Theriogenology 176 (2021) 94–103

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Antifreeze proteins for low-temperature preservation in reproductive medicine: A systematic review over the last three decades

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ARTICLE INFO

Article history:
Received 31 May 2021
Received in revised form
21 September 2021
Accepted 24 September 2021
Available online 25 September 2021

Keywords:
AFP
Embryo
Ice-binding proteins
Oocyte
Semen

ABSTRACT

Antifreeze proteins (AFPs) are synthesized by diverse non-mammalian species, allowing them to survive in severely cold environments. Since the 1990s, the scientific literature reports their use for low-temperature preservation of germplasm. The aim of this systematic review was to compile available scientific evidence regarding the use of AFP for low-temperature preservation of several reproductive specimens. Internet databases were consulted using the terms: "antifreeze protein" OR "AFP" OR "antifreeze glycoprotein" OR "AFGP" OR "ice-binding protein" OR "IBP" OR "thermal hysteresis protein" AND "cryopreservation". From 56 articles, 87 experiments testing AFPs in low-temperature preservation of gametes, embryos or reproductive tissues/cells were fully analyzed and outcomes were annotated. A positive outcome was considered as a statistically significant improvement on any parameter evaluated after low-temperature preservation with AFP, whereas a negative outcome included worsening of any evaluated parameter, in comparison to untreated groups or groups treated with a lower concentration of AFP. The findings indicated that research on the use of AFP as a cryoprotectant for reproductive specimens has increased markedly over the past decade. Some experiments reported both positive and negative results, which depended, on AFP concentration in the preservation media. Variation in the outcomes associated with species was also observed. Among the 66 experiments conducted in mammals, 77.3% resulted in positive, and 28.8% in negative outcomes after the use of AFP. In fishes, positive and negative outcomes were observed in 71.4% and 33.3% of 21 experiments, respectively. Most positive outcomes included preserving cell post-warming survival. The beneficial effect of AFP supports its use in cryobiological approaches used in human and veterinary medicines and animal protein industry. Moreover, combination of different AFP types, or AFP with antioxidants, or even the use of AFP-biosimilar, comprise some promising approaches to be further explored in cryopreservation.

1. Introduction

Long-term preservation of germplasm, often associated with assisted reproductive techniques (ARTs), have supported conservation of genetic resources in endangered species [1], accelerated genetic improvement of food-producing animals [2,3], and helped human patients to overcome infertility [4]. More recently, cryopreservation of gonads (or their fragments) has been investigated as an alternative strategy to preserve reproductive ability in human patients submitted to reprotoxic chemo- or radiotherapies [5,6]. The biotechnology approaches concerning low-temperature preservation of reproductive cells have helped to sustain biological diversity [7]. Moreover, the escalating use of ARTs in humans, including the transfer of frozen-thawed embryos, requires improvement in cryopreservation technologies to increase pregnancy rates [8,9].

Cryogenic lesions include cell membrane rupture [10], chromosome spreading [11], DNA fragmentation [12], abnormal gene expression [13], and damage to organelles such as mitochondria [14] and cytoskeleton [15]. These lesions ultimately derive from two main events: high non-physiological intracellular

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https://doi.org/10.1016/j.theriogenology.2021.09.025
0093-691X/

concentration of solutes, and formation of ice crystals, intra- and extracellularly. The formation of ice crystals is influenced by pressure and temperature during crystallization, while the growth of crystals is mainly determined by cooling rate and nucleation temperature [16]. Large ice crystals result from the aggregation of individual crystals, which can attain different shapes, hexagonal (Fig. 1A) being the most prevalent in cellular cryopreservation [17]. Ice recrystallization occurs during freezing and thawing, as a consequence of thermodynamically spontaneous change in crystal shape or coalescence of small ice crystals into larger ones [18]. Two techniques are routinely used for cryopreservation: slow-freezing, which aims to control the pace of freezing; or vitrification, which prevents crystallization, by creating a non-crystalline amorphous solid. For both, the control of ice crystal formation and coalition, during freezing and/or thawing, is a pivotal feature promoted by cryoprotectants. Many cell permeating or non-permeating molecules have been used as cryoprotectants [19].

Antifreeze proteins (AFPs) comprise a subgroup of ice-binding proteins, with variable structural characteristics, synthesized by diverse species that inhabit low-temperature environments, often colder than 0 °C [20,21]. Fish-isolated AFPs emerged in the early 1990s as promising extracellular cryoprotectants for preservation of mammalian germplasm [22]. The extraction and purification of these proteins were performed from fish plasma, and later from muscle homogenates, by high-throughput liquid chromatography, reaching purity levels higher than 95% [23]. The AFPs protect live organisms from freezing by reducing the freezing point below the melting point, creating a thermal hysteresis gap [24]. *In vitro* experiments [25,26] have demonstrated that AFP effects include thermal hysteresis induction (Fig. 1B) and ice recrystallization inhibition (Fig. 1C–E). Thermal hysteresis slows the kinetics of ice formation [27], and the magnitude of this effect is related to the physical properties of AFPs, such as their size, shape, concentration, and absorption resistance to ice [28]. Efficiency of ice recrystallization inhibition promoted by AFPs relies on their binding ability to ice crystals, which is related to the crystal shape and to the specific AFP binding motifs [29]. There are a diverse number of biological and recombinant AFP structures that share the same basic mechanism of interaction with ice crystals [30].

For the last 30 years, experiments describing the use of different types of AFP in low-temperature preservation of reproduction-associated cells and tissues have been published. Both positive and negative effects of AFPs were reported, generally related to increasing or decreasing post-thaw cellular survival, respectively. The use of AFP for low-temperature preservation of reproductive cells and tissues has expanded beyond the naturally-sourced types, including recombinant AFP derived from fish, yeast, bacteria, or beetle genomes, and *in vitro*-synthesized AFP. The emerging technology for AFP production by recombination, synthetic *in vitro* production, and the development of biosimilar proteins, have enhanced the enthusiasm for increasing their use. Understanding what has been elucidated up until now might support future directions in the use of AFP for cryopreservation, ultimately contributing to ARTs. This systematic review aimed to 1) compile results from the use of AFP as cryoprotectants for reproductive specimens, taking into account the species, cryopreservation methods, AFP type, and concentration; and 2) to observe approaches used for cryopreservation of reproductive specimens with emerging sources of AFP or biosimilar molecules.

2. Methods

2.1. Data source

For this systematic review, internet databases (MEDLINE – PubMed, Scielo, Web of Science, Google Scholar) were consulted in December 2020. Scientific articles from each bibliographic database were selected using the terms: “antifreeze protein” OR “AFP” OR

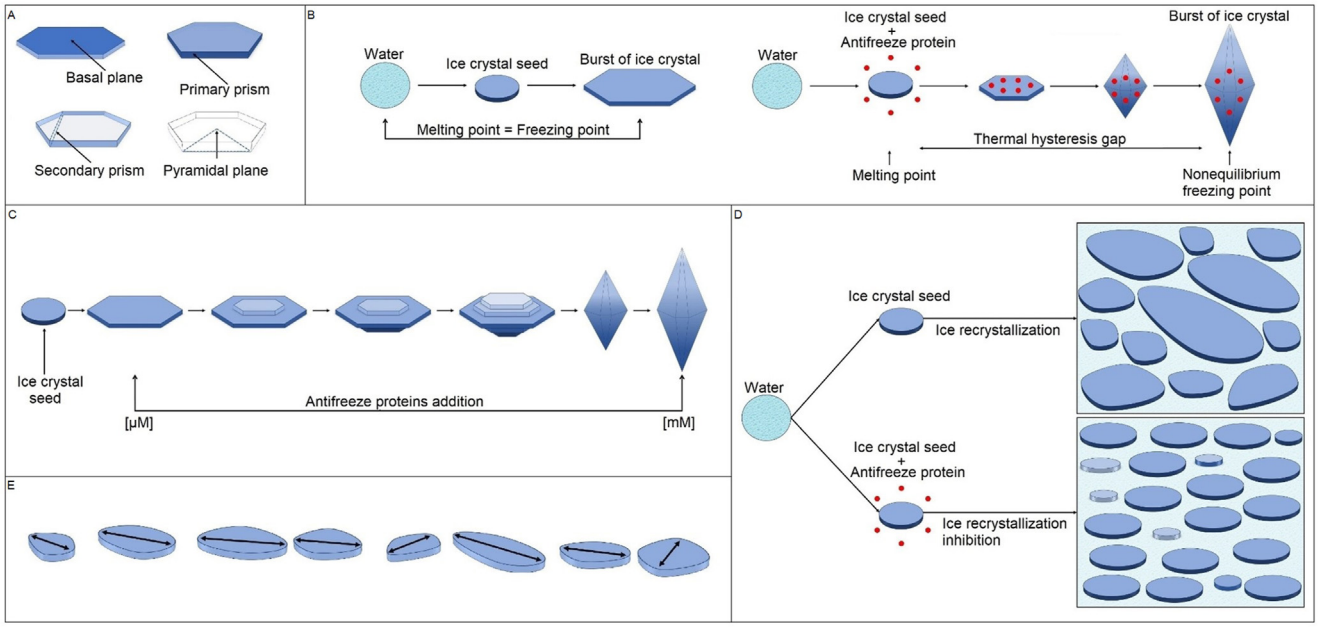

Fig. 1. Ice crystal structure and major effects of antifreeze proteins (AFPs). (A) Illustration of hexagonal ice crystal structure (basal, primary, and secondary prismatic and pyramidal plane structures); (B) Illustration of ice crystal formation and thermal hysteresis phenomenon in the presence of AFP. In left, the ice starts to grow rapidly where the melting point is equal to freezing point. In right, AFP adsorbs irreversibly to specific planes of ice (dependent on type), avoiding ice growth until nonequilibrium freezing point, separating melting and freezing points; (C) Illustration of relationship of ice crystals morphology and AFP concentration. The increase of AFP concentration promotes a change in ice crystal structure from hexagonal to bipyramidal ice crystals; (D) Illustration of Ice Recrystallization Inhibition. Above, spontaneous coalescence of small to larger crystals. Below, in the presence of AFP the small ice crystals maintain their structures without the formation of large ice crystal; (E) different irregular forms of ice crystal growth during ice recrystallization, in the AFP absence.

"antifreeze glycoprotein" OR "AFGP" OR "ice-binding protein" OR "IBP" OR "thermal hysteresis protein" AND "cryopreservation". No filters of date, interval of publication, or species were applied to the search. Language filter was only applied at the end of the survey to avoid bias. Studies that were not published in English were excluded.

2.2. Study selection and criteria

After the database search, articles were screened based on title and abstract. Multiple duplicated papers were excluded, as well as articles that did not report the use of AFP as cryoprotectants for reproductive-related cell/tissue. Then, full texts were obtained and analyzed, and the articles selected for full consideration were carefully screened according to the inclusion criteria: complete articles that contain scientific experiment(s) reporting the use of AFP for low-temperature preservation (cold-liquid storage or cryopreservation) of gametes, embryos, or tissues/cells of the reproductive system. From the selected articles, the "Materials and Methods" sections were carefully evaluated; a flow chart illustrating the data search and selection process is presented in Fig. 2. The subjects of our data compilation were experiments within the selected articles. The proportions of those experiments that reported positive outcomes and negative outcomes were calculated. Type and concentration of AFPs, cryopreservation method, and specimen used were considered, for categorizing experiments. The number of experimental groups (that included AFP in protocols for low-temperature preservation of specimens) were also compiled. The control groups were experimental groups to which treatment groups were compared to. Biological cells/tissue studied in control groups were of the same type as in the experimental group, but preserved at low temperatures without addition of AFP, and/or samples not kept at low temperatures ("fresh" samples) without addition of AFP. A positive effect was recorded when a group treated with AFP showed statistical improvement of viability, quality, and/or functionality of the post-warmed specimens, compared to control groups. A negative effect was recorded when those parameters showed statistical worsening compared to control groups; or when compared to other AFP-treated groups that used the same type of AFP at lower concentrations (denoting a dose-dependent detrimental effect).

3. Results

3.1. Overview

Out of the 342 articles obtained from the consulted databases, only 88 articles remained after initial screening. From these, 56 (63.6%) were eligible according to our inclusion criteria. A detailed list of data recovered is presented in Supplementary Table 1. The

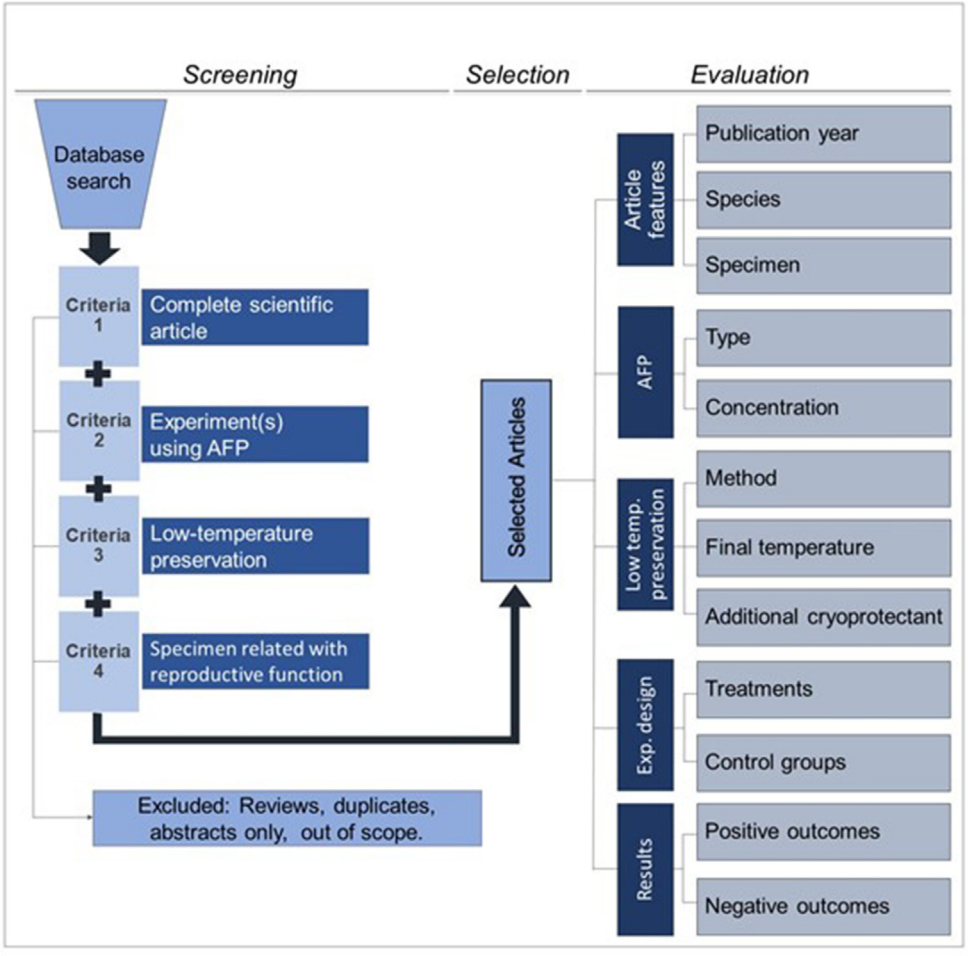

Fig. 2. Flow chart of screening, criteria and evaluation considered in this systematic review of antifreeze proteins (AFPs) in low-temperature preservation of reproductive tissues and cells.

number of experimental articles published increased more markedly during the last decade compared to the preceding ones (1990s: 15; 2000s: 7; 2010s: 34 articles). Every article described studies conducted in one single species, except one that reported experiments in pig and in mouse [31]. Forty percent of the articles (22/56) reported two or more experiments using AFP for low-temperature preservation of specimens. Experiments with no mention of the use of AFP in any treatment were not considered for this review. Eighty-seven experiments (66 in mammalians and 21 in fishes) of reproductive specimens low-temperature preservation with AFP were recorded.

The selected articles described the use of AFP from natural sources, recombinantly produced, or from synthetic manufacture (Supplementary Table 2). Methods for low-temperature preservation included cold-liquid storage (at 0 °C to 5 °C), slow-freezing (controlled low-speed cooling and kept at −10 °C and −196 °C), and vitrification (ultra-fast cooling and kept at −196 °C). Some articles also reported analysis after dilution with AFP at room temperatures (17 °C to 28 °C), to test AFP cytotoxicity. Treatment groups at these latter temperatures were not accounted in the low-temperature preservation, but were cited in the discussion when relevant.

3.2. Analysis of the outcomes

Some articles reported positive and negative effects of AFPs, and both were taken into account. Among the studies conducted in mammals, 90.5% (38/42) reported that AFP produced positive outcomes after low-temperature preservation; however, negative effects were also reported in 38.1% (16/42) of them. Regarding the articles in fishes, 85.7% (12/14) reported positive outcomes, whereas 50% (7/14) indicated at least one negative effect caused by AFP. When individual experiments were analyzed in mammalian and fish, respectively, 77.3% (51/66) and 71.4% (15/21) reported positive outcomes after the use of AFP. The main positive outcomes described in those articles were listed in Table 1. Negative results were observed in 28.8% (19/66) of experiments conducted in mammals, and in 33.3% (7/21) of experiments conducted in fishes and were mostly associated with higher AFP concentrations. The outcomes analysis of AFP on low-temperature preservation is presented in Table 2.

3.3. Semen

The AFP concentrations used in mammalian semen preservation ranged from 0.001 μg/mL to 500 μg/mL [32–47]. Cryopreservation media contained a cell-permeating cryoprotectant (acetamide or glycerol), except for the media used for mouse epididymal spermatozoa [47], which did not report any positive outcome after the use of AFP. Among the 15 articles that reported AFP beneficial outcome for mammalian semen slow-freezing, mitigation of the cryopreservation-induced reduction in sperm motility and/or viability were observed in 14. One exception was the study reporting that AFPs did not preserve motility but improved osmotic resistance in cattle spermatozoa [35]. Comparisons of multiple AFPs were reported in five studies, in which some AFP type was more advantageous in improving preservation, compared to others [32,34–36,41]. Dose-related effects due to increased concentrations of AFPs in the media were also observed [32,36,42,43]. In seven articles [33,34,38–41,47], mammalian semen diluted in AFP-containing media was evaluated at room temperature (approximately 24 °C), and no cytotoxicity was detected under these conditions.

Concentrations of AFP in preservation media of fish semen ranged from 0.1 μg/mL to 10 mg/mL [48–56]. The media utilized contained cell-penetrating cryoprotectants: dimethyl sulfoxide (DMSO) was used in six slow-freezing experiments [49,50,54–56], and DMSO plus glycerol was used in one vitrification experiment [51]. The use of DMSO with AFP elicited beneficial results (including mitigating loss of motility) in all but one experiment [54]. In the slow-freezing process a clear dose-related effect of AFP III was observed [54]. In seven experiments conducted in fishes, the uses of AFP I or III in slow-freezing were compared [49,50,56]. In one study, AFP I and III in combination produced a better result than either component alone for semen vitrification [51]. Semen cryopreserved with AFP was compared to fresh semen in nine experiments, and in three, AFP added to cold-preserved samples was able to maintain the same quality features as fresh semen [49,50,55].

3.4. Oocytes

Mammalian oocytes were preserved at low temperatures using AFP, in concentrations that varied from 0.1 μg/mL to 50 mg/mL

Table 1
The main positive effects of antifreeze proteins (AFPs) as cryoprotectant in low-temperature preservation of reproductive cells and tissues.

AFP effects in cryopreservation			
Semen	Oocyte	Embryos	Reproductive Tissue
• Reduce loss of motility [33,36–40,42–45,48,50–53,55,56]	• Protect oolemma structure [31,58,59,62–64]	• Enhance survival after *in vitro* culture [77,78]	• Maintain intact follicles [79,80]
• Increase post-thaw survival [43,44,48,50,55]	• Maintain maturation capacity [31,61,62,64]	• Higher viability [73]	• Reduce apoptotic follicles [79–81]
• Improve osmotic resistance [35,41,43]	• Increase viability [61,63,66,67]	• Increase embryo development [31,73]	• Maintain intact primordial follicles [81]
• Decrease loss in kinetic parameters [34,36,43,44,46,49,51,56]	• Preserve spindle structure [58,59,61,66]	• Increase survival rate [46,67,74,77,78]	• Increase cell viability [82,85]
• Support the lipid composition of plasma membrane [49]	• Maintain intracellular ATP [58]	• Increase expansion after warming [74,75]	• Maintain the survival after cryopreservation [78]
• Reduce changes in protein expression pattern [50]	• Increase embryo development [57,59,61,67,68]	• Maintain mitochondria membrane potential [74]	• Maintain the survival after transplantation [79,80]
• Improve plasma membrane integrity [34,37–40,44,55]	• Reduce caspase activity [59,66]		• Enhance blastomere viability [78]
• Improve fertility [39,51]	• Improve fertilization [57,60,64,68]		• Increase survival rate [82]
• Maintain acrosomal integrity [42,44,45]	• Stabilize microfilamentous morphology [60]		• Improve spermatogonia production [85]
• Maintain mitochondria membrane potential [34,45]	• Reduce ROS[a] production [61,66,67]		
• Higher sperm normal morphology [34]	• Maintain mitochondria membrane potential [58]		

[a] ROS: reactive oxygen species.

Table 2
Data of outcomes analysis on low-temperature preservation of germplasm and embryos with antifreeze proteins (AFPs) in 1990–2020.

			Articles	Experiments			
				Total	Cold Liquid	Slow-freezing	Vitrifi-cation
Semen	*Mammal*	Total number	16	23	8	21	0
		Positive outcomes (%)	93.8	82.6	0	90.5	.
		Negative outcomes (%)	43.8	30.4	25	23.8	.
	Fish	Total number	9	11	2	6	3
		Positive outcomes (%)	88.9	90.9	100	83.3	100
		Negative outcomes (%)	44.4	36.4	0	16.7	100
Oocytes	*Mammal*	Total number	14	18	6	0	15
		Positive outcomes (%)	92.9	88.9	66.7	.	86.7
		Negative outcomes (%)	28.6	27.8	16.7	.	26.7
Embryos	*Mammal*	Total number	10	19	6	5	9
		Positive outcomes (%)	80	52.6	33.3	0	88.9
		Negative outcomes (%)	50	31.6	0	80	22.2
	Fish	Total number	3	5	3	2	1
		Positive outcomes (%)	66.7	60	33.3	50	100
		Negative outcomes (%)	0	0	0	0	0

[22,31,57–68]. Antifreeze proteins used in cold-liquid storage processes promoted beneficial results [22,57,64,68], but at high concentrations of AFP elicited negative effects in human oocytes [68]. Cell-permeating cryoprotectants (glycerol, ethylene glycol, DMSO or propanediol) were added to vitrification media along with AFP [31,57–63,65–67]. Higher concentrations of AFP showed less benefits than lower concentrations for vitrified samples [58,67]. Cytotoxic effects were observed in mice oocytes kept at room temperature with AFPs added to media [57], but not in pig oocytes [31]. Six articles reported experiments comparing two or more AFP, promoting similar positive results [63,64,67,68], whereas other studies showed that some AFP type was more advantageous than others [31,61].

3.5. Embryos

Mammalian embryos were preserved with the aid of AFP at concentrations that varied from 0.1 μg/mL to 50 mg/mL [31,46,67,69–75]. The developmental stage of mammalian embryos varied from 2-cell to blastocyst. Both AFP I and nfeAFP11 (a recombinant AFP III-type) were beneficial for cold-liquid storage [71,73]. In sheep, embryos chilled in media with AFP I maintained a similar hatching rate, viability, and diameter as fresh embryos [71]. Slow-freezing with the aid of AFP resulted in no advantage compared to control, and, often a reduction in embryo survival was observed [69,70,72]. Although no adverse effect was observed with the use of 0.1 mg/mL AFPs, a dose-related negative effect was observed with the use of 1 mg/mL AFP I or III, which disrupted post-thaw survival, comparing to controls [69]. Embryos benefited from AFP added to vitrification media, showed higher survival rates than controls [31,46,66,67,75]. Dose-related negative effects of AFP on embryo vitrification were described [46,66]. Comparison of multiple AFPs for embryo vitrification allowed the observation of similar positive outcomes [31,67]. No evidence of cytotoxic effects was observed when AFP I or III were added in media at room temperature [70].

The AFP concentrations varied from 40 μg/mL to 10 mg/mL for preserving fish embryos [76–78]. Two articles reported better survival rates after the use of AFP for chilling embryos [77,78]. A positive effect of AFP I in mitigating reduction in embryo survival after slow-freezing was reported [78]. Both AFP I and III promoted positive effects on vitrified embryos; however, AFP I promoted superior results [78]. No cytotoxic effects of AFP on fish embryos were observed. Likewise, no evidence of cytotoxicity promoted by AFP was observed when embryos were microinjected with AFPs and submitted to chilling process [76,77].

3.6. Other reproductive specimens

For vitrification of mouse ovaries, concentrations of AFP ranged from 0.1 mg/mL to 20 mg/mL in the media combined with cell-permeating cryoprotectants (ethylene glycol with or without DMSO) [79–82]. Positive effects were reported in all articles with exception of one where a negative effect was observed related to the use of lower concentrations of AFP [80]. Association of two different AFPs, AFP III and *Flavobacterium frigoris* ice-binding protein (FfIBP), resulted in preservation of a higher proportion of intact ovarian primordial follicles compared to the use of each AFP alone [81]. Moreover, slow-freezing of hamster CHO-K1 ovarian cells with 0.1 mg/mL of *Glaciozyma* sp. antifreeze protein (LeIBP) in the media promoted a 10% increase in cell viability, in comparison to media with 5% DMSO [83].

In fishes, the use of AFP at concentrations of 10 mg/mL for blastomeres [78], 10 mg/mL to 20 mg/mL for primordial germ cells (PGCs) and gonadal ridges [84], and from 0.1 μg/mL to 10 μg/mL for testes [85], with ethylene glycol and/or DMSO were described. The AFP I helped mitigating viability loss after slow-freezing in blastomeres [78], but was detrimental to PGC viability in slow-freezing of gonadal ridges [84]. The use of AFP I or AFP III in slow-freezing of fish testes has been shown to reduce cell viability. When both AFPs were associated to hypotaurine or catalase, beneficial results on improvement of spermatogonia production and viability of frozen-thawed testes were observed [85].

4. Discussion

Reproductive-related specimens, including gametes, embryos, and gonads, from human, non-human mammalians, and fishes have been preserved at low temperatures in media containing AFP. Cryopreservation is often detrimental to the structure and function of those cells and tissues due to biophysical and physiochemical events, such as cold, osmotic shock, ice crystal formation, and cryoprotectant toxicity [86]. Antifreeze proteins have been shown to protect cell membranes by avoiding ion leakage [87], which might be helpful during the chilling process. When specimens are submitted to slow-freezing processes, adding AFP induces a thermal hysteresis reaction, which reduces the occurrence of ice crystal and controls the velocity that these are formed [25]. In the

vitrification process, AFP increases solution viscosity [88]. Moreover, during the process of freezing and thawing specimens, adding AFP inhibits ice recrystallization [26,83], which is helpful to avoid cell damage. Some AFPs may also protect cells by reducing the concentrations of other cryoprotectants added to media. As seen in ovarian CHO-K1 cells, AFP produced better results with a lower concentration (5% × 10%) of DMSO [83].

Antifreeze proteins have most often elicited positive outcomes in low-temperature preservation of reproductive specimens, including increase in cell viability after thawing/warming (Table 1). However, when AFPs were added in higher concentrations, detrimental effects were observed to those cells and tissues. It is noteworthy that high AFP concentrations may have cytotoxic effects [32,89]. Indeed, AFPs were shown to interfere with membrane permeability to ions [87] and enzymes [33] at chilling temperatures, which might help to explain how AFPs' cytotoxicity occurs. Such effects could also be related to alterations in ice crystals shape, from less to more harmful shapes, as AFP concentrations increase [29] (Fig. 1C).

Most of the studies on AFP in mammals and fishes aimed to enhance sperm cryopreservation. The use of AFP in sperm freezing seeks to improve spermatozoa post-thawing survival and fertilization rate preservation, being the majority of the outcomes reported herein. In mammals, AFP benefited the process of semen slow-freezing, whereas positive outcomes were not observed in semen kept at 4–5 °C. Fertilization potential after the use of AFP-treated semen was tested only in sea bream, sterlet, and buffalo [39,50,51,54]. More studies investigating fertilization rates of semen cryopreserved with AFP would help to clarify the role of AFP in improving sperm survival parameters that support its major functionality: fertilizing and supporting the development of a viable individual. Consequently, a more robust support for AFP use in semen cryopreservation media could be established. Despite that, the positive outcomes obtained after the use of naturally-sourced or recombinant AFP III for cryopreservation of semen from human or non-human primates [43,44], encourage its further application in clinical ART.

In mammalian semen, AFP cytotoxicity was not observed at room temperature, but at chilling: at 17 °C in cattle, or at 4–5 °C in both sheep and chimpanzee [32,36,44]. Detrimental effect of a higher concentration of AFP was clearly observed in frozen/thawed human semen [43]. No cytotoxicity was observed in one study that added AFP to fresh chilled fish semen [48]; however, others described inferior results associated with higher doses of AFP during freezing of sterlet and vitrification of Persian sturgeon semen [52–55]. Moreover, the AFP detrimental effect on sperm appears to depend mostly on concentration, but also on AFP type. In addition, the presence of cell-permeating cryoprotectant in the media might also have contributed to those effects [32]. Regarding the volume of AFP used, lower concentrations, in the nanogram to microgram per milliliter range, appear to be adequate for most mammalian species. For cold-liquid storage of fish semen, AFP concentrations in the range of milligrams per milliliter appear to work best, whereas in slow-freezing or vitrification of those specimens, micrograms of AFP (in concentrations similar to those found in the fluids from the source species) seem more appropriate. Hence, choosing the most appropriate AFP concentrations for low-temperature preservation of semen requires special attention because of the narrow range of AFP concentration that is capable of protect spermatozoa from cryodamage.

Preservation of oocytes at low-temperature allows cryobanking of female germplasm and supports chronological management of several ARTs, such as *in vitro* production of embryos, cloning, and transgenesis [3,90]. One of the main disadvantages of oocyte cryopreservation is the high sensitivity to cold exposure and the relative fragility of this oversized cell [91]. Preservation of oocyte at low-temperature with added AFP has been performed only in mammals. The addition of AFPs were shown to be effective in improving fertilization rates. Increased fertilization resulting from the use of AFP in media can be supported by observation of some effects of AFP at the molecular level, such as maintenance of normal meiotic spindle organization and chromosome alignment [58], reduction in ROS production [61], and maintenance of cell membrane structure [31]. In general, mammalian oocytes benefited from the use of AFP at a higher concentration range, when compared to semen. Although an ideal concentration of AFP must be chosen, as shown for cattle and mouse oocytes, cytotoxicity appears not to be as prevalent as it is for semen preservation. Keeping oocytes incubated with AFP at chilling temperature before vitrification appears to be the best approach, in comparison to keeping them at room temperature, as shown in the mouse [57]. Storing oocytes with AFP in cold-liquid was shown to be effective for all species tested [57,64,68,69], but a high-dose cytotoxicity should be taken into account, especially in human oocytes [68].

Embryo cryopreservation has been widely used in the fishery and livestock industry, particularly in domestic ruminants, and has helped to accelerate genetic improvement, or to alleviate infertility [2,92,93]. More recently, the interest in embryo cryopreservation has escalated in human medicine, particularly in the context of the increasing practice of single embryo transfer and pre-implantation genetic testing [94,95]. Antifreeze proteins have improved viability of zebrafish, pig, mouse, rabbit, and cattle *in vivo*-produced and vitrified embryos. Vitrification conducted in *in vitro*-produced sheep and cattle embryos also benefited from the use of AFP. Some of the valuable effects of AFP in embryos include higher survival and development, and lower apoptosis rates. The procedure of embryo vitrification has been performed for many years [96] and has increasingly become the cryopreservation method of choice in some species, especially, for *in vitro*-produced embryos [92]. In the fish industry, since the benefits of using AFP for embryo preservation appear to vary significantly among species, the positive results observed in sea bream and zebrafish should encourage further research aiming to adapt its use for other species of economic or diversity interest. In farm animals, the wide use of embryo vitrification has the disadvantage of in-straw dilution without the use of a microscope for thawing [97], and AFP could be helpful as a non-penetrating adjuvant in the warming process. This latter scenario deserves further investigation.

All the studied mammalian embryos, except for rabbits, tolerated concentrations of AFP in the milligram range. At room temperature, exposure to AFP might not disrupt embryo quality, as shown in mice [70]. Chilling with AFP is beneficial for sheep [71] and cattle embryos [73]. Although the results observed after the use of AFP for slow-freezing of mice [69,70] or horse [72] embryos, indicate a disruptive effect of AFP in these specimens, no other study on mammalian embryo slow-freezing with AFP is available. It might be worth to test AFP as slow-freezing adjuvants for embryos of other species, in which higher survival rates after cryopreservation are possible, such as bovine.

Ovary cryopreservation is a way of attempting restoring fertility by autografting preserved ovaries after reprotoxic anti-cancer treatment in young women [5], and to preserve fertility in domestic animals [98]. Promising results were provided by AFP for mouse ovaries cryopreservation. The outcomes included maintenance of ovarian follicles viability and cellular function after thawing, and they were more pronounced when higher (up to 20 mg/mL) AFP concentrations were used. In zebrafish, vitrification or freezing gonadal ridges, aiming the retrieval of PGCs for conservation of diploid genome, has been attempted [84]. The use of AFP was indifferent or even led to negative results when AFP was

present at higher concentrations. For slow-freezing of blue catfish testes, AFP alone did not improve spermatogonia production and viability; the positive outcomes were dependent on the concurrent presence of catalase or hypotaurine [85]. Zebrafish blastomeres [78] and PGC dissociated from gonadal ridges [84] were cryopreserved. While 10 mg/mL AFP I was helpful in the former specimen, it did not produce advantage in the latter. This result indicated that beneficial effects of AFP for cryopreservation also depends on cellular type. Those results encourage further investigation on complementary effects of AFP and antioxidants for cryopreservation of gonads.

Studies on cryopreservation of mammalian reproductive specimens were conducted using AFP from diverse sources: extracted from fish, recombinant, and synthetic (Supplementary Table 2). Among the few studies in mammalian semen that compared AFP types, it appears that the fish-extracted AFP I elicits better outcomes than AFP III or antifreeze glycoprotein (AFGP), at least in sheep and cattle. Despite this finding, AFP III (including the naturally-produced or recombinant) has been tested in the majority of studies, not only with mammalian semen, but also oocytes and embryos. Due to the scarcity of studies comparing different AFP sources, determination of the most recommended type of AFP for cryopreserving mammalian reproductive specimens are not possible to be made so far. Recombinant *Glaciozyma* sp. antifreeze protein (rLeIBP) has been used recently to cryopreserve mouse ovaries and cattle embryos and appears to lead to superior results than AFP III. Regarding preservation of mammalian oocytes and embryos, due to the high variation in AFP type and concentration used in these studies, it has become challenging to determine a protocol that appears more suitable to produce better outcomes. It is worth noting that the first report on the use of more than one type of AFP concomitantly in the media was performed in mouse oocytes [81]. In that study, the association of two AFP (recombinant AFP III - rAFP III plus FfIBP) promoted better results than the use of each one alone. However, in that specific design, it became difficult to infer if the effect was produced by a synergy-like effect of two different molecules, or simply because the total AFP dose was doubled. Research associating different types of AFP addressing possible synergistic effects on reproductive cells and tissues may be an interesting approach for further studies.

The only reliable sources of AFP used for low-temperature preservation of fish reproductive specimens are those naturally extracted from other species of fishes. While AFGP was tested only for chilling Middle Russian carp semen, AFP I and/or AFP III were used in all other studies. From the data retrieved, AFP III produces superior results for semen cryopreservation (slow-frozen and vitrification), AFP I is most suitable for embryo slow-freezing, whereas both types produce similar results for embryo vitrification. However, such inferences need further confirmation, since AFP efficiency seems to vary with species, and embryo developmental stage. The use of one type of AFP alone, although most often tested, was shown to be not as good as association of AFP I and III, at least as recently revealed for vitrification of sea bream semen [51]. Thus, the synergistic activity of more than one type of AFP is a feature that deserves further exploration.

From what has been addressed throughout the last three decades, it is clear that AFP can be helpful for low-temperature preservation, most significantly cryopreservation of reproductive specimens from food-producing mammalian, fishes, laboratory models and humans. Over the last five years, the development of recombinant technologies for producing AFP in bacteria (*Escherichia coli*) or yeast (*Pichia pastoris*) has broadened the possibilities of AFP use to a large extent, once it permitted access of higher quantities of AFP, sourced not only from fish genomes, but also from yeast, bacteria, and insects. However, there are still limitations for the use of AFP from those natural or recombinant sources, especially, for cryopreservation of human specimens.

The naturally-derived AFGP inspired the *in-vitro* synthesis of AFGP-8, which was studied as cryoprotectant in the vitrification media of cattle oocytes and embryos [66,74]. Corroborating the results of fish-isolated or recombinant AFP, the chemically synthesized AFGP-8 elicited beneficial results, regarding post-thaw survival of those specimens. Therefore, considering its cryoprotecting activity and source, AFGP-8 has an extensive potential to be used in human reproductive medicine, as well as in other commercial applications. Other molecules that have ice-binding activities, sometimes named AFP-biosimilar, have been tested as adjuvants for cryopreservation of reproductive specimens. Among them, one or more of the following: copolymer of polyvinyl alcohol, vinyl acetate and polyglycerol polymer were studied for cryopreservation of mammalian reproductive specimens such as mouse oocytes [99] and embryos [100], cattle [101] and horse [102] oocytes, rabbit embryos [103], goat [104] and macaque ovarian tissue [105], producing variable, but mostly positive results. More recently, the carboxylated ε-poly-L-lysine (CPLL), which has been shown to have AFP-like activity by inhibiting ice recrystallization [106], has been studied as non-penetrating cryoprotectant for reproductive specimens. Improvement in cell survival and other relevant features were demonstrated in mouse oocytes [107] and embryos [108–110], pig embryos [111], and semen from buffalo [112,113] and cattle [114]. These findings support the potential for the use of this AFP-like ampholytic polymer in vitrification and slow-freezing procedures on reproductive specimens. Another perspective for exploring AFP functions is on the use of transgenesis. Some studies had generated transgenic mice expressing the AFP III gene [115–117]. Improved microstructure of cell membrane was observed in ovarian cortex, as well as higher fertility after whole ovary vitrification, with the expression of AFP III in mouse tissues, including reproductive ones [115–117]. The results in transgenesis are promising to maintain expression of these proteins in reproductive tissues, leading to a better cryotolerance of tissues from transgenic organisms [116,117]. Nonetheless, the transgenesis approach remains only to research while the transgenesis use on food-producing animals could entail the risk of undesirable effects, such as development of food allergies [118].

5. Conclusions

Antifreeze proteins of diverse origins have been shown to be potentially efficient agents in cryopreservation of mammalian and fish reproductive specimens, being successfully applied in different techniques and animal models. Overall, the studies conducted over three decades showed an important role of AFP on improving reproductive cell survival and functionality after thawing/warming. However, detrimental effect of AFP on cells, such as changes in their ultrastructure and metabolism remain to be investigated in depth. A considerable potential for the use of chemically synthesized AFP or AFP-like molecules has emerged in the last years. The application of these molecules in human and veterinary medicines, as well as in genetic preservation programs, and animal protein industries deserves further exploration. Moreover, bioengineering using AFP genes may have an important application for the cryopreservation of reproductive tissues or slices, especially for cryobanking purposes.

CRediT authorship contribution statement

Lucas F.L. Correia: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing, equally contributors'

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Acknowledgements

LFLC was supported by CAPES (Finance Code 001), BRCA by FAPERJ, and RITPB by CNPq. JMGS-F is a FAPERJ and CNPq fellow. This project was supported by CNPq (Project 434302/2018-0), and FAPERJ. We thank Dr. Ligia Prezotto for help with critical English revision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2021.09.025.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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