



# Cephalotoxins: A Hotspot for Marine Bioprospecting?

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Molluscs provided one of the pioneering approved pharmaceuticals from the seas: the painkiller ziconotide, developed from an  $\omega$ -conotoxin isolated from cone snails. As marine biotechnologists are turning towards the immense range of novel bioproducts from marine invertebrates, little attention has been given to cephalotoxins, a group of obscure proteinaceous toxins produced by the salivary glands of coleoids, i.e., octopuses, squids and cuttlefishes. These toxins, for which there is empirical evidence for acting as immobilisers at least against crustaceans, are proteinaceous substances among the many that comprise the venomous mixtures secreted by these animals. Despite the ecological and economical importance of cephalopods, little is known about cephalotoxins, beginning with the actual span of taxa that secrete them. Indeed, cephalopods are long suspected for producing specific toxins as part of their predation and defence mechanisms, making them a promising group of marine animals for the bioprospecting of novel compounds. Despite scant or absent toxicological or otherwise experimental evidence for their bioreactivity, advances in “omics” methods have shed some light in the molecular structure of cephalotoxins. There are reports of cephalotoxins being complex glycoproteins that take part in a myriad of novel compounds being produced by the salivary glands. Still, there is no consensus of cephalotoxins being a conserved form of proteins. As Blue Biotechnology and marine bioprospecting for novel bioreactives are gaining momentum, the present review will provide the state-of-the-art on cephalotoxins, highlighting old and new research and existing gaps in the current knowledge.

**Keywords:** marine biotechnology, marine toxins, cephalopods, bioprospection, novel bioproducts, omics

## INTRODUCTION

Marine toxins are likely as diversified as the oceans’ immense biodiversity and result from co-evolutionary processes between the organisms that secrete them and their target recipients, whether prey, predators or parasites. Along with other types of natural products, these substances are considered to hold high-value bioproducts due to their potential specificity against molecular receptors and metabolic pathways. Many toxins from animals are peptides or high molecular weight proteins that interact with specific enzymes and ion channels (e.g., sodium and/or potassium ion channels), thus affecting, for example, neuromuscular, cardiovascular and immune systems (Zhang, 2015; Ponte and Modica, 2017). For these reasons, toxinology has been rising as a promising field with direct implications for drug discovery, novel therapeutic alternatives and even ecologically

friendly antifoulants and pesticides. Although still lagging behind research on their terrestrial counterparts, such as snakes, bees and scorpions, marine toxinology is on the rise as researchers and bio-based industrialists become increasingly aware for the uncanny value of the oceans as an almost limitless source of novel bioproducts (see for instance Molinski et al., 2009; Leal et al., 2020).

Venoms are complex mixtures of toxins, enzymes, peptides and salts, which renders molecule isolation, identification and determination of substance-specific bioreactivity challenging. Their efficacy results from the interaction of these compounds, not just from toxins as noxious agents but also from permeabilisers like proteolytic enzymes. Still, several venomous or poisonous species of snakes, lizards, and leeches have already been used to produce toxin-based drugs for clinical applications directed against disease-induced pain (e.g., from osteoarthritis and multiple sclerosis), as anticoagulants for specific coronary interventions and also for hypertension or specific types of diabetes (see Bordon et al., 2020 for a review). Additionally, studies on wasp venoms are revealing promising alternatives for traditional antibiotics and even as antiviral and anti-seizure therapeutics (Vila-Farrés et al., 2012; Sample et al., 2013; Castro e Silva et al., 2020). In turn, pushed by the recent premises of the “Blue Growth” revolution, the last decade witnessed a growing interest in marine bioproducts, toxins included. Indeed, marine ecosystems are nowadays acknowledged to be a highly valuable source of pharmaceutically active toxins (see Greener, 2020). Notwithstanding, marine invertebrates are considered the most promising group of animals possessing toxins in face of their richness. Marine animals such cnidarians (jellyfishes, sea anemones, hydrozoans), echinoderms (starfishes, sea urchins), annelids (polychaetes), nemertines, bryozoans (moss animals), sponges, tunicates and molluscs (gastropods, cephalopods) stand out as toxin-secreting eumetazoans (see Kem, 2005; Zhang, 2015; Rodrigo and Costa, 2019). As examples, recent investigations highlighted bryostatins, which are heterocyclic molecules produced by bryozoans, and other cyclic peptides produced by endosymbiotic micro-organisms from some tunicates, as high-potential compounds for cancer therapeutics (Kem, 2005; Watters, 2018). As yet another example, clinical trials on toxic peptides from sun anemone have been revealed favourable results for the treatment of autoimmune diseases, due to their effects as strong potassium channel blockers (Prentis et al., 2018). However, the most prominent example among biotechnological applications of marine bioproducts is the approved painkiller ziconotide (commercialised as Prialt), developed from a  $\omega$ -conotoxin found in the venom of the cone snail *Conus magus* (Williams et al., 2008). This case has recently been followed by trabectedin (Yondelis), an anti-cancer quinoline first isolated from a tropical tunicate (Cuevas and Francesch, 2009), which, albeit not primarily a toxin, yields leverage to drug development from marine bioproducts.

As more species of marine invertebrates join the ranks of toxin-secreting marine animals, attention is turning to one of the oldest-known venomous group of molluscs, the cephalopods. Perhaps surprisingly, only a few toxins have yet been described for these predators but the broad range of bioactive compounds

in their venomous saliva, which includes neurotoxic proteins and peptides, amines and permeabilising enzymes, yields high promises for marine bioprospecting (see Cooke et al., 2017). Recent advances in marine “venomics” (see von Reumont et al., 2014) can shed new lights on these obscure toxins. Cephalotoxins, in particular, appear to be a unique class of toxins whose exact nature remains elusive even though their discovery dates from the mid-XX century. In this review we will summarise the scant knowledge on these toxins that are produced by the salivary glands of cephalopods, endeavouring a fresh biotechnological perspective for the bioactives secreted by these important and ubiquitous animals.

## CEPHALOTOXINS AND OTHER COLEOID TOXINS

The interest on cephalotoxins, albeit when they were not termed as such, rose in the late XIX century when Lo Bianco (1888) studied the toxicity of octopus saliva to crabs. These observations showed that after injecting secretions from octopus’ posterior salivary glands directly into crab gills, the target experienced hindered locomotion, progressing rapidly towards complete immobilisation and eventually death (see also Ghiretti, 1960). The active substance that causes this paralysing action was later purified by Ghiretti (1959) from the posterior salivary glands of *Sepia officinalis*, identified as a proteinaceous compound and called cephalotoxin. From that time onwards, the composition of salivary secretions of cephalopods gained attention and several constituents have since been isolated and identified, with emphasis on neurotoxins and neuropeptides (Table 1). It is now clear that the complexity of these secretions is reflected in a broad range of biologically active compounds, proteinaceous or not. This includes biological amines, namely tyramine, histamine, *p*-hydroxyphenyl-ethanolamine (octopamine), 5-hydroxytryptamine (5-HT or serotonin), noradrenaline or dopamine (Henze, 1913; Bottazzi and Valentini, 1924; Erspamer, 1948; von Euler, 1952; Erspamer and Asero, 1953; Hartman et al., 1960). Several enzymes have also been identified in cephalopod saliva, such as hyaluronidase, chitinase, phospholipase A2, peptidase S1, metalloprotease, carboxypeptidase (Romanini, 1952; Fry et al., 2009; Cornet et al., 2014; Whitelaw et al., 2016), as well as tachykinins (Eledoisin, OctTK-I, and OctTK-II) and CAP domain- (CRISP [Cysteine-rich secretory proteins], Antigen 5 [Ag5], and Pathogenesis-related [PR-1]) -bearing proteins (Erspamer and Anastasi, 1962; Kanda et al., 2003; Fry et al., 2009). However, the number of cephalopod species covered in terms of its toxin identification is still acknowledged to be very low (Cooke et al., 2017). From both Superorders of Class Cephalopoda, Octopodiformes accounts for the highest number of investigated species (about 14 from a total of 300). As for Decapodiformes, i.e., cuttlefishes and squids, not even 2% of all known species have been studied for the characterisation of salivary gland secretions. The number of species for which there is detailed information on cephalotoxins is even smaller. The octopuses *Octopus vulgaris*, *Hapalochlaena maculosa*, and *Eledone cirrhosa*, were the first to have  $\alpha$ -

**TABLE 1** | A comparative overview cephalotoxins and other major proteinaceous or peptidic neurotoxins considered specific of cephalopods.

Species	Toxin name	Molecular weight (kDa)	Isoelectric point	Known mode-of-action/ Bioreactivity	Post-translational modifications	References
<i>Doryteuthis (Amerigo) pealeii</i> <sup>a</sup>	Unknown cytolytic protein	–	–	–	–	Kem and Scott, 1980
<i>Eledone cirrhosa</i>	Eledone toxin	30–70	>7	Neurotoxin	–	McDonald and Cottrell, 1972
<i>Eledone moschata</i> <i>Eledone cirrhosa</i> <sup>b</sup>	Eledoisin <sup>c</sup>	–	–	Hypotensive agent	–	Erspamer and Anastasi, 1962
<i>Enteroctopus doffeini</i> <sup>d</sup>	–	22.15	5.2–5.3	Potential neurotoxin	–	Songdahl and Shapiro, 1974
<i>Octopus vulgaris</i>	$\alpha$ -cephalotoxin	91.20	4.5–5.1	Neurotoxin (inhibitor of glutamate-mediated synaptic transmission)	–	Cariello and Zanetti, 1977
<i>Octopus vulgaris</i>	$\beta$ -cephalotoxin	33.90	1.8–2.5	Neurotoxin (inhibitor of glutamate-mediated synaptic transmission)	–	Cariello and Zanetti, 1977
<i>Octopus vulgaris</i>	OctTK-I and OctTK-II	–	–	Hypotensive agent	–	Kanda et al., 2003
<i>Sepia esculenta</i>	SE-Cephalotoxin <sup>e</sup>	100	9.08	Neurotoxin	Glycosylation	Ueda et al., 2008
<i>Sepia officinalis</i>	Cephalotoxin	–	–	Neurotoxin	–	Ghiretti, 1959
<i>Sepia pharaonis</i>	–	~50	–	Antibacterial agent	–	Karthik et al., 2015
<i>Sepia prashadi</i>	–	1.96	–	Potential antibacterial and antiviral agent	–	Karthik et al., 2019

<sup>a</sup>Name updated from *Loligo pealei*.

<sup>b</sup>Name updated from *Eledone aldrovandi*.

<sup>c</sup>12-amino acid neuropeptide.

<sup>d</sup>Name updated from *Octopus doffeini*.

<sup>e</sup>UniProt accession B2DCR8.

[–] Data not available/unknown.

and  $\beta$ -cephalotoxin, hapalotoxin, and Eledone toxin identified, respectively (McDonald and Cottrell, 1972; Cariello and Zanetti, 1977; Savage and Howden, 1977). Indeed, *Hapalochlaena* spp. (the famous blue-ringed octopus) is particularly known for the potency of its neurotoxic agents, which are lethal and devoid of known antidote. Later, Sheumack et al. (1978) was able to identify tetrodotoxin (TTX) in the posterior salivary glands of the octopus *Hapalochlaena maculosa* as well. This non-peptidic neurotoxin is not, however, exclusive to octopus' salivary glands since it was found in multiple body parts of the animal, including arms, cephalothorax and abdomen (Yotsu-Yamashita et al., 2007). It must be noted, though, that TTX is a secondary metabolite best-known from pufferfishes (Tetraodontidae) as well as in other marine animals and amphibians, with its synthesis being associated to endosymbiotic bacteria (Lago et al., 2015). Despite the aforementioned studies, information on the bioreactivity and structural properties of cephalotoxins is still very limited. However, cephalotoxins from octopuses and *Sepia esculenta* have been conclusively identified as functional proteins. The latter species, in particular, yielded, to date, the only complete cephalotoxin amino acid sequence available from a curated record at UniProt, which illustrates how little is known about these substances whose existence is known for more than a century. Toxins from *Sepia pharaonis* and *Sepia prashadi*, and from the squid *Doryteuthis (Amerigo) pealeii* have also been identified as unknown cytotoxic proteins secreted by posterior salivary glands (Kem and Scott, 1980;

and purified by Karthik et al. (2015, 2019), which may indicate potential cephalotoxin candidates.

## Secretion, Delivery, and Mode-of-Action

As carnivores, cephalopods use envenomation as a means for hunting, however, it may also confer defence against predators (Chichery and Chichery, 1988; Norman and Reid, 2000). Naturally, the secretion of cephalotoxins by salivary glands and their delivery via a wound created by the typical parrot beak of coleoids indicates a major role in feeding. The salivary glands (anterior and posterior) are the constituents of the digestive system responsible for secreting substances that intervene directly in the capture of prey, as well as in lubricating and protecting the digestive tract, besides contributing to the predigestive process (see Fernández-Gago et al., 2018 and references therein for details). The anterior salivary glands of cephalopods are located behind the buccal mass and are compound tubuloacinar glands mostly composed by granular and mucous secretory cells (Fernández-Gago et al., 2018). However, the disposition of the posterior salivary glands, where active toxins are secreted, is more variable. While octopuses and cuttlefishes possess a pair of posterior salivary glands, squids have only a single gland (see for instance Boucaud-Camou and Boucher-Rodoni, 1983). The posterior salivary glands are adjacent to the digestive gland and are tubular as well. In octopodids, granular and mucous cells are strongly present in glandular epithelia, the latter being responsible for

the production of acidic glycoconjugate mucins (Fernández-Gago et al., 2018). In sepoids (cuttlefishes), the mucous cells of salivary glands secrete tryptophan-rich proteinaceous substances (Boucaud-Camou, 1968). Interestingly, enterochromaffin cells (a type of neuroendocrine cells) have been detected in the posterior salivary glands of octopods as well, albeit absent in sepoids (Boucaud-Camou and Boucher-Rodoni, 1983). In turn, salivary gland morphoanatomy for the Teuthoidea (squids) is scarce. In any case, the relationship between salivary gland structure, biochemistry and toxin secretion remains somewhat elusive.

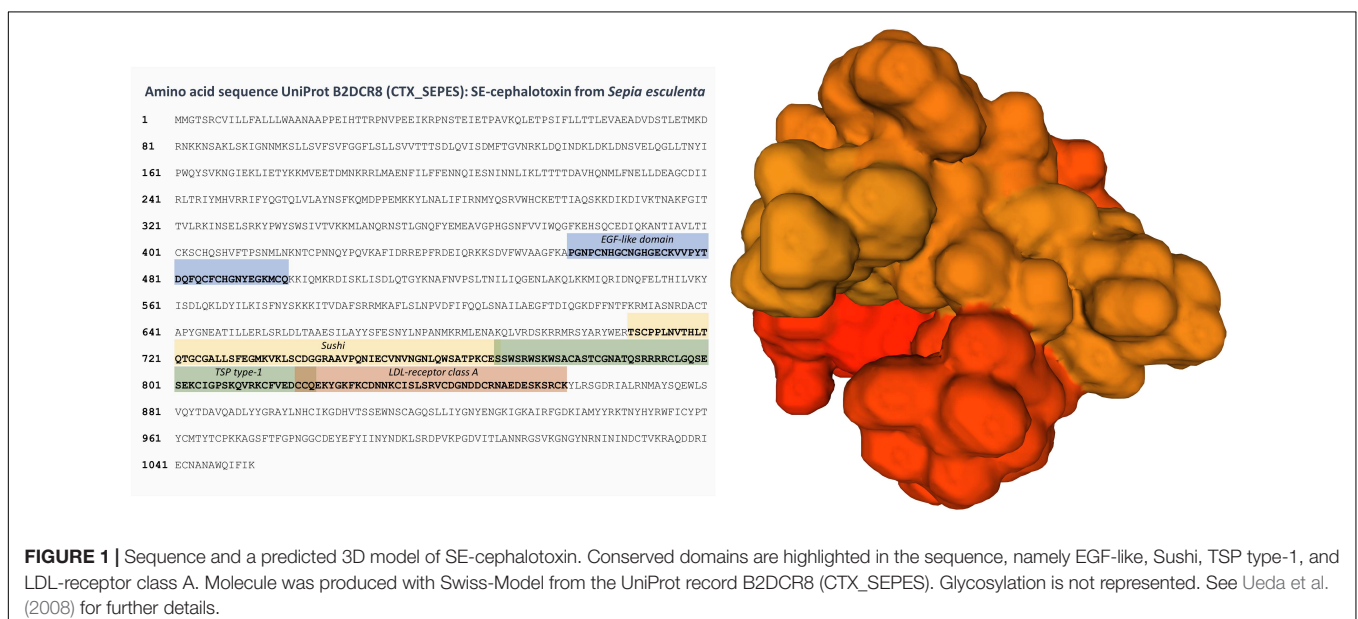
The buccal mass plays a paramount role in toxin delivery. Here is located the posterior salivary gland duct, running within the salivary papilla, and the anterior salivary gland duct, both opening into the buccal cavity. During an attack, the toxic secretions produced by the posterior salivary glands are delivered to the prey through a wound created by quick bite rather than by injection through the two-piece beak (also called rostrum). Toxins with immobilising properties, with the aid of permeabilising enzymes plus cardioexcitatory and vasodilatory substances, are promptly disseminated through the prey's circulatory system, rapidly affecting mobility and motor coordination (Zhang, 2015; Lobo-da-Cunha, 2019). Likely, the copious amount of mucus comprising neutral glycoproteins, sialic acid, dipeptidase and hyaluronidase secreted by the anterior salivary glands, is the delivery vehicle of the bioactive compounds secreted by the posterior salivary glands (Budelmann et al., 1997; Ponte and Modica, 2017).

The number of detailed studies on the toxicological effects of cephalopod salivary secretions is still considerably low. Most bioassays were performed on decapod crustaceans and in general report the paralysing properties of crude secretions from a few species of octopuses, squids and cuttlefishes (Ghiretti, 1959; McDonald and Cottrell, 1972; Songdahl and Shapiro, 1974; Cariello and Zanetti, 1977; Savage and Howden, 1977;

Ueda et al., 2008; Cornet et al., 2014). However, there are some studies performed on mice that suggest toxicity to vertebrates as well (Ueda et al., 2008). Whenever the chemical nature of these secretions was investigated, cephalotoxins were consistently present and considered the compounds with highest toxic activity. Cephalotoxins seem to have a powerful paralysing action against models as distinct as crustaceans and mice by blocking the electrical and mechanical responses of muscles (McDonald and Cottrell, 1972), even though the exact mechanisms are not known. Songdahl and Shapiro (1974) also stated that the speed of the toxicological reaction may be dose-dependent. Further, Ueda et al. (2008) demonstrated that distinct cephalopod species secrete toxins with different potencies against the same target species, i.e., whereas salivary extracts from cuttlefish were lethal to crabs and non-toxic to mice, extracts from squid revealed higher toxicity against mice. Cariello and Zanetti (1977) verified a similar toxicological reaction of the cephalotoxins upon octopus itself, causing significant lethality as well. Additionally, toxicological studies on strains of Gram-positive and Gram-negative bacteria confirmed that peptidic extracts of the *Sepia officinalis* posterior salivary glands revealed bactericidal and antimicrobial activities (Cornet et al., 2014). Disclosing allo- or orthosteric ligands for cephalotoxins in target organisms would provide a major leap in understanding mode-of-action of these toxins and eventually explore their biotechnological potential.

## Molecular Characterisation of Cephalotoxins

Knowledge on the molecular structure of cephalotoxins is scant or incomplete. The most complete characterisation of these toxins, so far, was obtained by Ueda et al. (2008), who disclosed a full 1052-amino acid sequence for a cephalotoxin variant





from the posterior salivary glands of the golden cuttlefish, *Sepia esculenta*. This form, termed SE-cephalotoxin by the same authors appears to be a  $\approx 100$  kDa monomeric protein whose amino acid sequence was determined from isolated cDNAs. The same authors also purified the protein by high-performance liquid chromatography (HPLC) and confirmed the findings by Western Blotting. Some of the most distinctive features of the protein is glycosylation at five distinct sites and the presence of four conserved domains, namely EGF-like, Sushi, TSP type-1 and LDL-receptor class A (Figure 1). Some of these, particularly the EGF- (epidermal growth factor) like domain have already been described in venom proteins such as in the gigantoxins from the cnidarian *Stichodactyla gigantea*, which interfere with sodium channels and have paralytic activities against crustaceans (Shiomi et al., 2003). Cephalotoxins may thus be considered as belonging to the class commonly referred to as EGF (neuro)toxins.

Despite the absence of further cephalotoxin mRNA or amino acid sequences from curated databases, cephalotoxin-like proteins (possibly non-toxic) have been found in a number of aquatic organisms, from fish to the exoskeleton of some corals (e.g., Ramos-Silva et al., 2013). However, the role and evolution of these proteins remains obscure. Still, important insights have been provided by Ruder et al. (2013), who used an RNA-Seq transcriptomic approach to study multiple toxins secreted by octopus, cuttlefish and squid, revealing for the first time the existence of cephalotoxin-like proteins in all these three major groups by homology matching.

## PROSPECTS FOR POTENTIAL BIOTECHNOLOGICAL APPLICATIONS

As mentioned earlier, it is believed that the immobilising or paralyzing activity of cephalotoxins against invertebrate prey, especially arthropods, can result from the interference with specific ion channels or receptors. On the very least, cephalotoxins could be explored for the development of natural and safer pesticides against arthropods (namely insecticides). Nonetheless, verified effects on murines open excellent prospects for biomedical applications, similarly to conotoxins and TTX, especially for the development of safer and non-addictive painkillers. In fact, Cariello and Zanetti (1977) reported cephalotoxins from *O. vulgaris* to be able to block glutamate-mediated synaptic transmission, a signalling process involved precisely in pain sensation (see for instance Wozniak et al., 2012). Nonetheless, only few bioactives from the cephalopod salivary glands are indeed under scientific scrutiny for potential applications. Among such studies, research on extracts from *Sepia pharaonis* and *Sepia prashadi* posterior salivary glands revealed antimicrobial activity against avian bacterial pathogens and also a potential function as an anti-metastatic agent (Karthik et al., 2015, 2017, 2019). These findings suggest that, adding to neurotoxic effects, biocidal and anti-proliferative properties of cephalotoxins and other components of cephalopod venoms can point to novel antibiotics and anti-cancer drugs, further confirming the rising interest of cephalopod toxins for biotechnology. It must be noted that even TTX, albeit

being ubiquitous among many animal taxa (cephalopods included) and one of the best-known and most lethal non-peptidic neural ion channel blockers, has also been considered for the development of analgesic and anaesthetic drugs in advanced cancer patients (Hagen et al., 2007). Indeed, under the trade name Tectin, TTX is already in phase III trials for the treatment of pain resulting from chemotherapy treatments (Newman and Cragg, 2014). To these applications we may add the potential deployment of TTX in the treatment for opioid dependence and management of withdrawal symptoms, with has already started phase IIa clinical trials as Tetrodin (Butler, 2005; Shi et al., 2009; Song et al., 2011). Similarly, the TTX-based drug Tocudin, has also started preclinical studies as a formulation intended for local anaesthesia (Butler, 2005). These findings highlight the high prospects set upon potential applications of marine animal toxins. Proteinaceous toxins such as cephalotoxins and conotoxins may offer advantages over those derived from primary or secondary metabolites, as they can be safer, more easily eliminated (by proteolytic activity) and, very importantly, directly cloned into adequate vectors for heterologous expression. In the latter case, constraints posed by protein secretion and post-translational modifications, such as glycosylation, can be circumvented by choosing eukaryote models, like yeast.

## LESSONS LEARNED AND FUTURE PERSPECTIVES

Even though the discovery and description of coleoid venomous secretions dates back to the XIX century, their toxins remain obscure. Cephalotoxins, albeit being consensually found to be the most bioactive substances in the venom secreted by the posterior salivary gland of these animals (whereas the anterior gland provides the mucin-rich vehicle of delivery), are no exception. Their potent neurotoxic and potential cytotoxic effects, together with the proteinaceous nature, makes them particularly appealing for drug discovery. Such endeavour requires, nonetheless, more solid knowledge on their molecular structure and their interaction with target receptors. With this respect, “omics” approaches, particularly proteomics and transcriptomics, can greatly assist in the filling-in of proteins secreted by the salivary glands of cephalopods by screening multiple peptides and proteins in single runs. These approaches may therefore provide a scaffold to support downstream finer structural details of the substances. The “big picture” must also be complemented with finely tuned toxicity and bioreactivity testing to provide the comprehensive bottom-up perspective needed to devise effective applications.

## AUTHOR CONTRIBUTIONS

CG and PC designed the conceptual framework of the manuscript. CG collected information and drafted the

manuscript with important input and supervision from PC. Both 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.

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