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REVIEW ARTICLE

A brief review of applications of antifreeze proteins in cryopreservation and metabolic genetic engineering

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Abstract
Antifreeze proteins (AFPs) confer the ability to survive at subzero temperatures and are found in many different organisms, including fish, plants, and insects. They prevent the formation of ice crystals by non-colligative adsorption to the ice surface and are essential for the survival of organisms in cold environments. These proteins are also widely used for cryopreservation, food technology, and metabolic genetic engineering over a range of sources and recipient cell types. This review summarizes successful applications of AFPs in the cryopreservation of animals, insects, and plants, and discusses challenges encountered in cryopreservation. Applications in metabolic genetic engineering are also described, specifically with the overexpression of AFP genes derived from different organisms to provide freeze protection to sensitive crops seasonally exposed to subzero temperatures. This review will provide information about potential applications of AFPs in the cryopreservation of animals and plants as well as in plant metabolic genetic engineering in hopes of furthering the development of cold-tolerant organisms.

Keywords Antifreeze proteins · Cold-tolerant organisms · Cryopreservation · Ice crystals · Metabolic genetic engineering

Introduction

Antifreeze proteins and discovery

Antifreeze proteins (AFPs) are biological antifreeze materials found in many organisms that live in extreme cold environments. These proteins bind to ice in such a way that inhibits the growth of the ice crystals, allowing the organisms to survive these harsh conditions. Scholander et al. (1957) first discovered antifreeze proteins during an investigation into why Arctic fish can survive in water colder than the freezing point of their blood. Similarly, DeVries and Wohlschlag (1969) isolated an antifreeze protein during an investigation of Antarctic fish. The presence of AFP in insects was discovered by Husby and Zachariassen (1980), and their existence in plants, fungi, and bacteria was uncovered by Griffith et al. (1992) and Duman and Olsen (1993). The naming of AFPs ranges from antifreeze proteins to ice structuring or binding proteins or thermal hysteresis proteins. For the purposes of this review, all such proteins will be referred to as AFPs.

Source of antifreeze proteins

Antifreeze proteins were first detected in Arctic fish (Scholander et al. 1957) and later grouped into types I, II, III, and IV based on their sequences and structures (Fig. 1). All these proteins share an ability to alter the freezing point of solutions. Duman and Olsen (1993) first discovered AFPs in microorganisms such as bacteria and fungi, and discoveries of other AFPs in bacteria and fungi followed (Gilbert et al. 2005; Hoshino et al. 2003; Kawahara et al. 2007; Muryoi et al. 2004; Newsted et al. 1994; Singh et al. 2014). Similarly, plant AFPs have been observed in 60 plant species, and among them, 11 of these proteins have been purified and characterized (Gupta and Deswal 2014). AFPs observed in plants, such as winter rye (Griffith et al. 1997), carrot (Meyer et al. 1999; Zhang et al. 2004), grass (Sidebottom et al. 2000), winter cereals (Yeh et al. 2000), peach (Wisniewski et al. 1999), and Japanese radish (Kawahara et al. 2009), demonstrate high sequence homology.

AFPs have also been discovered in insects, such as milkweed bugs (Patterson et al. 1981), budworm moths (Hew

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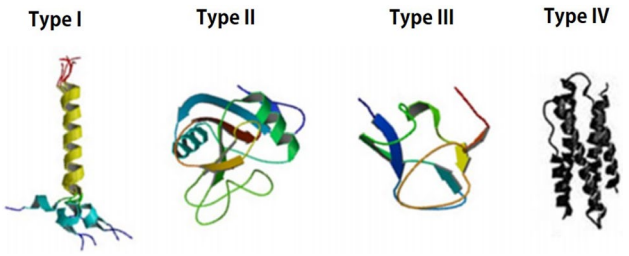

Fig. 1 Structural differences among types of fish AFPs (I, II, III, and IV)

et al. 1983), snow scorpionflies (Husby and Zachariassen 1980), stoneflies (Gehrken and Somme 1987), the beetle *Dendroides canadensis* (Wu et al. 1991), Alaskan insects and spiders (Duman et al. 2004), and wood cockroaches (Duman 1979). AFPs allow these insects to survive subzero winter temperatures by decreasing the freezing points of their bodily fluids and inhibiting recrystallization.

Antifreeze proteins have been isolated from many different organs, such as the liver, stomach, heart, seeds, stems, bark, leaves, and flowers (reviewed in Cheung et al. 2017). Although their structures and amino acid sequences vary, all bind to different faces of the ice crystal (Jia and Davies 2002).

Mechanism of action and role of antifreeze proteins

AFPs allow organisms to survive in harsh environments by lowering the freezing point of water through binding with ice nuclei and inhibiting recrystallization (Figs. 2, 3). Recently, Liu et al. (2018) discovered that AFPs from the fungus *Pichia pastoris* lowered freezing temperatures, controlled ice crystal sizes, and reduced damage from the freezing of hydrated gluten. Similar activities by plant AFPs in protecting plant cells from freezing damage have been reported, as well. AFPs from winter-hardy coniferous species have been shown to inhibit ice crystal formation (Jarzabek et al. 2009). The capacity for insect AFPs to reduce solution freezing

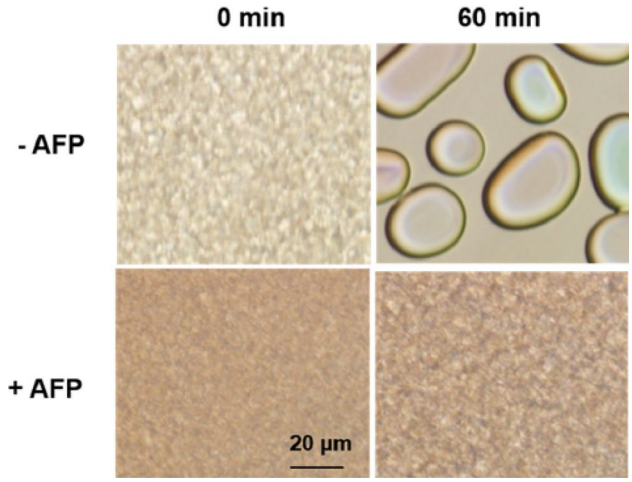

Fig. 3 Comparison of the status of ice recrystallization in solutions with (+) or without (–) AFP at – 6 °C for 60 min

points has also been well documented (Duman and Serianni 2002; Duman 2002; Olsen and Duman 1997; Graham et al. 1997; Tomczak et al. 2003). Interestingly, AFP structures in these organisms, such as the ocean pout, winter flounder, beetle, moth, and snow flea, are distinct from one another (Fig. 4, PDB 101).

Classification of antifreeze proteins based on activity

AFPs can be classified as moderately active or hyperactive based on their ice binding positions. Moderately active AFPs bind to the prism and pyramidal planes of the ice crystal and generate a hexagonal bipyramidal ice crystal shape, whereas hyperactive AFPs bind to these planes and the basal plane of the ice crystal (Fig. 5), resulting in a circular disk-like ice crystal morphology (Knight et al. 1991; Drori et al. 2014; Park et al. 2012). The binding of hyperactive AFPs to the basal plane may result in a greater inhibitory effect on ice growth from whole ice surfaces than that of moderately

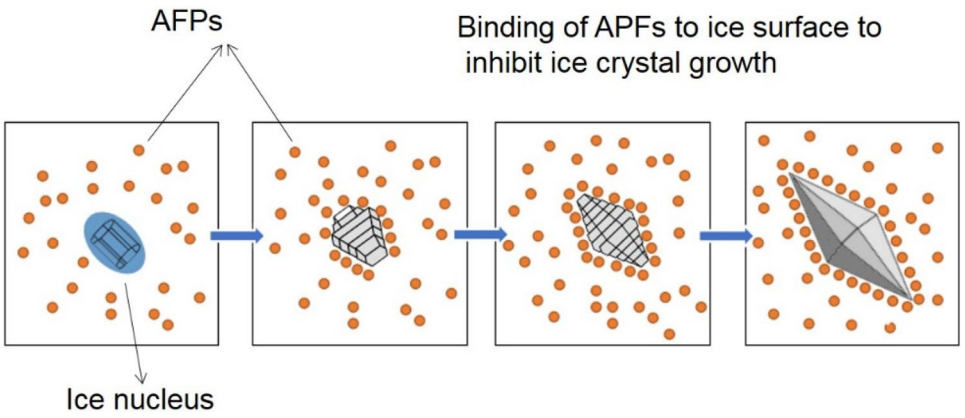

Fig. 2 Binding of antifreeze proteins (AFPs) to the ice nucleus to prevent the formation of large ice crystals

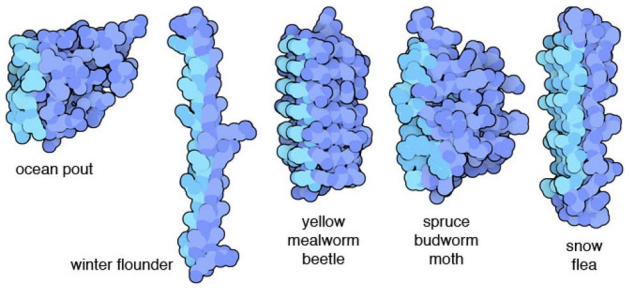

Fig. 4 Summary of structural differences among antifreeze proteins in different taxa (PDB 101)

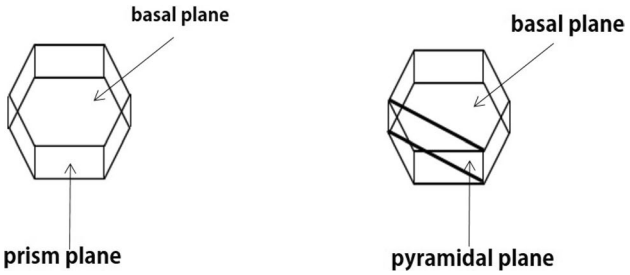

Fig. 5 Different ice crystal-binding sites for hyperactive versus moderately active antifreeze proteins

active AFPs, with much higher thermal hysteresis (TH) activity than that of their moderately active counterparts (Kong et al. 2016; Pertaya et al. 2008; Drori et al. 2014).

Classification of antifreeze proteins based on TH values

The changing of melting and freezing points conferred by AFPs is known as thermal hysteresis (TH). AFPs can also be classified based on TH values to indicate their level of antifreeze activity. AFPs with high TH values, such as insect AFPs (TH values 5−10 °C), are considered hyperactive (Lin et al. 2010), while those from plants and fish (TH values, 0.2−0.6 °C and 1−2 °C) are classified as moderately active (Sicheri and Yang 1995; Griffith and Yaish 2004). However, hyperactive AFPs do not necessarily ensure better cryopreservation than moderately active proteins; for example, moderately active AFPs have been shown to protect mouse ovarian tissue ten times more effectively than hyperactive proteins (Kim et al. 2015; Lee et al. 2015a, b).

Application of antifreeze proteins

The reduction of freezing points by AFPs is non-colligative and does not significantly alter melting points regardless of concentration (Raymond et al. 2007; Lee et al. 2010; Tomczak et al. 2003). Effective inhibition of ice recrystallization by low concentrations of AFPs has been reported in previous studies (Knight et al. 1984, 1988), and differs from common antifreeze agents like methanol, glycerol, or ethylene glycol that lower freezing points in proportion to their concentrations. AFPs are highly sought after for use in cryopreservation, biotechnology, and the food industry (Christner 2010) owing to their unique abilities. The addition of AFPs to cells, organs, and tissues of plants and animals has been shown to improve cryopreservation efficiency (Jeon et al. 2015; Seo et al. 2018). With regards to food, AFPs improve the texture of ice cream (Regand and Goff 2006) and the quality of preserved meat (Griffith and Ewart 1995). The expression of AFPs in transgenic plants increases ice growth therein (Griffith et al. 1997; Hoshino et al. 1999; Maunsbach et al. 2001; Holmberg et al. 2001). This review discusses the applications of diverse AFPs in plant and animal biotechnology in detail.

Application of AFPs in cryopreservation

Cell, tissue, and organ cryopreservation methods are well established for plants and animals through the use of cryoprotectants like dimethyl sulfoxide (DMSO), glycerol, and polyvinylpyrrolidone (PVP). However, as cell and organ membranes are extremely sensitive to freezing and thawing cycles, high concentrations of these compounds are necessary to dehydrate the cytosol and minimize the formation of intracellular ice crystals during freeze and thaw cycles (Taylor and Fletcher 1998, 1999). These high concentrations can also cause cytotoxicity by altering the epigenetic regulation of cells (Adler et al. 2006; Thaler et al. 2012) and has led to a demand for alternative cryoprotectants with less toxicity. As described above, AFPs can inhibit the growth of ice during freezing and thawing without significantly affecting the melting point. In addition, as these proteins lower the freezing point non-colligatively, they are considered less toxic than existing cryoprotectants. Relatively low concentrations of AFPs can inhibit the recrystallization of ice compared to other cryoprotectants. Many researchers have focused on the use of AFPs in the cryopreservation of cells, tissues, and organs of plants and animals, owing to their unique properties. According to a review by Kim et al. (2017), type III AFPs are most commonly used for cryopreservation, followed by type I AFPs. Type II AFPs are rarely used as cryoprotectants (Fig. 6).

Application of AFPs to the cryopreservation of animal cells, tissues, and organs

Different types of fish AFPs, especially types I and III, have been used to improve the cryopreservation of animal cells, tissues, and organs, such as oyster oocytes (Naidenko 1997), bovine and porcine oocytes (Rubinsky et al. 1991,

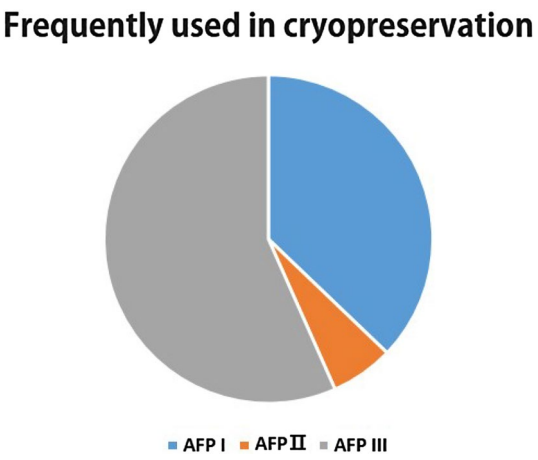

Fig. 6 Use of different types of AFPs in cryopreservation research

1992), vertebrate and invertebrate cell lines (Koushafar and Rubinsky 1997), intact livers (Lee et al. 1992; Rubinsky et al. 1994), and bull sperm (Prathalingam et al. 2006). Improved motility and reduced enzyme leakage have been observed in sperm cryopreserved with AFPs compared to those without (Uperti et al. 1996). The presence of AFPs also improves the quality of cryopreserved sheep embryos (Baguisi et al. 1997). Rubinsky et al. (1994) reported that hearts preserved with AFPs remained viable, as evidenced by electron microscopy, while those preserved without AFP did not. AFPs have been shown to protect the heart from freezing damage and improve viability during cryopreservation in other studies, as well (Amir et al. 2004, 2005; Soltys et al. 2001). Embryos from *Sparus aurata* injected with AFP exhibited improved tolerance to chilling at 0 and – 10 °C with a hatching rate of approximately 100% (Robles et al. 2006). Jo et al. (2011) found that the addition of fish AFP (500 ng/mL) to vitrification solution improved the survival rate of immature mouse oocytes, while Lee et al. (2015a) reported that a diverse array of proteins from yeast, bacteria, and fish improved murine oocyte quality and embryonic development. Zilli et al. (2014) reported that the addition of type III fish AFP to the cryopreservation medium protected sperm from freezing and improved their viability as compared to other treatments (control, DMSO, and DMSO + type I fish AFP). The cryoprotective effect of type III fish AFP has also been observed in rabbit embryos (Nishijima et al. 2014). Ideta et al. (2014) reported that bovine embryos stored in medium containing 10 mg/mL AFP survived for 10 days at 4 °C. Fish embryos incubated in a solution containing type I AFP exhibit a significantly increased survival rate upon exposure to 4 or 10 °C (Martínez-Páramo et al. 2008a, b, 2009). Type I AFPs block potassium and calcium ion channels, reducing ion leakage from lipid membranes at 4 °C (Rubinsky et al. 1992; Baguisi et al. 1997), thereby helping to maintain the transmembrane ionic gradient and improve cryopreservation of cells and tissues (Arav et al. 1993). Similarly, type III AFPs stabilize the plasma membrane by interacting with lipids (Wang and Huang 1996). Unlike type I and III AFPs, type II AFPs have been associated with cytotoxicity in cells, tissues, and organs during cryopreservation (Naidenko 1997; Pham et al. 1999; Wang et al. 1999) and use thereof is infrequent in cryopreservation (Fig. 6). Higher concentrations of AFPs can cause the formation of destructive, needle-like ice and lead to a decrease in the post-thaw survival of cryopreserved cells (Lee et al. 2015a, b; Hansen et al. 1993; Carpenter and Hansen 1992). Low concentrations of AFPs are thus preferred in cryopreservation studies. However, the optimal concentration for cryopreservation differs depending on cell type and AFP source, and the utilization of AFPs in cryopreservation requires fine-tuning depending on these different parameters.

Application of AFPs to the cryopreservation of plant cells, tissues, and organs

The cryopreservation of plant cells, tissues, and organs has also been attempted for long-term species conservation (Engelmann 2011; Jeon et al. 2015; Seo et al. 2018). However, since commonly used explants, such as the callus or shoot tips, contain high amounts of cellular water, freezing injuries are likely via the crystallization of this water into ice during freezing and thawing, leading to low survival rates. In addition, as mentioned above, commonly used cryoprotectants, such as glycerol, sugars, and DMSO, are toxic to certain plant tissues. In 1989, Cutler et al. (1989) investigated the effects of AFP vacuum infiltration into the leaves of potato (*Solanum tuberosum* L.), canola (*Brassica napus*), and *Arabidopsis thaliana* (L.) plants, and found that AFPs lowered their freezing temperatures significantly compared to that of water-infiltrated controls, with the amount of freezable water reduced across a range of low temperatures. In canola, the freezing temperature was decreased by an average of 1.8 °C, indicating that AFP infiltration could depress its freezing point to a level that would substantially improve crop survival in typical agricultural environments. Wang et al. (2001) further reported that the utilization of AFPs improves cryopreservation efficiency in rice embryogenic cells. Jeon et al. (2015) also observed that the addition of type I fish AFP to vitrification solution significantly increased cryopreservation efficiency in the chrysanthemum. Seo et al. (2018) found that the inclusion of type III fish AFPs in cryoprotection solutions improved the cryopreservation efficiency of potato shoot tips. Pe et al. (2019) observed involvement of AFPs in regulation of cold-responsive genes in *Hosta capitate* under low-temperature condition. Currently, however, AFPs are utilized less frequently

in plant cryopreservation than in that for animals and animal tissues.

Application of AFPs in metabolic genetic engineering

The introduction of genes encoding AFPs via metabolic genetic engineering is another promising strategy to improve freeze tolerance in transgenic plants and animals. Many studies have attempted to generate freeze-tolerant plants and animals via the overexpression of *AFP* genes in otherwise cold-sensitive organisms. Success in this area has recently increased substantially.

The production of transgenic plants overexpressing fish AFPs has been reported for many species, including *Arabidopsis*, tobacco, tomatoes, and potatoes (Hightower et al. 1991; Kenward et al. 1993; Wallis et al. 1997; Worrall et al. 1998). Ice recrystallization has been successfully inhibited in fish *AFP*-overexpressing transgenic tomato leaf extracts (Hightower et al. 1991), and Balamurugan et al. (2018) claimed that the overexpression of the *AFP* gene from rye grass (*Lolium perenne*) in tomato plants resulted in significantly higher freeze tolerance than in wild-type plants through a threefold increase in relative water content and 2.6-fold reduction in the electrolyte leakage index. Wallis et al. (1997) observed that transgenic potatoes overexpressing *AFP* genes exhibited significantly less electrolyte leakage than control plants after freezing at −2 °C. The overexpression of insect *AFP* in *Arabidopsis* resulted in significant antifreeze activity and improved frost resistance (Meyer et al. 1999), and transgenic *Arabidopsis* plants overexpressing insect *AFP* showed increased cold tolerance through a decrease in their freezing temperature (Huang et al. 2002). Lin et al. (2011) confirmed that the integration of insect *AFP* in transgenic *Arabidopsis* reduced its freezing temperature of by 2−3 °C as compared to wild-type plants. Similarly, improved freeze tolerance was observed in transgenic *Arabidopsis* overexpressing an insect AFP from a spruce bud worm; the transgenic lines had less ion leakage and malondialdehyde than wild-type lines in temperatures as low as −20 °C for 30 min and 4 °C overnight (Zhu et al. 2010). Similarly, the introduction of insect *AFPs* and type I fish *AFPs* into tobacco inhibits ice recrystallization (Holmberg et al. 2001; Kenward et al. 1993) in these plants. Deng et al. (2014) claimed that the heterologous expression of *AnAFP* in tobacco resulted in less wilting and less change in relative electrical conductivity under cold stress (−3 °C) compared to wild-type plants after a 16 h freeze and 1 h thaw. Conversely, the overexpression of type II fish *AFPs* in tobacco does not confer cold tolerance (Kenward et al. 1999). Wang et al. (2008) also observed that transgenic tobacco overexpressing *AFP* from the insect *Microdera punctipennis* demonstrated improved freeze tolerance over wild-type plants through reductions in ion leakage and malondialdehyde levels. Fan et al. (2002) confirmed that transgenic expression of the carrot *AFP* gene could enhance the tolerance of tobacco plants to cold through a significantly greater reduction in ion leakage (1−30%) than that of the wild type (1−80%). Transgenic wheat overexpressing *AFP* showed significant freeze tolerance at −7 °C, with high levels of antifreeze activity (Khanna and Daggard 2006). The most effective antifreeze proteins for different plants, however, depend on AFP expression level, localization, and stability.

The introduction of type I fish AFP to salmon fish eggs resulted in the generation of cold-tolerant transgenic salmon fishes (Hew et al. 1992). Hew et al. (1999) went on to report that the integration of fish AFP into salmon resulted in inherited expression of the *AFP* gene in the F_3 generation. Similarly, the microinjection of ocean pout type III *AFP* into goldfish oocytes resulted in transgenic goldfish with improved resistance to low temperatures (Wang et al. 1995), and Hobbs and Fletcher (2008) also transferred the *AFP* gene to salmon to improve freeze resistance.

The overexpression of type III fish AFP has been shown to improve antifreeze activity and protect against freezing damage during the cryopreservation of transgenic mouse ovaries (Bagis et al. 2006, 2008). Uhlig et al. (2011) also reported that the heterologous expression of *AFPs* in *Escherichia coli* yielded antifreeze activity and caused crystal deformation, recrystallization inhibition, and TH. Similarly, the expression of fish AFP in *Drosophila* increased antifreeze activity, and the transgenic flies were able to survive significantly longer in near freezing temperatures than controls through the prevention of apoptosis (Nicodemus et al. 2006; Neelakanta et al. 2012).

Conclusion

AFPs from diverse taxa, including fish, bacteria, fungi, insects, and plants, allow organisms to survive at subzero temperatures by reducing the freezing point for ice growth therein. AFPs derived from different species have been used successfully for the cryopreservation of plant and animal cells and organs, although their ability to reduce the freezing temperature is dependent on the species and cell type from which the AFP is derived, AFP type and concentration, and cryopreservation protocol. Extensive research has focused on the development of *AFP*-overexpressing transgenic animals or plants to improve their survival in extreme cold conditions. However, the cytotoxicity of certain AFPs somewhat limits their cryopreservation applications. As such, the identification of novel AFPs better suited to cryopreservation would have important practical implications. The development of a technique for direct delivery of the

AFPs into cells to control crystal growth without damaging the cells is also necessary for improving the applicability of AFP in future cryopreservation techniques.

Future perspectives

Successful application of AFPs in cryopreservation of animals and plants has been reported in several different studies. However, due to low yield and high cost, future applications for AFPs remain uncertain. Increasing production yields with different molecular biological techniques is needed, and lowering the cost of AFP use in cryopreservation and the food industry will increase future usage. The overexpression of AFP genes derived from different organisms has been shown to confer freeze protection to sensitive crops exposed to seasonally subzero temperatures, and costs associated with transgenic plants have been decreasing rapidly. We expect the production of AFP-expressing transgenic plants via metabolic genetic engineering to increase, especially to improve frost tolerance in garden plants and ornamental cut flowers, supporting further development in the global horticultural industry.

Authors' contributions AHN collected the literature and wrote the manuscript; CKK advised and assisted with the writing of the manuscript.

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Compliance with ethical standards

Conflict of interest statement The authors report no conflicts of interest.

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