, Hojan et al. (33) did not observe any significant changes in LVEF or 6-minute walk distance after a 9-week exercise program. Although the intervention period took place at the same time as in our study (3 to 6 months after the beginning of trastuzumab), the training program was more substantial than ours, because it included supervised aerobic and weight training activities (90 min 5-day/week). However, our intervention was longer (12 vs. 9 weeks) and personalized with intensities determined by an exercise test (vs 80% according to the HRmax = 220–age), making it possible to improve cardiorespiratory capacities (such as peak VO_2) and maintain cardiac parameters (LVEF and GLS).

Studies about left ventricular remodeling, cardiac events during trastuzumab therapy and clinical implications of fluctuations in LVEF are limited (11, 37). In patients with cardiac dysfunction, a slight decrease of LVEF may impact the physical ability to perform prolonged physical exercise,

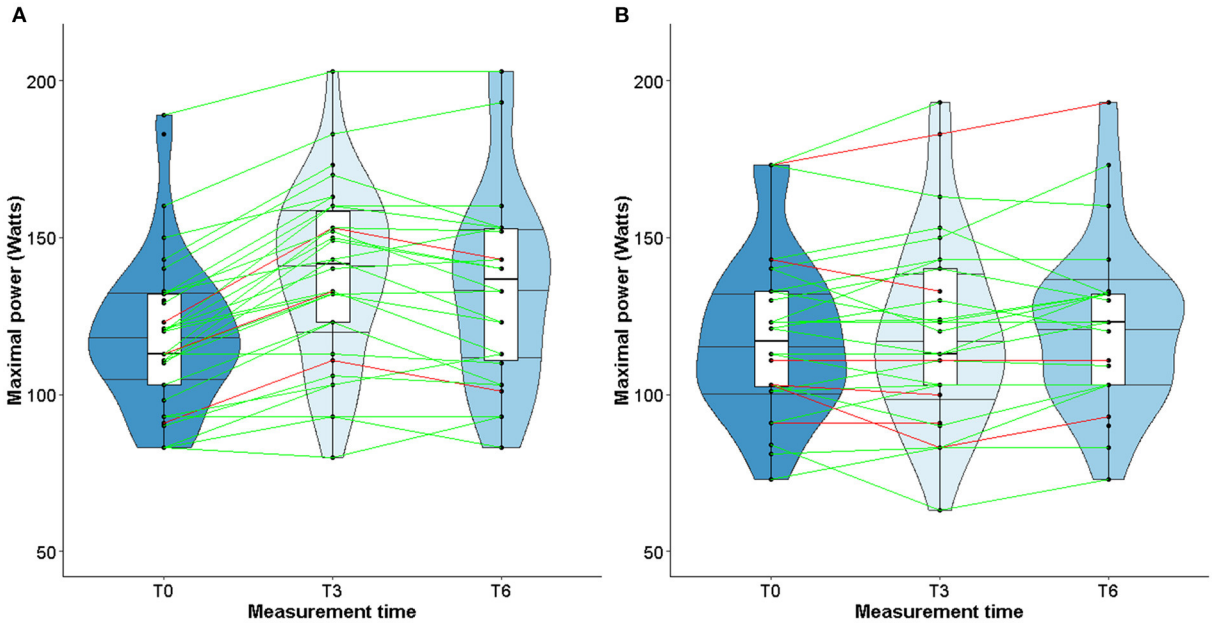

FIGURE 3
Violin plot for evolution peak VO_2 in TG **(A)** and CG **(B)**.

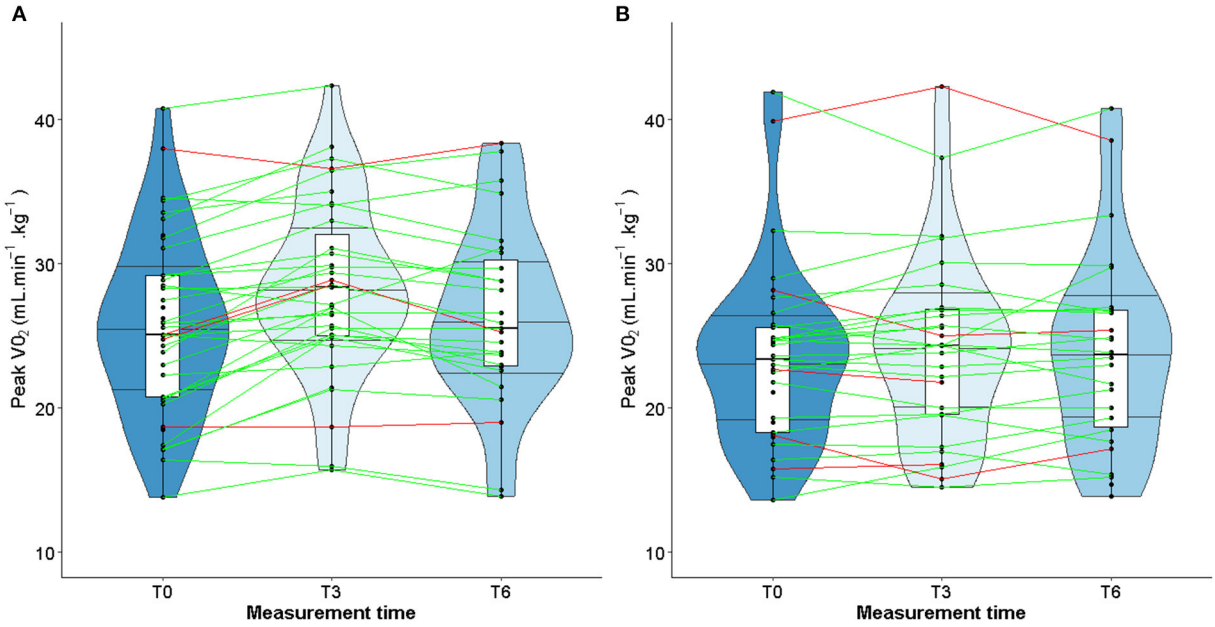

FIGURE 4
Violin plot for evolution of maximal power in TG **(A)** and CG **(B)**.

therefore impairing quality of life. More recently, studies conducted in patients with solid malignancies have shown that cardiorespiratory fitness, in particular peak VO_2, is a predictor of anthracycline- and trastuzumab-induced left ventricular dysfunction (62–64). A slight decrease of LVEF following exposure to trastuzumab might alter the cardiorespiratory fitness of patients when intense physical activity is required. However, our study did find any correlation between changes

TABLE 6 Subgroups analysis in patients who presented cardiotoxicity (TOX group) or not (NoTOX group).

	TOX group $n = 10$			NoTOX group $n = 65$			p		
	T0	T3	T3-T0	T0	T3	T3-T0	T0	T3	T3-T0
LVEF (%)									
Median (IQR)	68.0 (67.0–75.0)	56.0 (54.0–62.0)	−12.0 (−14.0,−11.0)	60.0 (56.0–63.0)	60.0 (57.0–64.0)	1.0 (−3.0, 5.0)	<0.01	0.04	<0.01
Peak VO_2 ($mL.min^{-1} \cdot kg^{-1}$)									
Median (IQR)	23.8 (18.7–28.2)	25.0 (18.7–28.9)	0.0 (−1.4,−2.4)	24.7 (20.6–28.0)	26.5 (22.9–30.1)	1.3 (0.1, 3.8)	0.84	0.64	0.08
Missing	0	1	1	1	11	11			
Maximal power (watts)									
Median (IQR)	112.0 (103.0–143.0)	111.0 (100.0–133.0)	0.0 (−3.0, 20.0)	120.0 (103.0–132.0)	132.0 (111.0–153.0)	12.0 (2.0–21.0)	0.95	0.34	0.17
Missing	0	1	1	2	11	11			

LVEF, left ventricular ejection fraction; Peak VO_2, Peak oxygen uptake.

in LVEF and peak VO_2 or maximal power. These observations could be explained by the fact that the median values of LVEF remained stable over time, despite heterogeneous individual trajectories (see Figures 3, 4). Furthermore, while peak VO_2 and maximal power were improved and followed the same trajectories in all the patients who participated in the exercise training, we did not observe variations in LVEF.

Moreover, peak VO_2 and maximal power were comparable in patients with and in those without cardiac toxicity (TOX; $n = 10$, NoTOX; $n = 65$) while relative LVEF was significantly different at T3 between groups. We suggest that compensatory mechanisms, including preservation of absolute stroke volume, chronotropic competence, increased oxygen extraction or augmented pulmonary lymphatic flow (65) may have preserved exercise tolerance despite left ventricular dysfunction or remodeling. These mechanisms could partly explain the absence of difference in peak VO_2 and maximal power between TOX and NoTOX groups and why LVEF changes did not influence cardiorespiratory fitness.

This study has some limitations that deserve to be underlined. First, diabetic patients or patients with an asymptomatic coronary pathology have not been identified in the study population, while these pathologies represent cardiotoxicity risk factors, like the side of radiotherapy that can affect the risk of cardiotoxicity development. In addition, it would have been interesting to study the markers in the measurement of cardiotoxicity such as those used in the ONCORE study (66). The use of a multimodal strategy that integrates several biomarkers with cardiac imaging could bring more information to detect early subclinical cardiotoxicity. Moreover, the number of subjects to meet the primary endpoint was not reached, and the overall population was likely too small to highlight significant effects in subgroup analyses. In addition, it would have been interesting to monitor the level of physical activity by actimetry during the first period (T0 to T3) in the CG and during the second period (T3 to T6) in both groups, to verify whether the total level of physical activity would have an effect on cardiotoxicity and physiological responses to exercise.

To conclude, a 12-week supervised exercise regimen was safe and improved the cardiopulmonary fitness in HER2-positive breast cancer patients treated with adjuvant trastuzumab therapy. The study is under powered to come to any conclusion regarding the effect on cardiotoxicity. Nevertheless, the lower limit of the binomial confidence interval in TG was higher than the null hypothesis rate (75%) confirming that this estimation was clearly higher than 75%. However, the results deserve further evaluations considering the use more important of targeted therapies, such as with trastuzumab emtansine in HER2-positive patients. Moreover, this study showed that exercise training enabled cardiopulmonary fitness, notably peak VO_2, to be rapidly improved. However, the changes in cardiorespiratory capacities were not correlated with changes in LVEF, particularly in patients with cardiotoxicity. This indicates that resting measurements of cardiac parameters (LVEF and GLS) are not sensitive to change and are not correlated with functional disabilities induced by chemotherapy treatments. Finally, exercise training was well tolerated without side-effects. It may therefore provide additional benefits on top of the usual cancer treatment and prevent exacerbation of cardiac toxicities that occur as a result of trastuzumab treatment.

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 Ethics Committee (Comité de Protection des Personnes Est-II), Besançon, France under the number P/2014/241 and by the National Health Products Safety Agency (N° ID RCB 2014-A01911-46). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: QJ, NM, DV, and FM. Administrative support: QJ, NM, MB, PR, BD, DV, XP, and FM. Inclusion of patients: NM, EC, LM, M-JP, FB, LC, ED, GM, and XP. Collection and assembly of data: QJ, NM, PR, BD, MC, and FM. Data analysis and interpretation: QJ, NM, AF, DV, and FM. Manuscript writing: QJ, AF, DV, and FM. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Pharmacogenomics in drug-induced cardiotoxicity: Current status and the future

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Drug-induced cardiotoxicity (DICT) is an important concern of drug safety in both drug development and clinical application. The clinical manifestations of DICT include cardiomyopathy, arrhythmia, myocardial ischemia, heart failure, and a series of cardiac structural and functional changes. The occurrence of DICT has negative impacts on the life quality of the patients, brings additional social and economic burden. It is important to identify the potential factors and explore the mechanisms of DICT. Traditional cardiovascular risk factors can only partially explain the risk of DICT. Pharmacogenomic studies show accumulated evidence of genetics in DICT and suggest the potential to guide precision therapy to reduce risk of cardiotoxicity. The comprehensive application of technologies such as third-generation sequencing, human induced pluripotent stem (iPS) cells and genome editing has promoted the in-depth understanding of the functional role of susceptible genes in DICT. This paper reviewed drugs that cause DICT, the clinical manifestations and laboratory tests, as well as the related content of genetic variations associated with the risk of DICT, and further discussed the implication of new technologies in pharmacogenomics of DICT.

KEYWORDS

drug-induced cardiotoxicity, pharmacogenomics, single nucleotide polymorphisms (SNPs), biomarker, new technologies in pharmacogenomics

Introduction

Drug induced cardiotoxicity (DICT) is a serious adverse drug reaction, which interferes with the normal physiological function of the cardiovascular system. The clinical manifestations of DICT are diverse and mainly include cardiomyopathy, arrhythmia, valve injury, myocarditis, pericarditis, cardiac insufficiency, and myocardial ischemia (1). Drug-induced prolongation of the QT interval can even lead to fatal severe ventricular tachycardia and sudden death (2). Cardiotoxicity has become an important issue in drug development and public health. Although the safety evaluations of all listed drugs have been obtained in clinical trials,

cardiotoxicity is still very common in clinical practice. Some cardiac injuries, such as acquired long QT syndrome (aLQTS), are the causes of relabeling and drug withdrawal. In recent years, cardiovascular complications of cancer therapeutics have even given rise to the new and unique interdisciplinary field of cardio-oncology (2).

The cardiotoxicity of drug is multifactorial. Traditional cardiovascular risk factors, such as gender, age, renal failure, iron overload, drug-drug interactions, and pre-existing cardiovascular diseases, can not fully explain the occurrence of DICT (3). Routine clinical monitoring indicators usually lack the specificity for the diagnosis of DICT. More and more evidence supports the importance of genetic components, which make some individuals more susceptible to DICT. Many risk genes have been identified, and some have been translated into clinic to optimize drug regimens (4). The integration of new technologies in life science and pharmacogenomics, a field focuses on exploring the genetic basis of interindividual difference in drug responses, has paved the way for the discovery and functional analysis of genetic biomarkers associated with risk of DICT.

Drugs with cardiotoxicity and the clinical evaluation

Cardiotoxic drugs and manifestations

Cardiotoxicity occurs in therapies with many of the drugs including the antineoplastic drugs (such as cancer chemotherapeutics, targeted therapies, cancer immunotherapies), anti-infective drugs, antiarrhythmics, and other non-cardiac drugs (such as antihistamines, bronchodilating, the lipid regulating agent, etc.) (Table 1). The manifestations are varied, which include arrhythmia (sinus bradycardia, atrial fibrillation, atrial flutter, ventricular arrhythmia, QT interval prolongation, even torsades de pointes ventricular tachycardia), cardiomyopathy, myocarditis, myocardial ischemia/myocardial infarction, heart dysfunction/heart failure, cardiogenic shock, and even sudden death (Table 1).

Indicators for clinical evaluation of cardiac toxicity

DICT is usually comprehensively evaluated by medication history, clinical manifestations, electrocardiogram (ECG), cardiac imaging, laboratory tests for cardiac biomarkers, and pathological examination with endomyocardial biopsy. Consensus definition of cardiac toxicities of cancer therapies has recently been coined by International Cardio-Oncology Society (IC-OS) (1). The cardiotoxicity of most drugs is cumulative, especially in high doses. Of course, cumulative use of low doses can also cause abnormal cardiac function on some occasions. For example, anthracyclines can cause cardiotoxicity at low-dose. During long-term follow-up, cardiac dysfunction was observed in patients received low-dose adriamycin, indicating "no safe" dose for anthracyclines (5, 6). Medication history with potential cardiotoxic drugs is an essential prerequisite for the diagnosis. Symptoms such as chest tightness, palpitation, exertional dyspnea, and in severe cases, upright breathing and syncope may occur (7). Physical examination may show signs of cardiac enlargement, tachycardia, galloping rhythm of the third heart sound, cardiac murmur, etc. ECG and echocardiography are routine non-invasive examinations for clinical monitoring of cardiac structural and/or function changes. The recovery of ECG, especially for QT intervals after drug withdrawal, is helpful for the diagnosis. Left ventricular ejection fraction (LVEF) is a commonly used cardiotoxicity monitoring index, but is insensitive and lacks specificity for early changes in systolic function. Noteworthy, a decrease in LVEF usually indicates more severe myocardial injury.

Serum cardiac biomarkers are also used in the diagnosis of DICT. For example, high sensitive troponin I (HS TnI) can be used to predict early cardiac injury. Troponin is a sensitive marker for detecting anthracycline-induced myocardial damage. Troponin I may be elevated in patients with myositis (8). The increase of Troponin above the 99th percentile limit supports the diagnosis of cardiac injury. B-type natriuretic peptide (BNP) and N-terminal pro-B-type natriuretic peptide (NT proBNP) are also commonly used to establish the diagnosis of heart failure (8). It is reported that serum NT proBNP correlated positively with cardiotoxicity, but the pre-treatment levels should be compared to confirm a drug-specific effect.

Endomyocardial biopsies provides direct histological evidence of cardiac injury and is the most sensitive to cardiotoxicity. However, due to its high risk and great trauma, it cannot be used as a routine examination for DICT. The pathological manifestations of DICT are complex, which requires comprehensive evaluation according to the drug used and clinical manifestations. It is worth noting that though many of the above mentioned clinical indicators are used, most of them lack specificity for drug toxicity.

Pharmacogenomics in DICT

There are significant individual differences in susceptibility to DICT. Genetics can partly account for this difference. Pharmacogenetic studies have shown that genetic variations represented by single nucleotide polymorphisms (SNPs) in drug disposition and response related genes can modify the risk of DICT through both pharmacokinetics (PK) and pharmacodynamics mechanisms [(9, 10, 15), Figure 1, Supplementary Table 1]. In recent decades, genetic

TABLE 1 Drugs that induced cardiotoxicity and the manifestations.

Types	Common drugs	Cardiotoxic manifestations
Antineoplastic drugs (1, 5)	Anthracyclines: adriamycin, aunorubicin, epirubicin	Arrhythmia, cardiomyopathy, heart failure
	Alkylating agent: cyclophosphamide	Hemorrhagic necrotizing pericardial myocarditis, heart failure, arrhythmia
	Anti microtubule: paclitaxel	Myocardial ischemia, sinus bradycardia, heart failure
	Antimetabolic drugs: 5-fluorouracil and capecitabine	Coronary spasm, heart failure
	Monoclonal antibody drug: trastuzumab, bevacizumab	Heart failure
	Small molecule protein kinase inhibitors: imatinib, sunitinib	Atrial fibrillation, heart failure
	Proteasome inhibitor: kafezomib	Heart failure
	Immunosuppressants: PD-1/PD-1L inhibitors	Myocarditis
Anti-infection drugs (6–8)	Macrolides: clarithromycin, azithromycin	Torsade de pointe, QT interval prolongation
	β Lactams: penicillin	Arrhythmia, myocarditis, heart failure
	Lincomrades: lincomycin and clindamycin	QT interval prolongation, ventricular tachycardia
	Quinolone: ciprofloxacin, levofloxacin, moxifloxacin, etc.	QT interval prolongation, ventricular tachycardia, occasionally develops to severe arrhythmias such as torsade de pointe
	Antifungal: imidazole antifungal agents (Itraconazole)	QT interval prolongation, ventricular tachycardia
	Antiparasitic: chloroquine	Heart block, congestive heart failure, cardiomyopathy
	Antiviral: α-Interferon (IFN-α)	Myocarditis, atrioventricular block, bradycardia
Antiarrhythmic drugs (9, 10)	Amiodarone	QT interval prolongation
	Digitalis	Atrioventricular block, ventricular arrhythmia
Antihistamines (9, 10)	Benamin, cetirizine, loratadine, desloratadine, levocetirizine,	Q-T interval prolongation, palpitations, arrhythmias, sinus bradycardia, supraventricular tachycardia, ventricular tachycardia, torsade de pointe, atrial fibrillation, etc.
Lipid lowering drugs (11)	Probucol	QT interval prolongation, ventricular tachycardia
Psychotropic drugs (12)	Thiazide antipsychotics, tricyclic antidepressants	Arrhythmia
Gastrointestinal motility promoting drugs (13)	Domperidone, cisapride	Q-T interval prolongation and arrhythmia
Bronchodilators (14)	Salbutamol	Arrhythmia (atrial fibrillation, sinus tachycardia, etc.)

polymorphisms of genes encoding drug transporters, drug metabolizing enzymes and drug targets have been extensively studies. Better understanding of the pharmacogenomics of DICT will help optimize the current treatment selection and dosing regimens, and minimize risk of DICT as well.

Pharmacokinetics (PK) gene polymorphisms

ATP-binding cassette (ABC) family transporters

The ABC family drug transporters play key roles in the transmembrane efflux of many cardiotoxic drugs, such as anthracyclines. For anthracyclines, association of genetic polymorphisms in ABC family members and risk of drug resistance or cardiovascular toxicity have been widely studied (16). Variations in family members including *ABCC1* (rs246221, rs4148350, rs45511401), *ABCC2* (rs8187710, rs8187694, rs3740066), *ABCB4* (rs1149222 and rs4148808), and *ABCC5* rs7627754 are associated with increased risk of persistent anthracycline cardiotoxicity in adults and children suffered from hematology and malignant tumors (17–24). SNP variants in these ABC genes that reduce or interfere with expression will lead to the accumulation of detrimental metabolites of anthracyclines in cardiomyocytes, thereby increasing the risk of DICT. ABCB1 (also known as P-glycoprotein) is a component of the heart endothelial blood barrier. Sissung et al. found that the *ABCB1* SNPs 1236C>T (rs1128503), 2677G>T/A (rs2032582), and 3435C>T (rs1045642) that alter protein folding can reduce intracardiac concentration of the ABCB1 model substrate romidepsin and drug-induced QT interval prolongation as well, which indicates cardioprotective of these SNPs toward ABCB1 substrates (25).

Transporters of the soluble carrier family (SLCs)

SLCs are the second largest membrane proteins family in human and play important roles in the absorption, distribution

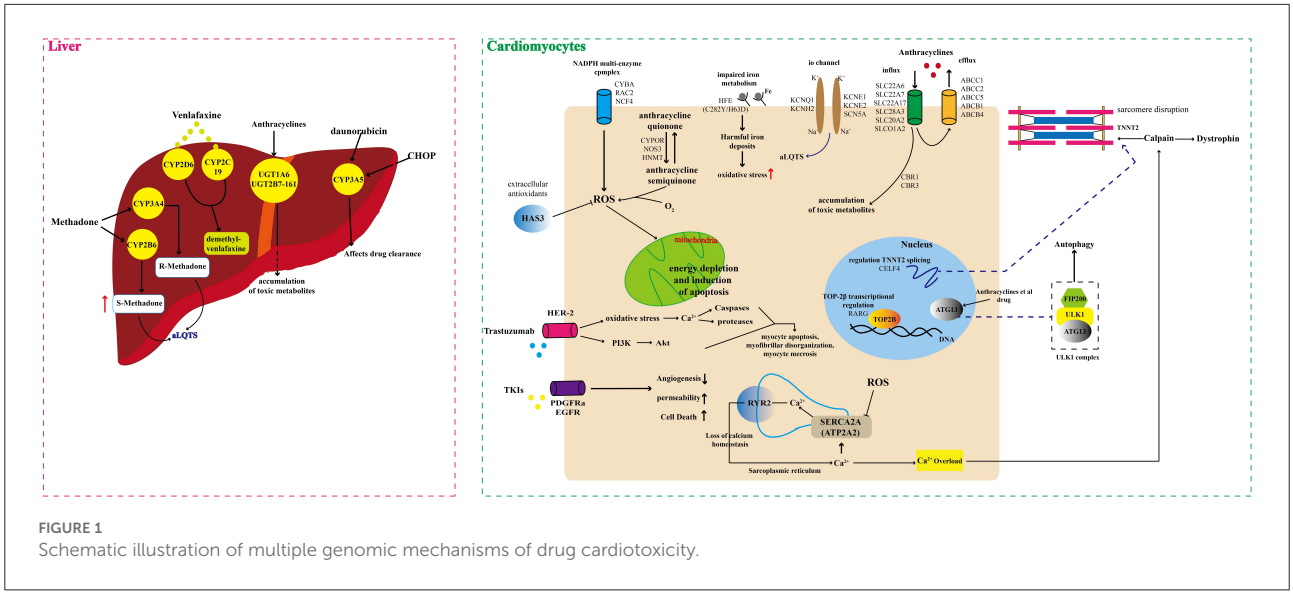

FIGURE 1
Schematic illustration of multiple genomic mechanisms of drug cardiotoxicity.

and excretion of drugs. Functional genetic variations in SLCs genes are also identified. In a study with 5–10-year follow-up in patients taking anthracyclines, the *SLC22A6* rs6591722 AA genotype showed increased risk of decreased left ventricular cardiac function (21). *SLC28A3* rs7853758 and rs4877847, *SLC10A2* rs9514091, *SLC22A7* rs4149178, *SLCO1A2* rs2857468, and *SLC22A17* rs4982753 were reported to be protective for anthracycline induced cardiotoxicity (22, 23, 26). These variants that lead to reduced gene expression can reduce cellular uptake of anthracyclines, reduce the production of harmful metabolites in cells, and protect cardiomyocytes. SLC28A3 plays a role in the influx of anthracyclines into cancer cells. The *SLC28A3* rs7853758 (G>A, Leu461Leu) polymorphism A allele can decrease its mRNA expression and is protective for anthracycline induced cardiotoxicity (21). Pharmacogenetic test for *SLC28A3* rs7853758 is suggested before treatment with anthracyclines in pediatric cancer patients (21). By using nanopore-based fine-mapping and base editing technologies, Magdy et al. identified the SNP rs11140490 at the *SLC28A3* locus was cardioprotective by regulating the expression of an antisense long non-coding RNA (*SLC28A3AS1*) that overlaps with *SLC28A3* (26).

Cytochrome P450 family of enzymes

Cytochrome P450 enzymes have the highest content in human liver and are responsible for the oxidative metabolism of 50% of clinical drugs. The genetic polymorphisms of CYP450 family members lead to huge individual differences in enzyme activities and drug metabolism, which eventually leads to adverse drug reactions (ADR) or unsatisfactory therapeutic efficacy. In past two decades, many of the interests have been focused on CYP450 genetic polymorphisms and individualized medicine.

Methadone is a racemic mixture of *R*- and *S*-methadone, which is considered to be the best antidote. CYP3A4 and CYP2B6 are the major CYP enzymes responsible for the metabolism of *R*- and *S*-methadone, respectively (26). High plasma concentration of *S*-methadone can lead to cardiotoxicity by prolonging the QT interval. A study of 125 death in Caucasians showed that *CYP2B6**9 (rs3745274, c516G>T), *CYP2B6**5 (rs3211371, c1459C >T), and *CYP2B6* rs8192719 (21563 C>T) were associated with the risk of fatal methadone cardiotoxicity (27). In order to identify genetic polymorphisms potentially associated with the risk of acquired long QT syndrome (aLQTS) in 153 cases with 216 QT-prolonging culprit drugs, Gray et al. recently observed that 22.2% and 7.8% of patients bearing rare variations in the LQTS genes and the CYP genes, respectively (28). Rare variant association studies indicated significantly higher burden of rare non-synonymous variants in CYP genes in the aLQTS cases. *CYP2B6* c.499C>G, c.1172T>A, c.415A>G, c.445G>A, and *CYP3A4* c.1000G>T might lead to increased risk for methadone-induced aLQTS (28). Some aLQTS cases can be explained by drug interactions in metabolism or pharmacodynamic synergy.

Venlafaxine (VEN) is a serotonin-norepinephrine-dopamine reuptake inhibitor. Excessive intake of VEN usually leads to mild cardiotoxicity. VEN is mainly metabolized by CYP2D6 and CYP2C19 to form demethylvenlafaxine (ODV). Patients with lower CYP2D6 or CYP2C19 activity are more likely to suffer from ADR events and have increased risk of developing cardiotoxicity during VEN treatment (29, 30). In patients developed VEN-related cardiotoxicity, *CYP2D6* poor metabolizer genotype **4/*9* and *CYP2C19* intermediate

metabolizer genotype *1/*2 are observed to show higher serum VEN levels (31).

CYP3A5 is involved in the clearance of daunorubicin (DNR) *in vivo*. *CYP3A5*3* is a common loss of function allele that results in the loss of CYP3A5 expression in adult liver. In children with acute lymphoblastic leukemia, an increase in area under the curve (AUC) for DNR plasma concentration was observed in *CYP3A5*3/*3* homozygotes (32). The *CYP3A5*3* polymorphism is also associated with increased risk of cardiotoxicity in patients with diffuse large B-cell lymphoma adopted combined therapy with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) (33). In addition, the *CYP3A5* rs4646450 polymorphism was observed to be a risk factor for doxorubicin induced cardiotoxicity, especially in males (21).

Carbonyl reductases (CBRs)

The CBRs oxido-reductase enzymes CBR1 and CBR3 are involved in the reduction of anthracyclines to the cardiotoxic ethanol metabolites, which play key roles in the induction of cardiovascular toxicity by anthracyclines. Genetic polymorphisms in *CBR1* and *CBR3* genes are reported to affect the production of the ethanol metabolites. Cancer patients with Down syndrome (DS) are prone to anthracyclines related cardiotoxicity, which could be explained by increased expression of CBR1 and increased metabolism of daunorubicin to the ethanol metabolites in the heart (34). *CBR1* 1096G>A (rs9024) is a 3′-UTR SNP that interferes with the inhibitory effects of hsa-miR-574-5p and hsa-miR-921 on its mRNA expression. The mutant 1096A allele was initially observed to increase the expression and activity of CBR1 (35). However, following studies with both liver cytosols and lymphoblastoid cell lines indicated that the *CBR1* 1096 G/A genotype showed a lower maximum rate of doxorubicinol synthesis than the GG genotype in the Whites (35). In support, a DS patient with the trisomic for the rs9024 A allele (A/A/A) exhibited low CBR1 enzymatic activity (34). In addition, among child cancer survivors receiving anthracyclines, the blacks showed relatively higher incidence of cardiotoxicity and lower frequency of the *CBR1* rs9024 A allele than the whites. *CBR1* rs9024 polymorphism may thus explain racial difference in susceptibility to anthracyclines induced cardiotoxicity (36). These findings suggest protective role of *CBR1* rs9024 A variant to anthracyclines induced cardiotoxicity. However, there are conflicting reports. For example, the rs9024 AA genotype is associated with increased risk of cardiotoxicity in children with acute lymphoblastic leukemia treated with UKALL 2003 protocol, and higher systemic doxorubicin exposure in this genotype is assumed (37, 38). Therefore, further studies are required to validate these associations.

CBR3 Val244Met (rs1056892) polymorphism can also affect the risk of anthracyclines induced cardiomyopathy in child cancer survivors and adult breast cancer patients (24). In child cancer survivors, *CBR3* Val244Met was dose-dependently associated with the risk of anthracyclines induced cardiomyopathy (39). Compared with *CBR3* Met244 carriers, rs1056892 GG (Val244) homozygotes showed 3.3-fold increased risk of cardiomyopathy in adults breast cancer patients when treated with low-dose anthracyclines (<250 mg/m^2). Functional study showed that the Val244 (rs1056892 G allele) catalyzed the synthesis of the cardiotoxic doxorubicinol with a rate 2.6-fold higher than the Met24 (40). However, in patients receiving high-dose anthracyclines (≥250 mg/m^2), no obvious association between the SNP and cardiotoxicity was observed (41). In a prospective single arm observational pharmacogenetic study with 155 breast cancer patients receiving doxorubicin, carriers of the *CBR3* Val244 allele showed significant reduction in LVEF at 6 months following initiation of doxorubicin, and Val244 homozygotes showed a further reduction (42). The *CBR3* Val244Met polymorphism was also associated with cardiotoxicity in breast cancer patients treated with trastuzumab (17).

Other drug metabolism genes

Some other drug metabolism genes can also influence the risk of DICT. For example, *Uridine diphosphate-glucuronosyltransferase 1A6* (*UGT1A6*) rs6759892, *UGT2B7*-161 rs7668258, *Histamine N methyltransferase* (*HNMT*) rs17583889, *P450 oxidoreductase* (*POR*) rs13240755 have been reported to be associated with increased risk of persistent cardiotoxicity after anthracycline therapy (22, 43, 44). In epidermal growth factor 2 (HER-2)-positive breast cancer patients treated with trastuzumab, the incidence of myocardial injury was reduced in carriers of the *UGT2B7-161* (rs7668258) T allele (45). This finding suggests *UGT2B7*-161 rs7668258 a potential predictor of cardiotoxicity in patients treated with trastuzumab therapy.

Pharmacodynamics (PD) gene polymorphisms

Human epidermal growth factor receptor type 2 (HER-2)

HER-2 is an important target of cancer targeted therapy. About 25–30% of breast cancer patients show HER-2 overexpression or *HER-2* gene amplification. The preferred treatment regimen for HER-2-positive breast cancer is based on trastuzumab and anthracycline/cyclophosphamide, which significantly improves the overall survival rate. However, at least 10–15% of the patients experienced anthracycline-induced cardiotoxicity, and 20–33% of patients also suffered from trastuzumab-induced cardiotoxicity (46). A genome wide association study (GWAS) carried out in 481 patients with (11 cases) and without (257 controls) trastuzumab-induced cardiotoxicity in Japanese showed that five SNPs including

rs9316695, rs28415722, rs7406710, rs11932853, and rs8032978 were independent predictors of trastuzumab cardiotoxicity ($P_{combined} = 7.82 \times 10^{-15}$, OR = 40.0) (47). The *HER-2* SNPs Ile654Val (rs1801201), Ile655/Val (rs1136201), and Pro1170Ala (rs1058808) were also associated with susceptibility to cardiotoxicity (48). In a meta-analysis of 344 patients with 43 developed drug induced cardiotoxicity, 67% of the patients carried the Ile/Val genotype, resulting in an OR of 5.35. *HER-2* Ile655Val is an independent predictor of cancer-therapy related cardiotoxicity (24, 48). By comparing *HER-2* genotype distribution in 29 cases with cardiotoxicity and 111 controls underwent trastuzumab treatment, Stanton et al., observed association between Pro1170Ala polymorphism and increased risk of trastuzumab induced cardiomyopathy (49). The frequency of *HER-2* Pro/Pro genotype in cases with cardiotoxicity (10/29, 34.5%) was higher than the controls (19/111, 17.1%) (49).

HER-2 SNPs inhibit HER4/HER4 homodimerization or HER4/HER2 heterodimerization through the HER-2 gene, thereby inhibiting a series of downstream signaling pathways, including PI3K-Akt. Blockade of the PI3K-Akt pathway will lead to the accumulation of ROS in cardiomyocytes, thereby triggering cardiomyocyte apoptosis (50). In addition, blockade of HER-2 signaling induces oxidative stress, leading to NO production and impairment of mitochondrial function, ultimately leading to myocyte apoptosis, myofibrillar disorder, and myocyte necrosis (50).

Tyrosine kinase receptor gene polymorphisms

Tyrosine kinases inhibitors (TKIs) are competitive inhibitors of the enzymes by binding to the adenosine triphosphate (ATP) binding pocket of the enzymes. TKIs are multi-target anticancer drugs with low specificity. Cardiovascular toxicity is one of the common ADR of TKIs. Using several publicly available datasets including drug-gene interaction database and GWAS database of heart failure, Li et al. found a group of overlap genes induced by TKIs and affect HF susceptibility (51). Comprehensive integrated analysis indicated that several SNPs potentially affect RNA binding protein-mediated regulation have the potential to affect cardiotoxicity of TKIs, among which the *PDGFRα* rs191188930 and *EGFR* rs142136033 have the potential to affect cardiotoxicity of multiple drugs including sunitinib, pazopanib, sorafenib, dasatinib and nilotinib (51). Of course, these findings require verification in clinic, and functional analysis of the suggested SNPs are also needed. The main mechanisms of TKIs-induced cardiotoxicity include inhibition of VEGF and PDGFR coronary microvascular dysfunction (52), through up-regulation of cardioprotective insulin and insulin-like growth factor (IGF) signaling and down-regulation of the phosphorylation of AKT and ERK affecting cardiomyocyte survival pathways, ultimately leading to cardiomyocyte death (53).

Others

In addition to genetic variants in PK and PD genes, some other genes that may modify risk of DICT have also been explored, such as genes related to regulation of oxidative stress, iron metabolism, autophagy, and myocardial sarcomere structure.

Oxidative stress related genes

Reactive oxygen species (ROS) produced by oxidative stress act as links between underlying cardiovascular disease and drug induced cardiotoxicity. NADPH oxidase (NOX) is the main endogenous source of ROS and a key mediator of cardiac oxidative damage. On the contrary, Hyaluronan synthase 3 (HAS3) is an enzyme that produces low molecular weight hyaluronic acid, which has antioxidant activity and protects the heart by reducing ROS-mediated cardiac damage (54). Several studies have focused on association of genetic variations in genes encoding enzymes involved ROS formation or clearance and risk of DICT.

NOX consists of five subunits, including two membrane-bound subunits (p22phox and gp91phox), three cytoplasmic subunits (p67phox, p47phox, p40phox), and a small G-protein Rac (52). The four NOX subunits are encoded by different autosomal genes: *CYBA* for p22phox, *NCF1* for p47phox, *NCF2* for p67phox, and *NCF4* for p40phox. Ras-related C3 Botulinum Toxin Substrate 2 gene (RAC2) is a small cytoplasmic GTPase that is required for NOX activation and regulation of ROS production (55, 56). Common polymorphisms in NOX subunit genes, such as *CYBA* rs4673, *NCF4* rs1883112, and *RAC2* rs13058338, are identified (57). Kopeva et al. divided 176 breast cancer patients who received anthracycline chemotherapy for 12 months into two groups: the anthracycline-induced cardiotoxicity (AIC) group (52 cases) and non-AIC group (124 cases), and observed that the *CYBA* rs4673 polymorphism was a risk factor for the occurrence of AIC (58). Alteration in *RAC2* can also lead to mitochondrial dysfunction and increased ROS production, and ultimately lead to cardiomyocyte damage (59). In a study aimed at identification of key genes affecting the risk of anthracycline-related congestive heart failure (CHF) in long-term survivors after haematopoietic cell transplantation (HCT), Armenia et al. observed that the odds of developing CHF after HCT was increased nearly 3 times in patients with the *RAC2* rs13058338 (7508 T>A) variants (4). Another study also supported association of *RAC2* rs13058338 variant with AIC in AML patients (44, 57).

By analyzing 2100 SNPs in genes associated with *de novo* cardiovascular disease in individuals exposed to high-dose anthracyclines (>250 mg/m^2), Wang et al. found that the *HAS3* rs2232228 AA genotype was associated with a 8.9-fold increased risk of cardiomyopathy compared with the rs2232228 GG genotype (60). In addition, *HAS3* mRNA expression in

heart samples of patients with the rs2232228 AA genotype was significantly lower than that of the GA heterozyotes (60). It is assumed that the *HAS3* AA genotype may increase the sensitivity of cardiomyocytes to ROS in the presence of high-dose anthracycline, and thereby increases the risk of AIC (60).

Iron homeostasis genes

Iron homeostasis is important for maintaining normal cardiac function. Iron-overload can lead to cardiomyopathy and heart failure. The *HFe* (high iron) gene on chromosome 6p encodes a protein that regulates iron transport and metabolism. HFE binds to transferrin receptors on the cell surface and promotes the uptake of transferrin bound iron. During anthracycline therapy, individuals with high HFE gene mutations may cause harmful iron deposition in the heart, causing more serious damage to cardiomyocytes (19). *C282Y* (rs1800562) and *H63D* (rs1799945) are two main functional SNPs of *HFE*. The rs1800562 polymorphism results in the substitution of tyrosine to cysteine at position 282 (C282Y), and the rs1799945 is a substitution of aspartate to histidine at position 63 (H63D) (61). A prospective association study of genetic mutations with anthracyclines induced cardiotoxicity in 184 child leukemia survivors observed positive associations for C282Y and H63D, with the H63D rs1799945 shows more prominent heart damage and cardiotoxicity (62).

Cardiac ion channel genes

KCNE1 encodes the β-auxiliary subunit of the voltage-gated slow cardiac potassium IKs current, whose dysfunction leads to cardiac arrhythmia. In a study of 153 aLQTS patients to explore possible rare variations related to TdP, four cases were observed to bear the *KCNE1*-c.253G>A (rs1805128) variant that is associated with increased risk of drug-induced TdP (28). Other variants in iron channel genes, such as *KCNE1*-c.253G>A, *KCNE2* c.22A>G, *SCN5A* (c.1715C>A, c.569G>A), *KCNQ1* (c.733G>A, c.727C>T), *KCNH2* -c.3163C>T are also found to induce aLQTS by causing QT prolongation (28).

Other pathways involved in myocardial function

Factors that affect myocardial sarcomere structure or transcriptional regulation may also modify risk of anthracycline induced cardiotoxicity. The cardiac sarcomere protein troponin T2 (TNNT2) is regulated by mRNA splicing, and different isoforms of TNNT2 (the fetal isoform, the adult cTnT3 isoform, for example) have different Ca^{2+} sensitivity (63, 64). Evidence shows that over-expression of Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A(DYRK1A) ameliorates the impact of daunorubicin on beating frequency in cardiomyocytes *via* increasing phosphorylation of the splicing factor Serine/arginine-rich splicing factor 6 (SRSF6), the latter plays a role in *TNNT2* mRNA splicing (65). CUGBP ELAV-like family member 4 (CELF4) is a mRNA binding protein that is also involved in regulating *TNNT2* mRNA splicing. The gene *TNNT2* also encodes cardiac troponin T (cTnT), an established biomarker of myocardial injury in the serum. cTnT is also important in Ca^{2+} signaling in the myocardium. GWAS in 430 childrens with (162 cases) and without (268 controls) cardiomyopathy after anthracycline therapy found that *CELF4* rs1786814 polymorphism was associated with risk of cardiomyopathy. In children exposed to >300 mg/m^2 of anthracyclines, the rs1786814 GG homozygotes showed a 10.2-fold increased risk of cardiomyopathy as compared with the G/A or A/A genotypes, while carriers of the *CELF4* rs1786814 A allele showed no change in risk of cardiomyopathy regardless of cumulative anthracycline exposure (66). This indicates that *CELF4* rs1786814 GG genotype is a risk factor of cardiovascular toxicity of anthracycline therapy (66).

The rs2229774 polymorphismin retinoic acid receptor gamma (*RARG*) gene (S427L) was associated with increased anthracycline cardiotoxicity with an OR of 4.7 (95% CI: 2.7–8.3) (67). In induced pluripotent stem cell-cardiomyocytes (iPSC-CMs), *RARG* rs2229774 variant was observed to increase double-strand DNA breaks, ROS production, and cell death, thereby increased susceptibility of the cells to doxorubicin-induced cardiotoxicity (68). Subsequent studies further revealed that RARG may increase DICT susceptibility by reducing mitochondrial numbers and attenuating DNA repair (69). Magdy et al. also observed that the *RARG* variation can function through disruption of RARG mediated inhibition on topoisomerase 2β (TOP2B) expression and activation of the extracellular regulated kinase (ERK) signaling upon doxorubicin treatment, emphasizing multiple pathways and mechanisms for the protective role of RARG in DICT (15).

Autophagy imbalance is also involved in the mechanism of DICT. To explore whether genetic polymorphisms in autophagy-related genes are associated with risk of DICT, 25 SNPs in genes related to autophagy regulation were genotyped in 147 triple-negative breast cancer (TNBC) patients with relatively complete ECG records during the chemotherapy cycles (70). The results showed that the rs10838611 G allele in autophagy-related 13 (*ATG13*) was significantly associated with abnormal ECG (OR: 2.258, 95% CI: 1.318–3.869), suggesting an increased risk of cardiac events (70).

New technologies in pharmacogenomics study of DICT

Candidate gene association studies (CGAS) and genome-wide association studies (GWAS) are two main methods in identifying drug response susceptible genes (71, 72). Although these studies have found genetic polymorphisms that may

lead to DICT, exploration of the the causal relationship and mechanism between the SNPs and DICT is difficult. Technologies such as human induced pluripotent stem cells (HiPSCs) and genome editing bring opportunities to make the functional analysis of the causative variants more feasible.

Whole genome or whole exon sequencing

In the 1990s, the implication of first-generation sequencing (FGS) technology pushed the completion of the sequencing of the first human genome (73). Subsequently, the rapid development of second-generation sequencing (SGS) promoted studies in genomics (73) as well as mapping of genetic polymorphisms in the human genome (74). Third-generation sequencing (TGS), also known as single-molecule sequencing, was also developed, such as single-molecule real-time (SMRT^{TM}) from Pacific Biosciences (PacBio), true single-molecule sequencing (tSMS^{TM}) from Helicos, and single-molecule nanopore DNA sequencing from Oxford Nanopore (75). TGS can not only adapt to the reading of longer genomes, identify complex structural changes in DNA samples, and accurately locate the position of sequence changes, but also can recognize DNA/RNA methyltransferase modifications, a successful step toward understanding the biology that occurs between DNA and proteins. Most importantly, TGS sequencing are as accurate as FGS and SGS for assembling complete genomes (76).

TGS has been applied in disease genomic and pharmacogenetics studies. For example, Wang et al. used single-molecule nanopore DNA sequencing to detect the serine/threonine protein kinase gene *BRAF* V600E mutation in thyroid cancer patient tissues with high sensitivity (77). Magdy et al. used nanopore DNA sequencing to pinpoint the association of GWAS-positive *SLC28A3* SNP with doxorubicin-induced cardiotoxicity. The results showed that single-molecule nanopore DNA sequencing can not only provide comprehensive and accurate information on precisely mapped GWAS-positive sites, but also help identify causal SNP/haplotype (78). It can be expected that in the future, TGS technology will be more widely used in the study of DICT.

Other omics-based technologies

In addition to genomics, emerging technologies for biomarker discovery include transcriptomics, metabolomics, proteomics, and gut microbiomics are developed. Application of these new methods can faciliated the identification of both predictive and diagnostic biomarkers for DICT and are prosperous.

RNA sequencing is a kind of high-throughput sequencing that is used to identify differentially expressed genes by detecting samples from different backgrounds (different species, tissues, and periods, etc.), discover potential biomarkers, and reveal the underlying molecular mechanisms for diseases. Combination of single cell RNA-seq (scRNA-seq) and spatial transcriptomics can bring RNA-seq technology into single-cell resolution and tissue-level transcriptomics, providing new insights for disease diagnosis, treatment, and prevention (79–82).

Metabolomics is a collection of small-molecule chemical metabolites that identify biomarkers primarily by methods such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. Both targeted and non-targeted metabolomics have been used to identify circulating metabolites related to drug induced cardiotoxicity. For example, Asnani et al. evaluated metabolite changes in 38 breast cancer female patients treated with anthracyclines and trastuzumab, and found that in patients with cardiotoxicity, citrate levels were reduced, while purine and pyrimidine metabolites were significantly increased, suggesting that metabolomics changes may also contribute to the development of DICT (83).

Proteomics has traditionally been dominated by methods of liquid chromatography-mass spectrometry (LC-MC), which is mainly used to identify and detect diagnostic markers, understand pathogenic mechanisms, and explain functional protein pathways in human diseases (84). Pilot study also used high-throughput proteomic analysis to the identify potential biomarkers associated with doxorubicin and trastuzumab-induced cardiac insufficiency in plasma (85–87). In an cohort of 35 patients treated with doxorubicin and trastuzumab, high baseline immunoglobulin E (lgE) levels was observed to be associated reduced risk of drug-induced cardiac dysfunction (85). These studies suggest that proteomics shed new light on the identification of novel molecular pathways and biomarkers for DICT.

The gut microbiome is another emerging field that attracts much interest in recent years. Liu et al. used 16rRNA gene and metagenomic sequencing to analyze the composition and function of the gut microbiota in mice with doxorubicin-induced cardiotoxicity (88). They observed that depletion of gut microbiota could alleviate adriamycin-induced myocardial injury and cardiomyocyte apoptosis, suggesting important role of gut microbiota in the pathogenesis of adriamycin-induced cardiotoxicity (88). It is also reported that intestinal flora butyric acid (BUT) derivative phenylalanine butyramide (FBA) can prevent anthracyclines induced left ventricular dilation, fibrosis and cardiomyocyte apoptosis in mice (89). FBA reduces anthracyclines induced damage of human cells and is protective against experimental doxorubicin cardiotoxicity by improving mitochondrial function and reducing oxidative stress (89). In conclusion, the gut microbiota may become a new target for the prevention of drug cardiotoxicity and cardiovascular disease.

Human induced pluripotent stem cells (HiPSCs) and 3D HiPSC-derived heart model

As the primary site of DITC, the human cardiac tissue is largely inaccessible and cannot be maintained in tissue culture. HiPSCs are a regenerative cell type that can be obtained by non-invasive manipulations. HiPSCs are also genetically identical to the patients from which they were obtained, which makes manipulating HiPSCs *in vitro* and comparing them with clinical phenotypes become possible. HiPSC can be used to determine the potential toxicity of drugs, study the mechanism of drug toxicity, verify the determinants of genetic variants in drug toxicity, and provide target information for new drug development. The use HiPSCs is emerging in pharmacogenomics study of DICT in recent years (90).

Patient-specific HiPSC-cardiomyocytes (HiPSC-CMs) develop similar characteristics to the human heart in genomics, transcriptomics, electrophysiology, biochemistry, contraction, and beating. Therefore, HiPSC-CMs have the advantage of reproducing human cardiac tissue in *in vitro* studies over other models such as animal models, non-human primary cells and immortalized cell lines (91, 92). Burridge and colleagues demonstrated that HiPSC-CMs recapitulate the susceptibility of individuals to doxorubicin-induced cardiotoxicity at the cellular level. They recruited 12 female breast cancer patients who had been treated with doxorubicin or equivalent, with 4 patients without clinical cardiotoxicity, 4 patients with established clinical cardiotoxicity, and 4 age-sex-matched healthy volunteers not received any medication (93). They observed that HiPSC-CMs from patients developed doxorubicin cardiotoxicity were consistently more sensitive to doxorubicin toxicity, suggesting HiPSC-CMs as a suitable cellular model to identify and characterize the genetic basis and molecular mechanisms of doxorubicin cardiotoxicity (93). Using HiPSC-CMs from patients treated with trastuzumab, Kitani et al. identified changes in metabolic pathways to be key important in cardiac dysfunction following trastuzumab treatment (94). The study also supports the use of *in vitro* HiPSC-CMs assays to investigate drug cardiotoxicity for antibody therapies (94). Non-specific HiPSCs are also used to study the mechanism of drug cardiotoxicity. For example, Sharma et al. using HiPSC-CMs, endothelial HiPSC-ECs, and cardiac fibroblasts HiPSC-CFs to detect the potention of 21 TKIs in inducing cardiotoxicity by high-throughput screening (95). HiPSCs have also been used in the study of cardiovascular toxicity of etoposide (96), arsenic trioxide (97, 98), lapatinib (99), and histone deacetylase inhibitors (100, 101).

Stem cell-derived cardiomyocytes are also used to develop *in vitro* 3-dimensional (3D) models, namely cardiac micro-tissues and organoids. These models can synergize with genetic engineering to provide tissue-level models for study drug cardiotoxicity. Richards et al. designed an *in vitro* organotypic disease model of cardiovascular disease based on the principle of tissue engineering (102), and observed that the human cardiac organoids can reproduce drug-induced or aggravate cardiac fibrosis at the tissue level (102). Truitt also used a 3D cardiac microtissue (CMT) model to study the cardiotoxicity of sunitinib. Compared with the 2D model HiPS-CMs, the CMT model is particularly preferable to evaluate the combined effects of drug treatment and afterload (103). Organ-on-a-chip models are micro-microfluidic-controlled 3D organoid models that not only accurately reproduce the physiological parameters of their *in vivo* counterparts, but also can be linked together by microfluidics in a manner similar to their arrangement *in vivo*, which make the study of multi-organ interactions possible. Compared with traditional 2D models, multi-organ models can predict human drug responses more accurately. Automated modular design platform based on multi-organ models has also been developed in mimicking the microenvironment in real time and *in situ*, which provide basis for in-depth study of the mechanism of drug cardiotoxicity (104).

CRIPSR/Cas9 genome editing

To exclude the influence of complex genetic backgrounds, the use of HiPSC-CMs from healthy volunteers or treated patients alone still does not meet the needs of current study. CRIPSR/Cas9 genome editing provides a solution to create HiPSCs genes by introducing targeted mutations in cell lines. The genome editing technology can provide precise control of genome conditions and make HiPSCs a powerful tool for studying drug-induced cardiovascular toxicity (105). Maillet et al. also performed CRISPR/Cas9 genome editing to disrupt the *TOP2B* gene in HiPSC-CMs to evaluate doxorubicin toxicity on the cells. It was found that disruption of *TOP2B* reduced the susceptibility of HiPSC-CMs to doxorubicin-induced cell death, and conformed that doxorubicin-induced double-strand DNA breaks (DSB) in HiPSC-CMs was TOP2B-dependent (106).

Expectation

DICT is a common ADR for diversity of drugs, especially in cancer therapies. Current studies have identified some genetic variants associated with risk of DICT through studies based on candidate genes or GWAS. Most variants mentioned above need further replication in different populations and clinical conditions. The availability of high throughput technologies such as whole genome/exon sequencing will facilitate the identification of additional genetic biomarkers potentially affecting DICT risk. Emerging of new models such as patient-derived HiPSC-CMs and genome editing cells or animals makes mechanism study of the variants more

approximate to the human myocardium. Of note, many potential pharmacogenomics biomarkers associated with risk of DICT are identified through case-control studies and few are translated into clinic practice in guiding drug therapies or individualized prevention of DICT. More studies, including well designed randomized clinical trials (RCTs), are required to confirm the utility of the genetic variants in future clinic practice. Translation of the pharmacogenomics findings into genotype-guided drug therapy is supposed to maximize drug efficacy and minimize cardiotoxicity for related drugs.

Author contributions

X-PC contributed to conception and design of the study. M-YL wrote the first draft of the manuscript. L-MP wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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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.2022.966261/full#supplementary-material

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Drug-induced torsades de pointes: Disproportionality analysis of the United States Food and Drug Administration adverse event reporting system

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Objective: This study aimed to identify the most common and top drugs associated with the risk of torsades de pointes (TdP) based on the United States Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS) database.

Materials and methods: We used OpenVigil 2.1 to query FAERS database and data from the first quarter of 2004 to the third quarter of 2021 were retrieved. The Medical Dictionary for Regulatory Activities (MedDRA) was used to identify TdP cases. We listed the most common drugs associated with the reported TdP cases. Then, the reporting odds ratio (ROR) and the proportional reporting ratio (PRR) for the reporting association between different drugs and TdP risk were calculated. Meanwhile, comparisons were conducted with the QT drug lists of CredibleMeds® in an attempt to identify drugs with a potential risk of TdP that were not on the list.

Results: A total of 9,217,181 adverse event reports were identified, of which 3,807 (0.04%) were related to TdP. TdP was more likely to occur in the elderly and females. Amiodarone (464 cases) was associated with most cases of TdP. According to the disproportionality analysis, the top five drugs with the highest ROR and PRR were tolazoline (ROR 1615.11, 95% confidence interval [CI] 455.59–5725.75, PRR 969.46, χ^2 2960.10), levomethadyl (ROR 1211.01, 95% CI 302.75–4844.04, PRR 807.67, χ^2 1677.03), ibutilide (ROR 1118.74, 95% CI 425.00–2944.91, PRR 765.77, χ^2 3845.27), halofantrine (ROR 660.55, 95% CI 184.21–2368.69, PRR 519.22, χ^2 1076.31), and isoproterenol (ROR 352.20, 95% CI 227.19–546.00, PRR 307.82, χ^2 6692.53). Approximately half of the top 50 drugs (22 for ROR, 30 for PRR) were not outlined on the QT drug lists of CredibleMeds®.

Conclusion: Approximately half of the top risk drugs (22 for ROR, 30 for PRR) were not outlined in the QT drug lists of CredibleMeds®. Notably, potential risks are of great importance and should be closely monitored in clinical practice. Also, further research is needed to investigate the association between these drugs and TdP.

KEYWORDS

long QT syndrome, torsades de pointes, FAERS, disproportionality analysis, pharmacovigilance

Introduction

Torsades de pointes (TdP) is a type of polymorphic ventricular tachycardia (1). The incidence of TdP is from 0.0032‰/year to 0.16‰/year (2–4), which is very low but often life-threatening. The mortality of TdP is approximately 10–20% (5). In general, TdP is associated with prolonged QT interval, for every 10 ms increase in QT interval, the risk of TdP increases by approximately 5–7% (6). Long QT interval can be congenital or acquired. The latter is most often drug-induced. In clinical practice, multiple drugs may cause TdP (7, 8), and several of them have been withdrawn from the market due to TdP, such as cisapride, droperidol, and terfenadine (9, 10).

It is particularly essential to assess drug-induced TdP. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) released a guideline for clinical evaluation of QT/QTc interval prolongation. This guideline could help us identify drugs that may cause QT prolongation before marketing (11). However, due to the limited sample size and follow-up time, it is difficult to observe rare adverse events (AEs) in such studies. Therefore, post-marketing surveillance is also very important, especially for rare but clinically significant AEs, such as TdP. Food and Drug Administration Adverse Event Reporting System (FAERS) database is believed to be a powerful tool for mining new or rare AEs and could reflect profiles of AEs in real-world clinical settings. It is a publicly accessible database that includes a large number of AE reports submitted by healthcare professionals, consumers, and manufacturers (12). Previous researchers have used the FAERS database to investigate TdP and have comprehensively assessed the specific class drugs in terms of TdP, mainly including H_1-antihistamines, antipsychotics, and antibiotics (13–16).

Increasing new drugs have been approved on the market in recent years, and it is of great significance to update the risk of TdP in currently available drugs based on spontaneous reporting AEs data. Therefore, this study aimed to comprehensively investigate the risk of drug-induced TdP across all drugs and identify drugs with a potential risk of TdP that were not on the QT drug lists of CredibleMeds®.

Materials and methods

Data source

This retrospective pharmacovigilance study was conducted based on the FAERS database. FAERS is a spontaneous reporting AEs database which is available to the public. A large amount of information of AEs can be found in this database, such as demographic and administrative information, drug information and reaction information (17, 18).

Data collection

OpenVigil 2.1[1] was used to retrieve FAERS data. OpenVigil 2.1 is a validated pharmacovigilance data extraction, cleaning, and mining tool of the FAERS database (19, 20). For this study, the AEs of TdP were searched from the first quarter of 2004 to the third quarter of 2021 using preferred terms (PTs) in the Medical Dictionary for Regulatory Activities (MedDRA) Dictionary (Version 24.0). We used Torsade de Pointes (PT: 10044066) to search.

Statistical analysis

First, the descriptive analysis was performed to summarize the clinical features of cases of TdP, including patients' gender, age and reporting country. According to the counts of reports, the top 50 drugs associated with TdP were selected.

Second, a disproportionality analysis was conducted. Disproportionality analysis is largely used to generate hypotheses on possible associations between drugs and AEs. It is based on the contrast between observed and expected numbers of reports, for any given drug and AE (21). Reporting odds ratio (ROR) and proportional reporting ratio (PRR) were two measures of disproportionality analysis. We calculated ROR

1 http://h2876314.stratoserver.net:8080/OV2/search/

and PRR to detect each drug's TdP risk signal. The equations and criteria for the algorithms were listed in Table 1 (22–24). ROR and PRR offer a rough indication of the strength of the signal. A relatively higher ROR or PRR indicates a stronger signal between the drug and TdP. The analyses were performed using the Microsoft EXCEL 2019.

Credible Meds (accessible at www.crediblemeds.org) has established a list of drugs that increase risk of QT prolongation and TdP. And these drugs were classified into three categories, "known risk," "possible risk," or "conditional risk" of TdP. In our study, comparisons were conducted with the QT drug lists of CredibleMeds® in an attempt to identify drugs with a potential risk of TdP that are not on the list (25).

Results

Descriptive analysis

From 2004 Q1 to 2021 Q3, there were 9,217,181 AEs in FAERS, of which 3,807 (0.04%) were TdP cases. The characteristics of cases were listed in Table 2. TdP was more likely observed in female patients (56.82%) and most frequently reported in the United States (41.27%). When stratified by age group, the majority of AE reports were distributed to the elderly (≥65 years) (33.44%).

Based on the counts of AE reports, the 50 most common drugs associated with TdP are summarized in Figure 1. Among the most frequently reported drugs, amiodarone (464 cases) was associated with the most cases of TdP, followed by furosemide (412 cases), methadone (292 cases), citalopram (260 cases) and loperamide (259 cases). Of these 50 drugs, 30 were included on the QT drug lists of CredibleMeds®.

TABLE 2 Characteristics of cases with torsades de pointes.

Characteristics	Cases, *n* (%) (Total cases: 3807)
Age	
≤18	154 (4.04)
19–40	646 (16.97)
41–64	1093 (28.71)
≥65	1273 (33.44)
Unknown or missing	641 (16.84)
Gender	
Male	1306 (34.31)
Female	2163 (56.82)
Unknown or missing	338 (8.87)
Reporting country	
United States	1571 (41.27)
United Kingdom	286 (7.51)
Canada	186 (4.89)
Japan	175 (4.60)
Germany	154 (4.05)
China	29 (0.75)
Other regions	1406 (36.93)

Disproportionality analysis

Based on the criteria for ROR, a total of 306 signals were detected for TdP. Drugs with the top 50 highest RORs are listed in Table 3. Tolazoline (ROR 1615.11, 95%CI 455.59–5725.75) reported the highest ROR for TdP, followed by levomethadyl (ROR 1211.01, 95% CI 302.75–4844.04), ibutilide (ROR 1118.74, 95% CI 425.00–2944.91), halofantrine (ROR 660.55, 95% CI 184.21–2368.69) and isoproterenol (ROR 352.20, 95% CI 227.19–546.00). Of the top 50 drugs, 28 were included on the QT drug lists of CredibleMeds®. According to the risk categories of CredibleMeds®, 19 drugs were classified as known risk of TdP, 5 drugs as conditional risk, 3 drugs as special risk, and the other one drug as possible risk of TdP.

The estimated PRRs for each of the individual drugs associated with TdP are summarized in the Supplementary Table 1. A total of 253 signals were detected, and the top five drugs with the highest PRRs were consistent with the results of RORs, including tolazoline (PRR 969.46, χ^2 2960.10), levomethadyl (PRR 807.67, χ^2 1677.03), ibutilide (PRR 765.77, χ^2 3845.27), halofantrine (PRR 519.22, χ^2 1076.31), and isoproterenol (PRR 307.82, χ^2 6692.53). Thirty drugs were included on the QT drug lists of CredibleMeds®. According to the risk categories, 22 drugs were classified as known risk of TdP, 5 drugs as conditional risk, 2 drugs as special risk, and the other one drug as possible risk of TdP.

TABLE 1 Summary of algorithms used for signal detection.

Algorithms	Equation	Criteria
ROR	$ROR = ad/bc$	lower limit of 95% CI > 1, a ≥ 2
	$95\%CI = e^{\ln(ROR) \pm 1.96(1/a+1/b+1/c+1/d)\wedge 0.5}$	
PRR	$PRR = a(c + d)/c(a + b)$	PRR ≥ 2, $\chi^2 \geq 4$, a ≥ 3
	$\chi^2 = [(ad - bc)^2](a + b + c + d)/[(a + b)(c + d)(a + c)(b + d)]$	

a, number of reports containing both the suspect drug and the suspect adverse drug reaction; b, number of reports containing the suspect adverse drug reaction with other medications (except the drug of interest); c, number of reports containing the suspect drug with other adverse drug reactions (except the event of interest); d, number of reports containing other medications and other adverse drug reactions. ROR, reporting odds ratio; PRR, proportional reporting ratio; χ^2, chi-squared.

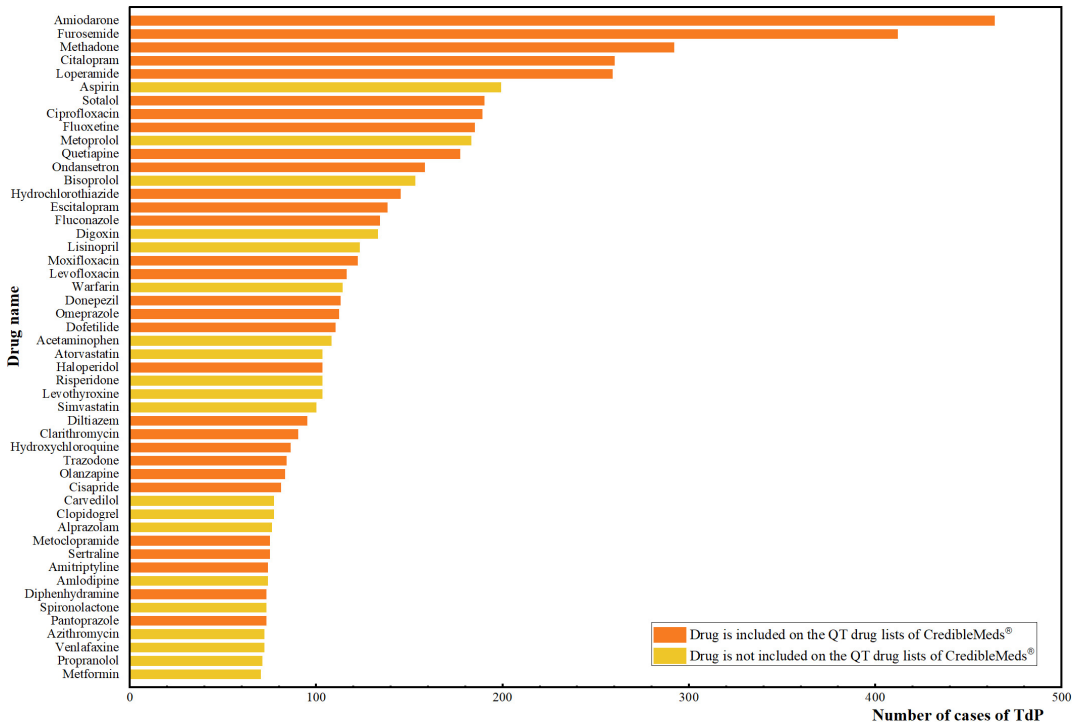

FIGURE 1
Top 50 drugs with the most number of cases of TdP. The vertical axis is the name of the drugs, and the horizontal axis is the corresponding number of cases of TdP for each drug. The orange color represents that the drug is included on the QT drug lists of CredibleMeds®. The yellow color represents that the drug is not included on the QT drug lists of CredibleMeds®.

Discussion

This study comprehensively assessed the AEs of drug-induced TdP in the real world based on the FAERS database. The results indicated that TdP was more likely to occur in the elderly and females. Amiodarone was associated with most cases of TdP. According to the disproportionality analysis, the top five drugs with the highest ROR and PRR were tolazoline, levomethadyl, ibutilide, halofantrine and isoproterenol. Approximately half of the top 50 drugs (22 for ROR, 30 for PRR) were not outlined on the QT drug lists of CredibleMeds®.

Drug-induced TdP is a non-negligible AE in pharmaceutical treatments, which can lead to cardiac sudden death. Previous studies have found multiple risk factors for TdP, including female, age (≥65 years), etc. (26–28), which was also observed in our study. Sex-related differences in TdP are increasingly recognized, but the mechanisms remain uncertain. Sex hormones may be a possible factor for sex-related differences. Testosterone appears to shorten the QT interval, and estrogen may lengthen QT interval. Thus, females have a longer QT interval compared to males after puberty, and the QT interval in males gradually lengthens and approximates that of females with aging (29, 30). In addition, QTc > 500 ms is considered a risk factor of TdP. Females have a higher baseline QT interval and are more likely to prolong over 500 ms when challenged by QT-prolonging drugs (7). In terms of the elderly, their prescriptions have more QT-prolonging drugs. A study conducted on 5,319 elderly outpatients in North Jordan showed that 58.5% of patients were consuming drugs that carry the risk of TdP (31). Therefore, the elderly are more likely to be exposed to high risk of TdP.

Based on data mining, we listed the top 50 drugs with the strongest signals, and compared these drugs to the QT drug lists of CredibleMeds®. Interestingly, about half of the drugs (22 for ROR, 30 for PRR) were not outlined in lists, representing potential new signals. The drugs involved in new signals include antihistamines (e.g., clemastine), antibiotics (e.g., cloxacillin), inhalation anesthetics (e.g., isoflurane), etc. Although some of these drugs have been on the market for a long time, there is inadequate attention to TdP. Therefore, further attention is needed to determine whether these drugs are needed to be included on the QT drug lists of CredibleMeds®. On the other hand, the post-marketing safety monitoring of newly marketed drugs is also an imperative topic. In our study, of the top 50 drugs, viloxazine was the most recently marketed (32). Due to the short time on the market, only two cases of TdP were reported, but with significantly higher ROR and PRR. As a result, physicians and pharmacists should be alert for viloxazine-induced TdP in clinical practice.

CredibleMeds is one of the most reliable sources of information on drug-induced QT prolongation, with close

TABLE 3 Reported odds ratios for the top 50 drugs.

Drug name	ROR (95% CI)	CredibleMeds® TdP risk
Tolazoline	1615.11 (455.59–5725.75)	N
Levomethadyl	1211.01 (302.75–4844.04)	KR
Ibutilide	1118.74 (425.00–2944.91)	KR
Halofantrine	660.55 (184.21–2368.69)	KR
Isoproterenol	352.20 (227.19–546.00)	SR
Chlorcyclizine	302.67 (69.57–1316.85)	N
Cisapride	273.60 (217.01–344.95)	KR
Viloxazine	134.52 (32.38–558.91)	N
Thiamylal	103.80 (32.67–329.81)	N
Procainamide	82.63 (36.60–186.55)	KR
Bepridil	78.68 (32.27–191.82)	KR
Safflower oil	78.11 (19.10–319.44)	N
Tandospirone	72.28 (17.70–295.12)	N
Sotalol	70.10 (60.47–81.26)	KR
Sertindole	62.09 (15.25–252.77)	KR
Amsacrine	61.33 (22.73—165.53)	CR
Esmolol	60.07 (29.76–121.22)	N
Amrinone	56.31 (13.85–228.88)	N
Droperidol	53.35 (30.09–94.62)	KR
Clemastine	52.90 (32.20–86.90)	N
Vorinostat	48.82 (32.27–73.85)	PR
Dofetilide	47.71 (39.40–57.78)	KR
Ferrous sulfate anhydrous	47.05 (24.31–91.07)	N
Disopyramide	46.90 (27.61–79.67)	KR
Amiodarone	46.88 (42.51–51.69)	KR
Fluphenazine	46.19 (31.94–66.78)	N
Ticarcillin	43.77 (13.97–137.17)	N
Almotriptan	38.68 (17.26–86.70)	N
Mexiletine	37.65 (20.74–68.36)	N
Terfenadine	37.25 (9.22–150.60)	KR
Ivabradine	35.39 (26.41–47.43)	CR
Pancuronium	34.24 (12.76–91.91)	N
Dopamine	32.59 (20.70–51.30)	SR
Pimozide	31.39 (12.98–75.89)	KR
Methadone	29.08 (25.79–32.79)	KR
Loperamide	29.03 (25.57–32.96)	CR
Sevoflurane	27.28 (19.61—37.95)	KR
Flecainide	26.84 (21.01–34.28)	KR
Acamprosate	26.53 (13.74–51.20)	N
Acenocoumarol	25.55 (18.61–35.08)	N
Flucytosine	25.50 (9.52–68.33)	N
Betahistine	24.04 (14.89–38.80)	N
Fluindione	23.85 (15.33–37.09)	N
Cimetidine	23.40 (16.49–33.19)	CR
Dronedarone	22.81 (17.28–30.11)	KR
Isoflurane	22.81 (10.83–48.05)	N
Chloral hydrate	22.09 (7.08—68.86)	CR
Ritodrine	21.06 (5.23–84.73)	SR
Cloxacillin	20.88 (6.70–65.08)	N
Chloroquine	20.51 (11.87–35.44)	KR

ROR, reporting odds ratio; CI, confidence interval; KR, known risk; PR, possible risk; CR, conditional risk; SR, special risk; N, not on the list.

monitoring of the FAERS database as well. Our results are slightly different from the QT drug lists of CredibleMeds® may be due to different methods. The CredibleMeds team used the Bayesian method to estimate the relative reporting ratio and we used the frequentist method. These two methods have their own strengths. The frequentist method has higher sensitivity, while the Bayesian method has higher specificity (33). Therefore, we hope our results could be a supplement to detect TdP of drugs.

Our study has several strengths. Above all, FARES is one of the largest public pharmacovigilance databases, and the sample size is sufficient to detect rare AEs of TdP and provide valuable suggestions to guide clinical decision-making. Further, we compared the 50 drugs with the strongest signals to the QT drug lists of CredibleMeds® and mined some new signals, which can be considered hypotheses to stimulate further research. Last but not least, given their simplicity and sensitivity, the choice of ROR and PRR for signal detection helps to detect new AEs signals more quickly.

Our study also has several limitations. First of all, due to the missing denominator data, the true incidence of TdP of each drug was not estimated based on the FAERS database (34). Second, considering that FAERS is a spontaneous reporting system, the causal relationship between AEs and drugs may not be determined. Thus, our research can only give clinicians and researchers some hints about which drugs may have a high potential risk of TdP, and further studies with more reliable methods are needed to verify. Third, due to the insufficient information in the FAERS database, it is hard to assess the effects of drug-drug interactions or route of administration (e.g., oral vs. intravenous) on TdP. Finally, underreporting may not be avoided because prolongation of the QT intervals is judged by an electrocardiogram, which is not routinely equipped in clinical practice. Nevertheless, the FAERS database remains an important tool for post-marketing surveillance.

Conclusion

In conclusion, we comprehensively assessed TdP reports and associated drugs using the FAERS database. Approximately half of the top risk drugs (22 for ROR, 30 for PRR) were not outlined on the QT drug lists of CredibleMeds®. Notably, the potential risks are of great importance and should be closely

monitored in clinical practice. Also, further research is needed to investigate the association between these drugs and TdP.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: http://h2876314.stratoserver.net:8080/OV2/search/.

Author contributions

ZW and PZ extracted and analyzed the data and drafted the manuscript. SZ provided pharmacological guidance. NH supported data analysis. All authors participated in the study design, contributed to the revision of the manuscript, and approved the final version.

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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.

The handling editor FS declared a shared affiliation with the authors at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.966331/full#supplementary-material

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Comparative cardiotoxicity risk of pembrolizumab versus nivolumab in cancer patients undergoing immune checkpoint inhibitor therapy: A meta-analysis

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Objective: Recently, several researchers have reported the incidence of cardiac-related toxicities occurring with nivolumab (Opdivo) and pembrolizumab (Keytruda). There is still a need for balance between oncology treatment efficacy and reduction of cardiotoxicity burden in immune checkpoint inhibitor (ICI)-treated patients. Thus, the primary aim was to determine whether pembrolizumab or nivolumab would present with a greater risk for cardiotoxicity reports.

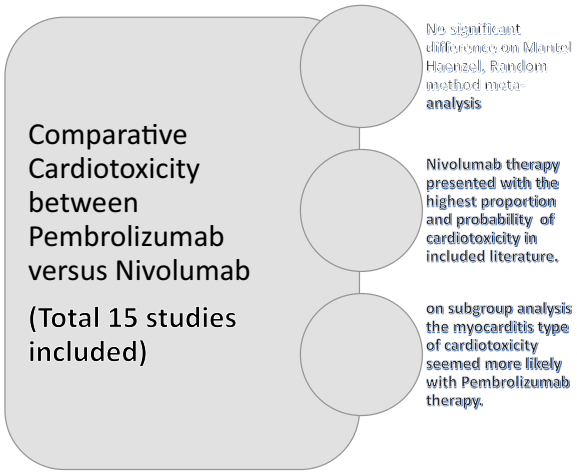
Materials and methods: This meta-analysis was performed with respect to the MOOSE reporting guidelines. Studies were retrieved by searching PubMed, Embase, and Google Scholar; the search terms were Keytruda or Pembrolizumab, PD1 inhibitors, anti-PD1 drugs, Nivolumab or Opdivo, and cardiotoxicities or cardiac toxicity. The study was restricted to original articles investigating ICI-induced cardiac immune-related adverse events (irAEs). The targeted population was cancer patients treated with either pembrolizumab or nivolumab monotherapy, of which those with records of any cardiac events following the therapy were labeled as events. The measures used to achieve the comparison were descriptive proportions, probabilities, and meta-analysis pooled odds ratios (ORs).

Results: Fifteen studies were included in this meta-analysis. Nivolumab accounted for 55.7% cardiotoxicity and pembrolizumab, for 27.31% (P = 0.027). The meta-analysis was based on the Mantel–Haenszel method, and the random-effect model yielded a pooled OR = 0.73 (95% CI [0.43–1.23] P = 0.24), with considerable heterogeneity (I^2 = 99% P = 0). Hence, the difference in cardiotoxicity odds risk between pembrolizumab and nivolumab was not statistically significant. On subgroup analysis based on cardiotoxicity type, the "myocarditis" subgroup in which there was no statistical heterogeneity was associated with a significant cardiotoxicity risk increase with pembrolizumab (OR = 1.30 [1.07;1.59], P< 0.05; I^2 = 0%, Ph = 0.4).

Conclusion: To our knowledge, this is the first meta-analysis to compare the cardiotoxicity potentials of nivolumab and pembrolizumab. In contrast to previous reports, the overall findings here demonstrated that nivolumab-induced cardiotoxicity was more commonly reported in the literature than pembrolizumab; however, myocarditis seemed more likely to occur with pembrolizumab therapy.

KEYWORDS

cardiotoxicity, meta-analysis, nivolumab, pembrolizumab, risk



GRAPHICAL ABSTRACT

1 Background

The World Health Organization's most recent data from 2021 has shown that cancer in general accounted for around 2 million of the nearly 10 million deaths worldwide (1). Therefore, it might be viewed as a significant health burden that can be reduced through early detection, precise diagnosis, and improved management and care. However, the pharmaceutical agents used to treat cancer, either alone or in combination therapy, have been linked to the emergence of toxicities affecting several vital organs, including the heart and vessels. In the growing field of cardio-oncology, which serves as a link between cardiologists and oncologists, the impact of cancer treatment on the heart and the management of cardiotoxicity are the main topics of interest (1, 2). New therapeutic agents have emerged over the past decade to improve cancer treatment and lessen the toxic side effects, including immune checkpoint inhibitors (ICIs), a special type of immunotherapy subclass whose cardiotoxicity potential was found to be lower than that of conventional chemotherapeutic drugs (3).

The development of these agents has been a revolutionary milestone that was associated with remarkable benefits and resulted in long-lasting tumor responses. They are now widely accepted as a key component of therapeutic strategies in cancer management (4, 5). The ICI subclasses include monoclonal antibodies that block programmed cell death receptors or their ligand (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). PD-1, PD-L1, and CTLA-4 immune checkpoints are markedly expressed in cancer cells and contribute to the inhibition of T-cell activation and are thought to represent one of many tumoral adaptive responses to escape from the immune system (6). The first successful use of CTLA-ICI therapy on mice was reported in 1996; then, in 2000, the first human CTLA-4 ICI, i.e., IPILUMAB, was introduced that got FDA approval in 2011 (7, 8). In 2006, nivolumab became the first PD1 ICI used in patients and also the first to obtain FDA approval in 2014 for melanoma (9) followed by pembrolizumab, another PD1 ICI (9). Nevertheless, their use also led to increased occurrences of different types of side effects (irAE), characterized by autoimmune reactions in various tissues that were rare but had serious side effects on the

Abbreviations: ICI, immune checkpoint inhibitors; iaAEs, immune-related adverse events; anti-PD1, monoclonal antibodies belonging to programmed cell death 1 inhibitor; anti-CTLA4, cytotoxic T lymphocyte-associated antigen 4.

heart (10). In recent years, several studies have consistently reported the incidence of cardiac-related toxicities such as myocarditis, athero-cardiovascular disease, and heart failure in the setting of PD1 ICI therapy among cancer patients (11–14).

The possible theories explaining the rationale of the occurrence of cardiac adverse events with ICI therapy among cancer patients have previously been explored in experimental animal models. It was found that PD1 and PDL1 upregulation had a cardioprotective role for cardiomyocytes; therefore, their blockades or inhibition with PD1 ICI would favor cardiomyocyte damage. Many research papers also reported that there can be an increased likelihood of rare but aggressive cardiotoxic events associated with the use of the PD1 inhibitor subclass of ICI compared with other ICIs. However, because PD1 ICI drugs were found to be safer in terms of the occurrence of other high-grade iRAes, they are being more frequently used than the rest. This gave rise to the need for establishing a balance between this novel oncology treatment efficacy and the burden of drug-adverse cardiac effects among treated patients. However, to our knowledge, no studies have yet been published directly to compare the risk of cardiotoxicity events between nivolumab and pembrolizumab, the two most commonly encountered PD1 ICI drugs used in cancer management. Logically, identification of the drug with a higher tendency for cardiac toxicities than others would be essential to helping specialist caregivers to select the better alternative for patients, taking into account both efficacy and cardiotoxicity indexes in their management and thereby possibly lessening the burden of PD1 ICI-induced cardiotoxicity.

1.1 Objectives

The primary aim of this study was to determine by means of a meta-analysis whether pembrolizumab or nivolumab would be associated with increased cardiotoxicity risk.

2 Materials and methods

This meta-analysis was conducted in accordance with the MOOSE reporting guidelines (15).

2.1 Types of participants

Patients with cancer who were receiving ICI treatment and whose cancer characteristics met the criteria for either pembrolizumab or nivolumab monotherapy, or both, were considered, and those who reported any cardiac pathologies after the start of monotherapy were categorized as events (cases).

2.2 Search methods for identification of studies

Relevant literature was obtained by searching PubMed, Embase, and Google Scholar, and an advanced search tool was used to restrict results to only human studies published between 2016 and 1/10/2022. The reference lists of some results were manually retrieved and screened to identify any study that can meet the inclusion criteria.

2.3 Electronic searches

All electronic searches were made on the Google Chrome version 105.0.5195.127 software.

The search entry terms were as follows: Keytruda or Pembrolizumab; PD1 inhibitors; anti-PD1 drugs; Nivolumab or Opdivo; cardiotoxicities or cardiac toxicity; and toxicities.

Our initial search strategy on PubMed was (((Keytruda) OR (pembrolizumab)) AND ((cardiotoxicities) OR (cardiac toxicity))) AND (((Opdivo) AND (Nivolumab)) AND ((cardiotoxicities) OR (cardiac toxicity))), which yielded few results. Therefore, another search strategy was implemented using (immune checkpoint inhibitors) AND (cardiotoxicity), which produced more results and was replicated in Embase and Google Scholar as well.

Reports containing the search terms were screened based on the relevance of their title and abstract; eligibility was assessed based on whether the studies addressed the issue of immune checkpoint-related cardiotoxicity. Therefore, irrelevant content was discarded according to the author's own opinion and purpose. Then, the full-text quality of the remaining articles was assessed to determine whether they would contribute to the study's aim.

2.4 Selection of studies

Study selection was done by two authors and was based on the following criteria:

i) Original studies: including prospective and retrospective studies that reported the cardiotoxicity due to ICI therapy in cancer patients irrespective of the type of cancer.
ii) Significant sample, VigiBase studies including all the cardiotoxicity reports after ICI therapy in cancer patients were also considered.
iii) Eligible studies should have disclosed any cardiac side effects that occurred during the course of pembrolizumab or nivolumab monotherapy.
iv) Eligible studies had to provide basic demographic data on the included participants.

2.5 Exclusion criteria

Case reports and case series were excluded because they did not provide the total number of patients treated with pembrolizumab and nivolumab monotherapy.

Because of the comparative nature of this study that targeted PD1 inhibitors, studies providing data only for subclasses of ICI not

including PD1 inhibitors or those including only one of the PD1 inhibitors (pembrolizumab or nivolumab) were excluded.

2.6 Quality assessment

To assess the quality of each included paper, the Newcastle–Ottawa Scale (NOS) scoring based on 10 points was implemented. A study was considered of good quality when the NOS score was >6; otherwise, it was considered of low quality.

2.7 Measures of comparator effect

The outcome of interest was the overall number of reports of cardiac side effects including myocarditis, pericarditis, heart failure, arrhythmia, coronary events, and major adverse cardiovascular events (MACE). For each study, the descriptive proportion of ICI-induced cardiotoxicity attributable to each drug and the probability of developing cardiotoxicity among the subset of patients treated with each drug was determined for description purposes, by the following formula.

$$cardiotoxicity\ \ proportion(x) = \frac{number\ \ of\ \ events\ \ imputed\ \ to\ \ x}{total\ \ number\ \ of\ \ events\ \ from\ \ all\ \ ICI}$$

$$cardiotoxicity\ \ probability(x) = \frac{number\ \ of\ \ cardiac\ \ events\ \ imputed\ \ to\ \ x}{total\ \ number\ \ of\ \ participants\ \ treated\ \ with\ \ x}$$

(Where (x) represents either nivolumab or pembrolizumab)

To compare the means of the cardiotoxicity proportions and probabilities between the two drugs, a one-tailed, P-value Student's *t*-test for independent samples was used. Given the retrospective nature of the study, the odds ratio on the random and fixed effect modality was estimated with its 95% confidence interval as the outcome measure effect of the meta-analysis (16).

2.8 Data extraction and management

Data were collected by the first author and cross-checked by the second author and then extracted into a Microsoft Excel file. For each included study, the collected data for the analyses were as follows: the name of the first author, the year of publication, patients' mean age (when available), type of malignancy, presence of cardiovascular risk factors, medical history, type of cardiotoxicity reported, total number of cardiotoxicity reports, number of patients treated with pembrolizumab, number of pembrolizumab-induced cardiotoxicity cases, number of patients treated with nivolumab, and its corresponding number of cardiotoxicity-induced cases. If an article did not provide the total number of patients treated for each drug but provided reporting the odds ratio and the number of events (cases), the number of treated patients was estimated by deducting from the ROR and related formulas provided within the study. For vigilance studies, the total number of each drug's adverse effect was considered as the total number of patients treated and the number of cardiac adverse effects was used as events.

2.9 Subgroup analyses

Subgroup analyses were performed to investigate the source of heterogeneity among studies; therefore, studies were grouped according to several criteria:

- According to the grading of the study's quality: based on their NOS score, studies were grouped into low and good quality.
- According to the report of cardiovascular disease history: grouped as (yes or no).
- According to the type of malignancy: we had the subgroup of studies reporting only on lung cancer (labeled OL) and studies reporting on lung cancer and other cancers (labeled ALL).
- According to the type of cardiotoxicity: we included studies that reported only on myocarditis, and the group reporting and myocarditis plus others such as pericarditis and arrhythmias.

2.10 Assessment of risk of publication bias

Publication bias was assessed by a funnel plot associated with Egger's test.

2.11 Sensitivity analysis

The robustness of the findings were explored with the "*cop*" argument in the "*metasens*" package in R statistical software. The Copas selection model described in Copas and Shi (2001) (<DOI: 10.1177/096228020101000402>) evaluates the sensitivity of meta-analysis, helping to determine the possible selection bias.

Statistical analyses were performed with Microsoft Office Excel 2016, and meta-analysis calculations were achieved with R statistical software version 4.1.3 (2022-03-10) ("One Push-Up" Copyright (C) 2022 The R Foundation for Statistical Computing Platform: i386-w64-mingw32/i386). We used the Cochran's Q statistic to estimate statistical heterogeneity and the I^2 statistic to quantify inconsistency. The assumption of homogeneity was considered invalid if $P< 0.10$.

3 Results

3.1 Results of the search

The search results are detailed in the flowchart below (Figure 1). A total of 1,368 records were identified by searching the database, of which 15 were deemed eligible for this analysis.

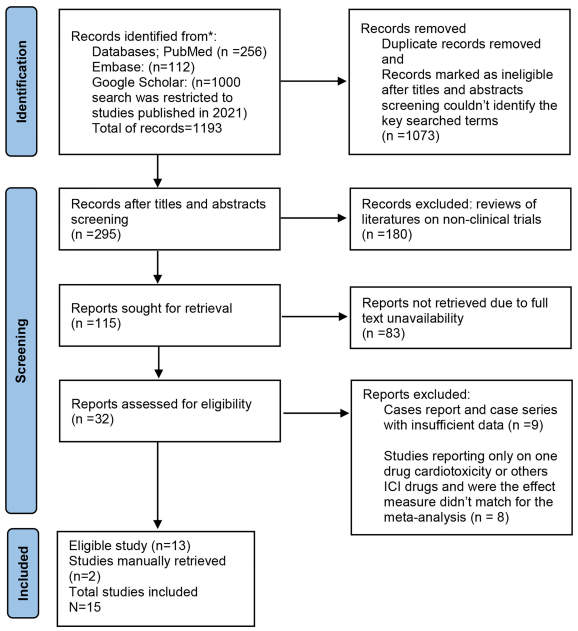


FIGURE 1
Prisma flow diagram.

For this meta-analysis, a total of 15 observational studies that appeared between 2018 and 2022 were included. The total number of participants in those studies was 7,517,257 (Table 1), and the mean age was estimated to be around 66 years. These participants all had malignancies that had been clinically diagnosed and confirmed, such as lung cancers, melanomas, Hodgkin lymphomas, endocrine cancers, and renal cancers, with lung cancers accounting for >60% of all cases (Table 1). Anti-PD1, anti-CTLA4, and anti-PDL1 were the immunotherapeutic drug classes reported in each study. Following monotherapy or combination therapy, a total of 18,833 ICI-induced cardiac adverse events were reported.

The total number of ICI-induced cardiotoxicity attributable to nivolumab was 55.7%, whereas that for pembrolizumab was for 27.31% (Figure 2). The difference in mean cardiotoxicity proportion between nivolumab and pembrolizumab was statistically significant (P = 0.027). Based on the available data, patients treated with pembrolizumab had a 4.6% chance of developing cardiotoxicity after treatment, whereas those treated with nivolumab had a 7.1% chance of developing cardiotoxicity, but the difference in cardiotoxicity probability means between the two drugs was not statistically significant (P = 0.28) (Table 2).

3.2 Meta-analysis results

The meta-analysis conducted with the recommended Mantel–Haenszel method when explored with the random-effect model for dichotomous outcome variables (16, 17) comparing the cardiotoxicity odds ratio between pembrolizumab and nivolumab yielded a pooled OR of 0.7347 (95% CI: 0.4371–1.2348, P = 0.24) (Figure 3), with considerable heterogeneity (I^2 = 99%, P_h = 0.00), showing that the difference between the risk of cardiotoxicity reports between pembrolizumab and nivolumab was not statistically significant. However, it would be significant for

TABLE 1 Characteristic of included studies.

Study	year	pem_eve	pem_tot	nivo_eve	nivo_tot	NOS_scale	Mean age	CVHX	typ_ca	cardiotoxity	total	C.Reports
Mahmood &al	2018	11	52	7	60	6	65	yes	OL	myocarditis	140	35
Shiori &al	2020	15	2148	24	4419	8	NA	no	A	others	4419	45
Serena&al	2020	7	15	4	8	8	68	yes	A	myocarditis	30	13
Qian &al	2019	69	493	125	968	7	65	no	A	myocarditis	43147	315
Nida&al	2021	22	123	66	217	8	62	yes	A	others	424	424
Nestor&al	2021	1013	25597	2014	46767	5	65	yes	A	others	13646	4401
Melissa&al	2020	4	47	19	137	7	64	no	OL	others	196	23
Joe et al	2018	43	10321	100	10321	6	NA	no	A	others	3121	1073
Chitturi&al	2019	6	45	10	71	10	68	yes	A	others	135	30
Chenxin &al	2021	2808	46251	6836	78047	6	NA	no	A	others	7443137	9271
Anna&al	2021	805	22378	1076	2791	6	69	no	A	others	2478	2478
Chenglui &al	2022	324	2181	196	1766	9	62	yes	A	myocarditis	5518	691
Zach&al	2022	17	243	8	220	8	65	yes	A	others	538	26
Maria&al	2020	1	8	3	52	8	70	no	OL	others	60	4
Luke &al	2021	0	39	2	37	7	71	yes	A	others	268	4
total		5145	109941	10490	145881		66.16667				7517257	18833

pem_eve, number of cardiotoxicity report with pembrolizumab as ICI monotherapy; pem_tot, number of patients treated with pembrolizumab; nivo_eve, number of cardiotoxicity reports with nivolumab as ici monotherapy; nivo_tot, patients treated with nivolumab; NOS, Newcasttle Ottawa scale; cvhx, history of cardiovascular disease; typ_ca, type of cancer; OL, only lung cancer, A, lung and others cancers such as melanoma, renal cancer, lymphoma,metastasis; c.reports, total number of ici induced cardiotoxicity cases.

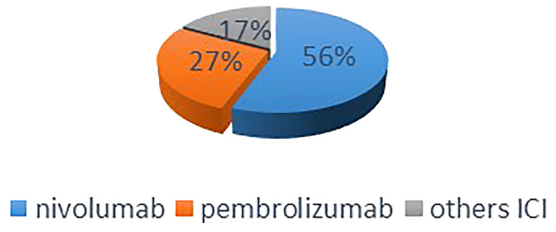


FIGURE 2
This pie chart shows that in overall included studies, nivolumab monotherapy accounted for half percent of the total ICI-induced cardiotoxicities.

pembrolizumab on the common (fixed model) OR = 0.5975 [0.5769–0.6190], P< 0.0001, in favor of pembrolizumab. Unfortunately, the fixed-effect model cannot be considered here because of the wide variability of effect measures among the included studies. Because of the high heterogeneity (I^2 = 99%), the pooled reporting odds ratio should not be given much consideration. Subgroup analyses were then performed as presented in the next subsection.

3.3 Subgroup analyses

Based on the data that was made available, subgroup analyses were carried out according to the following parameters: study quality (good and low); type of malignancy (only lung and all); presence of cardiovascular history or risk factors (yes and no); type of malignancy (myocarditis and other); and subgroup analysis results. High heterogeneity was present in the subgroups of "low quality," "other cardiotoxicities," and "all cancers," whereas moderate-to-low heterogeneity was present in the subgroups of "good quality," "only lung cancers," and "myocarditis." However, apart from the myocarditis group, where a higher risk of cardiotoxicity report was linked to pembrolizumab therapy (OR = 1.30 [1.07–1.59], P< 0.05) (Table 3 in the annex), the results among other subgroups remained insignificant.

3.4 Results of publication bias assessment

The funnel plot obtained displayed obvious asymmetry that was confirmed by linear regression Egger's test (P = 0.9963), indicating the strong presence of publication bias among the studies (Supplementary File).

3.5 Sensitivity analyses

The overall sensitivity analysis revealed that even when selection bias was assumed, the OR and its 95%CI did not vary significantly. The reliability of this meta-analysis is demonstrated by an unadjusted OR = 0.7347 (95% CI: [0.4371; 1.2348)], P = 0.2445, which did not differ much from the adjusted OR = 0.7328 (95% [0.4427; 1.2128] P = 0.2265. Moreover, the test for residual selection bias yielded a P-value = 0.4338.

TABLE 2 Ici induced cardiotoxicity proportion attributable to pembrolizumab and Nivolumab.

Study	year	pem_eve	pem_tot	nivo_eve	nivo_tot	quality_grade	Study design	total	C.Reports	Propor_P	Propor_N2	PROBP	PROBN
Mahmood &al	2018	11	52	7	60	low	analytic	140	35	0.314286	0.2	0.211538	0.116667
Shiori &al	2020	15	2148	24	4419	good	descriptive	4419	45	0.333333	0.533333	0.006983	0.005431
Serena&al	2020	7	15	4	8	good	analytic	30	13	0.538462	0.307692	0.466667	0.5
Qian &al	2019	69	493	125	968	good	descriptive	43147	315	0.219048	0.396825	0.139959	0.129132
Nida&al	2021	22	123	66	217	good	analytic	424	424	0.051887	0.15566	0.178862	0.304147
Nestor&al	2021	1013	25597	2014	46767	low	descriptive	13646	4401	0.230175	0.457623	0.039575	0.043065
Melissa&al	2020	4	47	19	137	good	analytic	196	23	0.173913	0.826087	0.085106	0.138686
Joe et al	2018	43	10321	100	10321	low	descriptive	3121	1073	0.040075	0.093197	0.004166	0.009689
Chitturi&al	2019	6	45	10	71	good	analytic	135	30	0.2	0.333333	0.133333	0.140845
Chenxin &al	2021	2808	46251	6836	78047	low	descriptive	7443137	9271	0.30288	0.737353	0.060712	0.087588
Anna&al	2021	805	22378	1076	2791	low	descriptive	2478	2478	0.324859	0.434221	0.035973	0.385525
Chenglui &al	2022	324	2181	196	1766	good	analytic	5518	691	0.468886	0.283647	0.148556	0.110985
Zach&al	2022	17	243	8	220	good	analytic	538	26	0.653846	0.307692	0.069959	0.036364
Maria&al	2020	1	8	3	52	good	analytic	60	4	0.25	0.75	0.125	0.057692
Luke &al	2021	0	39	2	37	good	analytic	268	4	0	0.5	0	0.054054
total		5145	109941	10490	145881			7517257	18833	0.273191	0.557001	0.046798	0.071908

Propor_p, cardiotoxicity proportion attributable to pembrolizumab; Propor_N, cardiotoxicity proportion attributable to Nivolumab; PROBP, probability with pembrolizumab; PROBN, probability with Nivolumab.

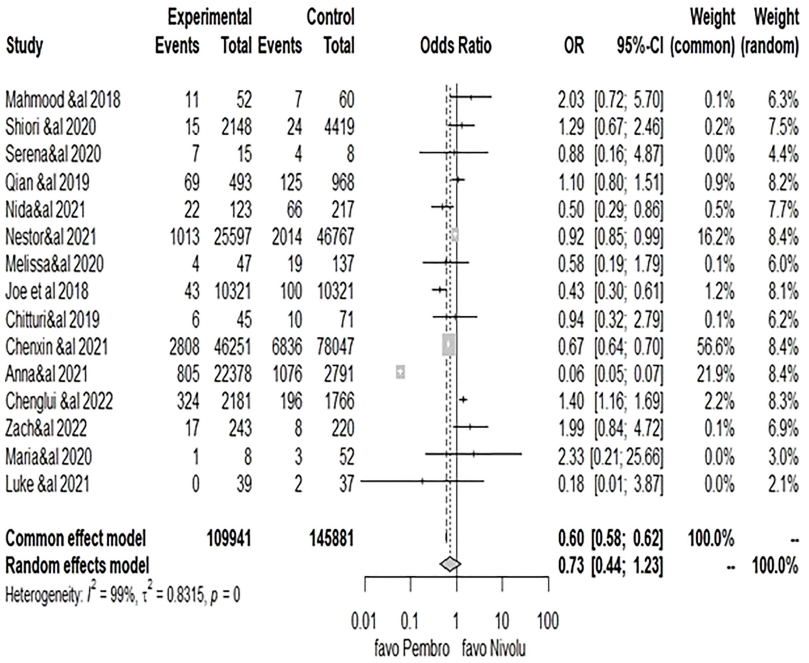


FIGURE 3
Forest plot of comparison of pembrolizumab vs. nivolumab, outcome: 1.1 cardiotoxicity odds ratio.

TABLE 3 Summary of meta-analyses and subgroup analysis.

Analysis modality	OR, 95%CI, P-value	I^2, P_h	observations
Over all analysis	0.7347 95%CI [0.4371-1.2348] P=0.24 (R.E)	I^2=99%, P_h=0	There could be a decrease risk of cardiotoxicity associated with Pembrolizumab than Nivolumab but the association was not statistically significant
Subgroup analysis based on the quality of included studies; Low quality(5 studies included)	OR=0.48[0.15; 1.52], P>0.005 OR=1,04 95%CI [0.75;1.45]	i^2=100%, P_h=0 i^2=50%, Ph=0.04	In the low quality group the cardiotoxicity risk appeared reduced with pembrolizumab but the result was not statistically significant and the heterogeneity was very high with In the good quality subgroup there was a non significant increased of cardiotoxicity with pembrolizumab than Nivloumab and the heterogeneity was moderate and tolerable
Good quality (10 studies included)			
Subgroup analyses based on the type of cancers Only lung Cancer(3 studies included)	OR=1.24 [0.46;3.32], P>0.005 OR=0.67 [0.37;1.19], P>0.05	i2=31%, Ph=0.24 i2=99%, Ph=0	although the assumption of homogeneity was kept within the (OL) subgroup, the results were still not significant but seemed to favour Nivolumab. Within the (A) subgroup the heterogeneity was considerable and the result remained not significant with tendency to favour Pembrolizumab.
All cancers(12 studies included)			
Subgroup analyses: presence of cardiovascular history YES(8 studies included)	OR=1,05 [0.72;1.63], P>0.05 OR=0.54 [0.22;1.31], P>0.05	i2=75%, Ph=0.01 i2=100%, Ph=0.0	Both subgroups did not resolve the heterogeneity issue and still reported a non statistically significant result. The "yes" subgroup favoured Nivolumab while the "No" subgroup favoured Pembrolizumab
NO(7studies included)			
Subgroup analysis: type of cardiotoxicity Myocarditis (4 studies included)	OR=1.30 [1.07;1.59], P<0.05 OR=0.59 [0.31;1.14], P>0.05	i2=0%, Ph=0.4 i2=100%, Ph=0.0	within the "myocarditis" subgroup the assumption of homogeneity was kept and the result was significant and favoured Nivolumab induced cardiotoxicity
Others			

4 Discussion

Our analyses found that the proportion of cardiotoxicity credited to nivolumab was the highest among ICI drugs; moreover, the probability of developing cardiotoxicity for someone treated with pembrolizumab was slightly lower than if the drug was nivolumab, but the difference was not statistically significant. However, although the results of the overall meta-analysis seemingly depicted a trend that favored pembrolizumab over nivolumab, it remained statistically insignificant even after performing subgroup analysis. The results remained inconclusive, and we observed that the considerable heterogeneity noted can have been because of the quality of studies, their designs, the great variation of outcome effect size, sample size, and other confounding factors such as cardiovascular history and type of cancers. With the exception of a subset of patients whose cardiotoxicity type was myocarditis, it can be observed that pembrolizumab therapy had a greater risk for cardiotoxicity than nivolumab.

Programmed cell death receptor inhibitor 1 is a protein found on T cells, which can bind to another ligand called PDL-1, thus preventing the T-cell-mediated destruction of other cells (18). Based on this mechanism, inhibitor agents for PD-1 and PDL-1 proteins have been manufactured to increase the T cells' ability to fight cancer cells and increase overall survival (19, 20). Furthermore, there has been substantial objective improvement in cancer outcomes with the increased use of these novel agents, and it has now become a trend in the oncology field (21–23). ICI drugs from the PD1 receptor inhibitor subclass are well known to be associated with side effects such as pneumonitis, pruritus, and neurological, endocrine, and gastrointestinal adverse effects, although it has been observed that the frequency of the irAEs was relatively lower than with the other subclasses (23–25, 51). However, the PCD receptor inhibitor subclass has recently been identified in previous studies as bearing a certain cardiotoxicity adverse effect potential, albeit accounting for<5% of all adverse effects.

The cardiotoxicity spectrum has in majority been represented by myocarditis (26, 27), but other cardiac conditions can also be observed such as arrhythmias, pericardial effusion, heart failure, coronary events, pericarditis, and heart block (28). Existing case studies and pharmacovigilance data showed that the irAEs mainly affect cardiac conduction and myocyte function, which would then lead to heart damage (29, 30). It also appears that cardiotoxicity incidences are more observable among patients undergoing combination therapy (31). The routinely and broadly used PD1 ICI are pembrolizumab and nivolumab. Hence, the core objective was to identify whether there were any differences between them regarding cardiac adverse effects despite belonging to the same subclass. Can those differences also be related to their clinical efficacy, structure, mechanisms of action, spectrum, and frequency of side effects? Moreover, with regard to the cardiotoxic adverse effects, which one can be better than the other? The two drugs belong to the same subclass and are used for similar therapeutic indications, the most common being non-small cell lung cancers, melanoma, and metastasis (20). However, from a previous report, it seemed as though nivolumab was potentially a more cardiotoxic ICI, as roughly 60% of cardiotoxicity reports in cancer-treated patients were associated with nivolumab (32, 33) in mono or combination therapy regimen, but this was observed more in studies in which the quality on the NOS scale was not satisfactory; however, recent cohort and case–control studies (also included in this meta-analysis) rather reported an increased risk associated with the use of pembrolizumab (see Zachary et al., 2022). The results after pooling all the included studies showed that although there were more reports of cardiotoxicity for nivolumab, the difference in cardiotoxicity odds ratio between pembrolizumab and nivolumab was not statically significant. This was consistent with some previously reported results. One study that aimed to compare the efficacy of the two drugs (50) reported in their survival analysis that despite the observation of a higher objective response rate with pembrolizumab than nivolumab, there was no significant difference in the progression of free survival between the patients treated with pembrolizumab and those treated with nivolumab (22). Another investigation on the overall incidence and risk of irAE between the two drugs (95% CI: 0.97–1.79) indicated that the difference was not statistically significant (33, 34). Moreover, there were no significant discernible differences in the mechanism of action of the two drugs: pembrolizumab and nivolumab are both humanized IgG4 monoclonal antibodies against PD-1, but with the distinction that they do not induce antibody-dependent cellular cytotoxicity, as would be the case for normal IgG antibodies; hence, their toxicities and side effects have been more characterized as immune-related than cytotoxic (35). Analysis of the included studies showed that nivolumab had the highest proportion of induced cardiotoxicity among all ICI-induced cardiotoxicities (13). In addition, the majority of patients with reports of cardiotoxicity had other factors such as conventional cardiovascular risk factors or cardiovascular history. Although this may explain the frequency of reports of cardiotoxicity, it cannot explain the large disparity in the proportions of cardiotoxicity between the two drugs, given that the distributions of cardiovascular risk factors or the history of the two groups were comparable (36). In this study, cardiotoxicity refers to any cardiac damage irrespective of extent or severity. The cardiotoxicity odds ratio between pembrolizumab and nivolumab was not statistically significant; however, this does not imply that there would not be any difference at all. Therefore, after consideration of the descriptive proportions reported for both drugs, it can be suggested that nivolumab is associated with more cardiotoxicity events than pembrolizumab. One plausible explanation for this finding can be that nivolumab was introduced first and gained recognition first before pembrolizumab. Another possible theory would be that because in general, the overall number of cases with cancer expressing high PDL1 (expression: >50%) seems to be lower than that of cases with a low PDL1 expression (expression: 1–49%). Per standard recommendation, pembrolizumab is known to be effective

in tumors expressing PDL1 at >50% and nivolumab in tumors expressing PDL1 at >1%. Therefore, there would naturally be more cases (low PDL1 expression) of cancer treated with nivolumab than with pembrolizumab. All these could have led to more reports of cardiac toxicity events with nivolumab than with pembrolizumab. Additionally, the small number of included studies and the high degree of heterogeneity between studies may have also played a role in the lack of statistical significance from the pooled odds ratio of cardiotoxicity effect between the two drugs. A few case series on ICI-induced cardiotoxicity also highlighted that cardiomyopathy, myocarditis, and conduction abnormalities were being underreported, which could have as well influenced the overall effect result of the current analysis (28). Another possibility can be that those considered to be developing cardiac toxicity were patients with cardiac clinical symptoms, resulting in non-involvement of the subclinical cases, which could have significantly influenced the results.

Numerous studies have shown that despite being relatively uncommon and few, the majority of myocarditis cases would present during the acute phase of therapy, with a propensity for seriousness and mortality, or the development of MACEs such as cardiac arrest, cardiac death, or stroke, but respond well to high corticosteroids for remission when administered in a timely manner (37–39). Although cardiac irAEs with ICIs are uncommon, the increased rate of mortality seen is an important factor to consider, as was also highlighted in another similar analysis (27). According to Dolladile et al.'s 2020 study, heart failure with left ventricular systolic dysfunction was seen among cases as a late adverse event. Therefore, patients treated with ICI should be monitored for at least 304 days. Additionally, because silent toxicities are possible (toxicity that manifests slowly before symptoms become obvious), such patients should also undergo routine cardiovascular screening for early detection of any abnormalities, especially for those aged >65 years and presenting at least two conventional cardiovascular risk factors or cardiovascular history (40). Nevertheless, the presumed advantages of early detection of cardiotoxicities through active screening, serial electrocardiograms, troponins, BNP, and echocardiography (which are helpful tools for the detection of subclinical cardiotoxicity during oncology therapy) should take into account the cost of testing as well as the possibility of false results, incorrect interpretation, and other related errors (31, 41). In a study, the highly sensitive troponin's prognostic value showed that a value higher than 14 ng/L before the administration of pembrolizumab was significantly associated with a high incidence of MACE, including stroke and cardiac death (42). However, this does not necessarily mean that highly sensitive troponin should be considered an eligibility criterion for pembrolizumab or nivolumab therapy, but rather it can be useful as a predictor of cardiotoxicity risk.

This study has some limitations. The included studies were observational in nature, with some data collected from electronic and registered databases, implying a high susceptibility to information and selection biases (43). Selection bias was a concern in the (39) study, which was of a retrospective design, because there was no prospective cardiovascular screening protocol across all sites, and screening for cardiac biomarkers and other tests was left to the discretion of each individual care provider. (26) study was distinguished by the small sample size, which resulted in confounder interference and reporting bias. The criteria for control groups in two of the included studies were dubious, making it difficult to select a group of cancer patients with similar cardiovascular comorbidity and who underwent adequate testing to exclude cardiac pathology, as controls (28, 44). The criteria for selecting pembrolizumab or nivolumab for patients were not detailed or obvious in the results of the included studies. The risk of bias across the included studies could not have negated the evidence found in each study, but it could have led to an underestimation of association or effect size. According to two VigiBase analyses, pharmacovigilance analyses generate hypotheses that must be tested, ideally in prospective studies. Adverse Reaction Reporting System databases may be biased given the significant overlap of Individual Case Safety Reports (ICSRs) between databases. In addition, some cases may not have been reported to State drug enforcement authorities (34). The only included prospective cohort study had a short follow-up period, which may have led to a limited number of events and biased interpretation of results (25). In addition, randomized clinical trials on cardiotoxicity issues were not available at the time of the search, resulting in a limited number of studies, small sample sizes, and low statistical significance. Moreover, unpublished records on the topic as well as articles published in languages other than English cannot be evaluated. To our knowledge, this is the first meta-analysis to directly compare the two drugs, and hence, these results are still important, because they highlight the need for additional, in-depth research on this topic in multiethnic, large-center settings to provide oncology patients with the best possible care while reducing the likelihood of cardiotoxicity.

5 Conclusion

Immune checkpoint inhibitors have revolutionized the treatment of advanced-stage cancers including metastases; however, the potential danger to vital organs (52) such as the heart cannot be overlooked. Therefore, it is paramount to look at every strategy with the potential to limit, reduce, or control the magnitude of this issue. Previous studies regarding the cardiotoxicity risks of ICIs and comparisons between anti-PD1 and anti CTLA4 (45) were made; however, this paper addressed the direct comparison between nivolumab and pembrolizumab cardiotoxicity potentials. Contrary to what was recently reported (46, 47), the descriptive proportions described herein have provided a clear indication that nivolumab-induced cardiotoxicities are reported more in the literature over the past years than pembrolizumab-induced cardiotoxicities. The discrepancy between these findings and previous ones highlights the need for

a prospective analysis on a larger sample cohort. However, the consensus on the need for proper cardiac screening before and after remains strong among researchers (48, 49). Therefore, the importance of multidisciplinary collaboration between oncologists, immunologists, and cardiologists in the management of cancer patients cannot be overemphasized.

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.

Author contributions

This manuscript was compiled and written by FN under the direction, guidance, and supervision of Z-QW. Proofreading and editing were done by CM. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1080998/full#supplementary-material

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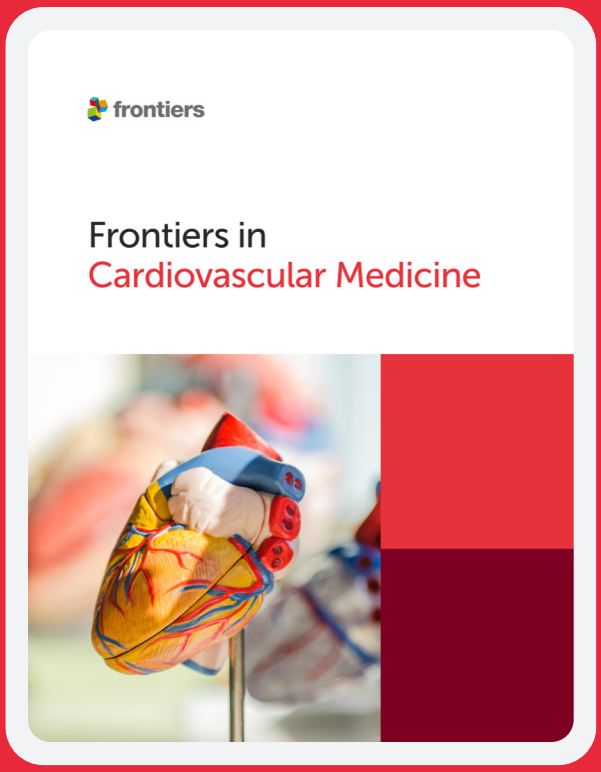

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