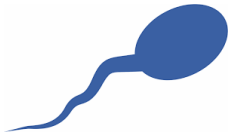
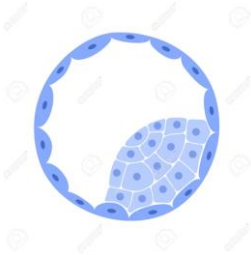
The 3rd CZU hybrid seminar - 2024
Biotechnology in small ruminant reproduction: an international experience

# Antifreeze proteins: potential cryoprotectant of gametes and embryos from small ruminants





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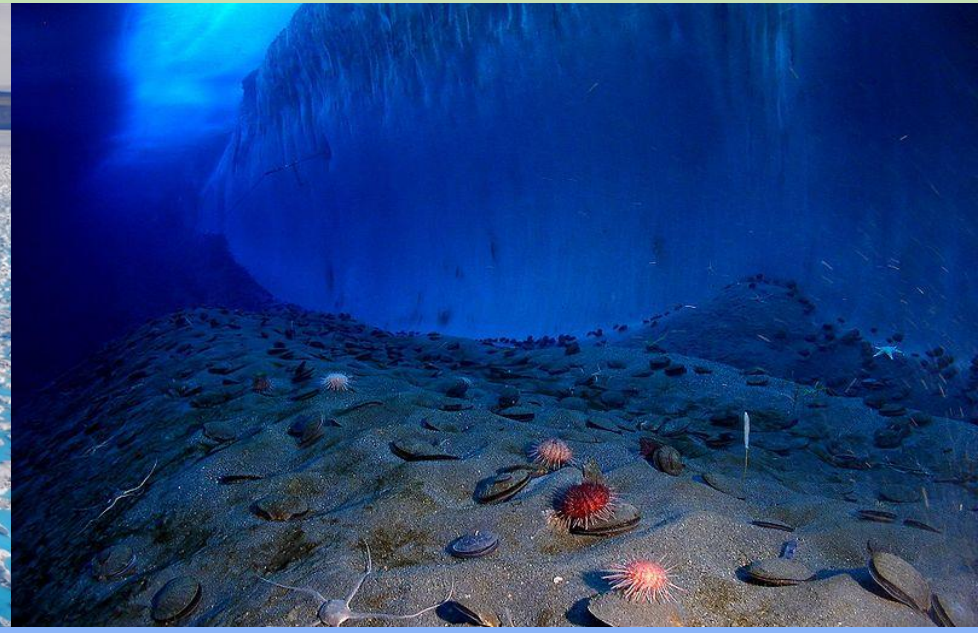
Universidade Federal Fluminense

# Introduction

McMurdo Strait
Water temperature
-1.87 °C



# Introduction

**Freezing Resistance in Some Antarctic Fishes**

Science AAAS (1969)

Abstract. *Measurements of serum freezing points in three Antarctic marine fishes indicated that they do not freeze in the −1.87°C seawater because their blood is isosmotic to seawater. Concentrations of sodium chloride, urea, and free amino acids in the serum accounted for only half of the freezing-point depression of the serum. A protein containing carbohydrate was isolated which accounted for 30 percent of the freezing-point depression of the serum.*

Arthur L. DeVries

*Paraliparis devriesi*

Donald E. Wohlschlag

Wohlschlag Bay

# Introduction

Table 1. Data on blood serum of three Antarctic fishes

| Fish | Serum freezing point (°C) | Serum chloride (mmole/liter) |
|---|---|---|
| *T. borchgrevinki* | − 2.07 ± 0.014(28) | 235 ± 1.6(26) |
| *T. hansoni* | − 2.01 ± 0.019(24) | 259 ± 4.3(24) |
| *T. bernacchii* | − 1.98 ± 0.007(25) | 254 ± 1.9(25) |
| *T. hansoni* | − 1.92 ± 0.015(13) | 258 ± 3.3(13) |
| *T. bernacchii* | − 1.87 ± 0.008(14) | 254 ± 4.4(14) |

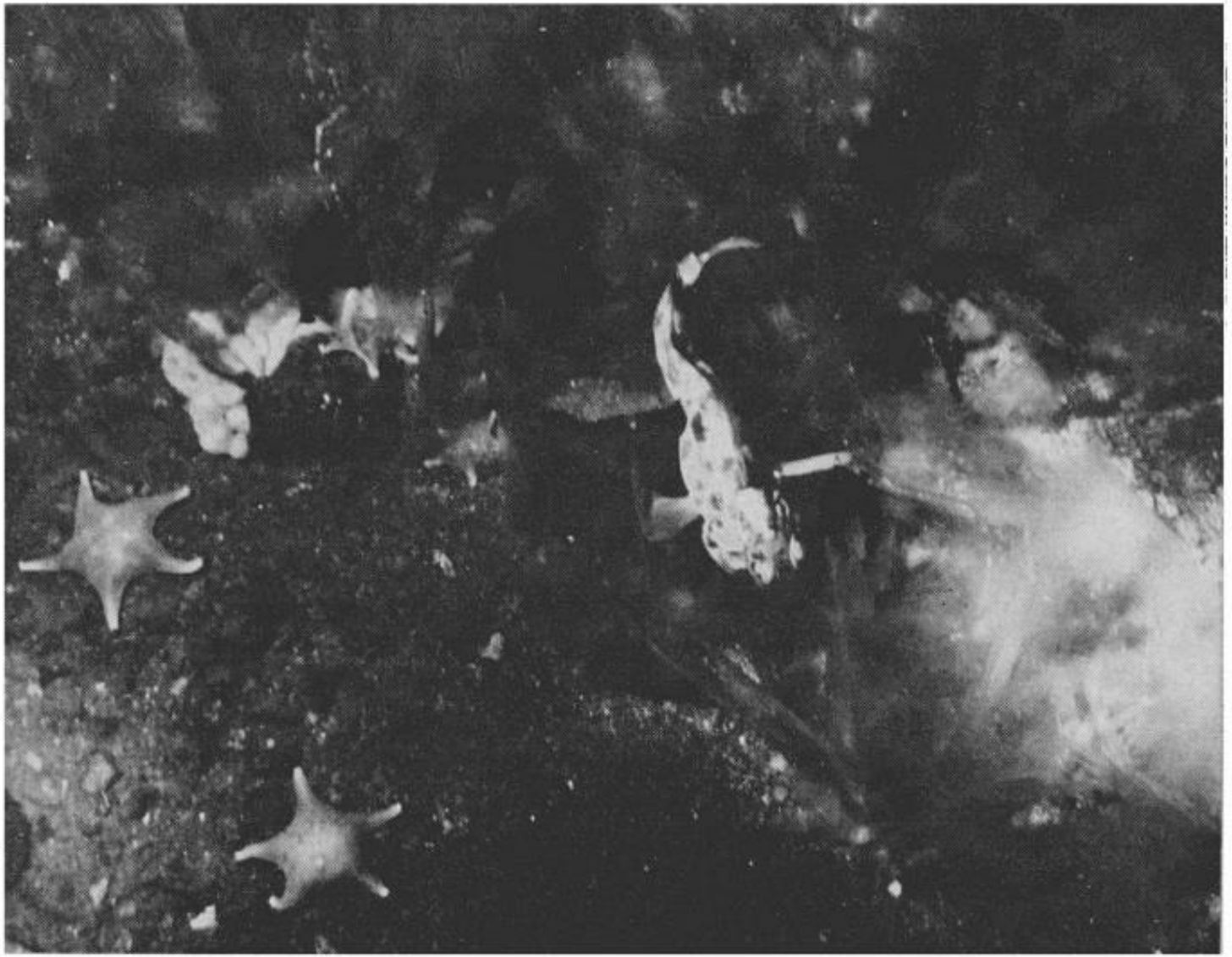
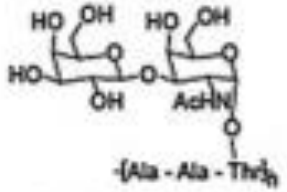
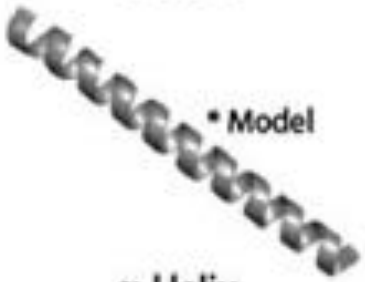


Fig. 1. *Trematomus bernacchii* resting on mass of anchor ice in 20 m of water in McMurdo Sound, Antarctica. [Photo by Paul Dayton]

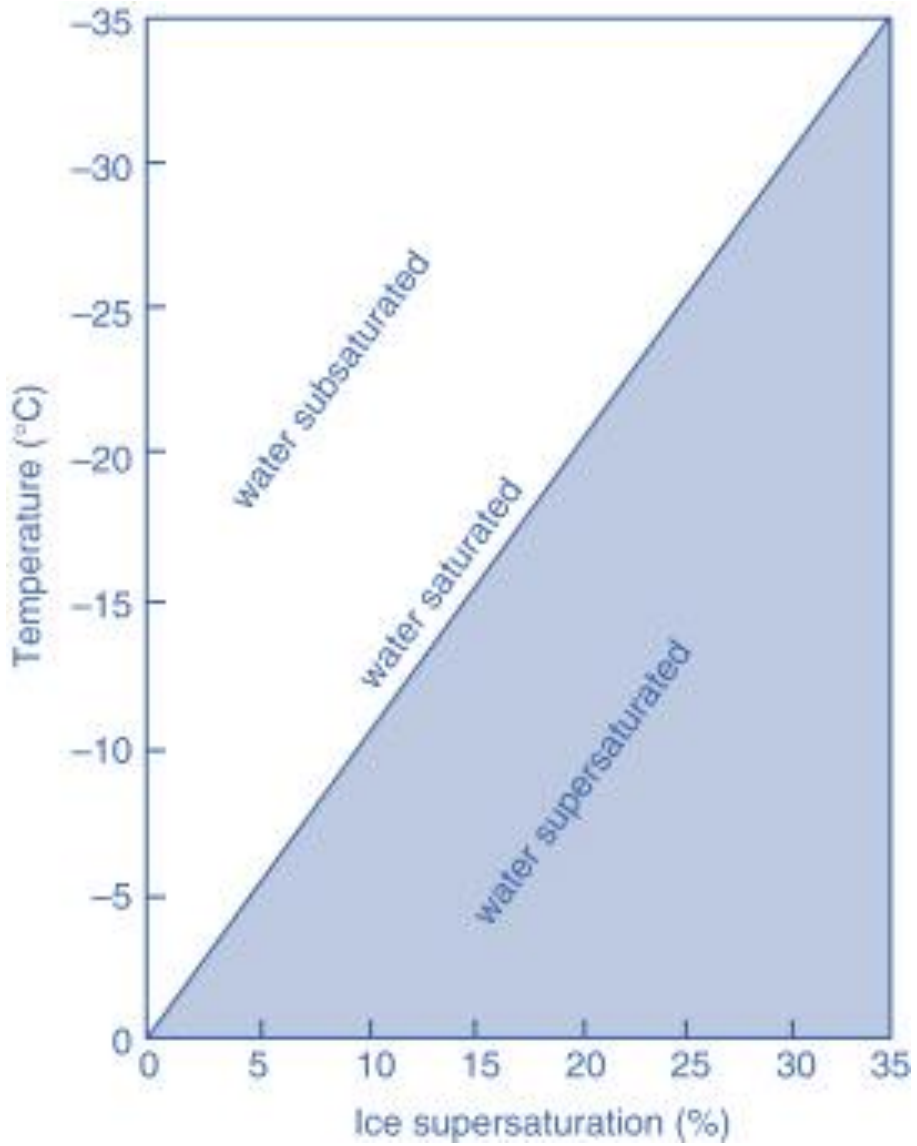
(DeVries; Wohlschlag 1969)

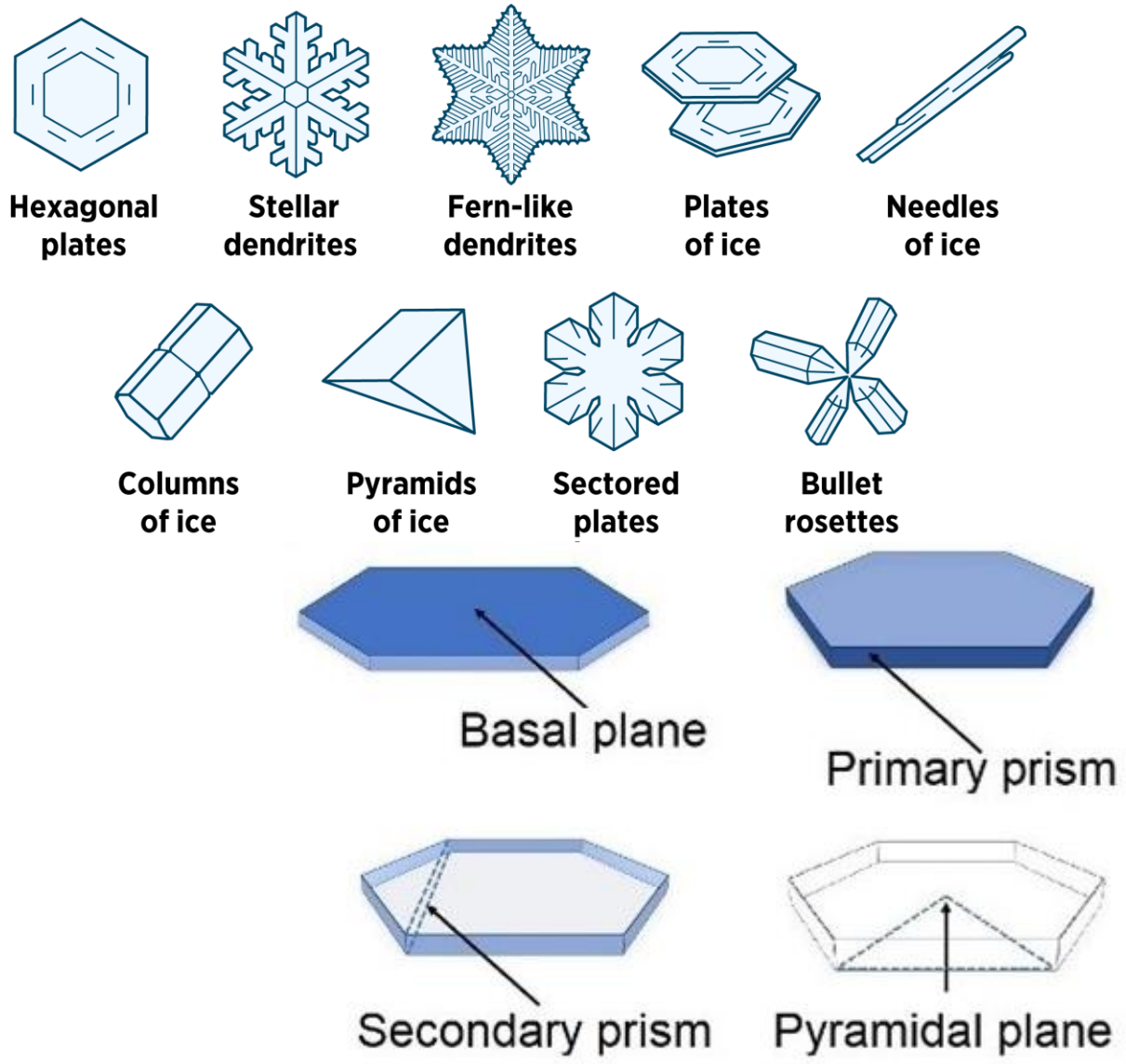
# Antifreeze Proteins (AFPs)

| Characteristic | AFGP | AFPI | AFPII | AFPIII |
|---|---|---|---|---|
| Typical Fish Source |  Emerald Rockcod *Notothenioidei, Gadidae* |  Winter Flounder *Pleuronectidae, Sculpin* |  Atlantic Herring *Clupeidae, Osmeridae* |  Ocean Pout *Zoarcidae* |
| Sequence Properties |  -(Ala - Ala - Thr)$_n$- | Ala-rich, 11-res. repeats | Cys-rich, C-type lectine-like | Unbiassed, SP and QAE-groups |
| Mass (Da) | 2.6 - 34 kDa | 3.3 kDa | 14 kDa | 7 kDa |
| Structure | N/A Polyproline II helix |  * Model α-Helix |  disulfide bonded |  β-Sandwich |

(Mahatabuddin; Tsuda 2018)

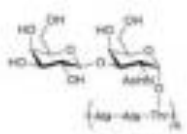
# Ice Crystals

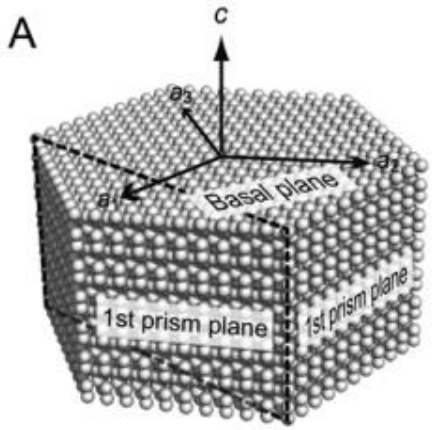


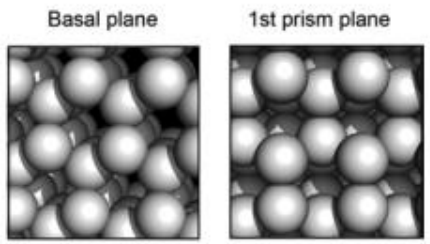




(Pruppacher and Klett, 1978; Cotton et al., 2011; Correia et al., 2021)

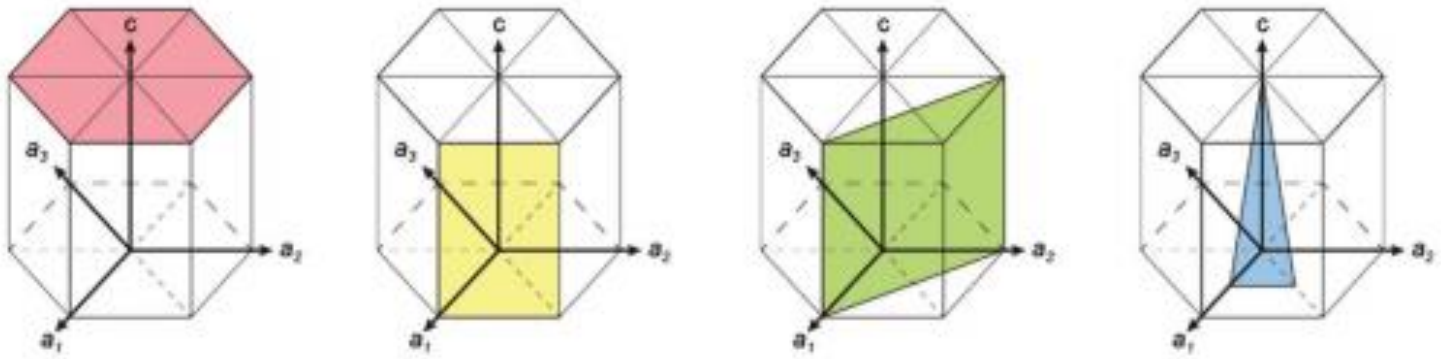
# AFPs and Ice Crystals

| | AFGP | Type I AFP | Type II AFP | Type III AFP | Insect AFP |
|---|---|---|---|---|---|
| Mass (kg mol$^{-1}$) | 3-33 | 3-4.5 | 11-24 | 6.5 | 9 |
| Representative structure |  |  |  |  |  |
| PDB | - | 1WFA | 2PY2 | 1HG7 | - |
| Binding plane |  |  |  |  |  |
| Origin | Antarctic notothenioids, northern cods | Right-eyed flounders, sculpins | Herring, sea raven, smelt | Eel pout, ocean pout, wolfish, | Darkling beetle, spruce budworm moth, midge, fly |




Crystal planes of hexagonal ice (Ih)




basal plane {0001} | primary prism plane {10$\bar{1}$0} | secondary prism plane {11$\bar{2}$0} | pyramidal plane {20$\bar{2}$1}

(Voets 2017; Mahatabuddin; Tsuda 2018)

# Mechanism of action

A- Freezing inhibition
B- Freezing tolerance
C- Ice binding

**Thermal Hysteresis**

**Inhibition of ice recrystallization**

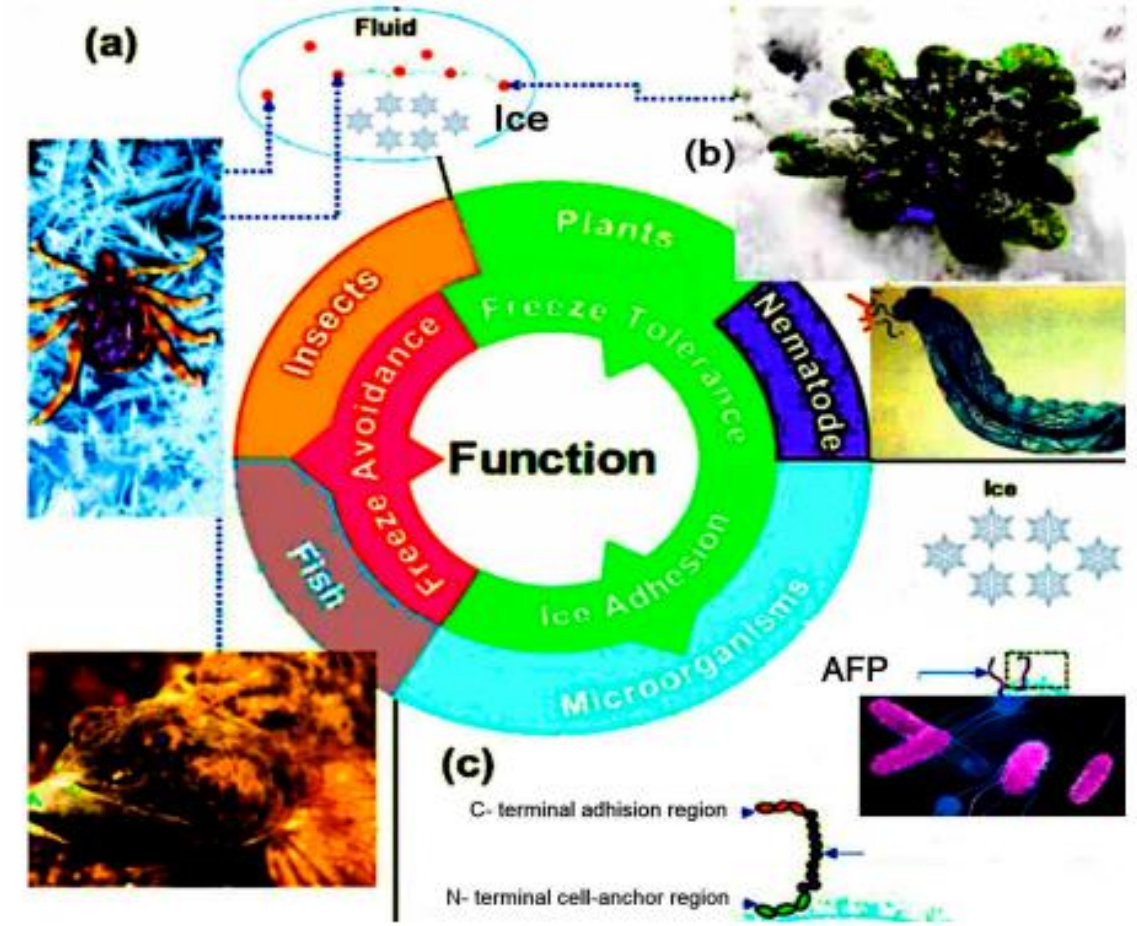
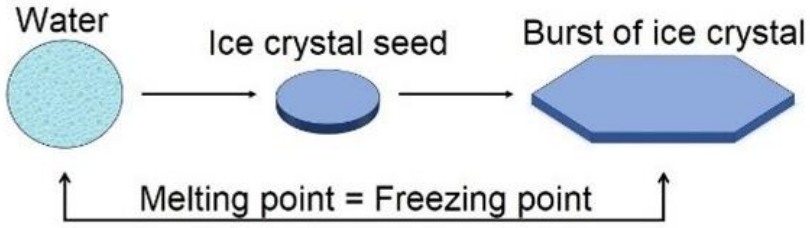
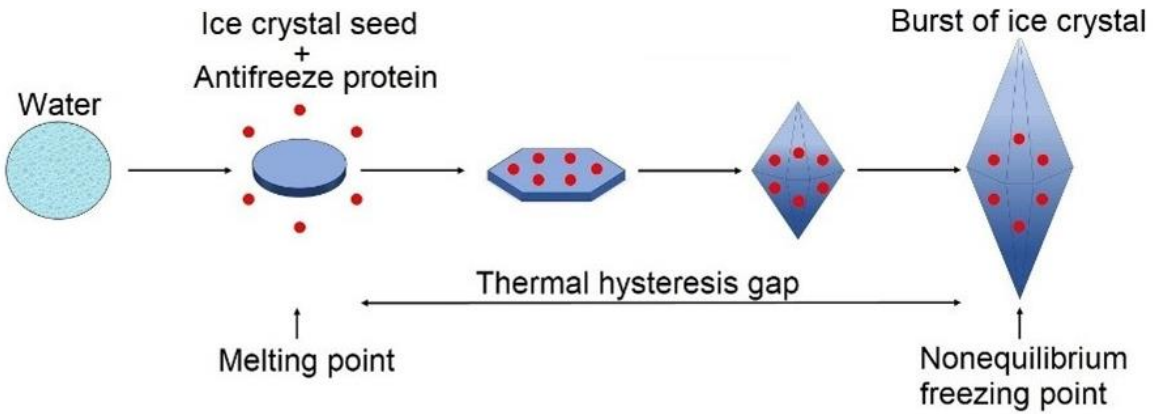



**Figure 1.** The natural properties of antifreeze proteins (AFPs) (thermal hysteresis and ice recrystallization inhibition) in various organisms in nature. (**a**) For freezing inhibition, AFPs can block forming of ice crystals in fish and insects by lowering down the freezing points in body fluids. (**b**) In plants and nematode, freeze-tolerating is carried out by binding the AFPs to the surface of ice and prevent it from becoming larger ice crystals. (**c**) AFPs of different microorganisms can adhere to the ice surface, like *Marinomonas primoryensis*, and inhibit the formation of ice crystals. Adhesion of AFPs to ice can be done from three regions: C terminal, N terminal, and intermediate repeat [10].

(Eskandari et al., 2020)

# Thermal hysteresis



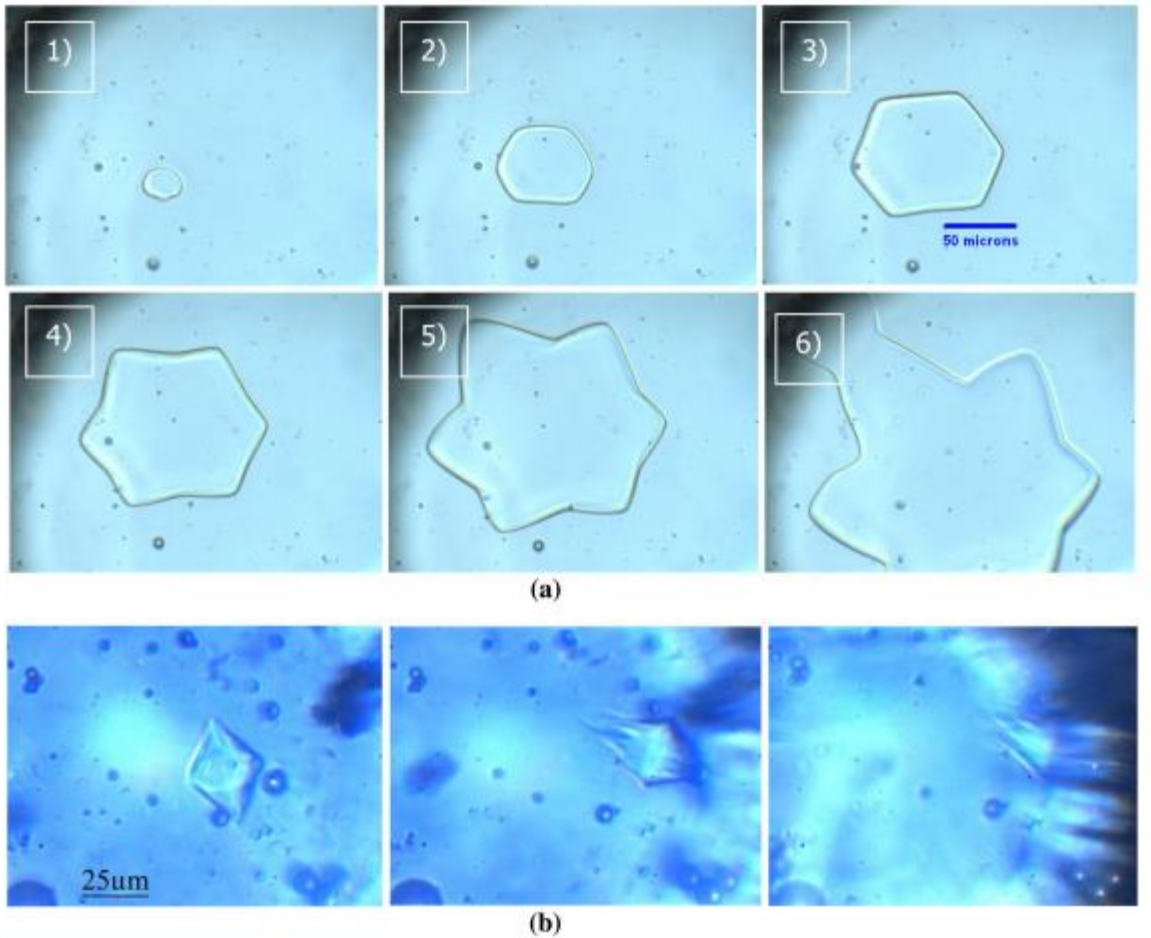
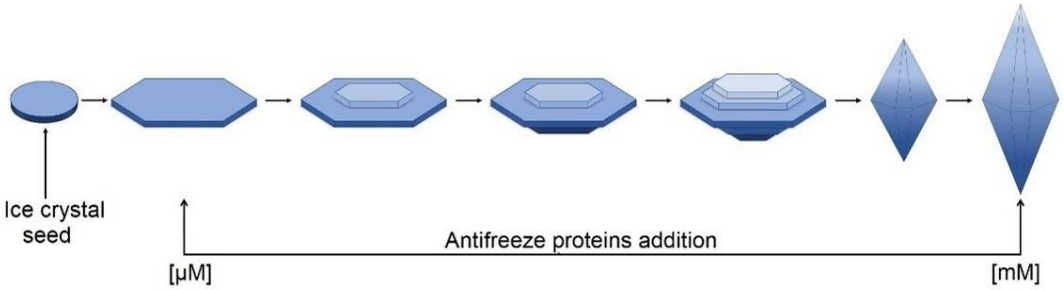




**Fig. 2** **a** Photo images of an ice crystal growing in water at 0 °C: (1) seed ice, (2) the ice crystal growing on the prism facets, (3) the ice crystal has formed a hexagonal shape, (4) the ice crystal further growing from the edges of the hexagon, (5) the ice crystal has formed a star-like shape, and (6) the ice crystal burst into the water from a tip of its star-like shape; **b** photo images of an ice crystal confined by the wild-type type-I AFP at a concentration of 6.0 mg/ml, undergoing decreases in temperature from − 0.09, to − 0.64 and to − 0.65 °C, respectively, from the left to right (the darker circles in the photo images represent air bubbles in the water and the solution)

(Flores et al., 2018; Correia et al., 2021)

# Thermal hysteresis



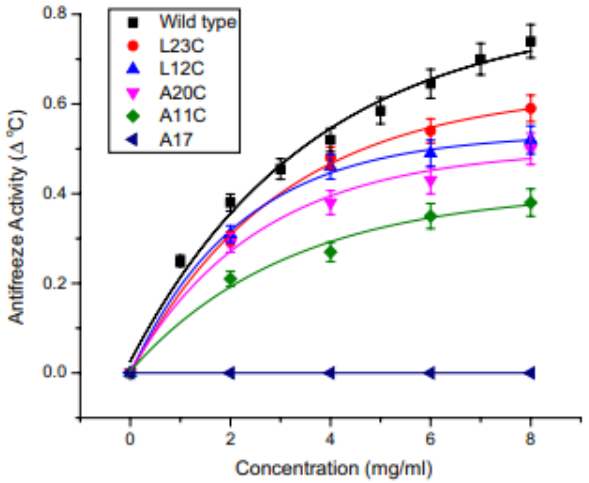




**Fig. 3** Antifreeze activity of the wild-type type-I AFP (black squares), spin-labeled L23C (red discs), spin-labeled L12C (blue, upward-pointing triangles), spin-labeled A20C (purple, downward-pointing triangles), spin-labeled A11C (green diamonds), and spin-labeled A17C (navy blue, leftward-pointing triangles)

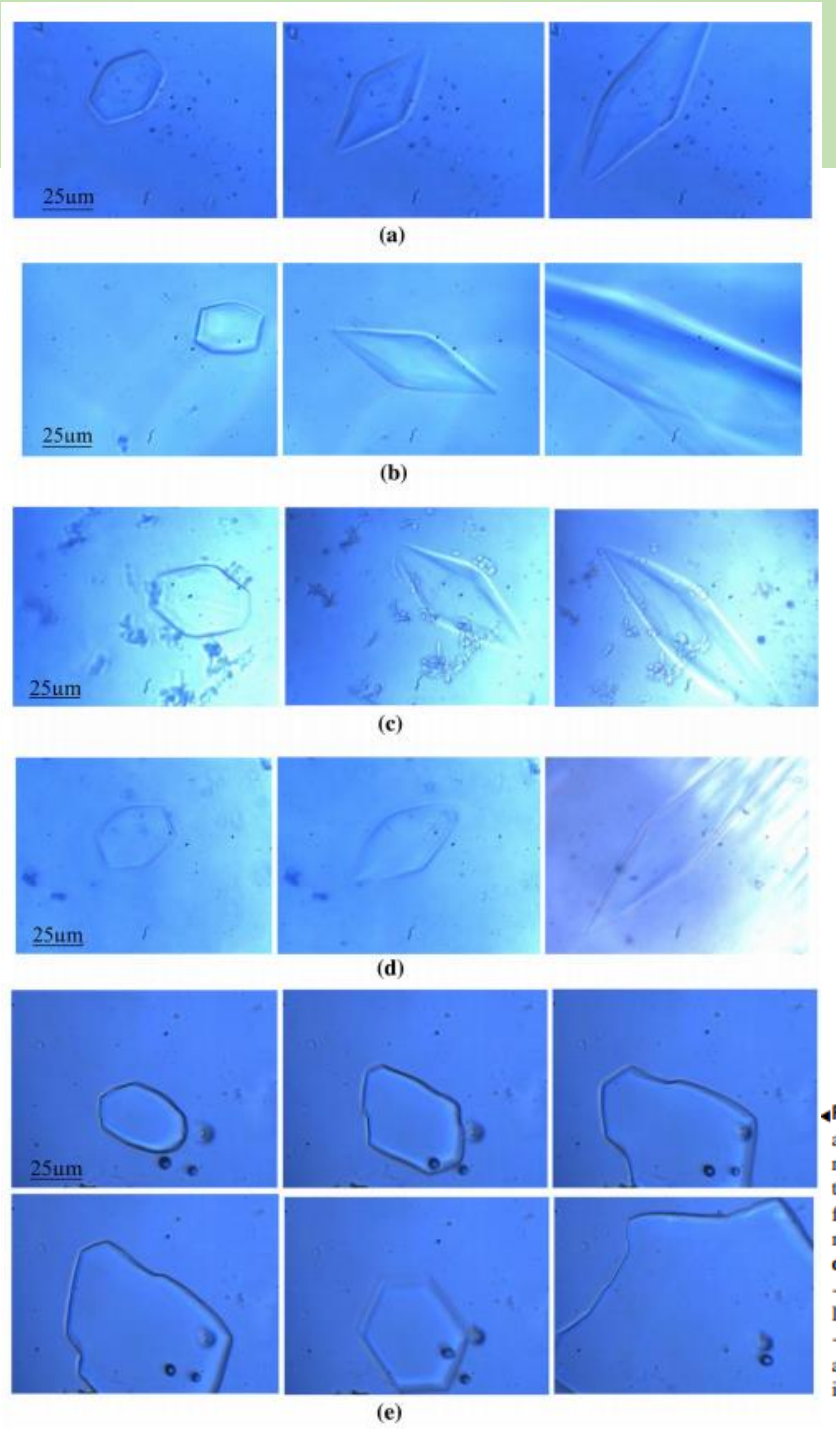



**Fig. 4** Photo images of ice crystals confined by **a** MSL-labeled L23C at a concentration of 8.0 mg/ml at − 0.15, − 0.63, and − 0.72 °C, respectively, from left to right; **b** MSL-labeled L12C at a concentration of 8.0 mg/ml at − 0.08, − 0.34, and − 0.52 °C, respectively, from left to right; **c** MSL-labeled A20C at a concentration of 8.0 mg/ml at − 0.10, − 0.65, and − 0.74 °C, respectively, from left to right; **d** MSL-labeled A11C at a concentration of 8.0 mg/ml at − 0.05, − 0.23, and − 38 °C, respectively, from left to right; and **e** MSL-labeled A17C at a concentration of 8.0 mg/ml at − 0.01, − 0.02, − 0.02 °C, respectively, from left to right (top), and − 0.02, − 0.01, and − 0.02 °C, respectively, from left to right (down) (the differences in hue were caused by the filters used when the images were taken)

(Flores et al., 2018; Correia et al., 2021)

# Thermal hysteresis

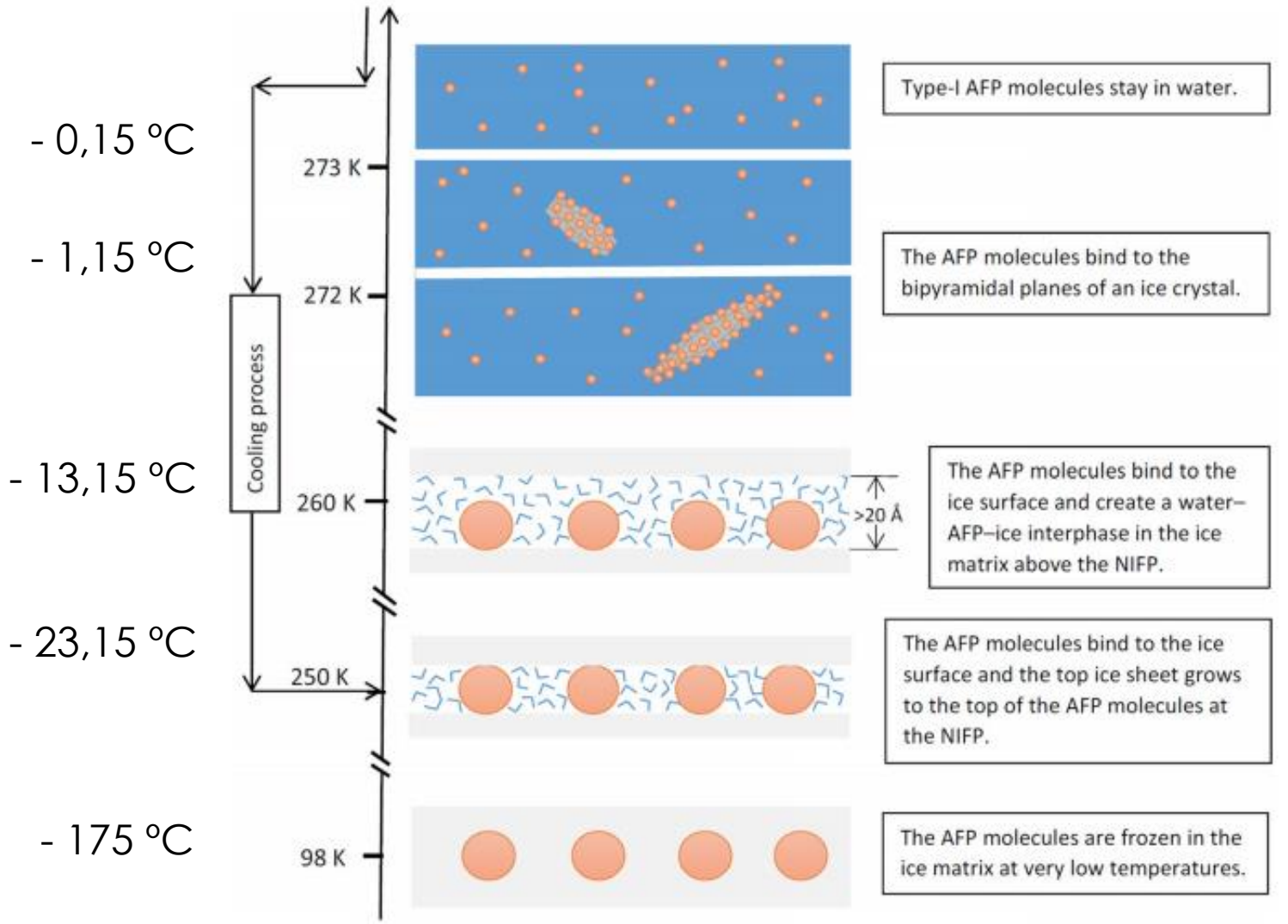
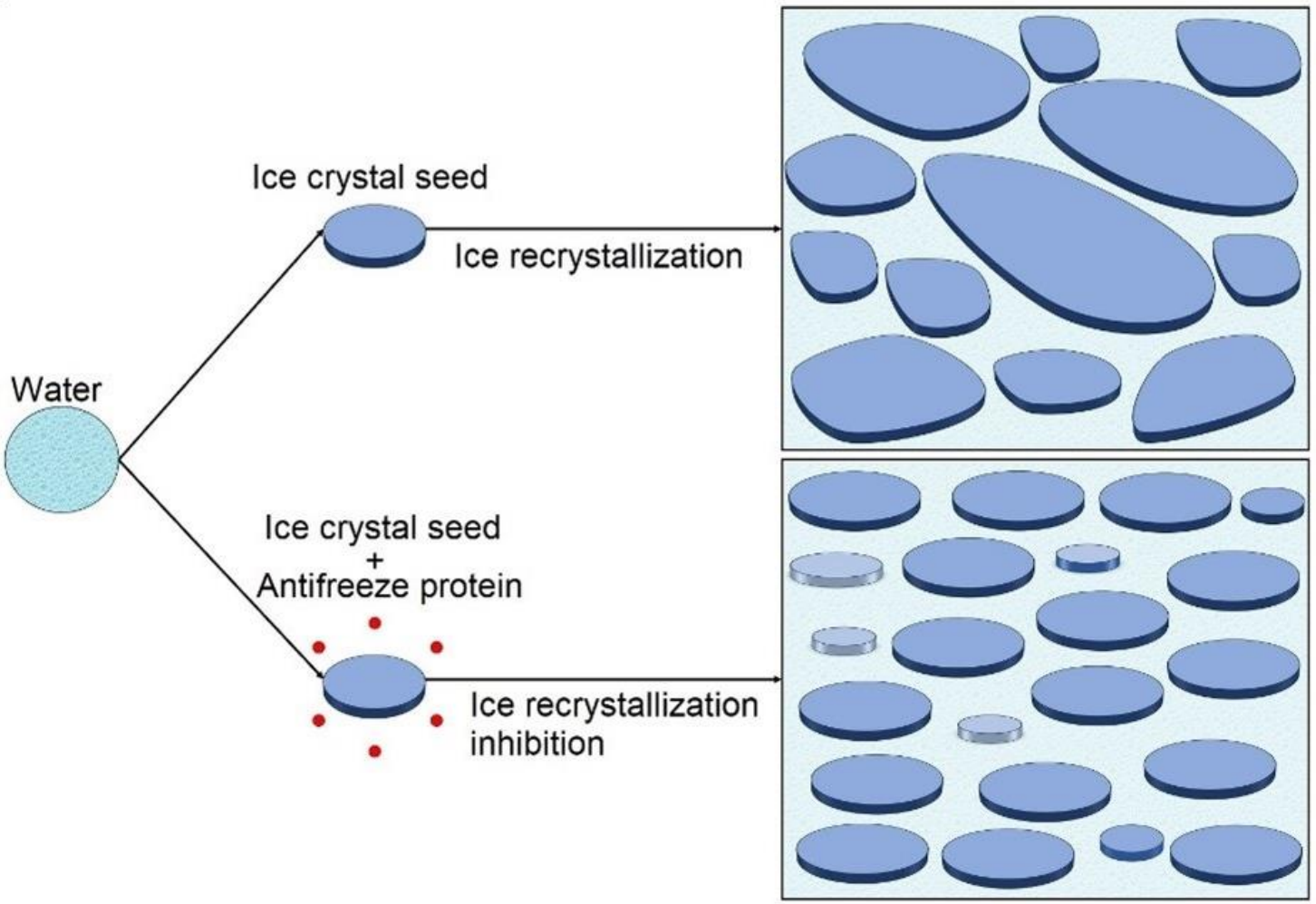



**Fig. 11** Cartoon presentations of the states of water and AFP molecules in the type-I AFP solution at different temperature ranges during the cooling (down) and heating (up) process. The gray color represents hexagonal ice, the blue color represents liquid water, and the orange color represents AFP molecules

(Flores et al., 2018)

# Inhibition of ice recrystallization



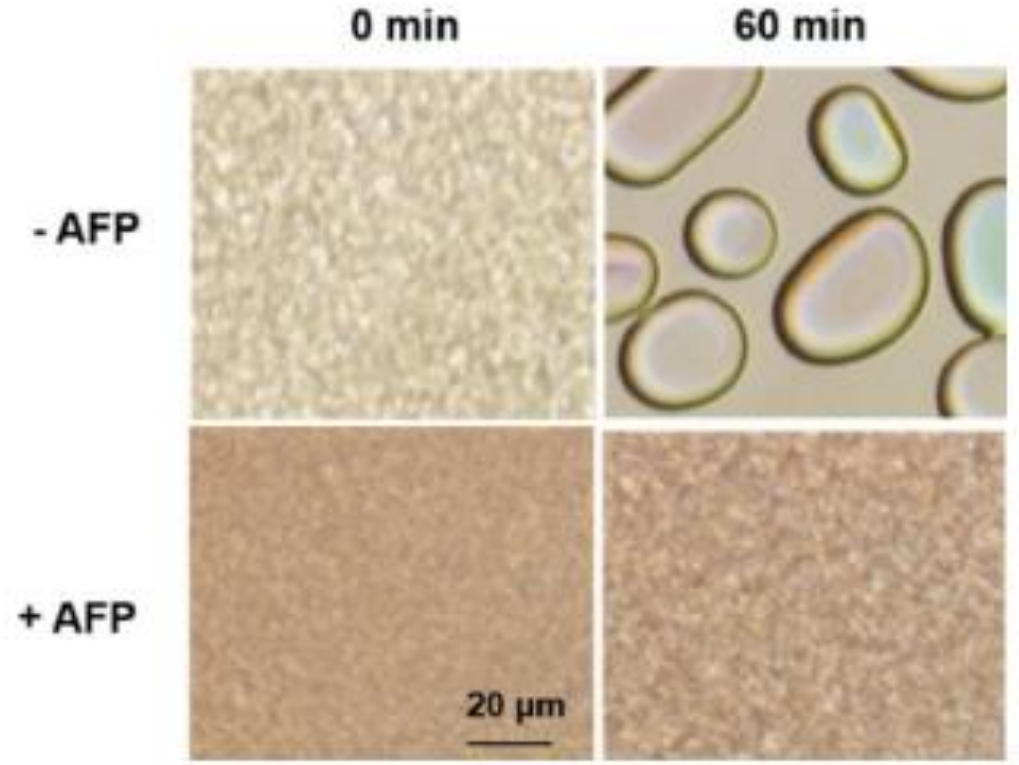
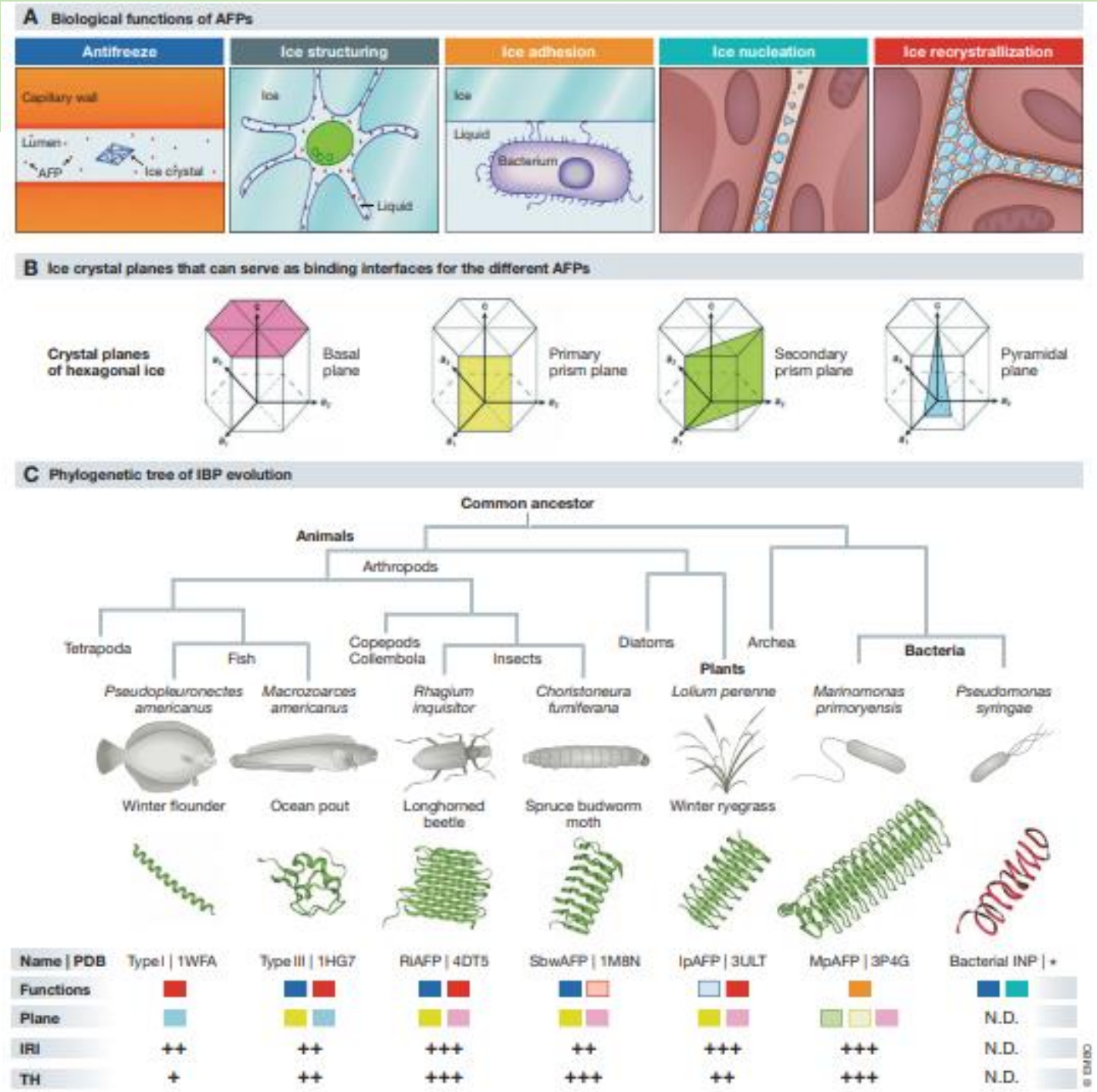



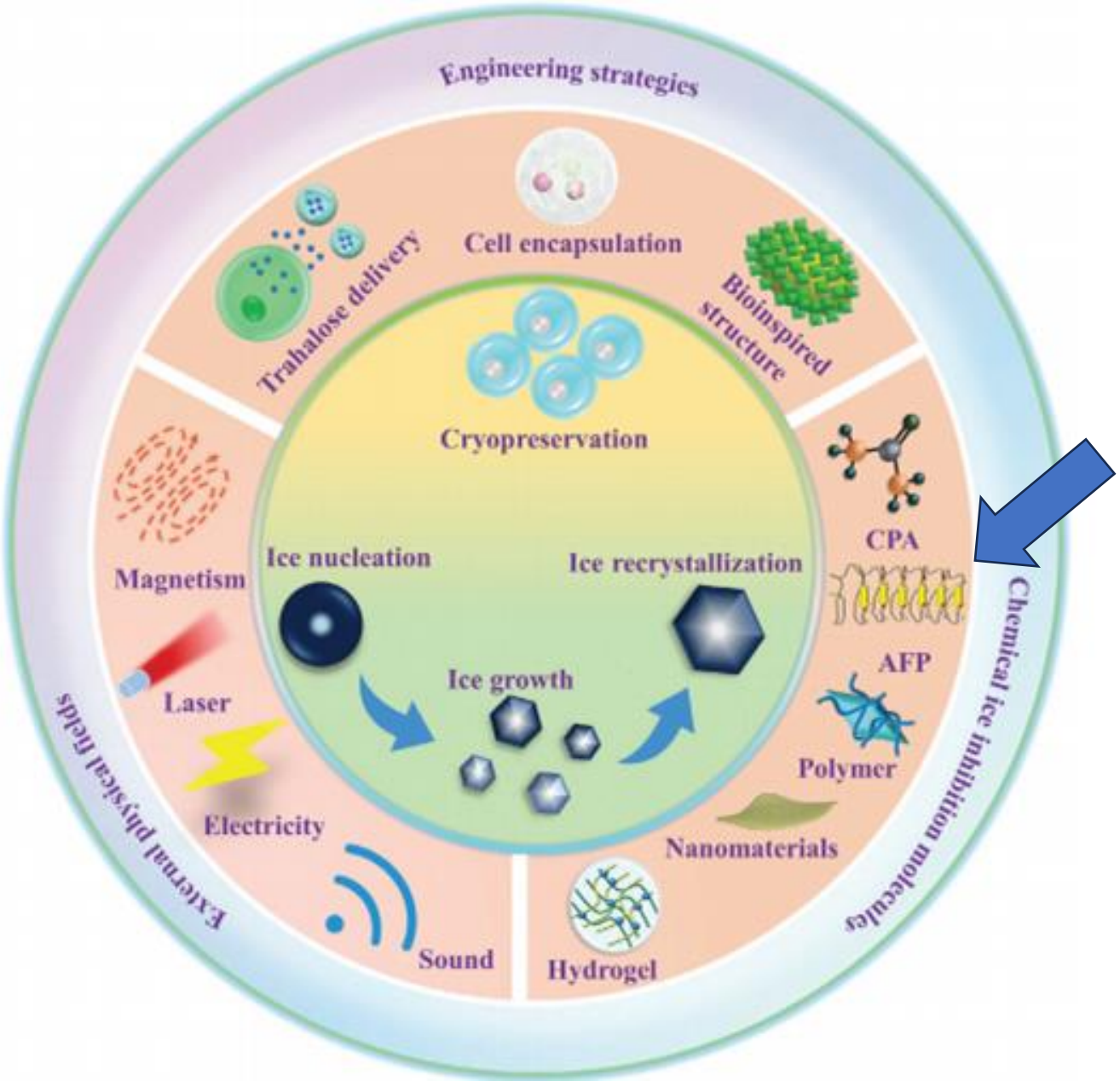

**Fig. 3** Comparison of the status of ice recrystallization in solutions with (+) or without (–) AFP at – 6 °C for 60 min

(Naing; Kim 2019; Correia et al., 2021)




(Tas et al., 2021)

# Cryopreservation strategies




(Tas et al., 2021)

# AFPs in reproductive cryopreservation

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

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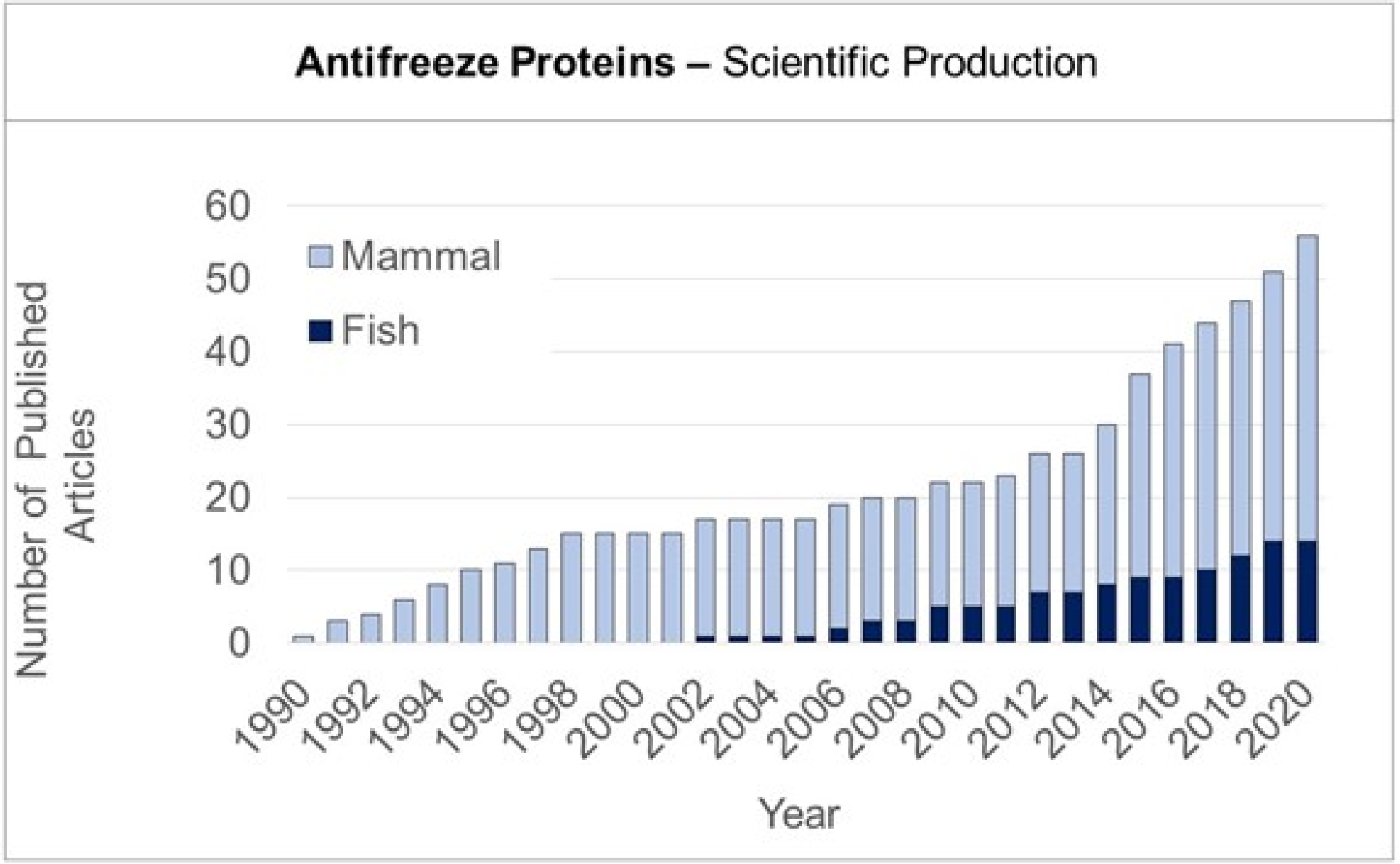
# AFPs in reproductive cryopreservation



# AFPs in reproductive cryopreservation

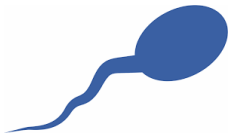
**Table 2.** Data of outcomes analysis on low-temperature preservation of germplasm and embryos with antifreeze proteins (AFPs) 1990-2020.

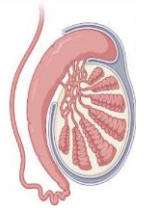
| | | | Articles | Experiments | | | |
|---|---|---|---|---|---|---|---|
| | | | | *Total* | *Cold Liquid* | *Slow-freezing* | *Vitrification* |
| **Semen** | *Mammal* | Total number | 16 | 23 | 8 | 21 | 0 |
| | | Positive outcomes (%) | 93.8 | 82.6 | 0 | 90.5 | . |
| | | Negative outcomes (%) | 43.8 | 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 |

**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* production [61,66,67] | | |
| • Higher sperm normal morphology [34] | • Maintain mitochondria membrane potential [58] | | |

*ROS: reactive oxygen species



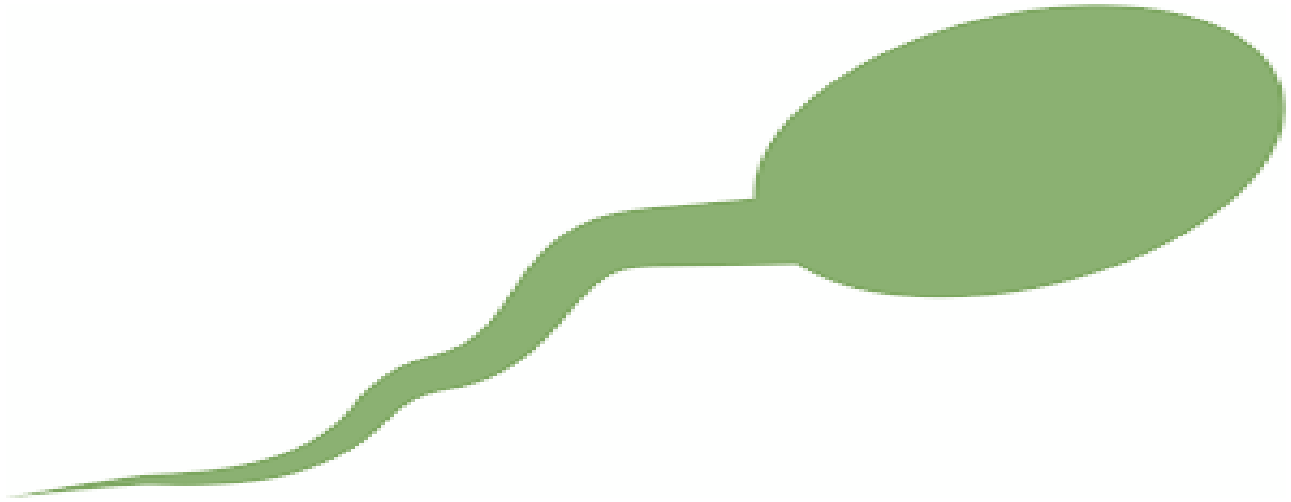




# AFPs in reproductive cryopreservation

**Supplementary Table 2. Characteristics of antifreeze proteins (AFPs) used in different experiments reported in this review.**

| Type | Source / genome | Synthesis | Structure | Mass (kDa) | References in this review |
|---|---|---|---|---|---|
| **AFP I** | *Pseudopleuronectes americanus* (fish) | Natural | Alanine-rich α-helices | 3.3 to 4.5 | [32-35,42,47,49-51,54-56,63,64,68-71,77,84,85] |
| | *Myoxocephalus scorpius* (fish) | | | | |
| **AFP II** | *Hemitripterus americanus* (fish) | | Alanin and cystein rich β-strands and α-helices, extensively disulfide bonded. | 11 to 24 | [63,64] |
| **AFP III** | *Macrozoarces americanus* (fish) | | Globular β-strands connected by large loops, packed orthogonally into a β-sandwich. | 6.5 to 14 | [31,34-37,43,44,46,47,49-52,54-56,58-60,63-65,67-71,76,78,79,85] |
| **AFGP** | *Trematomas borgrevinki* (fish) | | Ala–Ala–Thr repeats with a disaccharide attached to the threonyl hydroxyl group. Has 8 fractions. | 2.7 to 32 | [22,31,32,35,38,41,47,48,57,62,63] |
| | *Dissostichus mawsoni* (fish) | | | | |
| **LeIBP/LAFP** | *Glaciozyma* sp (yeast) | Recombinant | Irregular β helical structure | 27 | [36,42,61,67,80,82,83] |
| **FfIBP** | *Flavobacterium frigoris* (bacteria) | | Irregular β helical structure | 25.3 | [61,80,81] |
| **rAFP III** | *Macrozoarces americanus* (fish) | | Globular β-strands connected by large loops, packed orthogonally into a β-sandwich. | 7 | [40,45,61,80,81] |
| **nfeAFP11** | *Zoarces elongatus* Kner (fish) | | Globular β-strands connected by large loops, packed orthogonally into a β-sandwich. | 7 | [73] |
| **DAFP** | *Dendroides canadensis* (beetle) | | Hydrophilic amino acids-rich 12- or 13-mer repeats | 7.3 to 16.2 | [39] |
| **ApAFP914** | *Anatolica polita* (beetle) | | Parallel β-helix with six repetitive 12-amino acid loops, containing repeats of Thr-Cys-Thr | 20 | [75] |
| **AFGP 8** | not available | *In vitro* | Ala–Ala–Thr repeats with a disaccharide attached to the Thr-OH group. | 2.7 | [66,74] |

# AFPs in small ruminants sperm cryopreservation



Cryobiology. 1994 Apr;31(2):180-4. doi: 10.1006/cryo.1994.1021.

# Effect of antifreeze proteins on the motility of ram spermatozoa

S R Payne [1], J E Oliver, G C Upreti

TABLE 1
Effect of AFP and AFGP on the Motility[a] of Ram Spermatozoa after Cooling and after Freezing and Thawing

| Treatment (μg/ml) | Spermatozoal motility (%)[b] Prefreeze (cooling) | Freeze–thaw | Difference |
|---|---|---|---|
| 0 | 68 | 36 | −32 |
| AFP | | | |
| 0.1 | 54 | 40 | −14 |
| 1 | 51 | 31 | −20 |
| 10 | 68 | 50 | −18 |
| AFGP | | | |
| 0.1 | 43 | 28 | −15 |
| 1 | 57 | 38 | −19 |
| 10 | 60 | 43 | −17 |

[a] Motility scores of 4 were obtained for all treatments.
[b] The data are the average of nine independent determinations.
[c] SED = standard error for the differences between means.

Cryobiology. 1994 Apr;31(2):180-4. doi: 10.1006/cryo.1994.1021.

# Effect of antifreeze proteins on the motility of ram spermatozoa

S R Payne [1], J E Oliver, G C Upreti

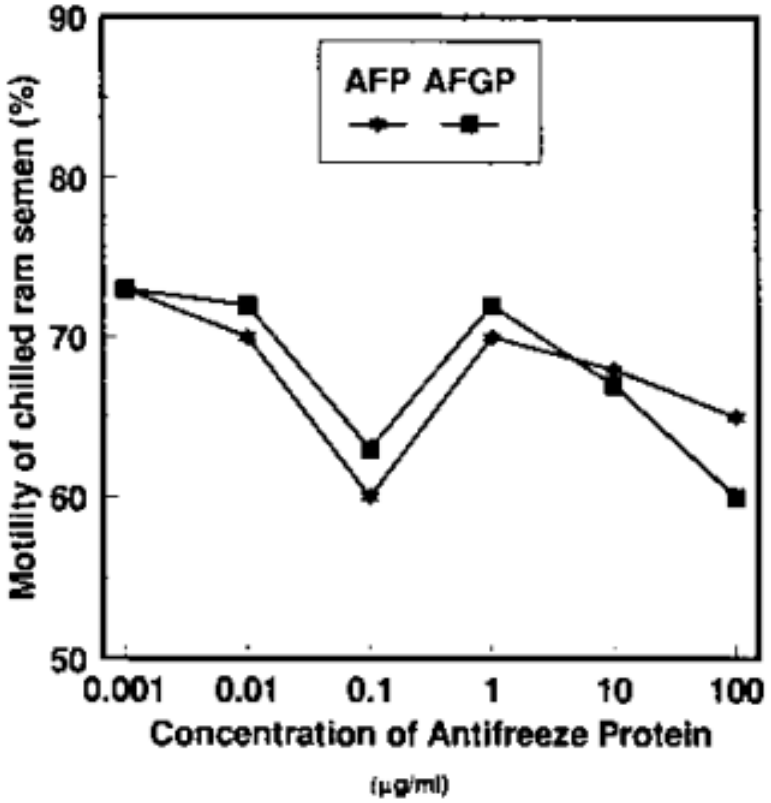



FIG. 1. Effect of various concentrations of AFP and AFGP on the motility of pooled ram spermatozoa cooled to hypothermic temperatures (5°C) for 3 h. (Control spermatozoal motility = 68%, SE = 3.7; the data are the average of three independent determinations on pooled semen samples from three rams)

**Antifreeze proteins slightly but significantly reduce the loss in ram spermatozoa motility** during the freeze-thaw process, suggesting that these proteins could be used to help protect spermatozoa from damage during freeze-thawing, particularly with the low concentrations required.

# Enzyme leakage during cryopreservation of ram spermatozoa

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Table 2
Effect of antifreeze proteins (AFP) on spermatozoal motility and levels of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) leaked in the supernatant

| Stage of process [a] | % Motile | | | ALP [b] ($Ul^{-1}$) | | | LDH [b] ($Ul^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 [c] | 10 [c] | SED [d] | 0 [c] | 10 [c] | SED [d] | 0 [c] | 10 [c] | SED [d] |
| Fresh | 89 | 89 | 1.7 | 175 | 221 | 20 | 211 | 321 | 144 |
| Chilled | 86 | 86 | 1.4 | 186 | 195 | 30 | 388 | 242 | 107 |
| Freeze–thaw | 37 | 42 | 2.3 | 204 | 209 | 8 | 166 | 170 | 17 |
| 6 h post-thaw | 13 | 22 | 3.6 | 10 | 11 | 1 | 86 | 122 | 16 |
| 24 h post-thaw | 5 | 13 | 2.1 | 3 | 2 | 1 | 284 | 374 | 52 |

[a] Activity was measured in supernatant (2000× $g$ for 10 min).
[b] Activities in $Ul^{-1}$ from a spermatozoal suspension of $400 \times 10^6$ sperm $ml^{-1}$.
[c] Levels of AFP in $\mu g\ ml^{-1}$.
[d] SED, standard error of the difference of the means of 15 replicates = three ejaculates from each of five rams.

**The presence of antifreeze protein did not influence ALP leakage, whereas LDH leakage increased during prolonged post-thaw incubations.**

Cryobiology 98 (2021) 194–200

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# Addition of antifreeze protein type I or III to extenders for ram sperm cryopreservation

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# Experimental design

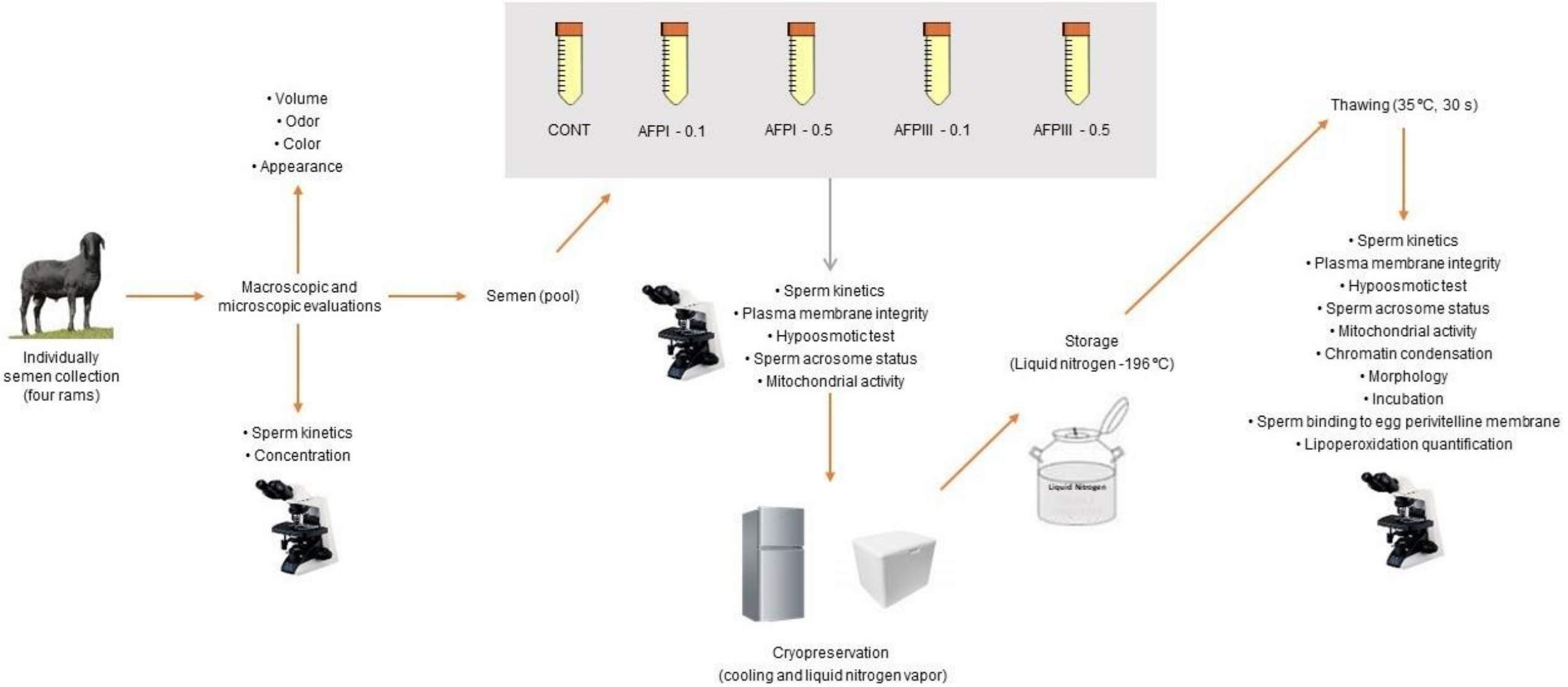

**Table 1.** Sperm parameters evaluated after dilution before freezing and immediately after (0 h) frozen-thawed ram semen subjected to extender containing different types and concentrations of antifreeze proteins (AFP) for cryopreservation (Mean + SEM).

| | | Before Freezing | | | Immediately (0 h) after frozen-thawed | | |
|---|---|---|---|---|---|---|---|
| | | 0 µg/mL | 0.1 µg/mL | 0.5 µg/mL | 0 µg/mL | 0.1 µg/mL | 0.5 µg/mL |
| Total Motility (%) | AFP I | 96.0 ± 3.0 $^{Aa}$ | 97.0 ± 1.6 $^{Aa}$ | 94.2 ± 4.5 $^{Aa}$ | 30.0 ± 2.1 $^{Aa}$ | 27.6 ± 4.8 $^{Aa}$ | 26.2 ± 2.0 $^{Aa}$ |
| | AFP III | 96.0 ± 3.0 $^{Aa}$ | 93.9 ± 3.0 $^{Aa}$ | 95.3 ± 2.6 $^{Aa}$ | 30.0 ± 2.1 $^{Aa}$ | 13.7 ± 1.4 $^{Ab}$ | 25.0 ± 2.3 $^{Aa}$ |
| Progressive Motility (%) | AFP I | 25.2 ± 4.0 $^{Aa}$ | 31.3 ± 6.4 $^{Aa}$ | 28.5 ± 6.2 $^{Aa}$ | 1.6 ± 0.3 $^{Aa}$ | 2.5 ± 0.2 $^{Aa}$ | 2.7 ± 0.6 $^{Aa}$ |
| | AFP III | 25.2 ± 4.0 $^{Aa}$ | 30.0 ± 4.8 $^{Aa}$ | 25.4 ± 4.8 $^{Aa}$ | 1.6 ± 0.3 $^{Aa}$ | 2.1 ± 0.5 $^{Aa}$ | 2.4 ± 0.4 $^{Aa}$ |
| Fast Sperm (%) | AFP I | 64.8 ± 4.8 $^{Aa}$ | 67.3 ± 1.8 $^{Aa}$ | 60.3 ± 4.4 $^{Aa}$ | 4.8 ± 1.1 $^{Aa}$ | 5.3 ± 1.3 $^{Aa}$ | 5.5 ± 1.9 $^{Aa}$ |
| | AFP III | 64.8 ± 4.8 $^{Aa}$ | 59.6 ± 7.1 $^{Aa}$ | 55.3 ± 10.0 $^{Aa}$ | 4.8 ± 1.1 $^{Aa}$ | 1.5 ± 0.6 $^{Aa}$ | 2.5 ± 1.1 $^{Aa}$ |
| Medium Sperm (%) | AFP I | 22.2 ± 4.2 $^{Aa}$ | 21.8 ± 4.1 $^{Aa}$ | 24.9 ± 6.1 $^{Aa}$ | 1.6 ± 0.2 $^{Aa}$ | 2.1 ± 0.4 $^{Aa}$ | 2.5 ± 0.8 $^{Aa}$ |
| | AFP III | 22.2 ± 4.2 $^{Aa}$ | 24.7 ± 7.5 $^{Aa}$ | 24.1 ± 7.5 $^{Aa}$ | 1.6 ± 0.2 $^{Aa}$ | 1.6 ± 0.3 $^{Aa}$ | 2.4 ± 0.5 $^{Aa}$ |
| Slow Sperm (%) | AFP I | 10.3 ± 2.6 $^{Aa}$ | 9.0 ± 1.9 $^{Aa}$ | 11.9 ± 2.5 $^{Aa}$ | 24.1 ± 1.1 $^{Aa}$ | 15.6 ± 1.4 $^{Ab}$ | 18.6 ± 3.1 $^{Aa}$ |
| | AFP III | 10.3 ± 2.6 $^{Aa}$ | 12.7 ± 2.2 $^{Aa}$ | 11.7 ± 2.5 $^{Aa}$ | 24.1 ± 1.1 $^{Aa}$ | 8.8 ± 0.6 $^{Ab}$ | 17.3 ± 1.3 $^{Ac}$ |
| VCL (µm/s) | AFP I | 89.0 ± 8.2 $^{Aa}$ | 95.5 ± 5.6 $^{Aa}$ | 86.3 ± 8.3 $^{Aa}$ | 36.2 ± 3.8 $^{Aa}$ | 44.2 ± 3.9 $^{Aa}$ | 41.2 ± 9.4 $^{Aa}$ |
| | AFP III | 89.0 ± 8.2 $^{Aa}$ | 87.2 ± 6.4 $^{Aa}$ | 87.0 ± 12.6 $^{Aa}$ | 36.2 ± 3.8 $^{Aa}$ | 42.9 ± 6.1 $^{Aa}$ | 37.0 ± 6.0 $^{Aa}$ |
| VSL (µm/s) | AFP I | 43.5 ± 7.8 $^{Aa}$ | 49.3 ± 9.4 $^{Aa}$ | 45.0 ± 10.6 $^{Aa}$ | 14.5 ± 2.1 $^{Aa}$ | 22.3 ± 3.2 $^{Aa}$ | 15.1 ± 5.2 $^{Aa}$ |
| | AFP III | 43.5 ± 7.8 $^{Aa}$ | 44.6 ± 7.2 $^{Aa}$ | 40.9 ± 7.7 $^{Aa}$ | 14.5 ± 2.1 $^{Aa}$ | 17.0 ± 3.1 $^{Aa}$ | 15.0 ± 2.8 $^{Aa}$ |
| VAP (µm/s) | AFP I | 63.8 ± 8.4 $^{Aa}$ | 63.5 ± 7.4 $^{Aa}$ | 55.1 ± 7.2 $^{Aa}$ | 21.4 ± 2.0 $^{Aa}$ | 34.3 ± 2.3 $^{Aa}$ | 23.7 ± 6.4 $^{Aa}$ |
| | AFP III | 63.8 ± 8.4 $^{Aa}$ | 65.2 ± 7.9 $^{Aa}$ | 54.4 ± 8.8 $^{Aa}$ | 21.4 ± 2.0 $^{Aa}$ | 27.1 ± 4.1 $^{Aa}$ | 28.6 ± 4.2 $^{Aa}$ |
| LIN (%) | AFP I | 47.1 ± 5.2 $^{Aa}$ | 50.1 ± 6.7 $^{Aa}$ | 49.1 ± 6.8 $^{Aa}$ | 36.8 ± 3.0 $^{Aa}$ | 56.6 ± 3.1 $^{Ab}$ | 56.9 ± 2.2 $^{Ab}$ |
| | AFP III | 47.1 ± 5.2 $^{Aa}$ | 49.6 ± 4.7 $^{Aa}$ | 46.2 ± 4.8 $^{Aa}$ | 36.8 ± 3.0 $^{Aa}$ | 53.3 ± 2.1 $^{Aab}$ | 64.7 ± 6.2 $^{Ab}$ |
| STR (%) | AFP I | 66.0 ± 3.6 $^{Aa}$ | 67.8 ± 4.7 $^{Aa}$ | 67.2 ± 4.9 $^{Aa}$ | 63.2 ± 0.8 $^{Aa}$ | 75.4 ± 0.9 $^{Ab}$ | 78.5 ± 2.8 $^{Ab}$ |
| | AFP III | 66.0 ± 3.6 $^{Aa}$ | 66.7 ± 3.0 $^{Aa}$ | 64.7 ± 2.8 $^{Aa}$ | 63.2 ± 0.8 $^{Aa}$ | 81.9 ± 3.2 $^{Ab}$ | 78.4 ± 4.1 $^{Ab}$ |
| WOB (%) | AFP I | 70.3 ± 4.0 $^{Aa}$ | 72.1 ± 5.0 $^{Aa}$ | 71.4 ± 4.6 $^{Aa}$ | 69.4 ± 7.2 $^{Aa}$ | 68.7 ± 4.5 $^{Aa}$ | 72.8 ± 4.2 $^{Aa}$ |
| | AFP III | 70.3 ± 4.0 $^{Aa}$ | 73.5 ± 3.8 $^{Aa}$ | 70.6 ± 4.7 $^{Aa}$ | 69.4 ± 7.2 $^{Aa}$ | 65.8 ± 4.2 $^{Aa}$ | 69.8 ± 6.4 $^{Aa}$ |
| ALH (µm) | AFP I | 3.0 ± 0.2 $^{Aa}$ | 3.0 ± 0.2 $^{Aa}$ | 2.7 ± 0.2 $^{Aa}$ | 1.9 ± 0.3 $^{Aa}$ | 2.1 ± 0.4 $^{Aa}$ | 1.6 ± 0.2 $^{Aa}$ |
| | AFP III | 3.0 ± 0.2 $^{Aa}$ | 2.8 ± 0.2 $^{Aa}$ | 2.8 ± 0.3 $^{Aa}$ | 1.9 ± 0.3 $^{Aa}$ | 1.6 ± 0.3 $^{Aa}$ | 2.1 ± 0.4 $^{Aa}$ |
| BCF (Hz) | AFP I | 7.0 ± 0.6 $^{Aa}$ | 7.4 ± 0.3 $^{Aa}$ | 6.7 ± 0.4 $^{Aa}$ | 5.2 ± 0.7 $^{Aa}$ | 7.1 ± 1.2 $^{Aa}$ | 6.9 ± 1.5 $^{Aa}$ |
| | AFP III | 7.0 ± 0.6 $^{Aa}$ | 7.7 ± 0.5 $^{Aa}$ | 7.4 ± 0.4 $^{Aa}$ | 5.2 ± 0.7 $^{Aa}$ | 4.9 ± 0.6 $^{Aa}$ | 7.1 ± 1.4 $^{Aa}$ |

**Table 1.** Sperm parameters evaluated after dilution before freezing and immediately after (0 h) frozen-thawed ram semen subjected to extender containing different types and concentrations of antifreeze proteins (AFP) for cryopreservation (Mean + SEM).

| | | Before Freezing | | | Immediately (0 h) after frozen-thawed | | |
|---|---|---|---|---|---|---|---|
| PM Integrity (%) | AFP I | 63.9 ± 7.6 $^{Aa}$ | 70.2 ± 5.7 $^{Aa}$ | 71.8 ± 7.2 $^{Aa}$ | 13.0 ± 4.4 $^{Aa}$ | 49.1 ± 4.6 $^{Ab}$ | 36.6 ± 7.3 $^{Ab}$ |
| | AFP III | 63.9 ± 7.6 $^{Aa}$ | 58.9 ± 3.2 $^{Aa}$ | 58.2 ± 1.4 $^{Aa}$ | 13.0 ± 4.4 $^{Aa}$ | 19.8 ± 3.6 $^{Ba}$ | 21.8 ± 4.0 $^{Aa}$ |
| Hypoosmotic (%) | AFP I | 85.3 ± 1.4 $^{Aa}$ | 86.3 ± 1.7 $^{Aa}$ | 79.6 ± 2.1 $^{Aa}$ | 13.6 ± 2.6 $^{Aa}$ | 16.9 ± 4.2 $^{Aa}$ | 22.2 ± 4.3 $^{Aa}$ |
| | AFP III | 85.3 ± 1.4 $^{Aa}$ | 81.6 ± 4.6 $^{Aa}$ | 87.4 ± 0.3 $^{Aa}$ | 13.6 ± 2.6 $^{Aa}$ | 15.7 ± 3.8 $^{Aa}$ | 11.9 ± 1.8 $^{Aa}$ |
| LSIA (%) | AFP I | 28.8 ± 4.4 $^{Aa}$ | 33.1 ± 8.8 $^{Aa}$ | 27.8 ± 4.8 $^{Aa}$ | 16.4 ± 6.2 $^{Aa}$ | 13.8 ± 6.8 $^{Aa}$ | 11.7 ± 6.1 $^{Aa}$ |
| | AFP III | 28.8 ± 4.4 $^{Aa}$ | 32.5 ± 5.6 $^{Aa}$ | 28.1 ± 4.8 $^{Aa}$ | 16.4 ± 6.2 $^{Aa}$ | 7.0 ± 2.1 $^{Aa}$ | 8.5 ± 1.4 $^{Aa}$ |
| LSAR (%) | AFP I | 65.8 ± 2.4 $^{Aa}$ | 57.8 ± 8.0 $^{Aa}$ | 56.4 ± 6.4 $^{Aa}$ | 8.3 ± 2.2 $^{Aa}$ | 10.7 ± 5.0 $^{Aa}$ | 10.6 ± 5.5 $^{Aa}$ |
| | AFP III | 65.8 ± 2.4 $^{Aa}$ | 56.8 ± 5.1 $^{Aa}$ | 57.3 ± 8.1 $^{Aa}$ | 8.3 ± 2.2 $^{Aa}$ | 8.7 ± 2.8 $^{Aa}$ | 10.0 ± 1.3 $^{Aa}$ |
| DSIA (%) | AFP I | 3.8 ± 0.4 $^{Aa}$ | 3.8 ± 0.7 $^{Aa}$ | 4.4 ± 1.5 $^{Aa}$ | 59.3 ± 14.1 $^{Aa}$ | 59.0 ± 17.5 $^{Aa}$ | 64.2 ± 14.1 $^{Aa}$ |
| | AFP III | 3.8 ± 0.4 $^{Aa}$ | 6.7 ± 1.9 $^{Aa}$ | 2.5 ± 1.1 $^{Aa}$ | 59.3 ± 14.1 $^{Aa}$ | 67.8 ± 11.0 $^{Aa}$ | 61.6 ± 10.7 $^{Aa}$ |
| DSAL (%) | AFP I | 6.1 ± 1.1 $^{Aa}$ | 6.1 ± 0.9 $^{Aa}$ | 3.4 ± 1.0 $^{Aa}$ | 3.9 ± 2.1 $^{Aa}$ | 11.3 ± 5.7 $^{Aa}$ | 7.4 ± 3.4 $^{Aa}$ |
| | AFP III | 6.1 ± 1.1 $^{Aa}$ | 4.2 ± 0.8 $^{Aa}$ | 4.7 ± 0.7 $^{Aa}$ | 3.9 ± 2.1 $^{Aa}$ | 7.2 ± 5.2 $^{Aa}$ | 8.8 ± 3.4 $^{Aa}$ |
| DAB I (%) | AFP I | 95.5 ± 0.9 $^{Aa}$ | 94.2 ± 1.2 $^{Aa}$ | 95.2 ± 0.8 $^{Aa}$ | 10.3 ± 2.2 $^{Aa}$ | 12.5 ± 3.0 $^{Aa}$ | 16.7 ± 2.5 $^{Aa}$ |
| | AFP III | 95.5 ± 0.9 $^{Aa}$ | 95.3 ± 1.1 $^{Aa}$ | 94.7 ± 1.0 $^{Aa}$ | 10.3 ± 2.2 $^{Aa}$ | 12.6 ± 0.7 $^{Aa}$ | 10.3 ± 1.7 $^{Aa}$ |
| DAB II (%) | AFP I | 0.7 ± 0.3 $^{Aa}$ | 0.9 ± 0.2 $^{Aa}$ | 0.8 ± 0.1 $^{Aa}$ | 9.4 ± 4.5 $^{Aa}$ | 13.3 ± 5.3 $^{Aa}$ | 11.8 ± 5.5 $^{Aa}$ |
| | AFP III | 0.7 ± 0.3 $^{Aa}$ | 1.0 ± 0.3 $^{Aa}$ | 0.7 ± 0.2 $^{Aa}$ | 9.4 ± 4.5 $^{Aa}$ | 8.8 ± 2.3 $^{Aa}$ | 8.0 ± 2.6 $^{Aa}$ |
| DAB III (%) | AFP I | 0.8 ± 0.3 $^{Aa}$ | 0.8 ± 0.3 $^{Aa}$ | 1.1 ± 0.3 $^{Aa}$ | 1.6 ± 0.3 $^{Aa}$ | 2.0 ± 1.0 $^{Aa}$ | 1.5 ± 0.4 $^{Aa}$ |
| | AFP III | 0.8 ± 0.3 $^{Aa}$ | 1.0 ± 0.5 $^{Aa}$ | 0.6 ± 0.3 $^{Aa}$ | 1.6 ± 0.3 $^{Aa}$ | 1.5 ± 0.3 $^{Aa}$ | 1.9 ± 0.6 $^{Aa}$ |
| DAB IV (%) | AFP I | 3.0 ± 0.6 $^{Aa}$ | 4.1 ± 1.2 $^{Aa}$ | 2.9 ± 0.6 $^{Aa}$ | 80.0 ± 5.7 $^{Aa}$ | 69.6 ± 7.6 $^{Aa}$ | 71.9 ± 6.0 $^{Aa}$ |
| | AFP III | 3.0 ± 0.6 $^{Aa}$ | 2.7 ± 0.6 $^{Aa}$ | 4.0 ± 0.6 $^{Aa}$ | 80.0 ± 5.7 $^{Aa}$ | 74.3 ± 5.3 $^{Aa}$ | 83.3 ± 3.0 $^{Aa}$ |
| Normal Chrom. (%) | AFP I | - | - | - | 99.0 ± 0.3 $^{Aa}$ | 98.9 ± 0.4 $^{Aa}$ | 98.5 ± 0.5 $^{Aa}$ |
| | AFP III | - | - | - | 99.0 ± 0.3 $^{Aa}$ | 98.8 ± 0.3 $^{Aa}$ | 98.8 ± 0.3 $^{Aa}$ |
| Normal Morphol. (%) | AFP I | - | - | - | 65.3 ± 1.9 $^{Aa}$ | 73.0 ± 1.0 $^{Ab}$ | 75.7 ± 2.2 $^{Ab}$ |
| | AFP III | - | - | - | 65.3 ± 1.9 $^{Aa}$ | 73.8 ± 1.6 $^{Ab}$ | 74.8 ± 0.5 $^{Ab}$ |
| Sperm binding (mm²) | AFP I | - | - | - | 186.7 ± 47.2 $^{Aa}$ | 178.3 ± 31.8 $^{Aa}$ | 175.0 ± 49.3 $^{Aa}$ |
| | AFP III | - | - | - | 186.7 ± 47.2 $^{Aa}$ | 238.3 ± 49.6 $^{Aa}$ | 191.6 ± 44.6 $^{Aa}$ |
| TBARS (ng/mL) | AFP I | - | - | - | 567.2 ± 20.0 $^{Aa}$ | 545.8 ± 29.3 $^{Aa}$ | 559.9 ± 18.3 $^{Aa}$ |
| | AFP III | - | - | - | 567.2 ± 20.0 $^{Aa}$ | 544.0 ± 15.6 $^{Aa}$ | 566.6 ± 19.2 $^{Aa}$ |

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# Addition of antifreeze protein type I or III to extenders for ram sperm cryopreservation

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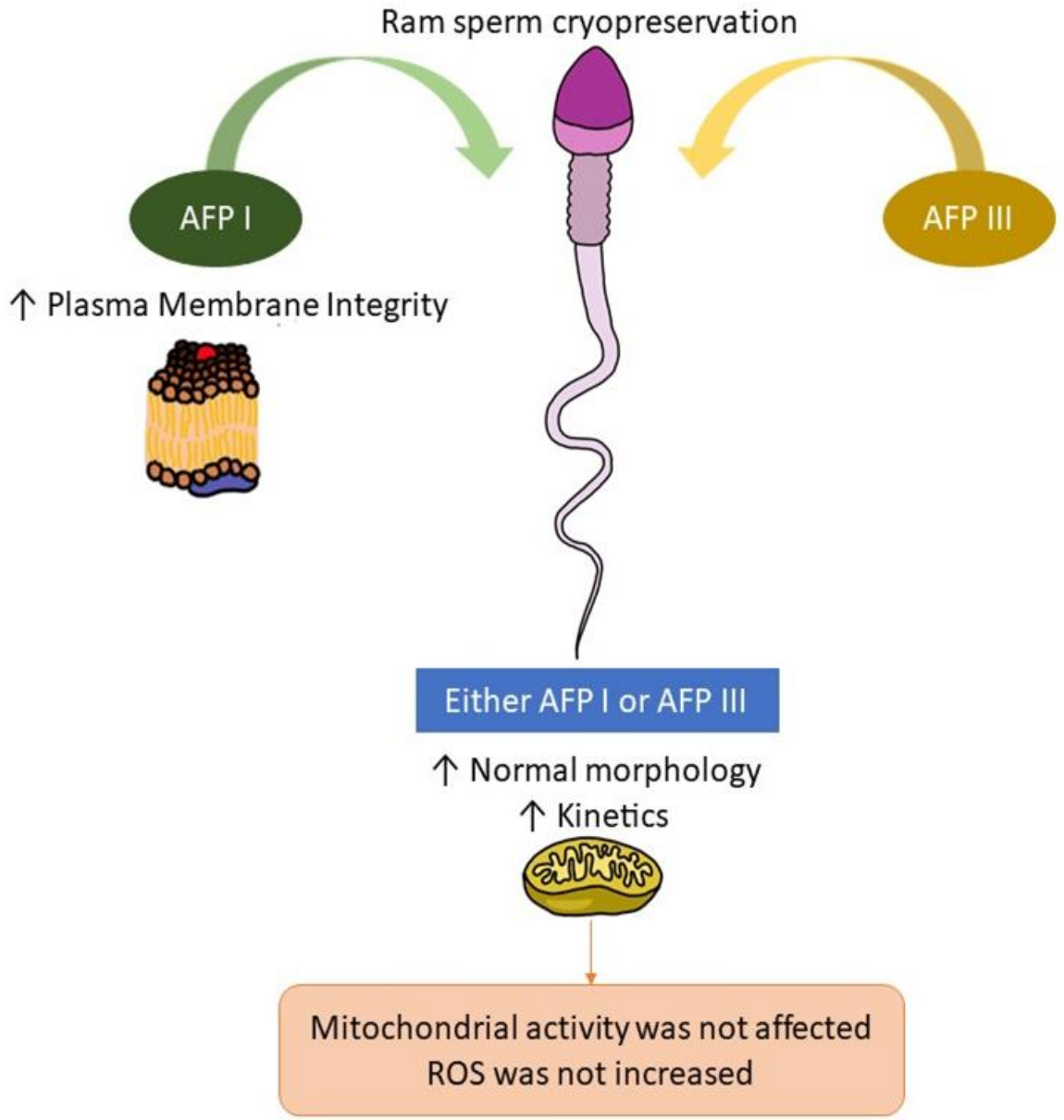
In conclusion, the **addition of AFP appears auspicious for cryopreserving ram sperm cells**. The use of **AFP, predominantly type I, increased sperm cell protection during cryopreservation, resulting in greater sperm kinetics, better plasma integrity and greater percentage of normal sperm cells**. These results open interesting possibilities to use AFP as a sheep semen cryoprotectant.



# To be published

**ROLE OF ANTIFREEZE PROTEIN TYPE I ON RAM SEMEN FREEZABILITY**

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# Experimental design

Six collection days

Individually semen collection (ten rams)

Sperm kinetics and concentration evaluations

Individual Semen

- Sperm kinetics
- Plasma membrane integrity/functionality

CONT AFP

Cryopreservation

**Total of cryopreserved ejaculates = 43**

Storage (Liquid nitrogen -196 ºC)


Thawing (35 ºC, 30 s)

- Sperm kinetics
- Plasma membrane integrity/functionality
- Sperm acrosome status
- Sperm binding to egg perivitelline membrane

**GLMM**
**P<0.05**

30

# Results

**Table 1.** Principal components analysis: eigenvalues, variances (%), accumulated variances, and eigenvectors identified in the Principal Components 1 to 4 (PC1 to PC4) evaluated in data obtained from frozen-thawed ram semen.

| | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| Eigenvalue | 7.05 | 4.20 | 1.83 | 1.02 |
| Variance (%) | 44.05 | 26.27 | 11.45 | 6.37 |
| Accumulated Variance (%) | 44.05 | 70.32 | 81.77 | 88.14 |
| Eigenvector | | | | |
| Total Motility | 0.85 | -0.04 | -0.51 | 0.03 |
| Progressive Motility | 0.89 | 0.14 | 0.19 | -0.24 |
| Fast Sperm | 0.83 | -0.08 | 0.25 | -0.34 |
| Medium Sperm | 0.92 | -0.19 | -0.05 | -0.09 |
| Slow Sperm | 0.78 | -0.00 | -0.60 | 0.07 |
| VCL | 0.81 | -0.26 | 0.41 | -0.06 |
| VSL | 0.52 | 0.80 | 0.24 | -0.02 |
| VAP | 0.77 | 0.52 | 0.30 | 0.01 |
| LIN | -0.02 | 0.99 | -0.05 | 0.03 |
| STR | -0.01 | 0.99 | -0.02 | 0.00 |
| WOB | 0.04 | 0.97 | -0.10 | 0.08 |
| ALH | 0.50 | -0.13 | -0.40 | 0.12 |
| BCF | 0.77 | -0.24 | 0.40 | -0.06 |
| PM Integrity | 0.35 | 0.06 | 0.26 | 0.80 |
| Hypoosmotic | 0.51 | -0.41 | 0.27 | 0.40 |

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: plasma membrane integrity.

**Table 2**. Pre-freezing ram spermatozoa parameters from ejaculates of poor (PF) or good freezability (GF) patterns in extenders without (CONT) or with 0.1 µg/mL AFP type I

| | | | | | Effects | | |
|---|---|---|---|---|---|---|---|
| Variables | PF - CONT | PF - AFP | GF - CONT | GF- AFP | AFP | Freezability | AFP x Freezability |
| Total Motility (%) | 94.7 ± 1.6 | 95.8 ± 1.6 | 99.5 ± 2.6 | 98.0 ± 2.6 | n.s. | n.s. | n.s. |
| Progressive Motility (%) | 15.3 ± 1.4 | 12.8 ± 1.4 | 14.5 ± 2.4 | 14.5 ± 2.4 | n.s. | n.s. | n.s. |
| Fast Sperm (%) | 27.0 ± 5.3 | 24.8 ± 5.3 | 32.3 ± 9.1 | 29.9 ± 9.1 | n.s. | n.s. | n.s. |
| Medium Sperm (%) | 29.6 ± 2.4 | 29.7 ± 2.4 | 37.1 ± 4.2 | 32.0 ± 4.2 | n.s. | n.s. | n.s. |
| Slow Sperm (%) | 38.1 ± 5.1 | 39.9 ± 5.1 | 30.2 ± 8.8 | 36.2 ± 8.8 | n.s. | n.s. | n.s. |
| VCL (µm/s) | 56.5 ± 4.3 | 56.7 ± 4.3 | 65.3 ± 7.3 | 61.4 ± 7.3 | n.s. | n.s. | n.s. |
| VSL (µm/s) | 24.0 ± 1.3 | 21.8 ± 1.3 | 23.7 ± 2.2 | 23.0 ± 2.2 | n.s. | n.s. | n.s. |
| VAP (µm/s) | 37.4 ± 2.4 | 35.0 ± 2.4 | 39.5 ± 4.1 | 37.6 ± 4.1 | n.s. | n.s. | n.s. |
| LIN (%) | 44.0 ± 1.7$^{a}$ | 40.6 ± 1.7$^{b}$ | 37.8 ± 2.9 | 39.8 ± 2.9 | n.s. | n.s. | 0.027 |
| STR (%) | 66.2 ± 1.4$^{a}$ | 63.7 ± 1.4$^{b}$ | 61.4 ± 2.4 | 62.9 ± 2.4 | n.s. | n.s. | 0.031 |
| WOB (%) | 65.7 ± 1.3$^{a}$ | 62.9 ± 1.3$^{b}$ | 61.2 ± 2.2 | 62.8 ± 2.2 | n.s. | n.s. | 0.024 |
| ALH (µm) | 3.7 ± 0.2$^{a}$ | 4.0 ± 0.2$^{b}$ | 4.2 ± 0.4 | 3.9 ± 0.4 | n.s. | n.s. | 0.014 |
| BCF (Hz) | 5.3 ± 0.5 | 5.4 ± 0.5 | 5.5 ± 0.9 | 5.5 ± 0.9 | n.s. | n.s. | n.s. |
| PM Integrity (%) | 68.6 ± 2.1 | 68.8 ± 2.1 | 70.9 ± 3.6 | 71.0 ± 3.6 | n.s. | n.s. | n.s. |
| Hypoosmotic (%) | 82.0 ± 1.9 | 83.3 ± 1.9 | 80.5 ± 3.3 | 82.4 ± 3.3 | n.s. | n.s. | n.s. |

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: Plasma Membrane integrity. n.s.: non-significant; $^{a,b}$ represents the differences of interaction within the same freezability category ($P<0.01$). Data is presented as LSMean ± SEM.

**Table 3**. Post-thawing ram spermatozoa parameters from ejaculates of poor (PF) or good freezability (GF) patterns in extenders without (CONT) or with 0.1 µg/mL AFP type I (AFP)

| | | | | | Effects | | |
|---|---|---|---|---|---|---|---|
| Variable | PF - CONT | PF - AFP | GF - CONT | GF- AFP | AFP | Freezability | AFP x Freezability |
| Total Motility (%) | 18.2 ± 2.2 | 25.3 ± 2.2 | 49.4 ± 3.8 | 48.1 ± 3.8 | n.s. | 0.001 | 0.100 |
| Progressive Motility (%) | 0.6 ± 0.3 | 0.9 ± 0.3 | 2.2 ± 0.5 | 3.3 ± 0.5 | 0.053 | 0.001 | n.s. |
| Fast Sperm (%) | 0.3 ± 0.2 | 0.4 ± 0.2 | 1.0 ± 0.3 | 1.6 ± 0.3 | 0.048 | 0.002 | n.s. |
| Medium Sperm (%) | 1.1 ± 0.7 | 2.0 ± 0.7 | 4.9 ± 1.1 | 6.6 ± 1.1 | 0.100 | 0.001 | n.s. |
| Slow Sperm (%) | 16.7 ± 1.7$^{aA}$ | 22.8 ± 1.7$^{bB}$ | 43.5 ± 2.9$^{A}$ | 39.9 ± 2.9$^{B}$ | n.s. | 0.001 | 0.014 |
| VCL (µm/s) | 22.0 ± 0.8 | 22.0 ± 0.8 | 25.1 ± 1.4 | 26.8 ± 1.4 | n.s. | 0.008 | n.s. |
| VSL (µm/s) | 9.3 ± 0.6 | 9.9 ± 0.6 | 11.3 ± 1.0 | 11.9 ± 1.0 | n.s. | 0.032 | n.s. |
| VAP (µm/s) | 13.6 ± 0.6 | 14.4 ± 0.6 | 16.5 ± 1.0 | 17.5 ± 1.0 | n.s. | 0.004 | n.s. |
| LIN (%) | 42.8 ± 2.2 | 45.4 ± 2.2 | 45.7 ± 3.8 | 44.7 ± 3.8 | n.s. | n.s. | n.s. |
| STR (%) | 66.7 ± 1.7 | 68.0 ± 1.7 | 68.5 ± 2.9 | 67.6 ± 2.9 | n.s. | n.s. | n.s. |
| WOB (%) | 62.5 ± 1.8 | 65.5 ± 1.8 | 66.2 ± 3.0 | 65.3 ± 3.0 | n.s. | n.s. | n.s. |
| ALH (µm) | 1.6 ± 0.2 | 1.7 ± 0.2 | 2.8 ± 0.3 | 2.9 ± 0.3 | n.s. | 0.001 | n.s. |
| BCF (Hz) | 3.4 ± 0.6 | 3.2 ± 0.6 | 5.8 ± 1.0 | 5.4 ± 1.0 | n.s. | 0.039 | n.s. |
| PM Integrity (%) | 26.4 ± 2.3 | 31.5 ± 2.3 | 27.3 ± 4.0 | 38.3 ± 4.0 | 0.001 | n.s. | n.s. |
| Hypoosmotic (%) | 15.1 ± 2.0 | 16.3 ± 2.0 | 20.4 ± 3.4 | 16.3 ± 3.4 | n.s. | n.s. | n.s. |
| Capacitated (%) | 16.5 ± 3.0 | 18.3 ± 3.0 | 26.0 ± 5.4 | 19.3 ± 5.4 | n.s. | n.s. | n.s. |
| Non-capacitated (%) | 8.2 ± 2.0 | 5.3 ± 2.0 | 4.6 ± 3.6 | 6.5 ± 3.6 | n.s. | n.s. | n.s. |
| Acrosome-reacted (%) | 75.4 ± 3.4 | 76.4 ± 3.4 | 69.4 ± 6.1 | 74.3 ± 6.1 | n.s. | n.s. | n.s. |
| Sperm binding ($mm^2$) | 484.6 ± 145.3 | 624.6 ± 145.3 | 1054.0 ± 234.3 | 1574.0 ± 234.3 | 0.005 | 0.005 | 0.094 |

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: plasma membrane integrity; Sperm binding: sperm binding to egg perivitelline membrane; n.s.: non-significant. $^{a,b}$ represents the differences of interaction within the same freezability category; $^{A,B}$ represents the differences of interaction within the same extender.

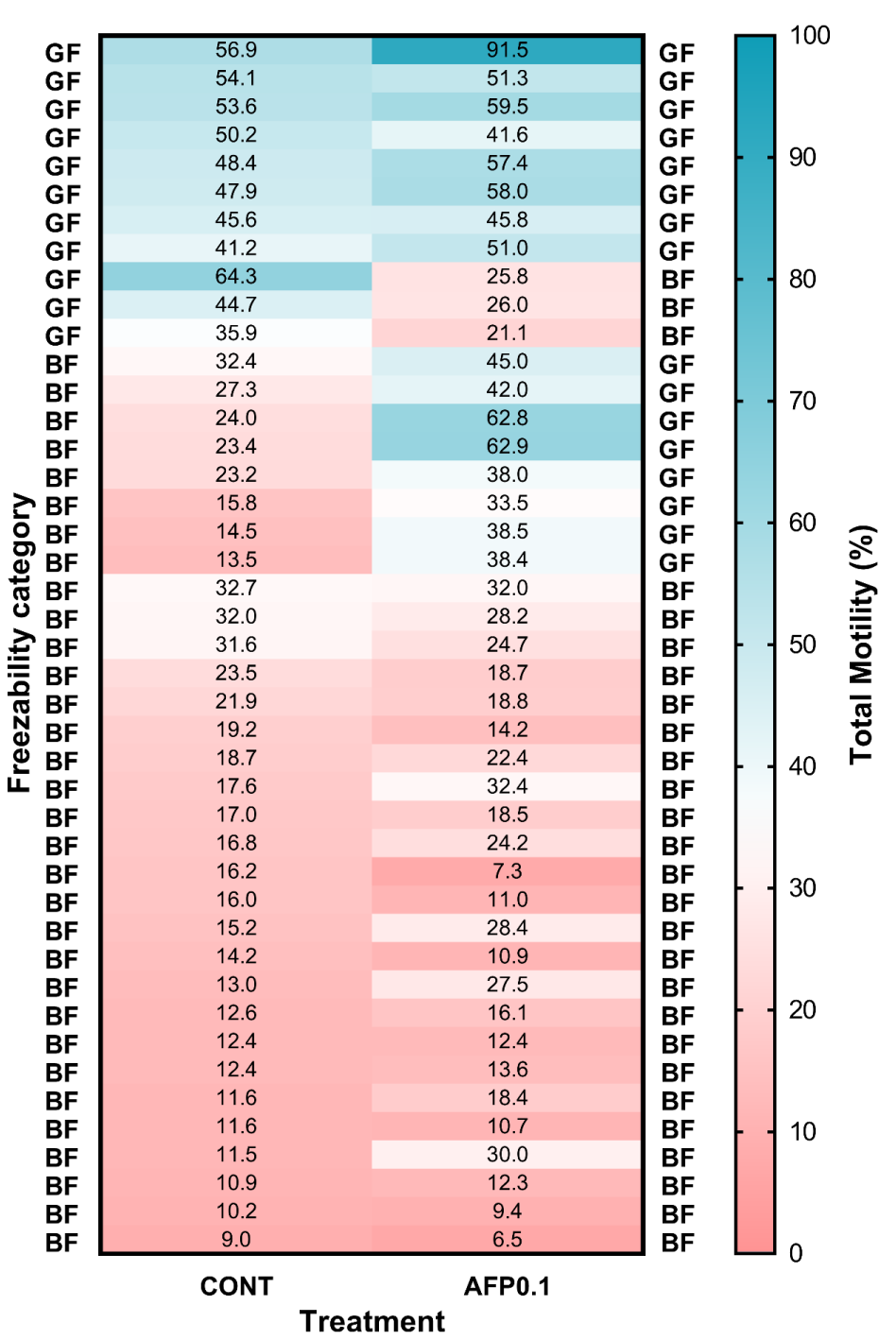
# Results

When samples treated with AFP were subjected to classification according to the developed freezability model, considering PCA and K-means clusters, it was detected that **AFP treatment did not alter semen freezability (p = 0.132).**

It was observed that **eight (25.0%) ejaculates of BF in the CONT extender were reclassified as GF** in the AFP extender.

Conversely**, three (27.3%) ejaculates of GF in the CONT extender were reclassified as BF in the AFP extender.**

Wilcoxon signed-rank tests



# ROLE OF ANTIFREEZE PROTEIN TYPE I ON RAM SEMEN FREEZABILITY

L.F.L. Correia[1]*, V.L. Brair[1], R.F. Braga[1], A.R. Taira[1], B.R.C. Alves[1], F.Z. Brandão[1], R Ungerfeld[2], R.I.T.P. Batista[1], J.M.G. Souza-Fabjan[1]*

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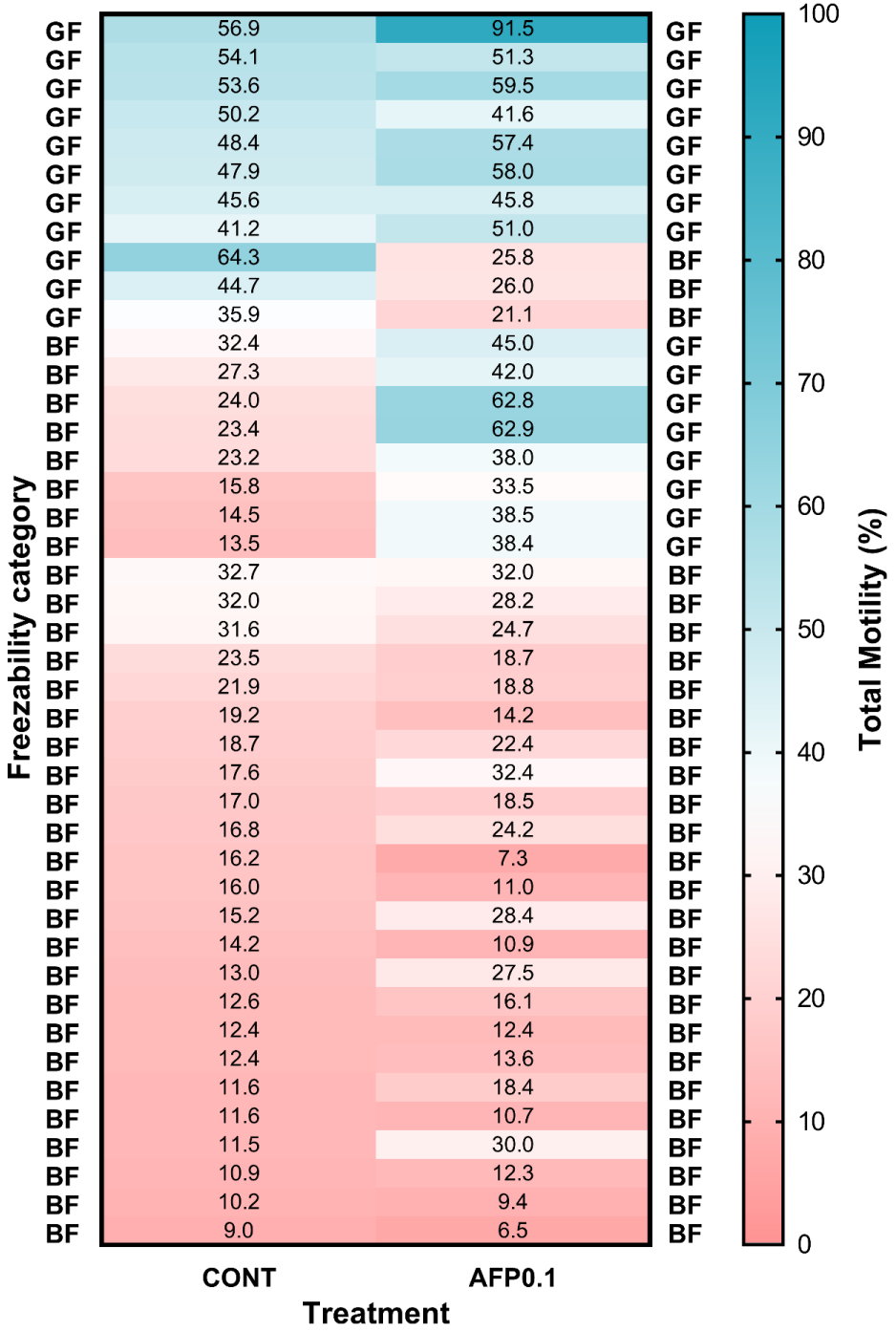
The addition of 0.1 µg/mL of **AFP I appears as having slightly different impacts in ram sperm with expected different freezability profiles**. However, the addition of **AFP I increased the percentage of sperm with fast velocity, sperm plasma membrane integrity, and sperm binding to the perivitelline membrane regardless of the freezability pattern of ejaculates in sheep.** These results indicate that AFP is **capable of mitigating some cryoinjuries independently of the cryoresistency pattern of the sperm samples**.



Animal Reproduction

ORIGINAL ARTICLE

# The association of resveratrol and AFPI did not enhance the cryoresistance of ram sperm

Viviane Lopes Brair[1], Lucas Francisco Leodido Correia[1], Nathalia Oliveira Barbosa[1], Rachel Ferreira Braga[1], Augusto Ryonosuke Taira[1], Andreza Amaral da Silva[2], Felipe Zandonadi Brandão[1], Rodolfo Ungerfeld[3], Joanna Maria Gonçalves Souza-Fabjan[1]*

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# Experimental design

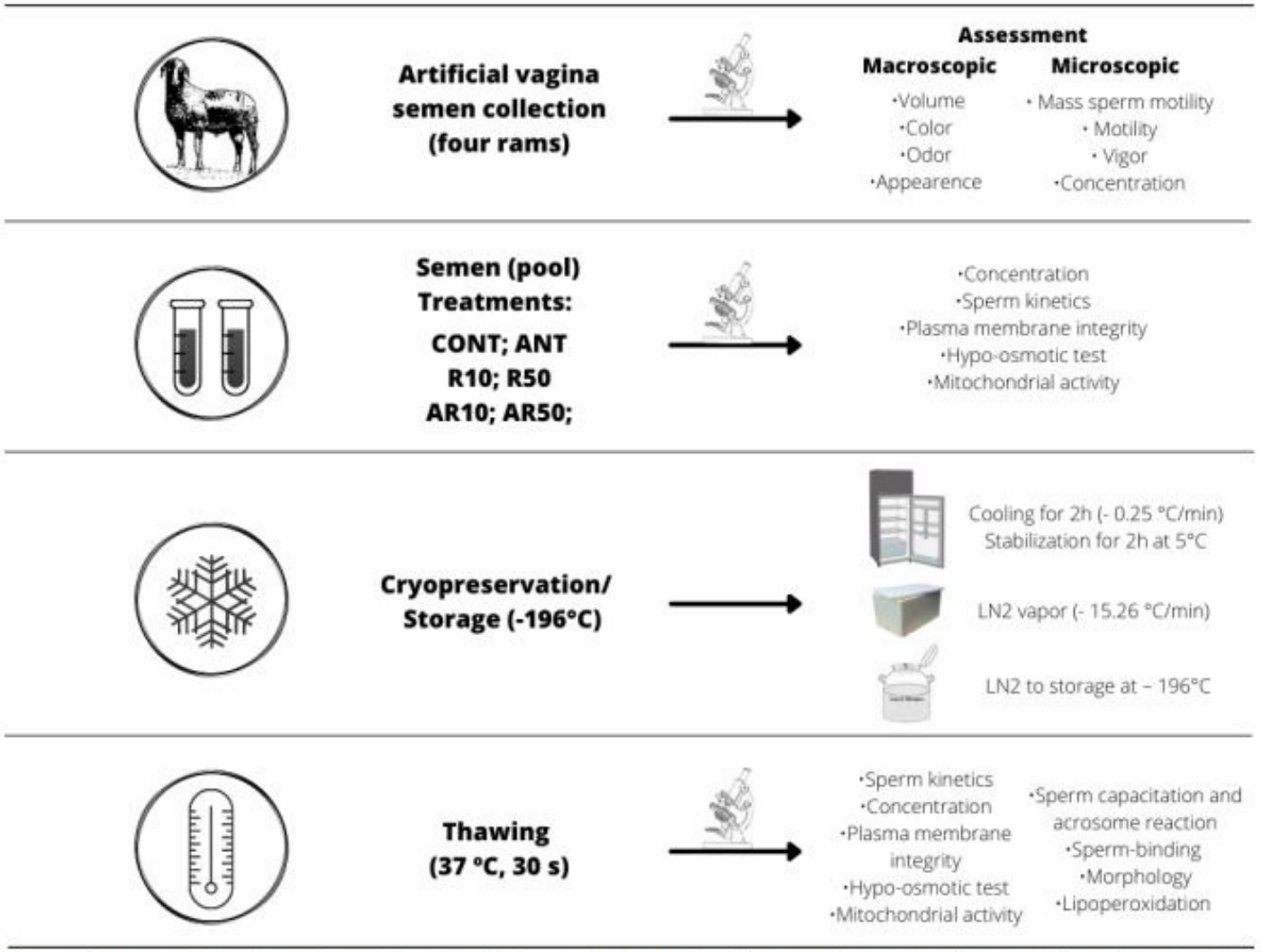



**Figure 1.** Experimental design scheme: CONT) control containing only extender; ANT) added of AFPI; R10) 10 µM/mL resveratrol; R50) 50 µM/mL resveratrol; AR10) AFP I with 10 µM/mL resveratrol; AR50) AFP I with 50 µM/mL resveratrol. The concentration of AFP I was: 0.1 µg/mL.

**Table 1.** Ram sperm endpoints after dilution (before freezing) and immediately after thawing in extenders containing (+) or not (-) antifreeze protein (AFP) type I (0.1 µg/mL), associated or not with different concentrations of resveratrol (0, 10, or 50 µM/mL) during cryopreservation (LSmeans ± SEM).

| Endpoints | AFP I (µg/mL) | Before cryopreservation | | | Frozen-Thawed | | | *P-value* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Resveratrol (µM/mL) | | | Resveratrol (µM/mL) | | | | | | |
| | 0.1 | 0 | 10 | 50 | 0 | 10 | 50 | AFP I | Resveratrol | Evaluation moment | AFP×Resveratrol ×Evaluation moment |
| Total motility (%) | - | 99.0 ± 4.0 | 99.4 ± 4.0 | 98.3 ± 4.0 | 25.3 ± 3.8 | 33.9 ± 3.8 | 24.3 ± 3.8 | n.s. | n.s. | 0.001 | n.s. |
| | + | 99.6 ± 4.0 | 99.1 ± 4.0 | 99.2 ± 4.0 | 28.7 ± 3.8 | 24.0 ± 3.8 | 23.9 ± 3.8 | | | | |
| VCL (µm/s) | - | 74.3 ± 6.0 | 72.1 ± 6.0 | 66.9 ± 6.0 | 23.7 ± 6.0 | 24.1 ± 6.0 | 22.6 ± 6.0 | n.s. | n.s. | 0.001 | n.s. |
| | + | 74.2 ± 6.0 | 69.9 ± 6.0 | 69.4 ± 6.0 | 24.8 ± 6.0 | 25.1 ± 6.0 | 21.8 ± 6.4 | | | | |
| VSL (µm/s) | - | 30.4 ± 2.5 | 27.1 ± 2.5 | 25.1 ± 2.5 | 15.5 ± 2.5 | 15.1 ± 2.5 | 13.2 ± 2.5 | n.s. | n.s. | 0.001 | n.s. |
| | + | 28.9 ± 2.5 | 28.3 ± 2.5 | 25.2 ± 2.5 | 15.3 ± 2.5 | 15.7 ± 2.5 | 13.9 ± 2.5 | | | | |
| VAP (µm/s) | - | 46.2 ± 3.7 | 42.7 ± 3.7 | 39.7 ± 3.7 | 18.8 ± 3.7 | 18.6 ± 3.7 | 16.8 ± 3.7 | n.s. | n.s. | 0.001 | n.s. |
| | + | 45.7 ± 3.7 | 43.1 ± 3.7 | 40.7 ± 3.7 | 19.0 ± 3.7 | 19.5 ± 3.7 | 17.2 ± 3.7 | | | | |
| LIN (%) | - | 37.8 ± 2.6 | 38.6 ± 2.4 | 36.3 ± 2.6 | 64.5 ± 2.4 | 62.1 ± 2.4 | 57.7 ± 2.4 | n.s. | n.s. | 0.001 | n.s. |
| | + | 38.7 ± 2.4 | 40.6 ± 2.4 | 37.1 ± 2.4 | 60.5 ± 2.4 | 62.3 ± 2.4 | 61.0 ± 2.4 | | | | |
| STR (%) | - | 62.8 ± 1.9 | 64.1 ± 1.8 | 62.2 ± 1.9 | 81.6 ± 1.8 | 80.7 ± 1.8 | 77.8 ± 1.8 | n.s. | n.s. | 0.001 | n.s. |
| | + | 63.2 ± 1.8 | 65.4 ± 1.8 | 62.7 ± 1.8 | 79.5 ± 1.8 | 80.3 ± 1.8 | 80.5 ± 1.8 | | | | |
| WOB (%) | - | 59.8 ± 1.8 | 59.7 ± 1.7 | 58.4 ± 1.8 | 78.9 ± 1.7 | 76.8 ± 1.7 | 73.8 ± 1.7 | n.s. | n.s. | 0.001 | n.s. |
| | + | 61.2 ± 1.7 | 61.5 ± 1.7 | 59.0 ± 1.7 | 75.8 ± 1.7 | 77.4 ± 1.7 | 75.7 ± 1.7 | | | | |
| ALH (µm) | - | 3.3 ± 0.3 | 3.5 ± 0.3 | 3.4 ± 0.3 | 2.9 ± 0.3 | 3.4 ± 0.3 | 3.3 ± 0.3 | n.s. | n.s. | 0.001 | n.s. |
| | + | 4.0 ± 0.3 | 3.3 ± 0.3 | 3.8 ± 0.3 | 2.7 ± 0.3 | 2.7 ± 0.3 | 2.7 ± 0.3 | | | | |
| BCF (Hz) | - | 7.7 ± 0.9 | 7.6 ± 0.9 | 7.5 ± 0.9 | 2.3 ± 1.0 | 1.9 ± 1.0 | 1.7 ± 1.0 | n.s. | n.s. | 0.001 | n.s. |
| | + | 7.2 ± 0.9 | 7.8 ± 0.9 | 7.8 ± 0.9 | 1.4 ± 1.0 | 1.2 ± 1.0 | 1.4 ± 1.0 | | | | |

**Table 1.** Ram sperm endpoints after dilution (before freezing) and immediately after thawing in extenders containing (+) or not (-) antifreeze protein (AFP) type I (0.1 µg/mL), associated or not with different concentrations of resveratrol (0, 10, or 50 µM/mL) during cryopreservation (LSmeans ± SEM).

| Endpoints | AFP I (µg/mL) | Before cryopreservation | | | Frozen-Thawed | | | *P-value* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Resveratrol (µM/mL) | | | Resveratrol (µM/mL) | | | | | | |
| | 0.1 | 0 | 10 | 50 | 0 | 10 | 50 | AFP I | Resveratrol | Evaluation moment | AFP×Resveratrol ×Evaluation moment |
| Plasma Membrane Integrity (%) | - | 81.1 ± 2.3 | 81.1 ± 2.3 | 82.6 ± 2.3 | 24.1 ± 2.4 | 30.2 ± 2.3 | 28.7 ± 2.3 | n.s. | n.s. | 0.001 | n.s. |
| | + | 78.6 ± 2.7 | 81.3 ± 2.3 | 81.9 ± 2.3 | 27.5 ± 2.3 | 30.3 ± 2.3 | 27.9 ± 2.5 | | | | |
| Hypo-osmotic (%) | - | 90.7 ± 2.1 | 92.9 ± 2.1 | 92.3 ± 2.1 | 19.7 ± 2.1 | 24.9 ± 2.1 | 22.4 ± 2.1 | 0.06 | n.s. | 0.001 | n.s. |
| | + | 91.1 ± 2.1 | 90.3 ± 2.1 | 91.3 ± 2.1 | 17.4 ± 2.1 | 18.9 ± 2.1 | 20.0 ± 2.1 | | | | |
| High MMP (%) | - | 69.5 ± 3.5 | 69.5 ± 3.5 | 69.6 ± 4.2 | 23.6 ± 3.8 | 29.4 ± 3.5 | 23.3 ± 3.8 | 0.07 | n.s. | 0.001 | n.s. |
| | + | 72.5 ± 3.5 | 74.5 ± 3.5 | 71.8 ± 3.5 | 30.6 ± 3.5 | 25.3 ± 3.8 | 33.0 ± 3.5 | | | | |
| Low MMP (%) | - | 19.5 ± 3.8 | 20.5 ± 3.5 | 20.0 ± 3.8 | 35.0 ± 3.5 | 35.9 ± 3.5 | 40.5 ± 3.8 | n.s. | n.s. | 0.001 | n.s. |
| | + | 22.0 ± 3.5 | 19.6 ± 3.5 | 17.8 ± 3.5 | 36.5 ± 3.5 | 37.0 ± 3.5 | 30.6 ± 3.8 | | | | |
| Inactive MMP (%) | - | 6.9 ± 5.4 | 7.1 ± 5.4 | 9.0 ± 5.4 | 38.9 ± 5.0 | 34.7 ± 5.0 | 29.4 ± 5.4 | n.s. | n.s. | 0.001 | n.s. |
| | + | 6.4 ± 5.4 | 9.4 ± 5.0 | 10.3 ± 5.0 | 32.9 ± 5.0 | 34.6 ± 5.0 | 32.4 ± 5.0 | | | | |

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross-frequency; MMP: mitochondrial membrane potential; n.s.: non-significant.

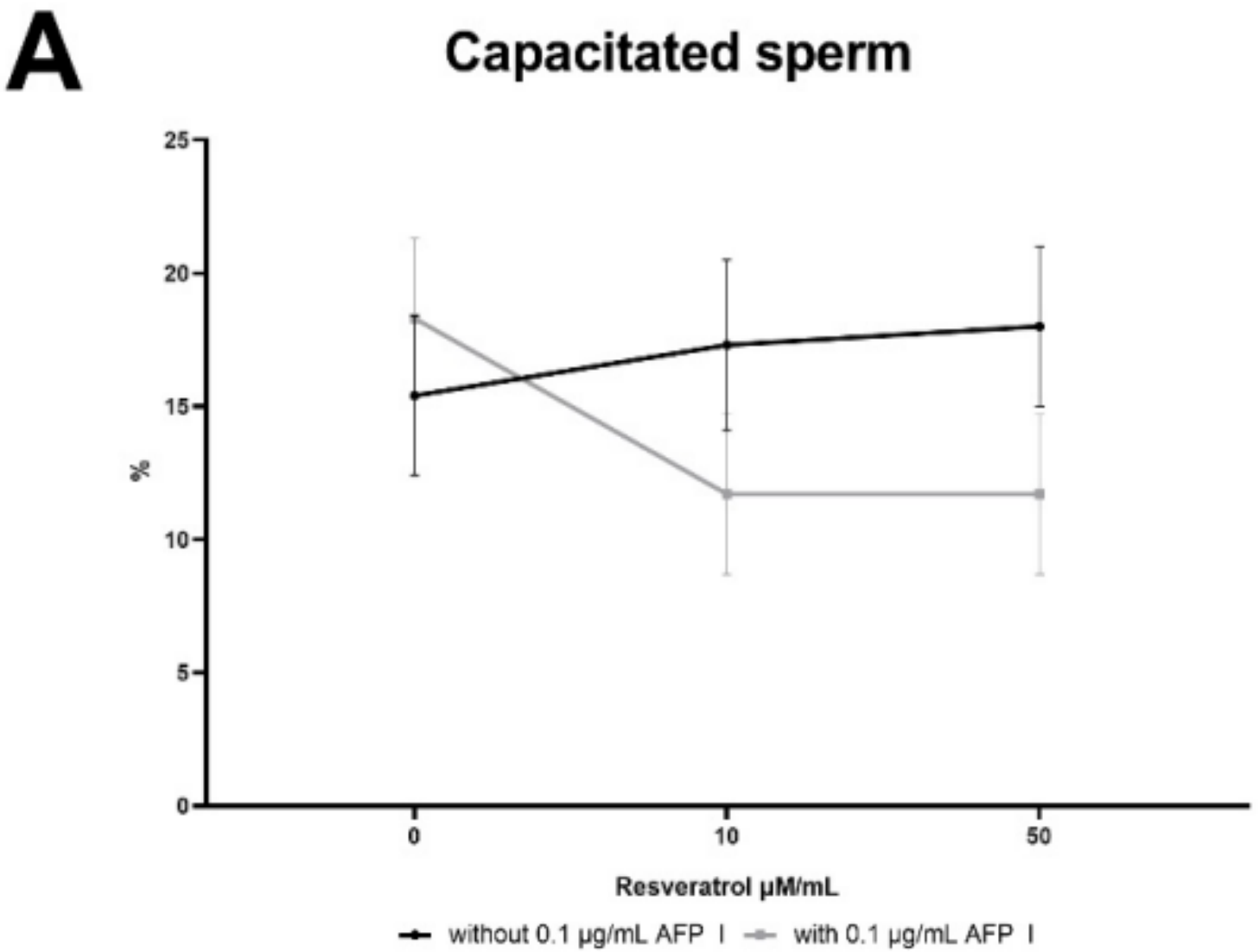
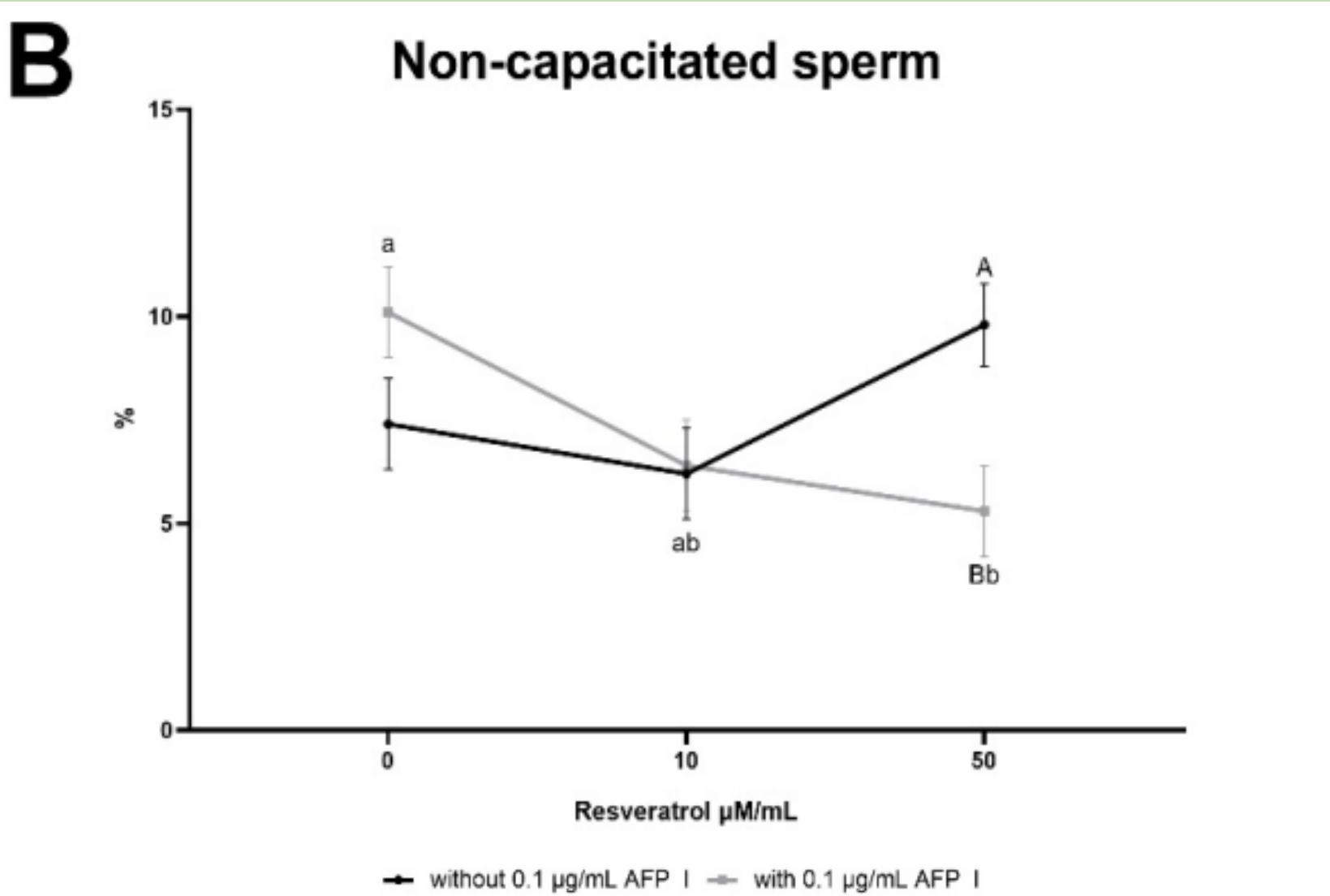



**Figure 2.** Interaction of (A) capacitated sperm, (B) non-capacitated sperm, and (C) acrosome-reacted sperm of frozen-thawed ram semen with or without the association of AFP I and different concentrations of resveratrol, immediately after thawing. Within a column or row, values with different superscripts differ significantly ($P < 0.05$). A,B differs between the absence or presence of AFP I (0.1 µg/mL). [a,b]differs among resveratrol concentrations (0, 10, or 50 µM/mL).

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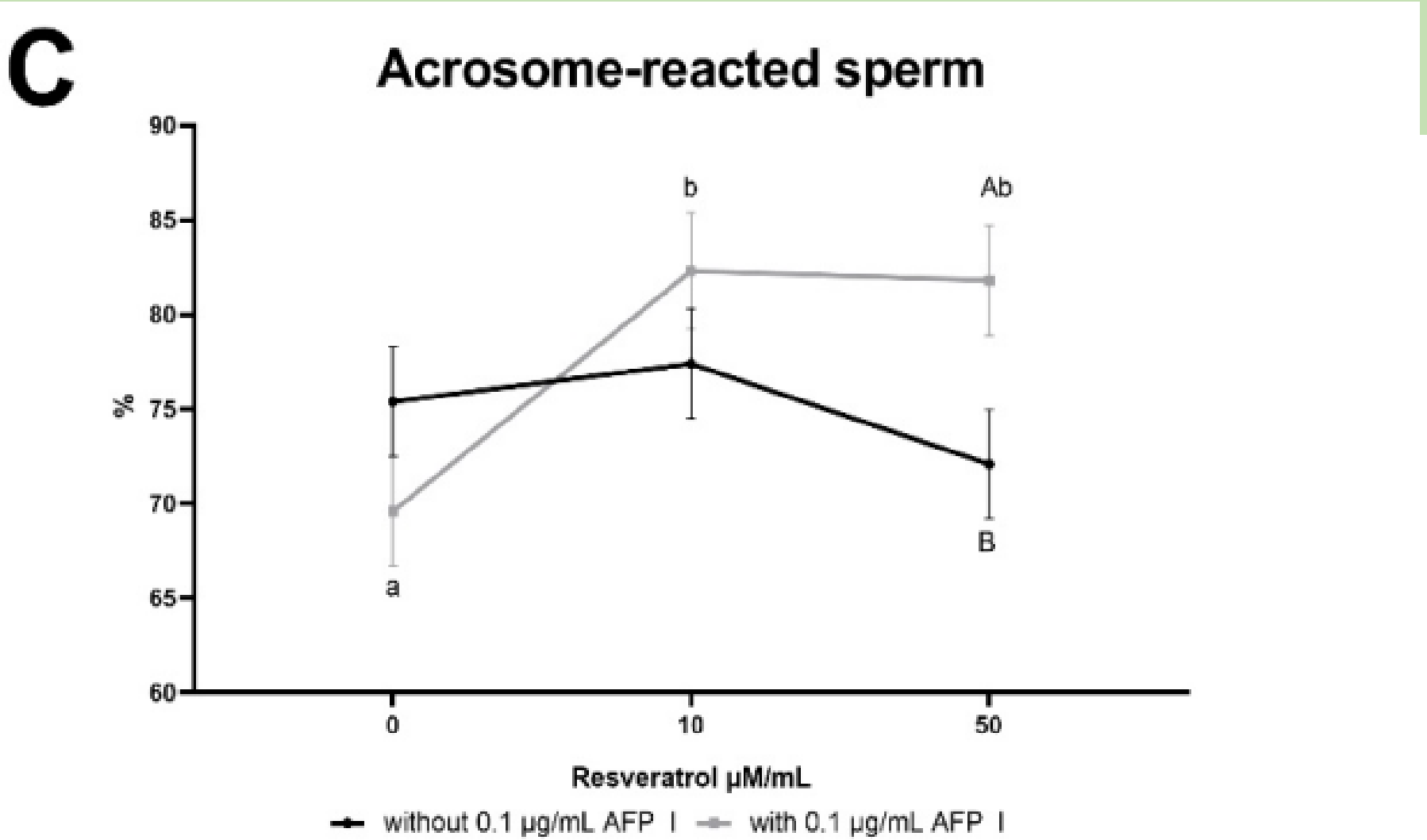



**Figure 2.** Interaction of (A) capacitated sperm, (B) non-capacitated sperm, and (C) acrosome-reacted sperm of frozen-thawed ram semen with or without the association of AFP I and different concentrations of resveratrol, immediately after thawing. Within a column or row, values with different superscripts differ significantly ($P < 0.05$). A,B differs between the absence or presence of AFP I (0.1 µg/mL). [a,b]differs among resveratrol concentrations (0, 10, or 50 µM/mL).

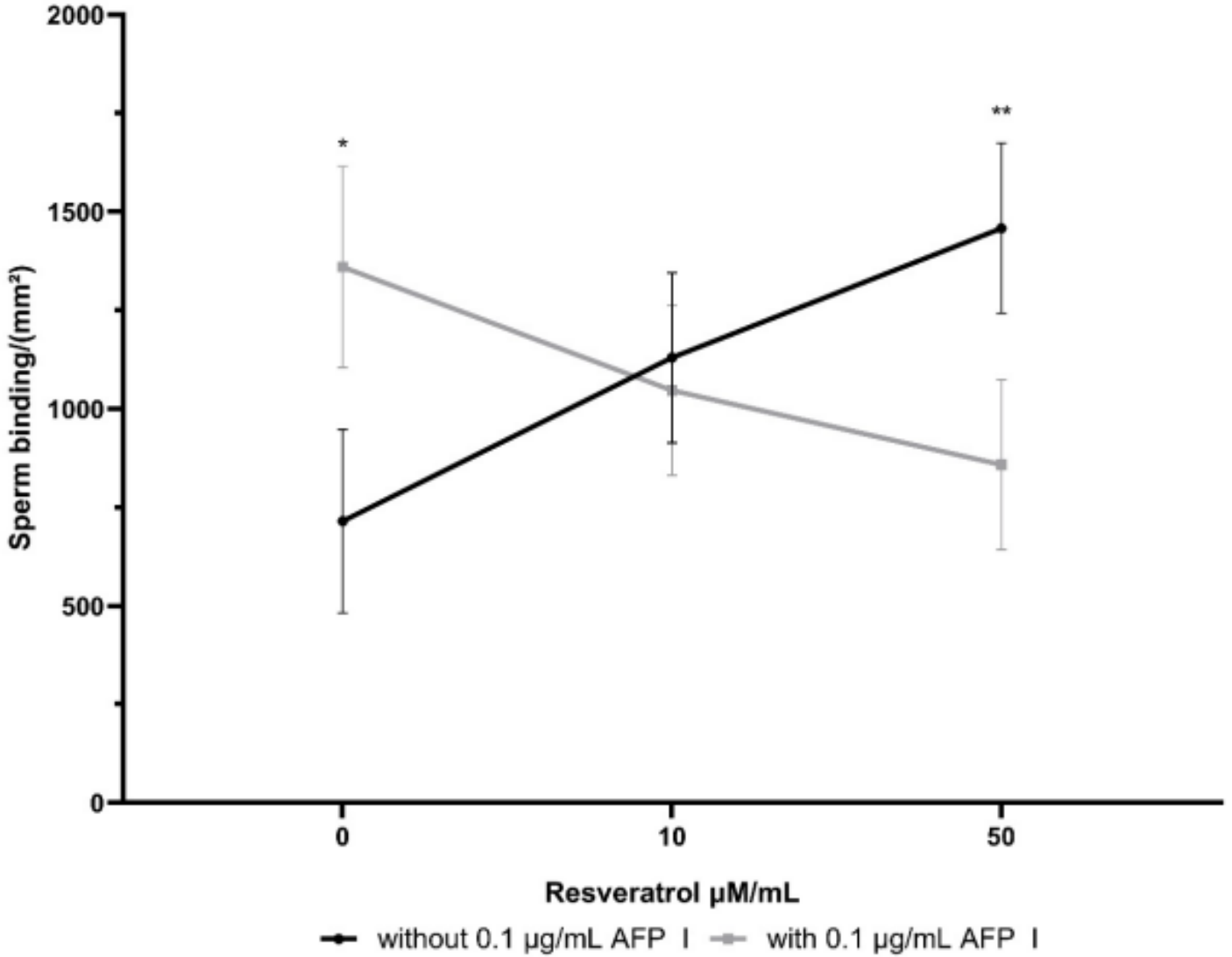



**Figure 3.** Interaction between the association of AFP I and resveratrol concentrations in semen extender on frozen-thawed ram sperm bound to egg perivitelline membrane test, immediately after thawing. *represents the high probability ($P$ = 0.071) of sperm bound in a single addiction of 0.1 μg/mL of AFP I in interaction analysis; **represents the high probability ($P$ = 0.058) of sperm bound in a single addiction of 50 μM/mL of resveratrol in the interaction analysis.

# Results

**Table 2.** Normal morphology and lipid peroxidation assessed by thiobarbituric acid reactive substances (TBARS) levels of cryopreserved ram sperm in extenders containing (+) or not (-) antifreeze protein type I (0.1 µg/mL), associated or not with different concentrations of resveratrol (0, 10 or 50 µM/mL) during cryopreservation (LSmeans ± SEM).

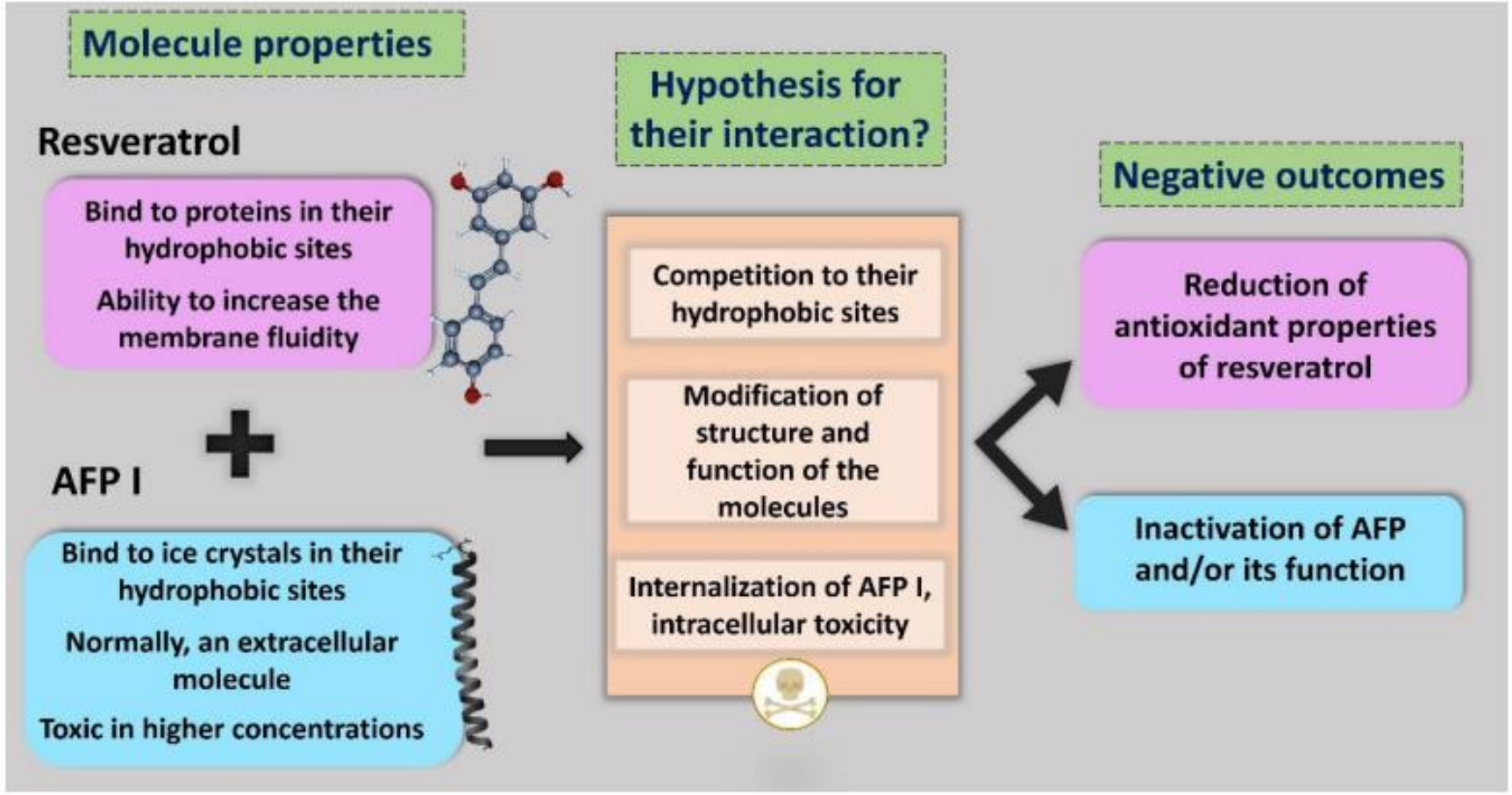
| Endpoints | AFP I (µg/mL) | Resveratrol (µM/mL) | | | *P-value* | | |
|---|---|---|---|---|---|---|---|
| | 0.1 | 0 | 10 | 50 | AFP I | Resveratrol | AFP×Resveratrol |
| Normal Morphology (%) | - | 79.8 ± 2.1 | 77.8 ± 2.1 | 77.1 ± 2.3 | n.s. | n.s. | n.s. |
| | + | 81.7 ± 2.3 | 80.1 ± 2.6 | 79.5 ± 2.3 | | | |
| TBARS (ng/mL) | - | 477.2 ± 66.2 | 613.9 ± 60.5 | 534.7 ± 66.2 | 0.085 | n.s. | n.s. |
| | + | 568.2 ± 60.5 | 666.1 ± 60.5 | 664.6 ± 60.5 | | | |

Abbreviations: n.s.: non-significant.

ORIGINAL ARTICLE

# The association of resveratrol and AFPI did not enhance the cryoresistance of ram sperm

Viviane Lopes Brair[1], Lucas Francisco Leodido Correia[1], Nathalia Oliveira Barbosa[1], Rachel Ferreira Braga[1], Augusto Ryonosuke Taira[1], Andreza Amaral da Silva[2], Felipe Zandonadi Brandão[1], Rodolfo Ungerfeld[3], Joanna Maria Gonçalves Souza-Fabjan[1]*




The **association of resveratrol and AFP I did not improve the quality of frozen-thawed ram semen and induced some deleterious effects** compared to the single addition of each one in the semen extender.

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Type: research-article

ORIGINAL ARTICLE

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# The Effects of Antifreeze Protein III Supplementation on the Cryosurvival of Goat Spermatozoa During Cryopreservation

Chunrong Lv,[1,2,*] Allai Larbi,[1,*] Sameeullah Memon,[1] Jiachong Liang,[1,2] Xiangwei Fu,[3] Guoquan Wu,[1,2] and Guobo Quan[1,2]

TABLE 1. EFFECTS OF ANTIFREEZE PROTEIN III SUPPLEMENTATION ON THE MOTILITY, MEMBRANE AND ACROSOME INTEGRITY OF GOAT SPERMATOZOA AFTER EQUILIBRATION AND BEFORE FREEZING

| *Concentrations of AFP (μg/mL)* | *Total motility (%)* | *Progressive motility (%)* | *Progressive motility (%)* | *Acrosome integrity (%)* |
|---|---|---|---|---|
| 0 | $71.57 \pm 5.43^{a}$ | $52.43 \pm 4.33^{a}$ | $56.18 \pm 5.11^{a}$ | $61.51 \pm 5.17^{a}$ |
| 1 | $72.73 \pm 3.59^{a}$ | $54.72 \pm 4.07^{a}$ | $56.47 \pm 5.26^{a}$ | $59.74 \pm 1.96^{a}$ |
| 10 | $69.10 \pm 3.23^{a}$ | $52.91 \pm 3.51^{a}$ | $54.91 \pm 3.30^{a}$ | $62.39 \pm 4.39^{a}$ |
| 50 | $70.74 \pm 6.29^{a}$ | $51.03 \pm 2.06^{a}$ | $55.26 \pm 4.89^{a}$ | $61.46 \pm 2.72^{a}$ |
| 100 | $69.02 \pm 5.86^{a}$ | $52.47 \pm 5.49^{a}$ | $57.33 \pm 5.06^{a}$ | $59.00 \pm 3.01^{a}$ |

Data are presented as mean ± SEM. Same superscripts within a column indicate no significant difference ($p > 0.05$).
AFP, antifreeze protein; SEM, standard error of the mean.

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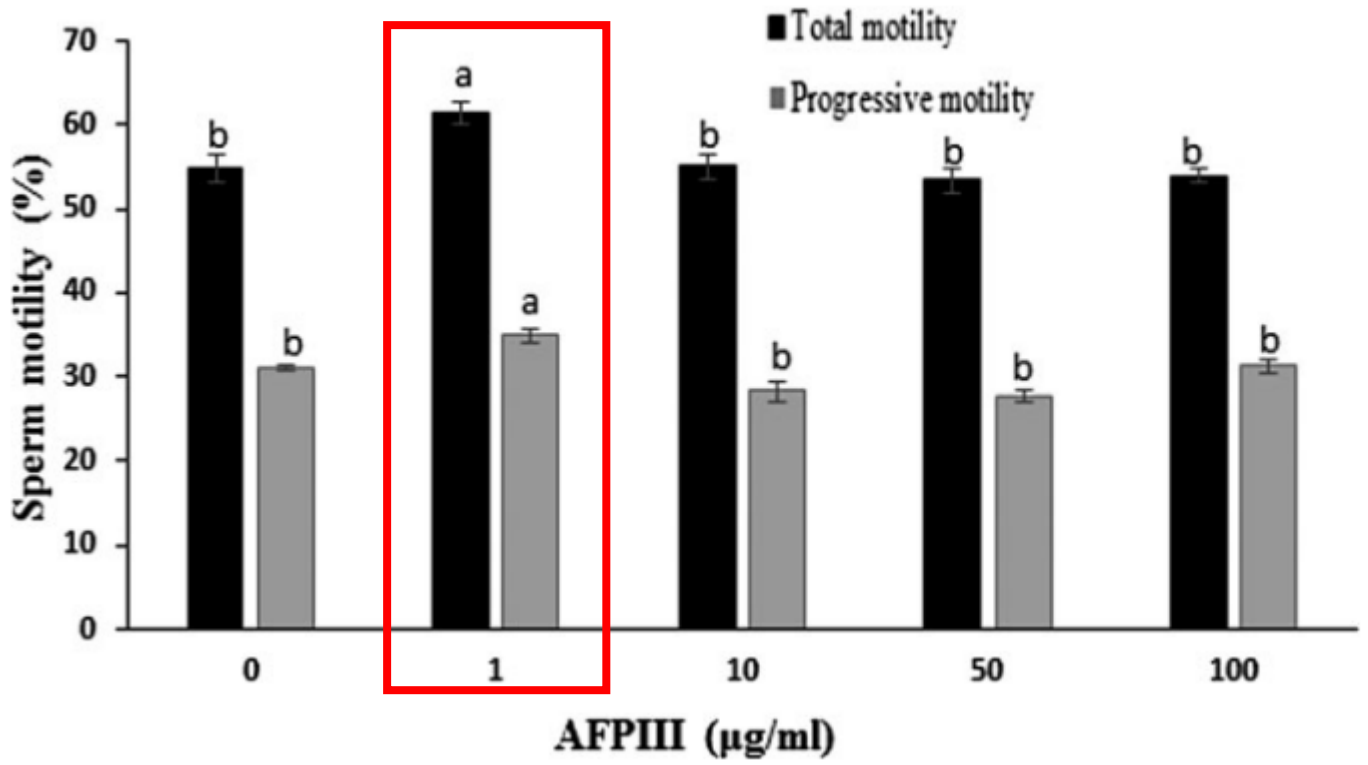


**FIG. 2.** Effects of AFPIII supplementation on the post-thaw motility of goat sperm. All data are expressed as mean ± SEM. Different superscript letters represent a significant difference ($p < 0.05$). AFP, antifreeze protein; SEM, standard error of the mean.

# The Effects of Antifreeze Protein III Supplementation on the Cryosurvival of Goat Spermatozoa During Cryopreservation

Chunrong Lv,[1,2,*] Allai Larbi,[1,*] Sameeullah Memon,[1] Jiachong Liang,[1,2] Xiangwei Fu,[3] Guoquan Wu,[1,2] and Guobo Quan[1,2]

TABLE 2. EFFECTS OF ANTIFREEZE PROTEIN III SUPPLEMENTATION ON MEMBRANE INTEGRITY, ACROSOMAL INTEGRITY, AND MITOCHONDRIAL ACTIVITY OF FROZEN–THAWED GOAT SPERMATOZOA

| *Concentrations of AFP (μg/mL)* | *Sperm with curved tail (%)* | *Acrosome integrity (%)* | *Mitochondrial activity (%)* |
|---|---|---|---|
| 0 | $33.06 \pm 1.17^{b}$ | $43.09 \pm 1.15^{b}$ | $47.40 \pm 1.25^{a}$ |
| 1 | $39.55 \pm 0.58^{a}$ | $48.43 \pm 1.22^{a}$ | $52.71 \pm 1.55^{b}$ |
| 10 | $24.24 \pm 1.20^{c}$ | $43.15 \pm 0.67^{b}$ | $47.27 \pm 1.73^{a}$ |
| 50 | $25.07 \pm 1.56^{c}$ | $40.08 \pm 1.47^{b}$ | $44.39 \pm 1.14^{a}$ |
| 100 | $26.72 \pm 2.44^{c}$ | $40.83 \pm 1.73^{b}$ | $44.75 \pm 2.15^{a}$ |

Data are presented as mean ± SEM. Different superscripts within a column indicate a difference ($p < 0.05$).

TABLE 3. EFFECTS OF ANTIFREEZE PROTEIN III SUPPLEMENTATION ON PHOSPHATIDYLSERINE DISTRIBUTION OF FROZEN–THAWED GOAT SPERMATOZOA

| *Concentrations of AFP (μg/mL)* | *Apoptosis-like (%)* | *Viable (%)* | *Dead (%)* |
|---|---|---|---|
| 0 | $15.76 \pm 0.69^{a}$ | $29.67 \pm 0.76^{b}$ | $54.93 \pm 0.64^{b}$ |
| 1 | $15.09 \pm 1.01^{a}$ | $35.11 \pm 0.71^{a}$ | $50.31 \pm 0.87^{c}$ |
| 10 | $16.98 \pm 0.56^{a}$ | $29.26 \pm 0.72^{b}$ | $54.21 \pm 1.09^{b}$ |
| 50 | $14.43 \pm 0.91^{a}$ | $27.60 \pm 1.12^{b}$ | $58.03 \pm 1.05^{a}$ |
| 100 | $14.33 \pm 0.95^{a}$ | $27.29 \pm 0.96^{b}$ | $59.34 \pm 1.00^{a}$ |

Data are presented as mean ± SEM. Different superscripts within a column indicate a difference ($p < 0.05$).

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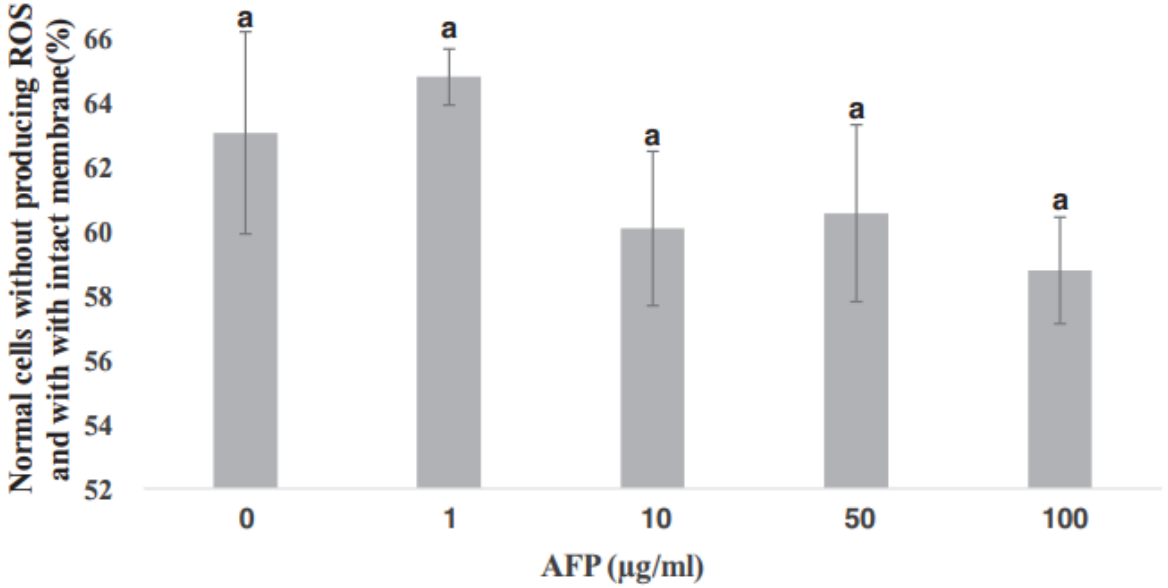


**FIG. 3.** Effects of AFPIII supplementation on oxidative stress of frozen–thawed goat sperm following staining with 2′,7′-dichlorodihydrofluorescein diacetate. All data are expressed as mean ± SEM. Different superscript letters represent a significant difference ($p < 0.05$). ROS, reactive oxygen species.

The presence of AFPIII at 1 μg/mL in freezing extenders can **enhance the post-thaw motility, membrane integrity, acrosome integrity, and viability of goat spermatozoa.** However, it should be noted that the positive effects of AFPIII may be dependent on the specific cryopreservation approach used in this study, such as the extender used and the freezing process.

Research in Veterinary Science 154 (2023) 108–112

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# Effect of antifreeze protein type III on frozen/thawed of spermatozoa recover from goat epididymis

Millena Maria Monteiro [a,*], Desirée Coelho de Mello Seal [a], Jerônimo Hugo de Souza [a], Mariana Trevisan [a], Lúcia Cristina Pereira Arruda [a], Sildivane Valcácia Silva [b], Maria Madalena Pessoa Guerra [a]

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[b] *Department of Biotechnology, Biotechnology Center, University Federal of Paraíba (UFPB), João Pessoa, Paraíba, Brazil*

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**Table 1**

Kinematics (CASA) of cryopreserved epididymal sperm of goats in extender supplemented with different concentrations of AFP III. Data are expressed as mean ± standard deviation.

| Variables | AFP III (μg/mL) | | | |
|---|---|---|---|---|
| | 0 | 1 | 10 | 100 |
| MT (%) | 67.41 ± 4.71 | 64.61 ± 9.01 | 66.63 ± 10.83 | 63.91 ± 8.54 |
| MP (%) | 25.51 ± 7.06 | 23.36 ± 4.14 | 23.66 ± 12.56 | 21.95 ± 7.62 |
| LIN (%) | 52.35 ± 5.34 | 52.91 ± 4.36 | 53.60 ± 7.04 | 50.84 ± 4.44 |
| STR (%) | 77.06 ± 2.97 | 77.53 ± 3.55 | 78.19 ± 4.21 | 77.18 ± 2.67 |
| WOB (%) | 67.78 ± 4.44 | 68.19 ± 3.10 | 68.34 ± 5.47 | 65.79 ± 3.87 |
| VCL (μm/s) | 71.41 ± 12.79 | 68.75 ± 16.43 | 73.80 ± 10.38 | 69.23 ± 6.63 |
| VSL (μm/s) | 42.60 ± 7.27 | 40.44 ± 5.25 | 41.08 ± 8.02 | 38.59 ± 6.61 |
| VAP (μm/s) | 55.09 ± 7.98 | 52.28 ± 6.87 | 52.31 ± 8.20 | 49.96 ± 8.24 |
| ALH (μm/s) | 2.86 ± 0.39 | 2.71 ± 0.43 | 2.78 ± 0.42 | 2.78 ± 0.45 |
| BCF (Hz) | 11.44 ± 0.89 | 11.38 ± 0.63 | 11.70 ± 0.82 | 11.79 ± 0.82 |

AFP III - antifreeze protein type III; MT - total motility; MP - progressive motility; LIN- linearity; STR- straightness; WOB: wobble; VCL - curvilinear velocity, VSL - straightline velocity; VAP - average path velocity; ALH- amplitude of lateral head displacement; BCF- beat cross frequency. $^{A, B, C, D}$: different letters on the same line represent statistical difference between treatments ($p < 0.05$).

# Effect of antifreeze protein type III on frozen/thawed of spermatozoa recover from goat epididymis

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**Table 2**

Plasma and acrosome membrane integrity, mitochondrial membrane potential, intracellular ROS (flow cytometry) levels of cryopreserved epididymal spermatozoa of goats, in extender supplemented with different concentrations of AFP III. Data are expressed as meean ± standard deviation.

| Variables | AFP III (μg/mL) | | | |
|---|---|---|---|---|
| | 0 | 1 | 10 | 100 |
| iMPA (%) | 36.17 ± 7.57$^{a}$ | 30.69 ± 4.49$^{ab}$ | 29.02 ± 6.69$^{ab}$ | 26.51 ± 6.17$^{b}$ |
| MMP (%) | 14.61 ± 8.84 | 18.81 ± 12.05 | 18.88 ± 11.44 | 22.16 ± 13.16 |
| iROS (%) | 79.02 ± 35.51 | 88.67 ± 23.13 | 86.54 ± 32.00 | 85.35 ± 31.69 |

AFP III: antifreeze protein type III; iPMA: percentage of cells with intact plasma and acrosome membrane; MMP: mitochondrial membrane potential; iROS: intracellular ROS levels. Average ± standard deviation; $^{a, b, c, d:}$ different letters on the same line represent statistical difference between treatments ($p < 0.05$).

In conclusion, the **addition of AFP III to Tris-egg yolk extender, used for the freezing of sperm obtained from the epididymis of goats, did not improve the preservation of these cells.**

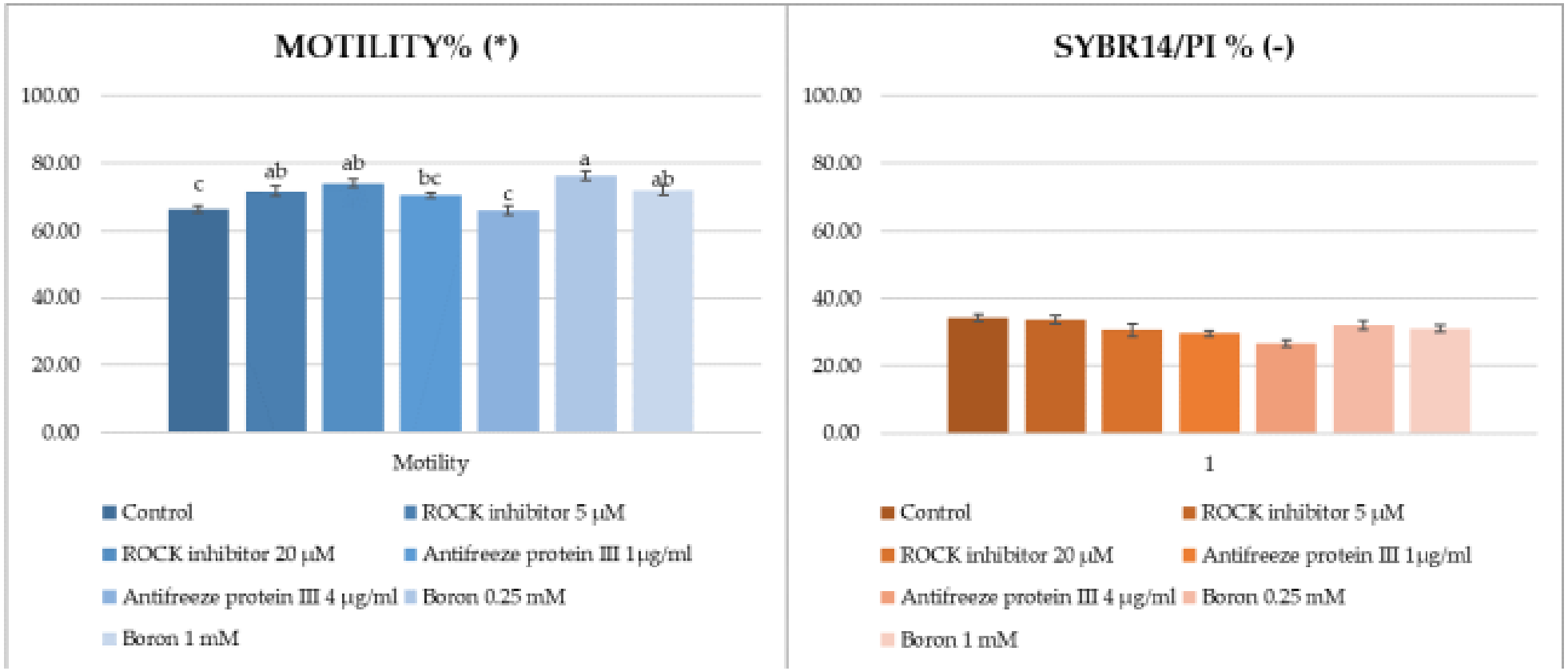
molecules

MDPI

*Article*

# The Effects of Different Doses of ROCK Inhibitor, Antifreeze Protein III, and Boron Added to Semen Extender on Semen Freezeability of Ankara Bucks

**Ömer Faruk Karaşör [1,*], Mustafa Numan Bucak [2,*], Mihai Cenariu [3], Mustafa Bodu [2], Mehmet Taspınar [4] and Filiz Taspınar [4]**



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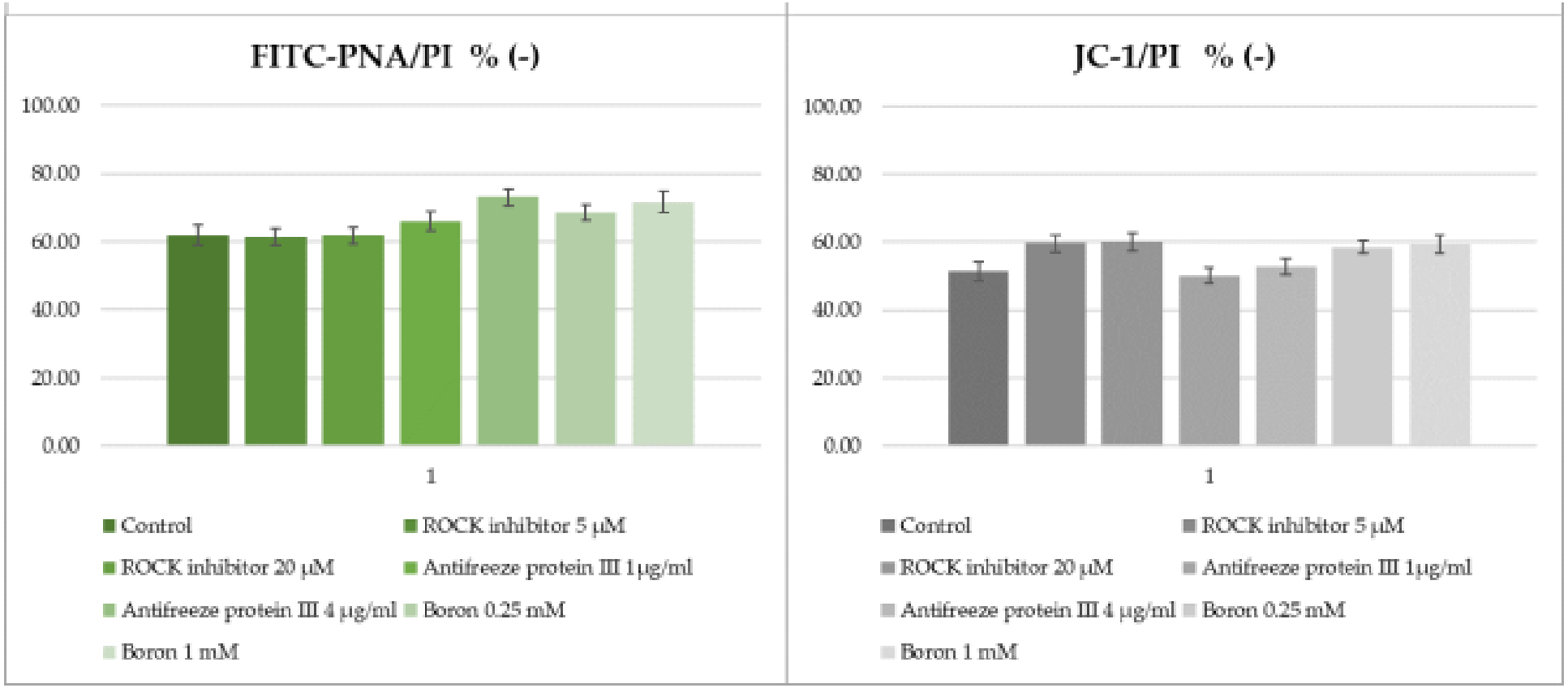

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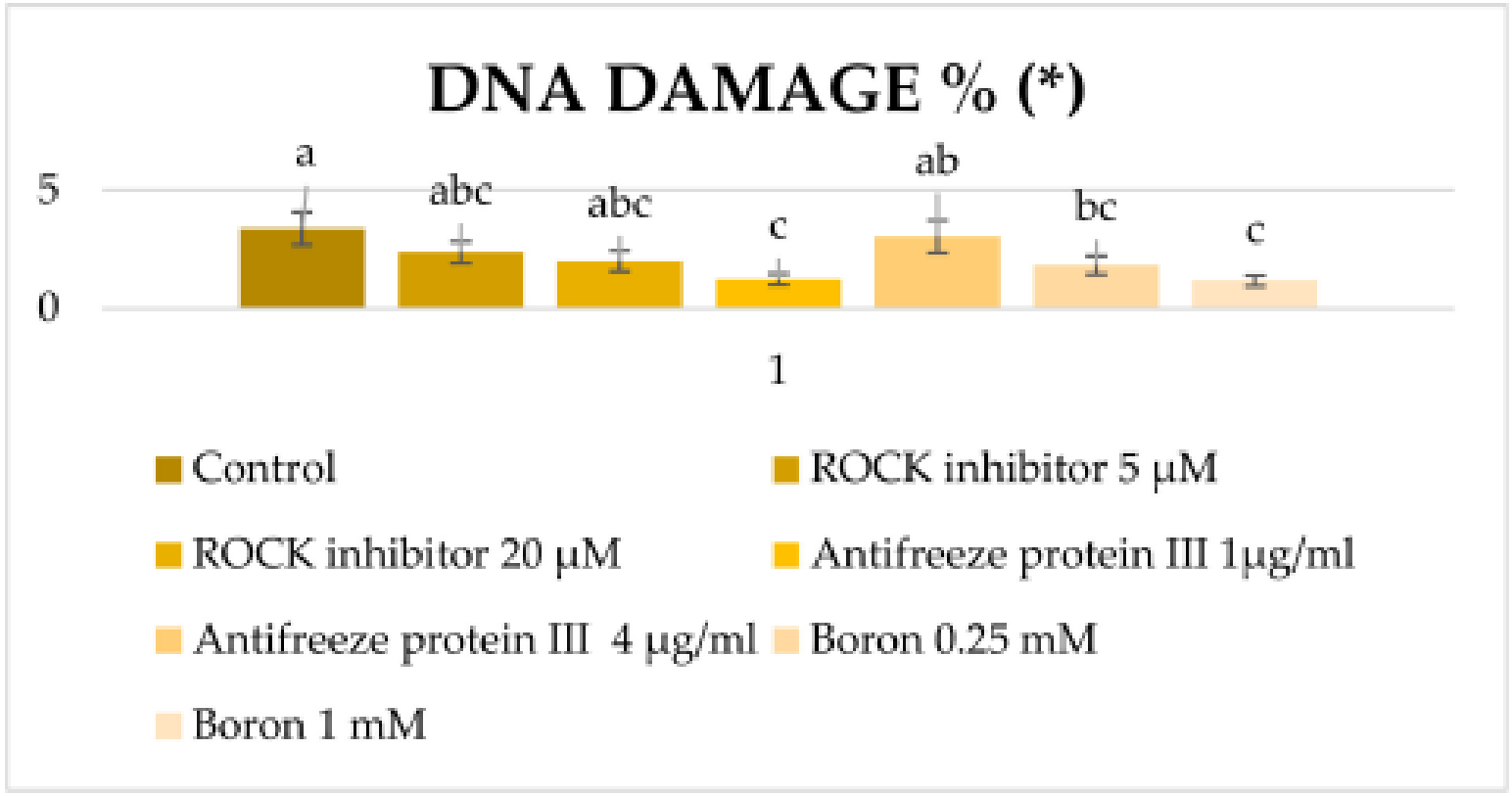



It was determined that some of the additives added to the Tris-based extender (ROCK inhibitor 5 and 10 µM; boron 0.25 and 1 mM) provided a significant improvement in sperm motility while others, **antifreeze protein III 1 µg/mL** and boron (0.25 and 1 mM), **decreased the damage to DNA**

# AFPs in small ruminants oocyte cryopreservation

Anais do XXV Congresso Brasileiro de Reprodução Animal (CBRA-2023), Belo Horizonte, MG, 24 a 26 de maio de 2023.

# Antifreeze protein type I affected positively the MATER gene expression in vitrified cumulus-oocyte complexes of ewes

*A proteína anticongelante tipo I afetou positivamente a expressão do gene MATER em complexos cumulus-oócitos vitrificados de ovelhas*

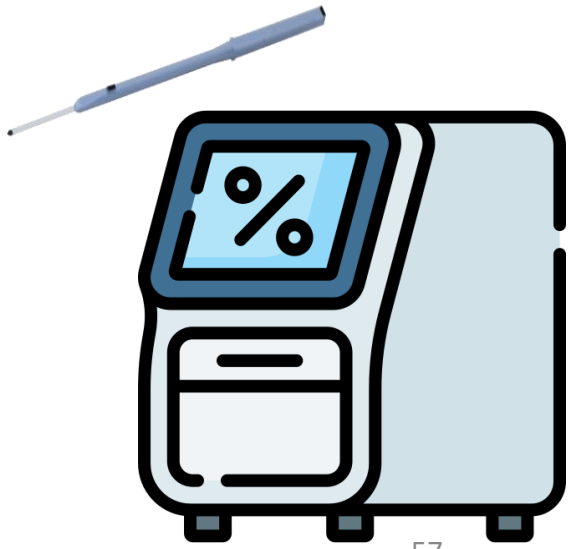
**Thais de Almeida Oliveira[1], Mariana Pedrosa de Paula Guimarães[1], Débora Fernanda Santos de Pinho[1], Leonardo Novaes Cajaiba[1], Ana Lucia Rosa e Silva Maia[1], Gabriela Ramos Leal[1], Felipe Zandonadi Brandão, Ribrio Ivan Tavares Pereira Batista[1], Joanna Maria Gonçalves Souza-Fabjan[1]**

[1]Universidade Federal Fluminense, Niterói, RJ, Brasil
*E-mail: joannavet@gmail.com

**Sheep Ovaries (n= 48)** → **COCs recovery** → **COCs selection** → Grade I, Grade II **(n= 45)** →

**FRESH CONTROL**
**Without vitrification (n=15)**

**VITRIFIED**
**G0: Without AFP (n=15)**
**G25: With 0.25 µg/mL AFP (n=15)**



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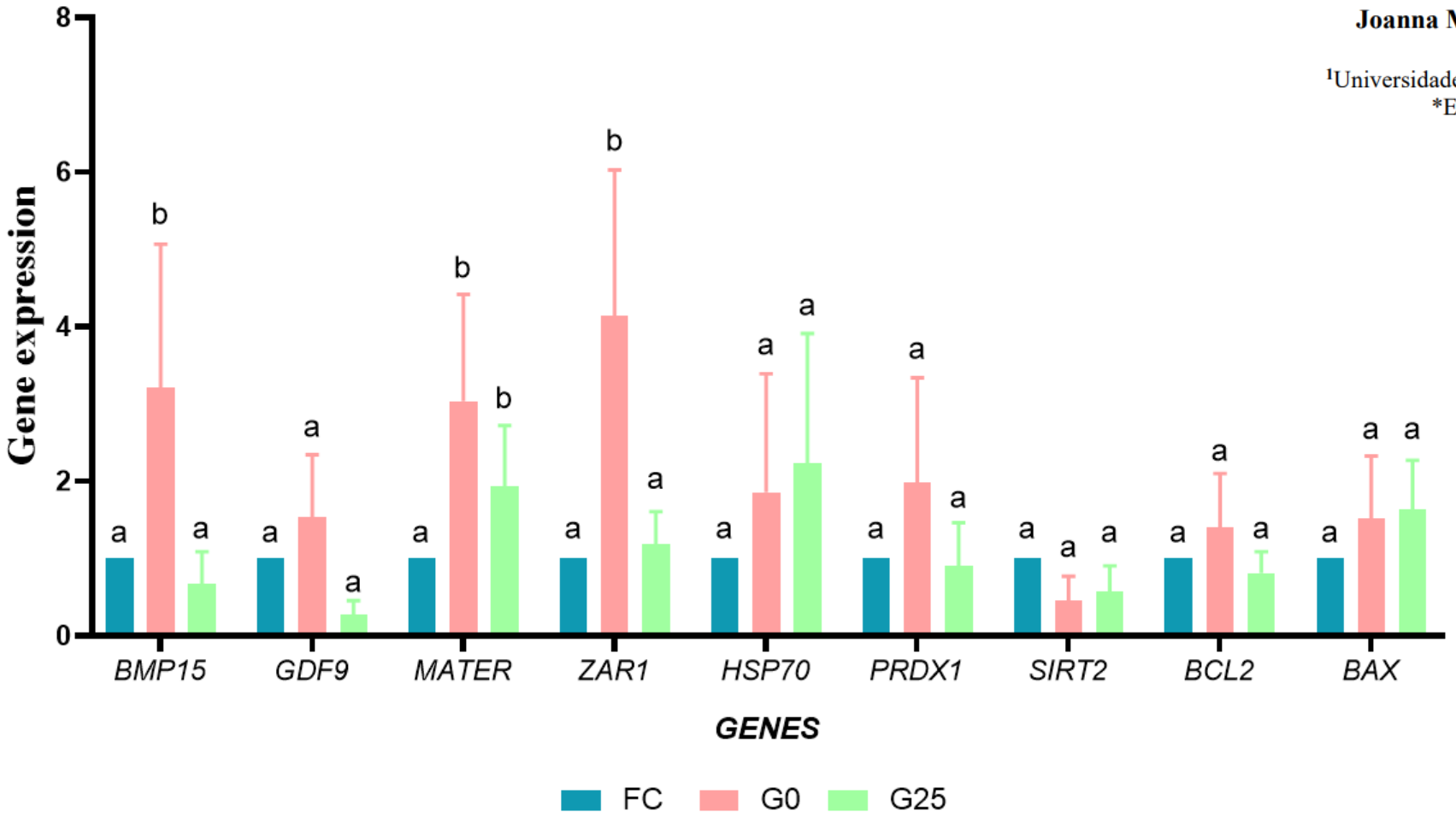



In conclusion, **the addition of 0.25 µg/ mL of AFP type I in the vitrification process seemed to affect positively the expression of the *MATER* gene.**

# Under analysis

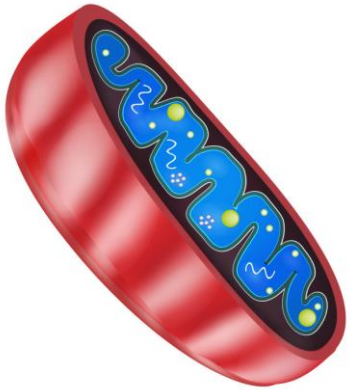
Sheep Ovaries → COCs recovery → Grade I / Grade II →

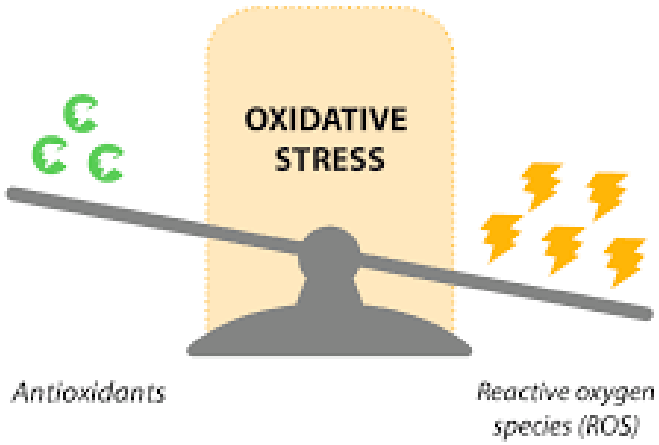
**FRESH CONTROL**
**Without vitrification**

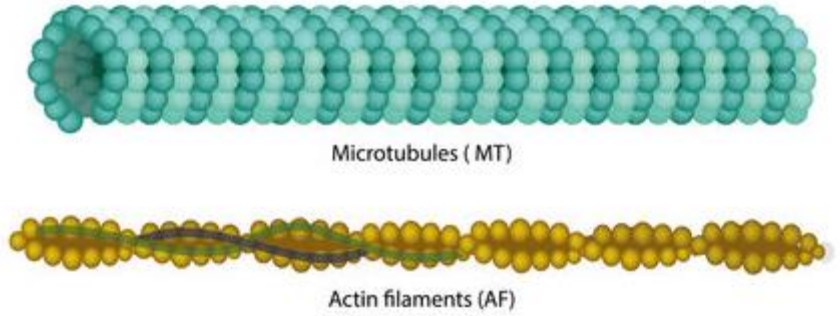
**VITRIFIED**

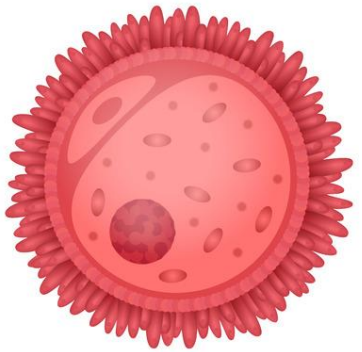
**G0: Without AFP I**

**G25: With 0.25 µg/mL AFP I**

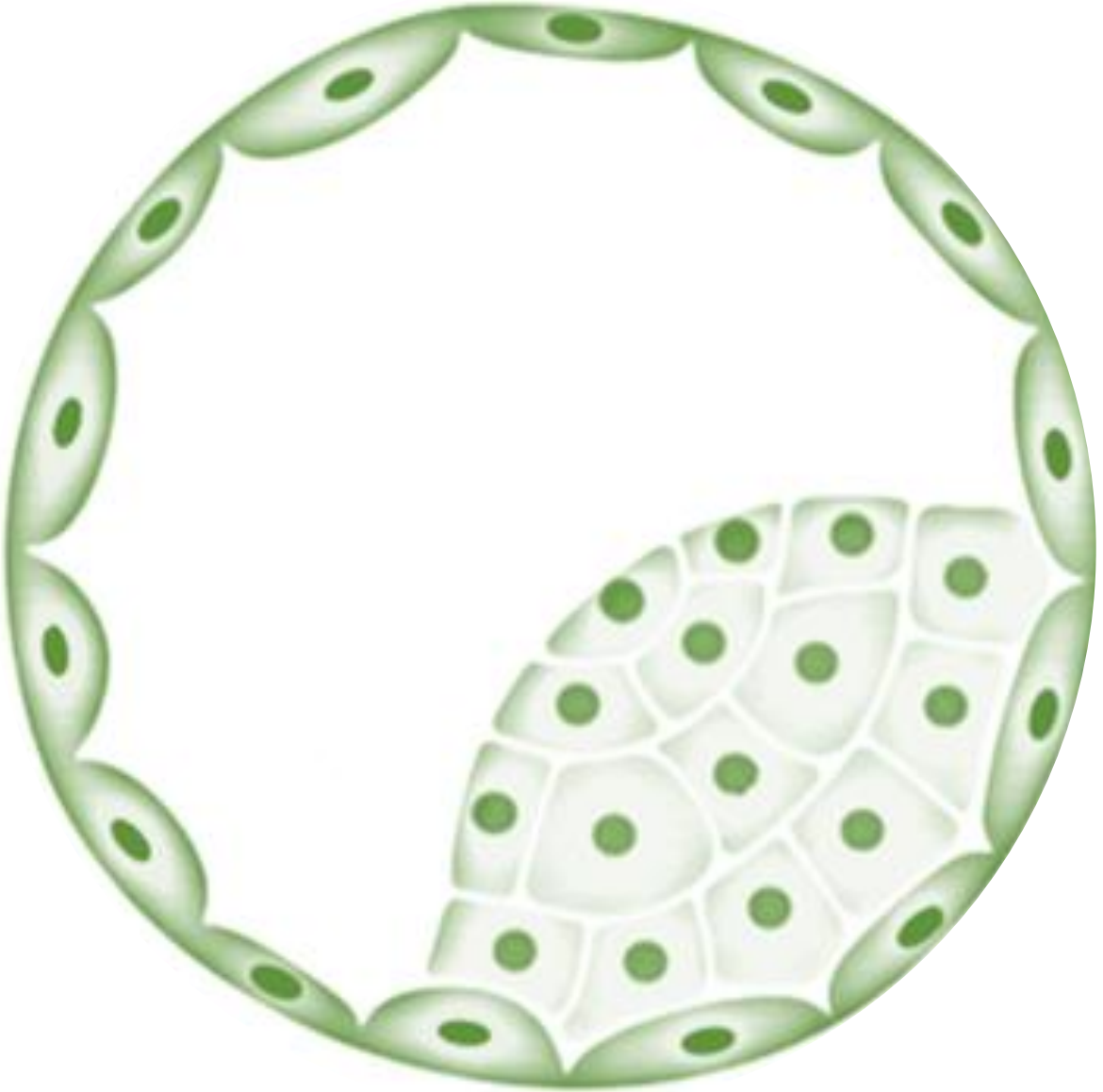




# AFPs in small ruminants embryo cryopreservation



Theriogenology. 1997 Oct 15;48(6):1017-24. doi: 10.1016/s0093-691x(97)00328-2.

# Hypothermic storage of sheep embryos with antifreeze proteins: development in vitro and in vivo

A Baguisi [1], A Arav, T F Crosby, J F Roche, M P Boland

Table 1. Viability rates in vitro (72 hours) of ovine embryos following storage at 4°C for 4 days in PBS supplemented with bovine serum albumen (BSA) at 4 mg/ml or in Winter Flounder (Type 1) or Ocean Pout (Type 3) antifreeze proteins at 1 and 10 mg/ml

| Treatment group | Viability rate (%) | Hatching rate (%) | Diameter (μm) |
|---|---|---|---|
| T1-Control | 18/21 (86)$^{a}$ | 15/21(7 1)$^{a}$ | 453±70$^{a}$ |
| T2-BSA | 9/15 (60)$^{ab}$ | 7/15(47)$^{a}$ | 260±10$^{d}$ |
| T3-OP1 | 15/21 (7 1)$^{ab}$ | 12/21 (57)$^{a}$ | 376±74$^{bc}$ |
| T4-OP10 | 11/20 (55)$^{b}$ | 9/20 (45)$^{a}$ | 337±33$^{c}$ |
| T5-WF1 | 14/15 (93)$^{a}$ | 11/15(73)$^{a}$ | 415±125$^{ab}$ |
| T6-WF10 | 13/15 (87)$^{a}$ | 9/15 (60)$^{a}$ | 353±137$^{c}$ |

$^{a,b,c}$ Values within columns without common superscripts are different ($P<0.05$).

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Table 2. Viability and hatching rates of ovine embryos following hypothermic storage for 4 days at 0°C or 4°C in PBS containing either bovine serum albumen (BSA) at 4 mg/ml or Winter Flounder (WF) at 1 mg/n-d and subsequent culture in vitro for 72 hours

| Treatment group | Viability rate | Hatching rate | Diameter (μm) |
|---|---|---|---|
| T1-controls | 10/10 (100)$^{a}$ | 10/10 (100)$^{d}$ | 432±46$^{a}$ |
| T2-BSA 0°C | 6/10 (60)$^{ab}$ | 3/10 (30)$^{e}$ | 263±12$^{c}$ |
| T3-BSA 4°C | 8/11 (73)$^{ab}$ | 6/11 (54)$^{e}$ | 275±20$^{c}$ |
| T4-WF 0°C | 6/11 (54)$^{b}$ | 5/11 (45)$^{e}$ | 345+62$^{b}$ |
| T5-WF 4°C | 9/12 (75)$^{ab}$ | 8/12 (67)$^{de}$ | 383±35$^{ab}$ |

$^{a-e}$ Values within columns with no common superscript are significantly different: $^{a-c}$ P<0.025; $^{d,e}$ P<0.01.

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Table 3. Pregnancy rates (Days 28 to 32) following surgical transfer of ovine embryos either fresh or following storage at 4°C for 4 d prior to transfer

| Treatment group | Pregnancy rate (%) | No. viable fetuses (%) | No.(%) viable fetuses from pregnant ewes |
|---|---|---|---|
| Fresh (control) | 6/8 (75) | 11/21 (52) | 11/16 (68) |
| BSA > 2°C/m | 10/11 (91) | 14/27 (52) | 14/25 (56) |
| WF > 2°C/m | 6/10 (60) | 10/23 (44) | 10/14 (71) |
| WF < 1°C/m | 8/10 (80) | 15/27 (56) | 15/21 (71) |

**No difference**

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# Hypothermic storage of sheep embryos with antifreeze proteins: development in vitro and in vivo

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In conclusion these data indicate that **AFPs and BSA can protect ovine embryos during storage at O° or 4°C in vitro for 4 d and result in high survival after in vitro culture or in vivo transfer**. If the mechanism of protection against hypothermia-related perturbations could be extended for longer periods (beyond 2 d) and across a wider range of biological cell types (gametes, tissues and organs), then it would have enormous theoretical and practical implications. **A specific role for AFPs has not been fully established**, and it will require further elucidation to optimize their properties.

Cryobiology 93 (2020) 109–114

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# Antifreeze protein from *Anatolia polita* (ApAFP914) improved outcome of vitrified *in vitro* sheep embryos

Xiaolin Li [a,b,1], Liqin Wang [b,1], Chen Yin [a,b,1], Jiapeng Lin [b], Yangsheng Wu [b], Dayong Chen [c], Chunjuan Qiu [c], Bin Jia [a,*], Juncheng Huang [b,**], XiangJu Jiang [d], Lan Yang [b], Li Liu [b]

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[b] Key Laboratory of Genetics Breeding and Reproduction of Grass Feeding Livestock, Ministry of Agriculture and Rural affairs, Urumqi, 830000, PR China
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[d] HouBo College of Xinjiang Medical University, Karamay, 834000, China

**Table 7**
Effect of ApAFP914 on the rapid development of *in vitro* embryos in sheep.

| Concentration (μg/mL) | No.thawed embryos | No. surviving embryos | No. hatching embryos | Survival rate (%) | Hatching rate (%) |
|---|---|---|---|---|---|
| Control | 126 | 122 | 72 | 95.14 ± 2.55 | 56.74 ± 5.51 |
| 5 | 68 | 64 | 41 | 96.23 ± 2.46 | 66.16 ± 6.13 |
| 10 | 239 | 227 | 138 | 95.09 ± 1.74 | 62.92 ± 5.79 |
| 15 | 103 | 97 | 53 | 94.12 ± 1.87 | 55.56 ± 3.93 |
| 30 | 134 | 125 | 74 | 93.50 ± 2.13 | 51.86 ± 5.81 |

**No difference**

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**Table 8**
Effect of ApAFP914 concentration on slower *in vitro* embryo freezing efficiency in sheep (repeated 3 times).

| Concentration (μg/mL) | No. thawed embryos | No. surviving embryos | No. hatching embryos | Survival rate (%) | Hatching rate (%) |
|---|---|---|---|---|---|
| Control | 60 | 48 | 11 | 77.37 ± 3.32 | 22.95 ± 5.95a |
| 5 | 64 | 51 | 12 | 78.66 ± 1.92 | 23.22 ± 0.81a |
| 10 | 72 | 60 | 24 | 84.95 ± 5.55 | 35.63 ± 7.59b |
| 15 | 59 | 48 | 5 | 82.43 ± 2.12 | 9.11 ± 3.23a |
| 30 | 63 | 54 | 5 | 85.62 ± 2.89 | 7.95 ± 1.62a |

As a cryoprotectant, **ApAFP914 can increase the hatching rate, indicating that AFP can be used for sheep embryo cryopreservation**. However, high concentrations of AFP tend to reduce developmental efficiency.

Research in Veterinary Science 168 (2024) 105132

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# Effect of antifreeze protein I in the freezing solution on *in vivo*-derived sheep embryos

Lucas F.L. Correia *, Gabriela R. Leal, Felipe Z. Brandão, Ribrio I.T.P. Batista, Joanna M. G. Souza-Fabjan *

*Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340 Niterói, RJ, Brazil*

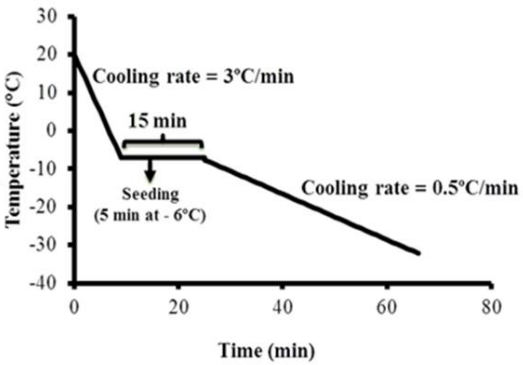
# Experimental design

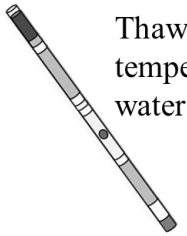
37 Santa Inês ewes

Superovulation and non-surgical embryo recovery

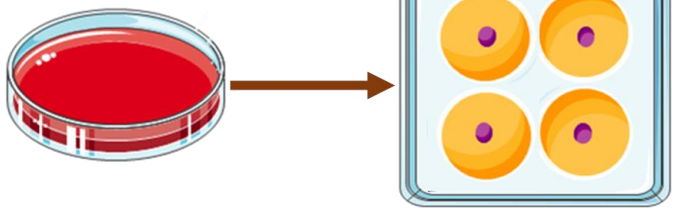
| 39 embryos | 53 embryos | 43 embryos |
|---|---|---|
| CONT | AFP0.1 | AFP0.5 |
| AFP I free | 0.1 µg/mL AFP I | 0.5 µg/mL AFP I |

Slow freezing



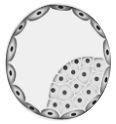



Thawing was performed at room temperature for 5 s followed by a water bath at 37 ºC for 30 s.



Embryos were kept in HEPES synthetic oviductal fluid amino acids (SOFaa) medium for less than 1 h. Afterwards, they were *in vitro* cultured in drops containing a ratio of 2.5 µL of SOFaa medium per embryo covered with mineral oil.

Embryos were *in vitro* cultured (IVC) at 38.5 ºC, 5% CO2, and 5% O2 during 48 h in benchtop incubator.



Embryo survival rate

24 h

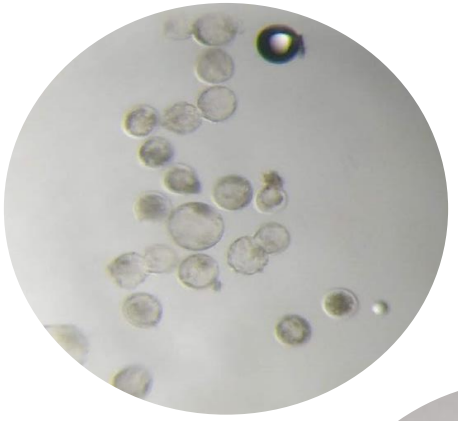
48 h

Gene expression:
- metabolism (*CDX2*, *OCT4*, *PRDX1, SIRT2*)
- quality genes (*HSP70*, *BAX*, *BCL2*, *CDH1*, *AQP3*)
- housekeeping (*GAPDH, ACTB)*

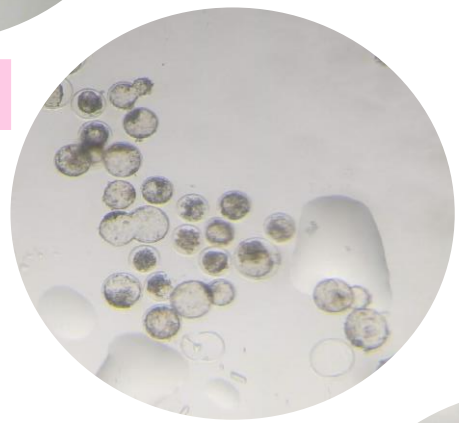
Epifluorescence microscopy (five embryos per analysis):
- Mitochondrial activity
- Reactive oxygen species (ROS)
- Gluthatione (GSH)
- Apoptosis and Dead Cells

Embryo hatching rate

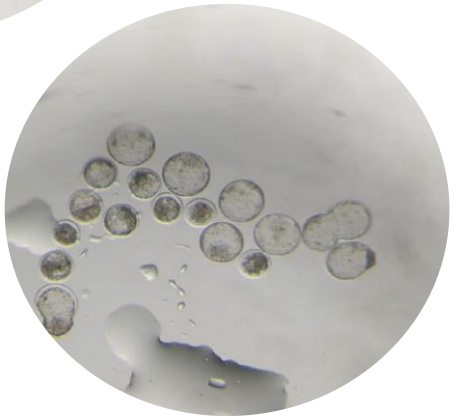
# Results



CONT



AFP0.1



AFP0.5

**Table 2**

Survival, morulas development, and hatching rates of *in vivo*-derived sheep embryos previously submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 48 h after thawing.

| Group | Embryo survival rate at 24 h after thawing (%) | Total embryo survival rate (%) | Total of morulas development (%) | Total hatching rate/Total of viable blastocysts[1] (%) |
|---|---|---|---|---|
| CONT[#] | 19/39 (49) | 22/39 (56) | 3/19 (16) | 11/22 (50) |
| AFP0.1[†] | 28/53 (53) | 32/53 (60) | 3/19 (16) | 21/34 (62) |
| AFP0.5[‡] | 19/43 (44) | 23/43 (53) | 3/16 (19) | 9/27 (33)[*] |
| TOTAL | 66/135 (49) | 77/135 (57) | 9/54 (17) | 41/83 (49) |

[#] CONT = Control group, which contained - 3 morulas, 16 compact morulas, 4 early blastocysts, 4 blastocysts, 11 expanded blastocysts, 1 hatched blastocyst.

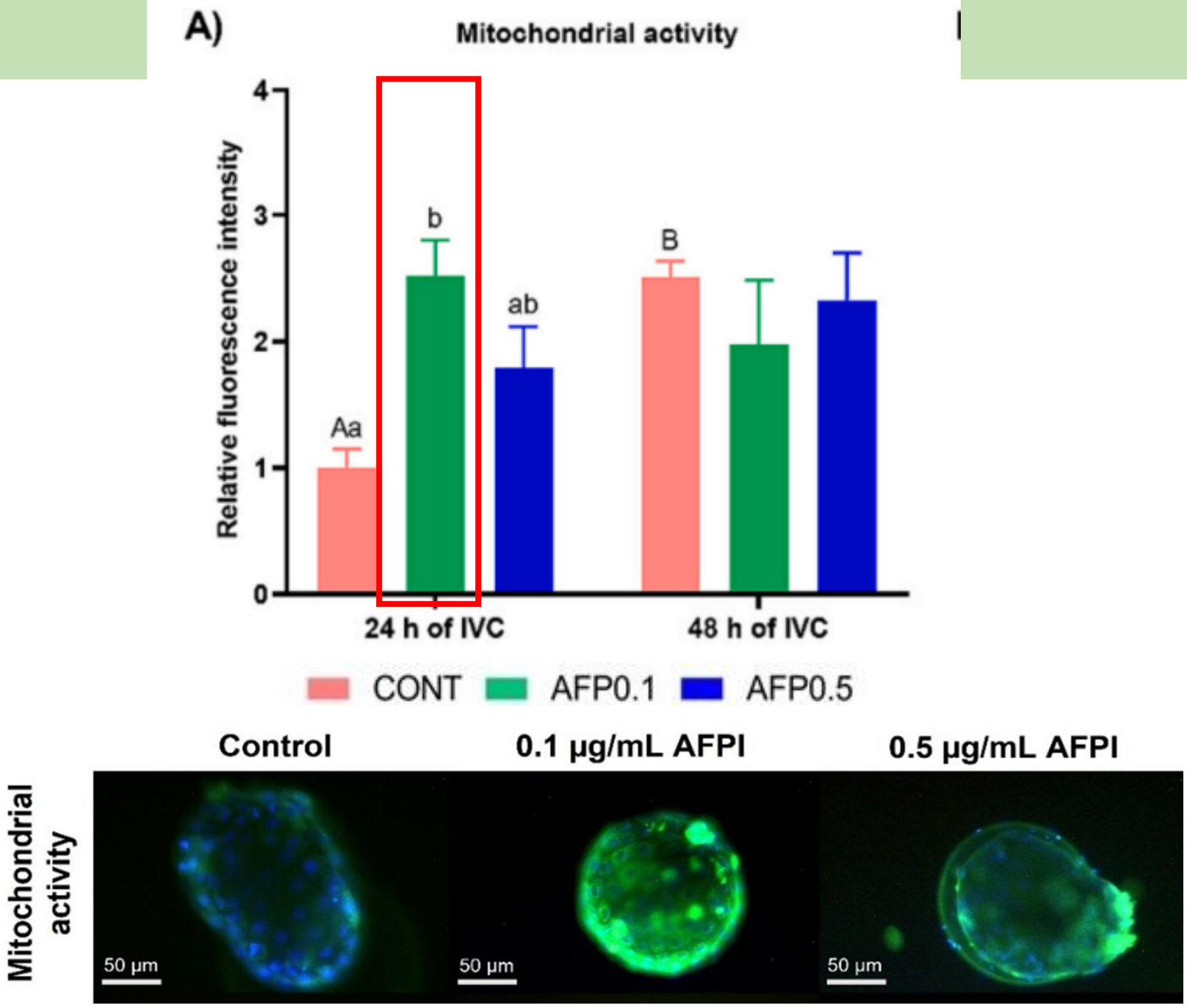
[†] AFP0.1 = 0.1 μg/mL of AFP I group, which contained – 4 morulas, 15 compact morulas, 7 early blastocysts, 8 blastocysts, 16 expanded blastocysts, 3 hatched blastocysts.

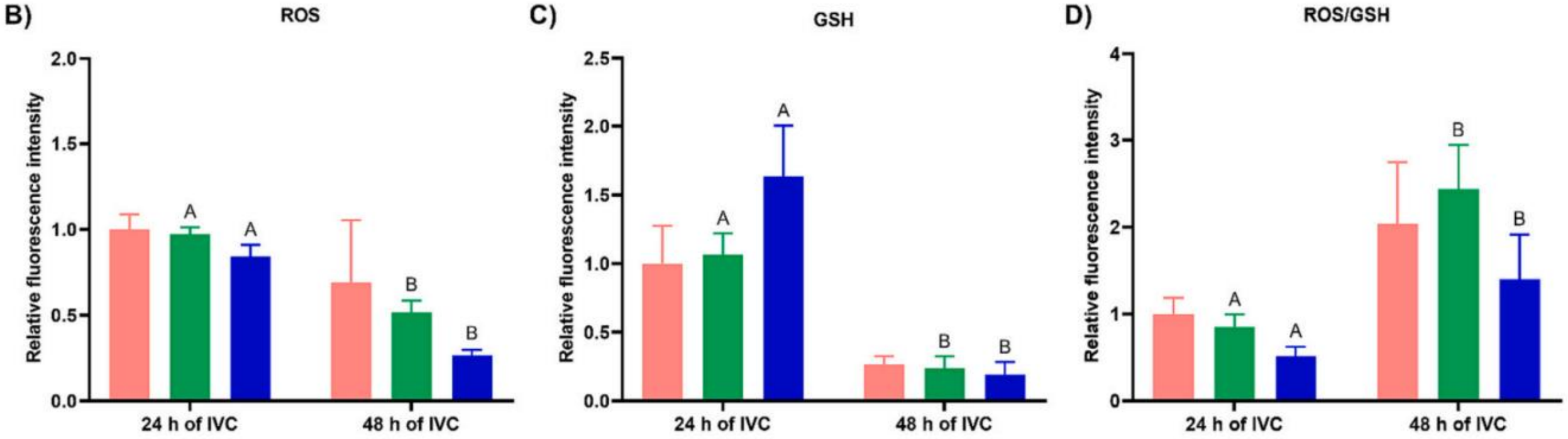
[‡] AFP0.5 = 0.5 μg/mL of AFP I group, which contained – 3 morulas, 13 compact morulas, 9 early blastocysts, 6 blastocysts, 11 expanded blastocysts, 1 hatched blastocyst.

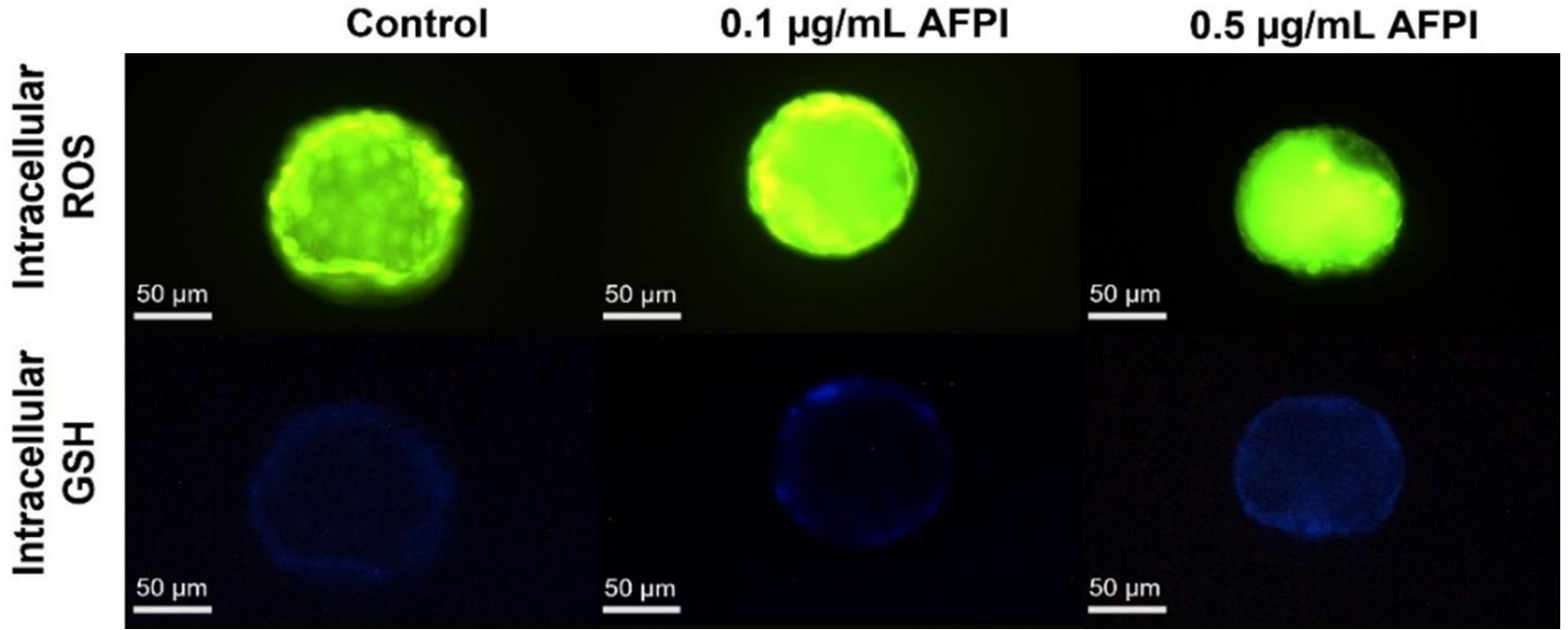
[1] Only viable blastocysts were considered to calculate the hatching rate (morulas and compact morulas that blocked their development, and blastocysts already hatched before cryopreservation were not considered for the hatching rate).

[*] Tendency for a lower hatching rate in AFP0.5 compared to AFP0.1 ($p$ = 0.09).

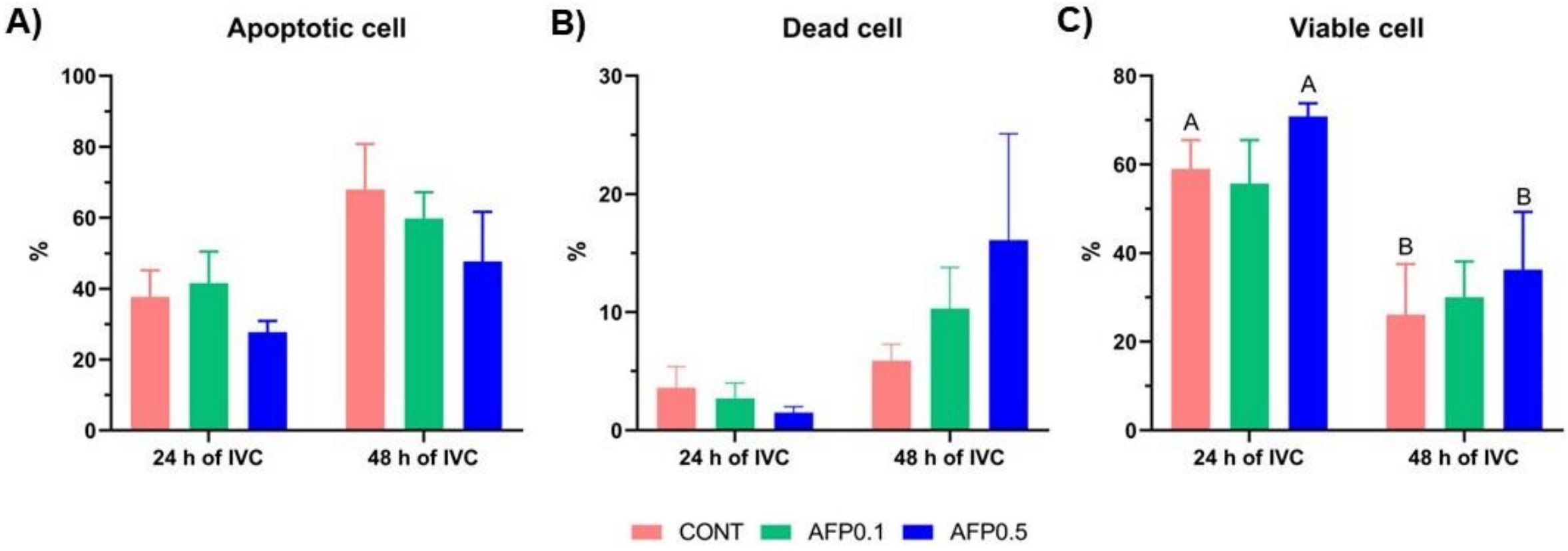
# Results

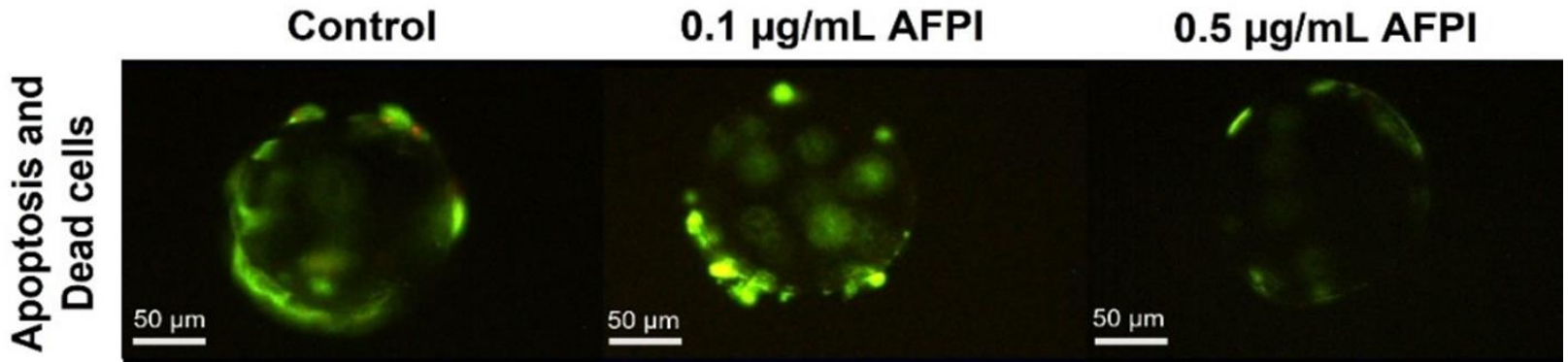

A)
Mitochondrial activity
Relative fluorescence intensity
0
1
2
3
4
Aa
b
ab
B
24 h of IVC
48 h of IVC
CONT
AFP0.1
AFP0.5
Control
0.1 µg/mL AFPI
0.5 µg/mL AFPI
Mitochondrial activity
50 µm
50 µm
50 µm


B)
ROS
Relative fluorescence intensity
2.0
1.5
1.0
0.5
0.0
A
A
B
B
24 h of IVC
48 h of IVC
C)
GSH
Relative fluorescence intensity
2.5
2.0
1.5
1.0
0.5
0.0
A
A
B
B
24 h of IVC
48 h of IVC
D)
ROS/GSH
Relative fluorescence intensity
4
3
2
1
0
A
A
B
B
24 h of IVC
48 h of IVC


Control
0.1 μg/mL AFPI
0.5 μg/mL AFPI
Intracellular ROS
Intracellular GSH
50 μm
50 μm
50 μm
50 μm
50 μm
50 μm

# Results


A)
Apoptotic cell
100
80
60
40
20
0
%
24 h of IVC
48 h of IVC
B)
Dead cell
30
20
10
0
%
24 h of IVC
48 h of IVC
C)
Viable cell
80
60
40
20
0
%
A
A
B
B
24 h of IVC
48 h of IVC
CONT
AFP0.1
AFP0.5


Control
0.1 µg/mL AFPI
0.5 µg/mL AFPI
Apoptosis and Dead cells
50 µm
50 µm
50 µm

# Results

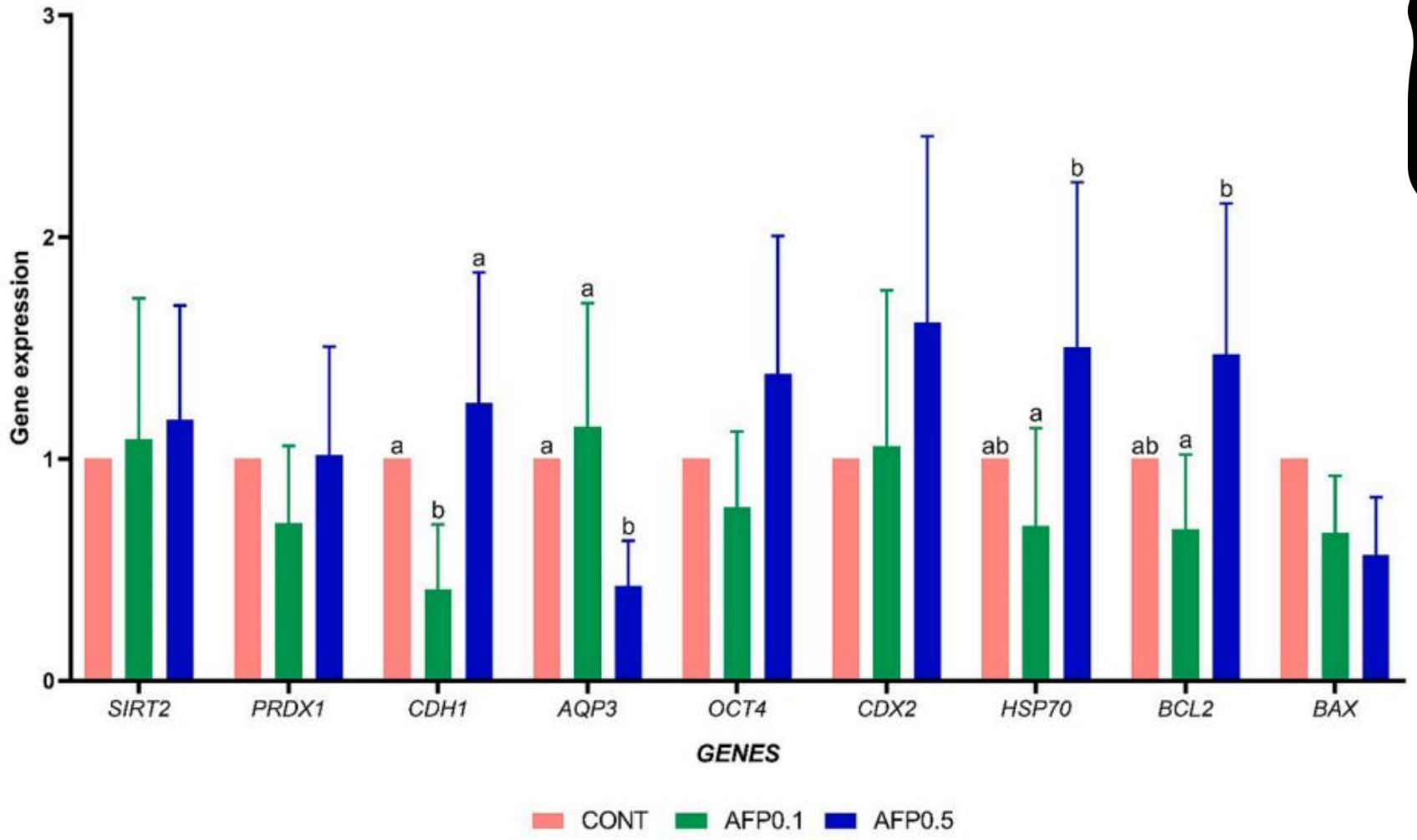



**Fig. 4.** Gene expression (Mean ± SEM) of sirtuin 2 (*SIRT2*), peroxiredoxin 1 (*PRDX1*), cadherin-1 (*CDH1*), aquaporin 3 (*AQP3*), octamer-binding transcription factor 4 (*OCT4*), caudal type homeobox 2 (*CDX2*), 70 kilodalton heat shock protein (*HSP70*), BCL2 associated X (*BAX*), and B-cell lymphoma protein 2 (*BCL2*) on *in vivo*-derived sheep embryos submitted to slow freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h after thawing. Different letters show statistical differences ($p < 0.05$).

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# Effect of antifreeze protein I in the freezing solution on *in vivo*-derived sheep embryos

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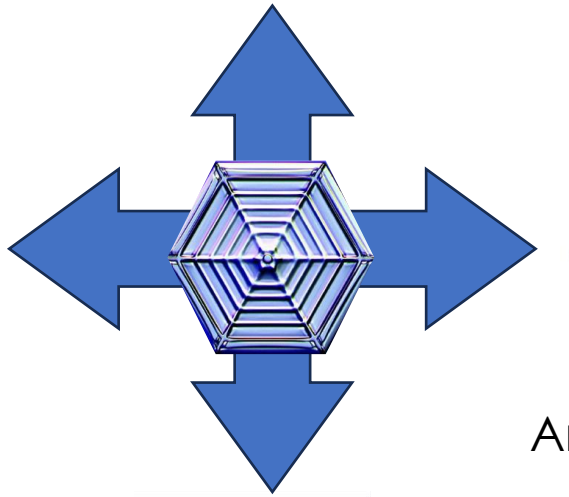
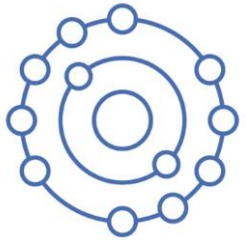
The addition of AFP I does not affect the survival and viability of *in vivo*-derived sheep embryos cryopreserved. The **supplementation of 0.1 µg/mL of AFP I in slow freezing solution enhances mitochondrial activity within 24 h of IVC, maintaining oxidative stress homeostasis and gene modulation**, being a potential supplementation to be applied in slow freezing medium of sheep embryos.

# New strategies

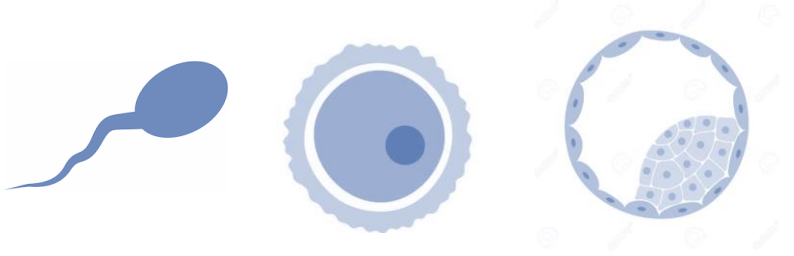
Extracellular vesicles

AFP I





Antioxidants

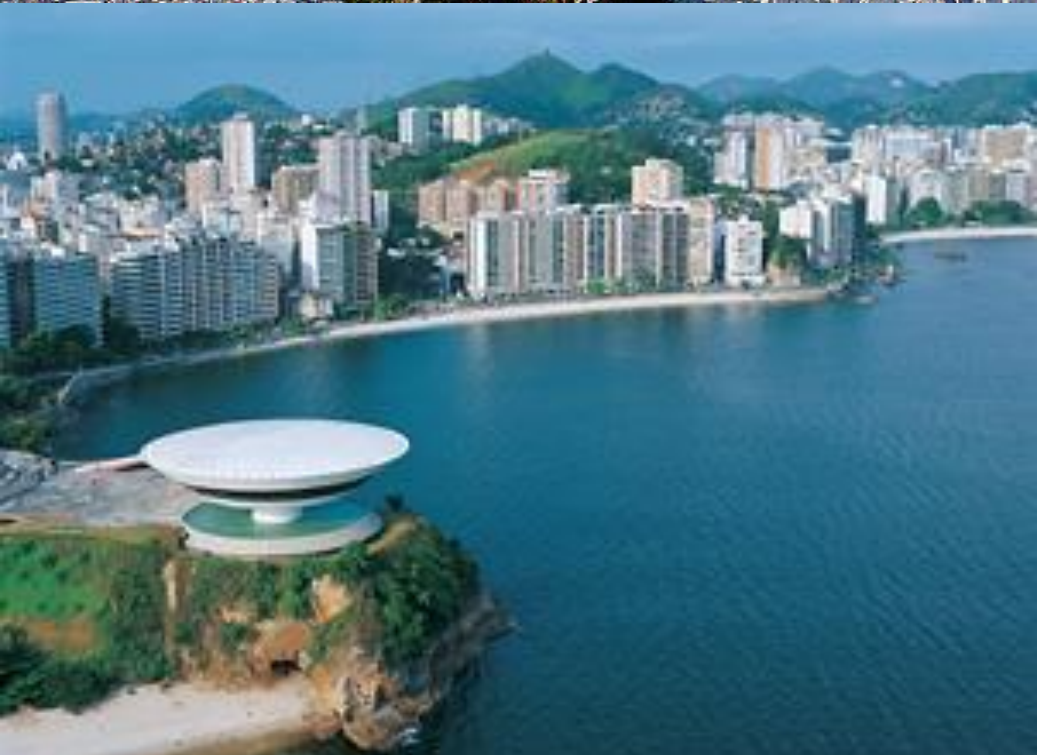



AFP III

# Conclusion

- Potential cryoprotective agent
- Different types of antifreeze proteins
- Best results at low concentrations
- Effects on the cryosurvival of gametes and embryos

• There are still gaps to be answered...




ORDEM E PROGRESSO


80 anos
VETERINÁRIA
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FAZENDA ESCOLA

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# Antifreeze proteins: potential cryoprotectant of gametes and embryos from small ruminants

## Thank you for your attention!

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GERAD uff