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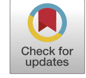

Antifreeze protein type I in the vitrification solution improves the cryopreservation of immature cat oocytes

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ABSTRACT

Oocyte cryopreservation is not yet considered a reliable technique since it can reduce the quality and survival of oocytes in several species. This study determined the effect of different concentrations of antifreeze protein I (AFP I) on the vitrification solution of immature cat oocytes. For this, oocytes were randomly distributed in three groups and vitrified with 0 μg/mL (G0, 0 μM); 0.5 μg/mL (G0.5, 0.15 μM), or 1 μg/mL (G1, 0.3 μM) of AFP I. After thawing, oocytes were evaluated for morphological quality, and compared to a fresh group (FG) regarding actin integrity, mitochondrial activity and mass, reactive oxygen species (ROS) and glutathione (GSH) levels, nuclear maturation, expression of *GDF9*, *BMP15*, *ZAR-1*, *PRDX1*, *SIRT1,* and *SIRT3* genes (normalized by *ACTB* and *YWHAZ* genes), and ultrastructure. G0.5 and G1 presented a higher proportion of COCs graded as I and while G0 had a significantly lower quality. G1 had a higher percentage of intact actin in COCs than G0 and G0.5 ($P < 0.05$). There was no difference ($P > 0.05$) in the mitochondrial activity between FG and G1 and they were both higher ($P < 0.05$) than G0 and G0.5. G1 had a significantly lower ($P < 0.05$) mitochondrial mass than FG and G0, and there was no difference among FG, G0, and G0.5. G1 had higher ROS than all groups ($P < 0.05$), and there was no difference in GSH levels among the vitrified groups ($P > 0.05$). For nuclear maturation, there was no difference between G1 and G0.5 ($P > 0.05$), but these were both higher ($P < 0.05$) than G0 and lower ($P < 0.05$) compared to FG. Regarding gene expression, in G0 and G0.5, most genes were downregulated compared to FG, except for SIRT1 and SIRT3 in G0 and *SIRT3* in G0.5. In addition, G1 kept the expression more similar to FG. Regardless of concentration, AFP I supplementation in vitrification solution of immature cat oocytes improved maturation rates, morphological quality, and actin integrity and did not impact GSH levels. In the highest concentration tested (1 μg/mL), AFP maintained the mitochondrial activity, reduced mitochondrial mass, increased ROS levels, and had the gene expression more similar to FG. Altogether these data show that AFP supplementation during vitrification seems to mitigate some of the negative impact of cryopreservation improving the integrity and cryosurvival of cat oocytes.

1. Introduction

According to the Red List of Threatened Species, several members of the *Felidae* family are classified as vulnerable or threatened with extinction [1], and the domestic cat has been used as an important experimental model for assisted reproductive technologies focused on endangered felid conservation [2]. Oocyte cryopreservation is considered an essential tool for preserving the female genome [3,4]. Improving the efficiency of oocyte cryopreservation using domestic cats could impact wildlife considering that in the event of a wild female's death, collecting and cryopreserving the oocytes may be one of the only options for preserving that genetic material. However, this technique still represents a great challenge due to the differential permeability property of oocytes among species [5], and the oocyte large cell size [6], since those characteristics can impair the penetration of cryoprotectants, increasing their sensitivity to the cryopreservation process.

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As a result, cryopreservation can lead to morphological and physiological damages involving the membrane, cytoskeleton, and mitochondria since the process can cause mitophagy [7], and the increase of oxidative stress, generating a reduced developmental competence [8,9]. For this reason, oocyte cryopreservation is not yet considered a reliable technique, since it usually reduces the quality and survival of oocytes in several species, including cats [9–13].

The use of substances that can mitigate the damage inherent to the cryopreservation process, such as antifreeze proteins (AFPs), naturally found in organisms that live at glacial temperatures [14], becomes an interesting alternative. According to their sequence and structural characteristics, AFPs are classified into four categories (antifreeze glycoproteins (AFGP), AFP I, AFP II, and AFP III) [14]. They bind to ice crystals, promoting thermal hysteresis, inhibiting recrystallization during warming, and stabilizing biological membranes [15–18], interacting and protecting the oolemma [19].

Few studies have demonstrated the role of AFPs on the vitrification of murine and bovine oocytes [20,21], reporting increased fertilization rates, reduced oxidative stress, and preserved membrane structure in species such as pigs, cattle, and mice [14]; and most of these studies, used AFP III [14]. These promising results and success, however, can be influenced by AFP type and concentration, as well as the vitrification protocol [22]. Moreover, each animal species presents differential expression levels of water and cryoprotectant channels in the membrane resulting in a differential permeability to water and cryoprotectants in the oocyte [5,17]. Considering that AFP I has a smaller molecular size, the present study aimed to determine the effect of different concentrations of AFP I in the vitrification solution during the cryopreservation of immature cat oocytes through the evaluation of (*i*) morphological quality, actin integrity, and ultrastructure; (*ii*) metabolism; (*iii*) expression of genes related to quality and oxidative stress; and (*iv*) nuclear maturation.

2. Material and methods

2.1. Ethics and experimental conditions

Cat ovaries were obtained from elective sterilization procedures at local veterinary clinics, and for this reason, this study did not require the approval of the Ethics Committee since the source of oocytes is considered biological waste. Only ovaries from non-pregnant cats were used in this study, and ovaries were kept in saline solution at 4 °C for up to 4 h until cumulus-oocyte complexes (COCs) recovery.

2.2. Reagents

Unless stated otherwise, all reagents used were from Sigma (St. Louis, MO, USA). The AFP type I (purified from *Myoxocephalus scorpius*) was obtained from A/F Protein Inc (Waltham, MA, USA).

2.3. Experimental design

As can be seen in Fig. 1, COCs were randomly distributed in four groups. One was used as a fresh control (FG), and the other three were vitrified with 0 μg/mL (G0, 0 μM), 0.5 μg/mL (G0.5, 0.15 μM), or 1 μg/mL (G1, 0.3 μM) of AFP I. The AFP I concentrations were previously chosen according to Correia et al. (2021) [14]. After warming, vitrified COCs were submitted to the morphological viability assessment at 0 and 2:30 h post-incubation, before being submitted to the following analysis. For epifluorescence microscopy analysis, approximately 40 vitrified-warmed COCs per group were evaluated for mitochondrial mass and activity, glutathione (GSH) and reactive oxygen species (ROS) levels, and actin integrity. The same analysis was performed on fresh COCs. The expression of heat shock protein (*HSP70*), peroxiredoxin 1 (*PRDX1*), growth/differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), zygote arrest 1 (*ZAR1*), sirtuin 1 (*SIRT1*) and sirtuin 3 (*SIRT3*) genes was performed in 15 COCs per group in vitrified-warmed (G0, G0.5, and G1) and fresh (FG) COCs. A total of 210 vitrified-warmed COCs and 80 fresh COCs were submitted to *in vitro* maturation (IVM). For the ultrastructure analysis, 7 to 10 COCs per

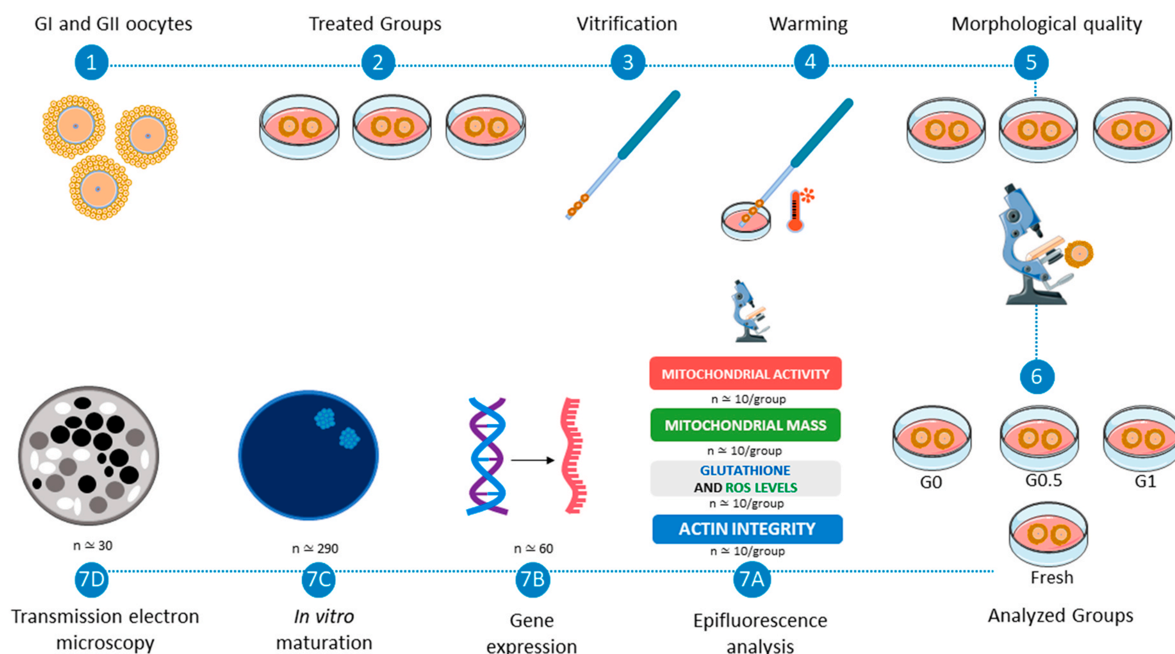

Fig. 1. Experimental Design. Schematic illustration of COCs (1) from three different groups (2) according to AFP concentration (G0, G0.5, and G1) subjected to vitrification/warming (3–4), evaluated for morphological quality (5), and compared with a fresh group, FG (6) regarding actin integrity; mitochondrial activity and mass; reactive oxygen species (ROS) and glutathione (GSH) levels (7), expression of genes related to oocyte quality and metabolism (8), nuclear maturation (9) and Transmission Electron Microscopy (TEM) (10). *Experimental groups: FG: fresh control; G0: 0 μg/mL; G0.5: 0.5 μg/mL and G1: 1 μg/mL AFP I.

group (vitrified-warmed and fresh) were evaluated through Transmission Electron Microscopy (TEM).

2.4. COC recovery and selection

Ovaries were washed with saline solution upon arrival, sliced with a scalpel, and washed again with a solution of phosphate-buffered saline (PBS) with 0.1 % polyvinyl alcohol and 100 μg/mL of antibiotic/antimycotic solution to recover the COCs that fell from the opened follicles. Only COCs presenting dark, homogenous cytoplasm and at least two layers of *cumulus* cells were classified as having good morphological quality and used in the experiment [23].

2.5. Vitrification and warming

COCs were vitrified using the Cryotop® device (Kizato, Fuji, Japan), according to Colombo and Luvoni (2020) [24], with minor modifications. COCs were equilibrated in a solution containing 7.5 % EG, 7.5 % DMSO, and 20 % fetal bovine serum (FBS) for 12 min, with or without AFP I, according to the experimental group (G0, G0.5, G1). They were then transferred to a vitrification solution containing 15 % EG, 15 % DMSO, and 0.5 M sucrose with 20 % FBS, with or without AFP I, as described above, and were vitrified for up to 90 s of contact with the vitrification solution.

For warming, COCs were recovered from the Cryotop® by immersing them in the thawing solution (TCM 199 supplemented with 20 % FBS and 1 M sucrose) for 1 min, then transferred to the dilution solution (TCM 199 with 20 % FBS and 0.5 M sucrose) for 3 min and washed (TCM 199 with 20 % FBS) for 5 min at room temperature.

2.6. Morphological quality

After warming, COCs were placed in a solution of TCM 199 with 20 % FBS and incubated for 2:30 h, at 38.5 °C in a 5 % CO_2 atmosphere until posterior analysis. COCs were evaluated for their morphology under a stereomicroscope immediately after warming and after incubation in three replicates. The COCs containing dark and homogenous cytoplasm with at least two compact *cumulus* cell layers were considered of good morphological quality (grade I and II) [23]. Those with irregular morphology, disrupted zona pellucida, and signs of cytoplasmatic degeneration were considered abnormal and were not used [25]. After the morphological assessment, only intact COCs were used for the following analyses.

2.7. Epifluorescence microscopy

The COCs were washed in PBS with 0.1 % bovine serum albumin (BSA), denuded with 10 mg/mL hyaluronidase, and incubated with the respective probe for each analysis. The analyses were carried out under a fluorescence microscope in four replicates.

2.7.1. Actin integrity

Oocytes (n = 10–12 per group) were fixed in PBS with 0.1 % BSA, 0.5 % Triton X 100, and 2 % paraformaldehyde for 30 min at 38.5 °C. They were then washed and incubated in PBS with 0.1 % BSA containing Alexa Fluor 350 Phalloidin (Invitrogen, Waltham, MA, USA) for 20 min at 38.5 °C. Oocytes were classified as intact when stained blue and damaged when they were unstained (dephosphorylated actin), and the percentage of intact oocytes per group was calculated [9,26].

2.7.2. Mitochondrial activity and mass

For the mitochondrial activity analysis, oocytes (n = 8–11 per group) were incubated at 38.5 °C for 30 min with 0.5 nM of MitoTracker Red FM (Invitrogen, Waltham, MA, USA) an excitation/emission (ex-em) of 579/599 nm in PBS with 0.1 % BSA. The mitochondrial mass analysis was adapted from Gutnisky et al. (2020) [27]. Oocytes (n = 10 per group) were incubated at 38.5 °C for 30 min with 0.5 nM of MitoTracker Green FM (Invitrogen, Waltham, MA, USA), a probe that evaluates the whole mitochondria regardless of their activity, at an excitation/emission (ex-em) of 490/516 nm, in PBS with 0.1 % BSA. Subsequently, they were washed in PBS, mounted on a slide, and analyzed under the epifluorescence microscope. Images were captured (Fig. 2) for later analysis of the intensity of fluorescence by ZEN 3.6 Blue Edition 32 software. The images were converted to grayscale and the intensity was adjusted by subtracting the mean intensity from the background. Each oocyte was measured (area in μm^2) and had its average intensity calculated (arbitrary units, A.U.), thus obtaining the intensity per area (A.U./μm^2) [28].

2.7.3. Reactive oxygen species (ROS) and glutathione (GSH) levels

Oocytes (n = 10) were incubated in 100 μL of PBS with 0.1 % BSA containing 10 μM H_2DCFDA (Invitrogen, Waltham, MA, USA) for ROS detection (ex-em 485/535 nm), and 10 μM of CellTracker Blue CMF_2HC (Invitrogen, Waltham, MA, USA) for GSH detection (ex-em 371/464 nm), for 30 min, at 38.5 °C, according to Piras et al. (2018) [29]. The oocytes were then washed and mounted for evaluation epifluorescence (Fig. 2) as described above.

2.8. Gene expression

The expression of *HSP70*, *PRDX1*, *GDF9*, *BMP15, ZAR1, SIRT1* and *SIRT3* was evaluated using quantitative PCR (qPCR) associated with reverse transcription [30]. *GDF9, ZAR1,* and *BMP15* were chosen to evaluate oocyte quality and competence; *SIRT1,* and *SIRT3* are related to the mitochondria function that is pivotal for oocyte development. *HSP70* and *PRDX1* are responsive to cellular stress, as well as oxidative stress, which can give an overview of the metabolic status of the oocyte. Primer sequences are described in Table 1. Three pools of five COCs were obtained in three replicates and used for mRNA extraction using a RneasyMicro Kit (Qiagen, Valencia, USA), according to the manufacturer's instructions, and the total mRNA extracted in each pool was quantified with a spectrophotometer (FG: 6.5 ± 1.1; G0: 3.7 ± 1.2; G0.5: 5.6 ± 2.4; G1: 12.9 ± 12.1). The amount of mRNA used for cDNA synthesis was 3 ng/mL. Reverse transcription utilized SuperScript IV (Invitrogen, Carlsbad, CA, USA). Samples were incubated at 65 °C for 5 min, 4 °C for 3 min, 50 °C for 10 min, and 80 °C for 10 min. Samples were kept at −20 °C until amplification. cDNA samples were amplified by qPCR using FastStart Universal SYBR Green PCR Master Mix. The reaction occurred at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 60 °C for 30 s. Fluorescence levels were obtained during the extension and the specificity of the reaction was checked using the melting curve. Primer's efficiency was checked by LinRegPCR [31], and it was 1.99 for *YWHAZ*; 1.98 for *ACTB*; 1.96 for *HSP70*; 1.97 for *PRDX1*; 1.97 for *GDF9*; 1.98 for *ZAR1*; 1.97 for *BMP15*; 1.99 for *SIRT1*, and 2.00 for *SIRT3*. CT values were compared by REST 2008 [32] after normalization with the reference genes values (*YWHAZ* and *ACTB*). The stability (smaller variation due to the treatment) of the reference genes was calculated according using the BestKeeper – Excel tool. The values of the Pearson correlation coefficient observed for β-actin ($r2 = 0.70$), GAPDH ($r2 = 0.80$) and ($r2 = 0.80$), and YWHAZ ($r2 = 0.79$) genes were indicative of stability ($P < 0.01$) of these reference genes.

2.9. In vitro maturation and nuclear maturation assessment

Vitrified-warmed COCs were washed in a TCM-199 medium containing 2.2 g/L of sodium bicarbonate, 3 mg/mL of BSA, 0.25 mg/mL sodium pyruvate, 0.15 mg/mL L-glutamine, 0.6 mg/mL sodium lactate and 0.055 mg/mL of gentamicin. About 10–20 COCs per group were placed in 500 μL of IVM medium (washing medium supplemented with 0.02 IU/mL of FSH/LH, 100 μM of cysteamine, and 0.5 mg/mL L-carnitine [13]), and cultured for 28 h at 38.5 °C in a 5 % O_2, 5 % CO_2 and

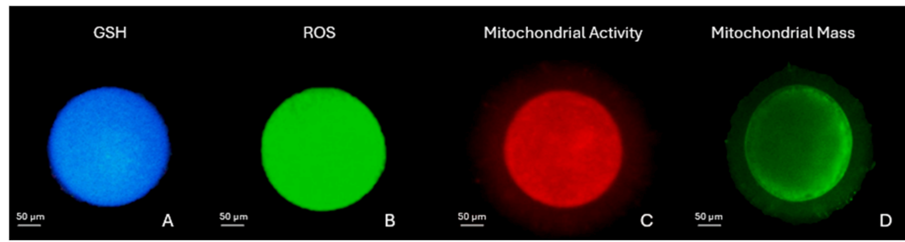

Fig. 2. Epifluorescence analyses. Illustration of post-warming cat oocytes after incubation with probes for evaluation of (A) intracellular levels of glutathione (GSH) and (B) reactive oxygen species (ROS), (C) mitochondrial activity, and (D) mitochondrial mass and evaluated under the epifluorescence microscope for later analysis of the intensity of fluorescence.

Table 1
Primer sequences, sizes, and references used for qPCR of fresh and vitrified-warmed cat cumulus-oocyte complexes vitrified with or without antifreeze protein type I.

Genes	Sequences (5′-3′)	Size (bp)	References
YWHAZ	**F**: GAAGAGTCCTACAAAGACAGCACGC **R**: AATTTTCCCCTCCTTCTCCTGC	115	Filliers et al., 2012
ACTB	**F**: GCCAACCGTGAGAAGATGACT **R**: CCCAGAGTCCATGACAATACCAG	128	Ishikawa et al., 2013
HSP70	**F**: GGCATCGATGTCGAAGGTCA **R**: ATCCAGGTGTACGAGGGTGA	129	XM_019830386.3
PRDX1	**F**: CCCCACGGAGATCATTGCTT **R**: AATGGTACGCTTGGGGTCTG	181	XM_003990035.5
GDF9	**F**: AGTTCAGATTGCAGCGGGTG **R**: GATGTGAAGAGCCGAGCAGT	264	NM_001165900.1
ZAR1	**F**: CATCCGATGGGAAAGTGCCT **R**: GCTGTCACAGGATAGGCGTT	241	XM_023252238.2
BMP15	**F**: GACAGCCCTCTAATGCCCTC **R**: CCAATGGTGCGGTTCTCTCT	187	NM_001165898.1
SIRT1	**F**: CGCCTTGCAATAGACTTCCC **R**: TGAATTTGTGACAGAGAGATGGTTG	145	Ishikawa et al., 2013
SIRT3	**F**: TGCTTCTGCGGCTCTACAC **R**: TGTCTCCCCAAAGAACACGA	229	Ishikawa et al., 2013

*F: forward; R: reverse; *YWHAZ*: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; *ACTB*: beta-actin; *HSP70*: 70 kDa heat shock protein; *PRDX1*: peroxiredoxin-1; *GDF9*: growth differentiation factor 9; *ZAR1*: zygote arrest 1; *BMP15*: bone morphogenetic protein 15; *SIRT1*: sirtuin 1; *SIRT3*: sirtuin 3.

90 % N_2 atmosphere in three replicates. COCs were then denuded, fixed in 4 % paraformaldehyde, and stained with Hoechst 33342 for evaluation of nuclear status under fluorescence microscopy, when they were classified according to the stage of development, namely, as germinal vesicle (GV; spherical nucleus surrounded by an intact nuclear membrane and decondensed chromatin), germinal vesicle breakdown (GVBD; chromosomal condensation and disintegration of the nuclear membrane), metaphase I (MI; metaphase plate peripherally located in the ooplasm), and metaphase II (MII; metaphase chromosomes at the ooplasm periphery and extrusion of the first polar body).

2.10. Transmission Electron Microscopy

COCs (n = 7–10 per group) were fixed (in 4 % paraformaldehyde, +2.5 % glutaraldehyde + calcium chloride (5 mM) in 0.1 M cacodylate buffer, pH 7.4) for 2 h at room temperature and, then, at 4 °C overnight. After, they were washed three times in 0.1 M sodium cacodylate buffer with 5 % sucrose per 15 min each time. Subsequently, the oocytes were individually post-fixed in 1 % OsO_4 in 0.1 M sodium cacodylate buffer with 0.8 % potassium ferricyanide, 5 mM calcium chloride, and 5 % sucrose for 30 min at room temperature, protected from light. Then, the samples were five times washed in 0.1 M cacodylate buffer and added with 5 % sucrose per 10 min each time. Oocytes were placed in 5 % aqueous uranyl acetate for 3 h, protected from light at room temperature, and then dehydrated by passing through an acetone series in increasing concentrations; infiltrated in Epon 812 resin. Semithin sections (1 mm) stained with 1 % toluidine blue were prepared in the slide and evaluated in the Light Microscopy. The ultrathin sections were collected on 200 mesh grids, stained with uranyl acetate and lead citrate, and viewed under a JEM-1400Plus transmission electron microscope (TEM). The ultrastructural characteristics were compared between vitrified and fresh groups.

2.11. Statistical analysis

All variables were submitted to Shapiro-Wilk's normality test and Levene's homoscedasticity test. The non-parametric data (mitochondrial activity and massd, ROS, and GSH levels) were submitted to the Kruskal Wallis followed by Dunn. Other non-parametric variables (actin integrity, morphological quality, and the nuclear maturation status) were evaluated by Fisher's exact test. The electron microscopy results were evaluated via descriptive statistical analysis The software used was SPSS 25 Statistics Base (IBM), with a 95 % confidence interval and $P < 0.05$ as significant.

3. Results

3.1. Morphological quality

Immediately after warming, all the groups showed a great reduction in COC quality rate without significant differences among them ($P > 0.05$), as described in Table 2. After 2:30 h, groups vitrified with AFP (G0.5 and G1) improved the morphological quality of COCs compared to G0 ($P < 0.05$).

Table 2
Effect of different concentrations of antifreeze protein (AFP) type I added in the vitrification solution on the visual morphological quality of immature vitrified-warmed cat cumulus-oocyte complexes immediately after warming (0 h) or after a short-term incubation (2.5 h).

Group	0 h	2:30 h
G0	63.4 % (26/41)^{a,A}	56.1 % (23/41)^{a,A}
G0.5	77.5 % (31/40)^{a,A}	90.0 % (36/40)^{b,B}
G1	66.7 % (30/45)^{a,A}	84.4 % (38/45)^{b,B}

COCs containing dark and homogenous cytoplasm with at least two compact cumulus cell layers were considered of good morphological quality. Analysis after warming (0 h) and post-incubation (2:30 h) in TCM supplemented with 20 % FBS.
^{a,b} differ between time points (0 vs 2.5 h) at the same group (within a row).
^{A,B} differ among groups (G0 vs G0.5 vs G1) at the same timepoint (within a column) ($P < 0.05$).
This analysis was performed in three replicates and the percentages were obtained by pooling the data from all replicates.
*Experimental groups: G0: 0 μg/mL; G0.5: 0.5 μg/mL and G1: 1 μg/mL AFP I.

3.2. Fluorescence microscopy

3.2.1. Actin integrity

G1 had a higher ($P < 0.05$) percentage (91.7%[a];11/12), of intact COCs than G0 (41.7%[b]; 5/12), and similar ($P > 0.05$) to G0.5 (63.6%[ab];7/11) and FG (100%[a];10/10).

3.2.2. Mitochondrial activity and mass

There was no difference ($P > 0.05$) in the mitochondrial activity between GF and G1 and they were both higher ($P < 0.05$) than G0 and G0.5. There was no difference among FG, G0, and G0.5 regarding mitochondrial mass. On the other hand, G1 had a significantly lower ($P < 0.05$) mitochondrial mass than FG and G0 (Fig. 3).

3.2.3. Reactive oxygen species (ROS) and glutathione (GSH) levels

The FG, G0, and G0.5 had similar ROS levels, while G1 had significantly higher levels, as presented in Fig. 4 ($P < 0.05$). All vitrified groups had lower ($P < 0.05$) GSH levels, and a higher ($P < 0.05$) redox balance (ROS/GSH) compared to FG.

3.3. Gene expression

In G0 and G0.5, most genes were downregulated compared to FG, except for *SIRT1* and *SIRT3* at G0 and *SIRT3* at G0.5. In contrast, in G1, most genes showed no significant difference compared to FG, except for *HSP70* and *ZAR1*, which were downregulated, and SIRT3, which was upregulated. G0 had no significant difference in its gene expression compared to G0.5 and was downregulated compared to G1 for the genes associated with the oocyte developmental competence (*GDF9, ZAR1*, and *BMP15*). The G0.5 was also downregulated in comparison with G1 for *PRDX1, GDF9, BMP15, SIRT1,* and *SIRT3*, and the only gene with no difference was *HSP70* (Fig. 5).

3.4. In vitro maturation (IVM)

Regarding nuclear maturation rates, there were no differences ($P > 0.05$) between G1 and G0.5, and they were both higher ($P < 0.05$) than G0 and lower ($P < 0.05$) when compared to FG, as seen in Table 3. In addition, there were no differences ($P < 0.05$) in the degeneration rates among the three vitrified groups.

3.5. Transmission Electron Microscopy

3.5.1. Light Microscopy

About 85.7 % (6/7) of COCs in the FG were covered by layers of cumulus cells, with those from the corona radiata connecting to the zona pellucida. The cytoplasm was filled with numerous large-diameter vesicles and lipid droplets. It was possible to visualize the projections of cumulus cells attached to the zona pellucida. The cumulus cells were well preserved and only one COC from this group showed characteristics of cellular degeneration (Fig. 6A). All COCs (10) in G0 showed characteristics of intense cellular destruction, such as loss of cytoplasmic content, which was evidenced by weak toluidine blue staining. Fifty percent (5/10) did not have cumulus cells (Fig. 6B). COCs from G0.5 also showed intense cell destruction. In approximately 43 % (3/7) of them, it was possible to identify the presence of large vacuoles, but in much smaller quantities than in the FG. In the remaining, the identification of these structures was not possible. Only two (28 %) had cumulus cells attached to the ZP, but these cells showed signs of intense destruction (Fig. 6C). In general, COCs from G1 showed a similar degree of destruction to those in the G0.5 group. Sixty-two percent (5/8) had cumulus cells attached to the zona pellucida, however, the cumulus cells from these COCs had a better degree of preservation compared to those in the G0 and G0.5 groups evidenced by the greater number of cell layers and by a stronger cell color after staining (Fig. 6D).

3.5.2. Ultrastructural evaluation

Oocytes from the FG were surrounded by the zona pellucida, which was crossed by many cytoplasmic projections coming from the cumulus cells and attached to the surface of the oolemma. The perivitelline space (PvS) was quite narrow, with microvilli compressed between the oolemma and the zona pellucida. The oocytes had a preserved cytoplasmic architecture and easily identified organelles. A large number of large electron-lucent vesicles and many lipid droplets were distributed throughout the ooplasm. Among the large vesicles and close to the lipid droplets were a large number of mitochondria, which were distributed in greater quantities in the peripheral region. Round mitochondria predominated over other forms of this organelle. Close to the oolemma, clusters of cortical granules were located. In general, the organelles of oocytes from the fresh group were easy to identify and showed a pattern of location (Fig. 6E).

Oocytes from G0, G0.5, and G1 groups showed a high degree of injury caused by freezing. PvS was absent in the vast majority of oocytes,

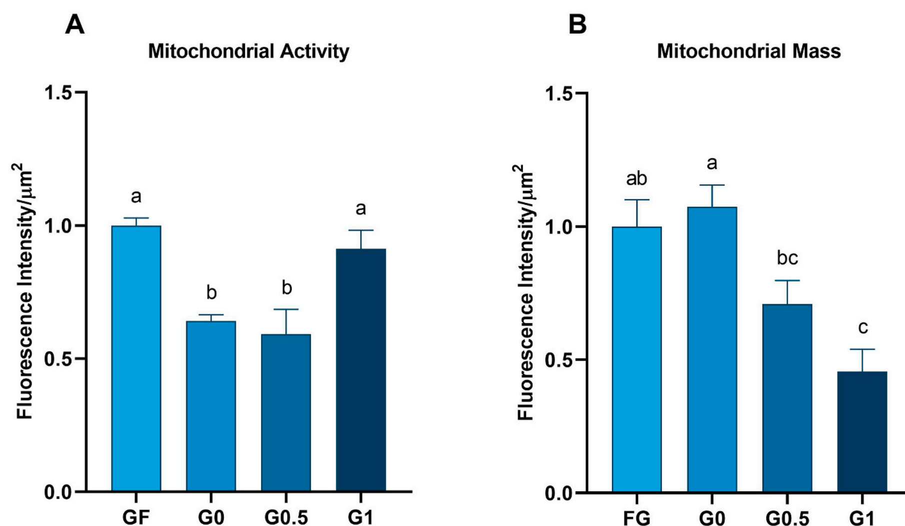

Fig. 3. Oocyte mitochondrial status. Graphs showing the relative fluorescence intensity levels (Mean ± SEM of arbitrary units) regarding **(A)** mitochondrial activity and **(B)** mass. Different letters show statistical differences ($p < 0.05$).
*Experimental groups: FG: fresh control; G0: 0 µg/mL; G0.5: 0.5 µg/mL and G1: 1 µg/mL AFP I.

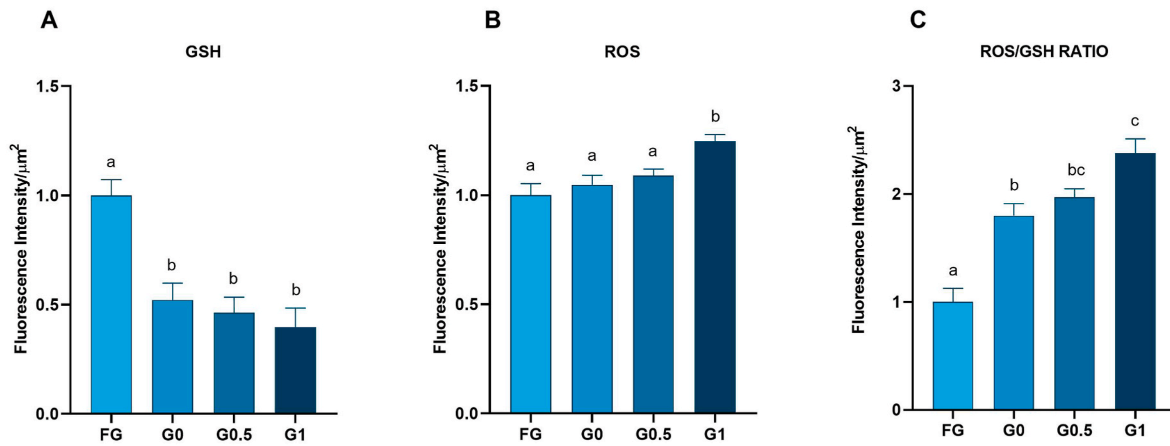

Fig. 4. Oocyte oxidative metabolism. Graphs showing the relative fluorescence intensity levels (Mean ± SEM of arbitrary units) regarding intracellular levels of **(A)** GSH and **(B)** ROS, and the **(C)** redox balance between oocytes derived from FG, G0. G0.5 and G1 groups at 2:30 h after warming. Different letters show statistical differences ($p < 0.05$).
*Experimental groups: FG: fresh control; G0: 0 μg/mL; G0.5: 0.5 μg/mL and G1: 1 μg/mL AFP I.

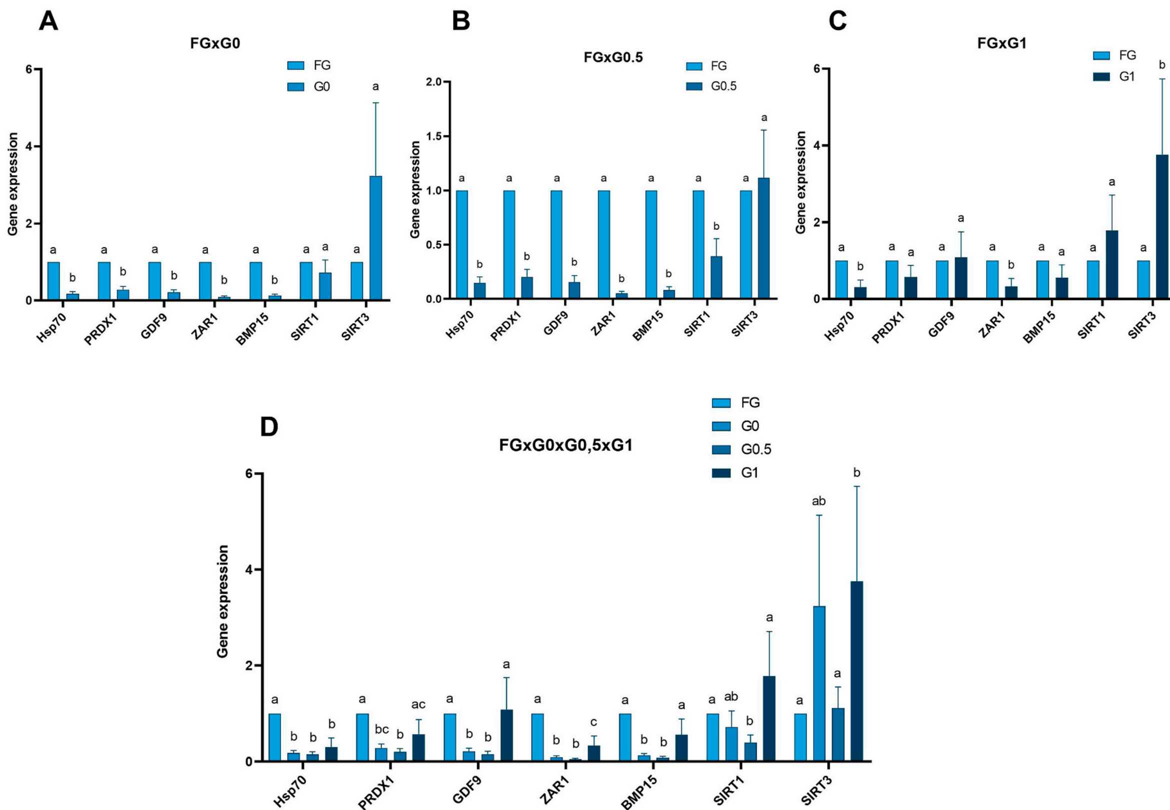

Fig. 5. Gene expression of 70 kDa heat shock protein (*HSP70*), peroxiredoxin-1 (*PRDX1*), sirtuin 1 (*SIRT1*), sirtuin 3 (*SIRT3*), growth/differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), zygote arrest 1 (*ZAR1*) on oocytes derived from **(A)** FG vs G0, **(B)** FG vs G0.5, **(C)** FG vs G1 groups and **(D)** showing the comparison only among the vitrified and the fresh group. Different letters show statistical differences ($p < 0.05$).
*Experimental groups: FG: fresh control; G0: 0 μg/mL; G0.5: 0.5 μg/mL and G1: 1 μg/mL AFP I.
n = 15 COCs were used per group, in three pools (3 replicates).

due to extensive areas without the plasma membrane. Transzonal projections and microvilli were intensely reduced, becoming absent in some areas. Many membrane fragments and a high number of vacuoles of different sizes and variable content were observed in the ooplasm (Fig. 6F-H). The cytoplasmic architecture was completely altered, and it was difficult to identify the organelles, especially in oocytes from G0. In this group, it was not possible to visualize the mitochondria cristae, only their outline could be seen (Fig. 6G), while in G0.5 and G1.0 oocytes, these organelles were less damaged, their shape was preserved and it was possible to observe the presence of their matrix and cristae (Fig. 6G-H), but they were less electron-dense than the FG mitochondria.

4. Discussion

Some studies have shown that the supplementation of different AFP types in cryopreservation solutions has been associated with positive

Table 3
Effect of different concentrations of antifreeze protein (AFP) type I added in the vitrification solution on nuclear maturation of good morphological quality oocytes from the domestic cat species.

Groups	GV (GVB) %/(n)	MI %/(n)	MII %/(n)	DG %/(n)	Total
FG	0.0 (0)	4.8 (4)	92.5 (74)[a]	2.5 (2)	(80)
G0	0.0 (0)	67.5 (54)	20.0 (16)[c]	12.5 (10)	(80)
G0.5	0.0 (0)	36.3 (24)	60.6 (40)[b]	3.0 (2)	(66)
G1	3.1 (2)	40.6 (26)	43.7 (28)[b]	12.5 (8)	(64)

(n), number of oocytes evaluated; GV, germinal vesicle; GVB, germinal vesicle breakdown; MI, Metaphase I; MII, Metaphase II; DG, degenerated. Fisher's exact test. Different letters mean statistical difference among groups, within a column ($P < 0.05$).
The analysis was conducted in three replicates and the percentages were obtained by pooling the data from all replicates.
*Experimental groups: FG: fresh control; G0: 0 μg/mL; G0.5: 0.5 μg/mL and G1: 1 μg/mL AFP I.
FG was not exposed to the vitrification solutions.

outcomes in oocyte cryopreservation in mammalian species [14]. As far as we know, this is the first study testing AFP supplementation during cat oocyte vitrification. Immature COCs were subjected to vitrification using different concentrations of AFP I, and after 2:30 h of thawing, the COCs vitrified with AFP in both concentrations presented similar morphological quality to fresh COCs and a significantly higher morphological quality than the group without AFP. In addition, both concentrations tested also improved the maturation rates, preserved the organelle's appearance, and reduced the signs of cellular destruction in the COCs under ultrastructural evaluation. Furthermore, the highest concentration increased ROS levels, reduced mitochondrial mass, and maintained mitochondrial activity and gene expression similar as of fresh COCs.

Cat oocytes have a high content of lipid droplets in the ooplasm that may reduce oocyte permeability to the cryoprotectant solution [33], and it has already been demonstrated that the lipid reduction in cat oocytes ooplasm can improve their cryosurvival [13]. In addition to this challenge related to the species, the vitrification process itself causes damage to the membrane and cytoskeleton [7] related to the cell size and the permeability of cryoprotectants [5,6]. Vitrified oocytes often present lesions such as rupture of ZP, plasma membrane, or cytoplasmic degeneration, which was confirmed by the electron microscopy images from the vitrified groups.

An increase in the viability of vitrified oocytes due to AFP supplementation has already been reported in other species, since it could reduce chilling sensitivity by conferring low-temperature protection to cellular membranes [34]. The morphological analysis of COCs is an empirical way to evaluate the effect of vitrification without the possibility of using other methods [25]. The better morphological quality in the AFP presence is probably due to its capacity to connect to cell membranes, promoting their stabilization during exposure to low temperatures due to the interaction between the antifreeze peptides and integral membrane proteins of the cell membranes, thus reducing their damage [34,35]. Also, in electron microscopy, AFPS groups maintained a pattern of maintenance of the transzonal projections due to less mechanical damage since AFP can inhibit recrystallization during warming [14].

As a consequence of external protection and better stability for the oocyte membrane, AFP I supplementation also promoted the maintenance of the integrity of actin microfilaments. Oocytes treated with AFP showed results similar to fresh oocytes, while only 41 % of oocytes vitrified without AFP remained with intact actin microfilaments. Luciano et al. (2009) [36] demonstrated that the vitrification of immature oocytes from domestic cats affects actin integrity. In the current study, the increase in the actin integrity rate was dose-dependent and was just observed in comparison to G0, in the higher concentration (G1). The protective role of the AFP has already been demonstrated in the preservation of the cytoskeleton during cryopreservation of oocytes from other species: Liang et al. (2016) [21] showed a protective effect of AFGP on actin integrity as well as meiotic spindle organization and alignment of chromosomes from matured bovine oocytes vitrified with AFGP and the same was observed in matured vitrified oocytes from mice using AFP III (Wen et al., 2014)

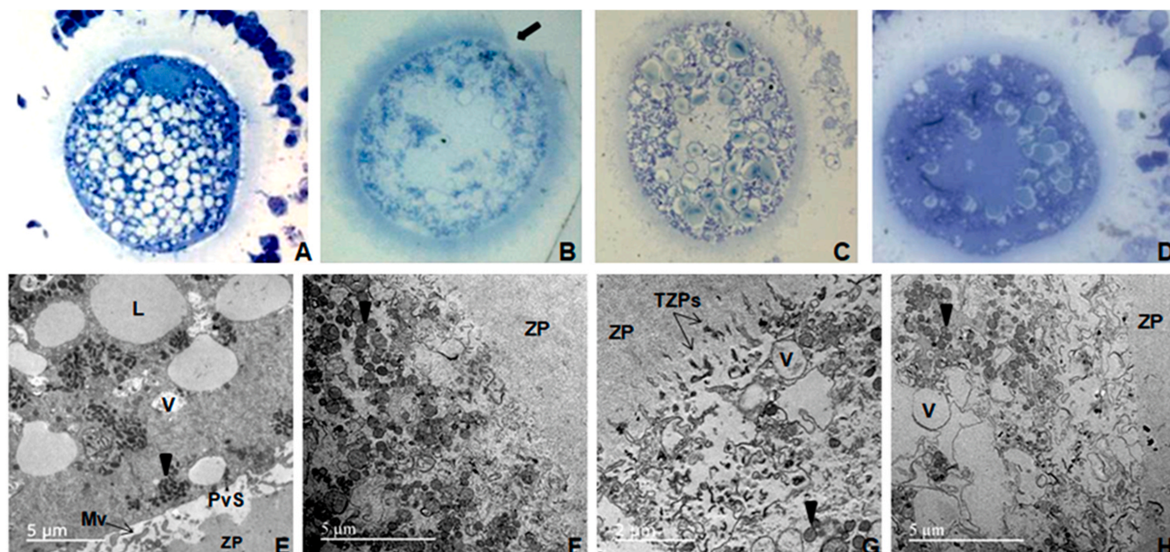

Fig. 6. Transmission Electron Microscopy. Light (A–D) and electron (E–H) micrographs of immature cat oocytes. Semi-fine cuts (A–D) show general aspects of control oocytes from FG (A-non-vitrified) and vitrified on absence (B; G0) and presence of 0.5 μg/mL (C; G0.5) and 1.0 μg/mL (D; G1)) of AFP. Notice a large number of vesicles in control oocytes, distributed throughout the cytoplasm, while on oocytes from other treatments (B, C, and D), these structures virtually disappeared. The arrow (B) refers to the loss of integrity of the zona pellucida. Ultrastructural imaging of control oocytes (E) allows observing the presence of clusters of mitochondria in cortical regions and close to lipid drops. These oocytes present a narrow perivitelline space, where there are many microvilli. Vitrified oocytes (F, G, and H) have evident signs of cellular destruction, with loss of cellular architecture. (E–H): ZP – Zona Pellucida, PvS – Perivitelline Space, Mv – Microvilli, TZPs – Transzonal projections, L – Lipid droplet, V – Vacuole, arrowhead – mitochondria.
*Experimental groups: FG: fresh control; G0: 0 μg/mL; G0.5: 0.5 μg/mL and G1: 1 μg/mL AFP I.

[37]. In both studies, the use of AFPs also contributed to better rates of embryonic development compared to the vitrified groups without AFPs, demonstrating that the protective role of the cytoskeleton (both actin and tubulin) is fundamental for subsequent embryonic development.

In cats, the meiotic maturation stage (immature or *in vitro* matured) did not affect the oocyte development competence [12] regarding cryopreservation. Even so, the use of *in vitro* matured oocytes for vitrification seems to present better results in some species [38]. Immature cat oocytes are considered even more challenging to cryopreserve although they are considered more resistant to cold damage, since the success depends on the maintenance of the ability to undergo IVM after thawing [33,39]. However, considering that immature gametes can be retrieved from endangered individuals post-mortem, the improvement of outcomes of oocyte cryopreservation in the immature stage is crucial to a better diffusion of this biotechnology. Our results showed that the structural protection caused by AFP, as evidenced by the morphological quality and the actin integrity, also influenced the rate of immature oocytes that presented a meiotic progression. The vitrification process (G0) reduced the nuclear maturation rate compared to fresh oocytes (FG); however, the supplementation of AFP, regardless of concentration, improved these rates (G0.5 and G1), probably reflecting in the competence of these oocytes.

Both genes *GDF9* and *BMP15* have been associated with oocyte quality and competence due to their important role in oocyte maturation. In the current study, the expression of genes related to oocyte competence (*GDF9, BMP15, ZAR1*) was reduced in G0 and G0.5 compared to FG. It is important to consider that a gene expression similar to FG may be indicative of oocyte quality and greater development potential, and this pattern was observed just in G1. The reduced levels of these genes can reveal a lower quality of oocyte and a lower probability of reaching maturation which can reflect in the developmental competence; however, G0 and G0.5 presented different results regarding maturation: G0.5 was better than G0 and similar to G1, suggesting that gene expression should be evaluated in a more widely, considering the general gene expression and all analyses related.

Altogether, gene expression also indicated a difference in quality among the vitrified groups regarding the ability to maintain the ideal level of transcripts (as in FG). It could be observed that vitrification significantly affected the major gene expression since in both G0 and G0.5, most gene expressions were reduced compared to the FG. However, the use of AFP I at a concentration of 1 μg/mL was enough to maintain the major expression of genes since G1 was the most similar to the FG, indicating a beneficial effect that allowed the maintenance of transcripts and the possible ability to handle the stress caused by vitrification. Other studies with AFPs in oocyte vitrification also showed the greatest similarity between AFP-vitrified groups and fresh ones compared to untreated groups in gene expression [20,35,37], suggesting that the supplementation of AFP on vitrification is associated with a more similar transcript pattern to oocytes that were not submitted to vitrification. On the other hand, at a concentration of 0.5 μg/mL, vitrification still induced a reduction of oocyte transcripts similar to in the absence of AFP.

Another important aspect is oocyte metabolism since it can directly impact the oocyte developmental competence, and the mitochondria function, distribution, and mass are crucial factors in this sense [40]. In mice, it was already reported that germinal vesicles from oocytes with damaged mitochondria transplanted into normal oocytes can be rescued and produce blastocysts [41], suggesting that the mitochondria have an essential role in oocyte competence. The G1 presented lower mitochondrial mass and similar mitochondrial activity to FG. The *SIRT1* gene expression was reduced in G0.5 and *SIRT3* was upregulated in G1. The *SIRT1* protein located in the nucleus can be translocated to the cytoplasm and is involved in the regulation of mitochondrial biogenesis and mitophagy; *SIRT3* is located in the mitochondria and can be translocated to the nucleus under stress and can control the expression of many mitochondrial proteins and influence major mitochondrial functions [42]. In this scenario, it was noticeable in the ultrastructural images that the G0.5 and G1 mitochondria had a more preserved aspect than the G0, compared to FG.

Mitochondrial activity keeps oocyte redox balance, ATP production, and metabolic status, and some data suggest that mitochondrial number, activity, and distribution may be related to oocyte quality [43]. Taken together, the results of the current study showed that the G1 oocytes presented a better-preserved mitochondrial aspect, a lower mitochondrial mass, a similar activity to fresh oocytes, and a higher expression of *SIRT3* compared to the FG. The AFP did not seem to interfere in the GSH level, but in the highest concentration (G1) elevated ROS levels. Mitochondrial activity is also related to ROS production [44,45], which was higher in G1 compared to all groups. Furthermore, *SIRT 3* is also indicative of oxidative stress since it can act as a sensor when ROS is increased, and G1 showed an upregulated expression of this gene. In addition to that, peroxiredoxins and HSPs are proteins involved in defense against oxidative stress [45,46], that can be increased by the cryopreservation process [8]. It was already reported that there is a lower abundance of these transcripts in oocytes after *in vitro* maturation compared to the ones that underwent *in vivo* maturation [47]. Considering that *in vitro* conditions can also increase oxidative stress, a reduction in these transcripts can be related to oocytes being more sensitive to stressful conditions [47], which can lead to a reduction in future developmental competence. As could be evidenced, *HSP70* expression was reduced in all vitrified groups compared to fresh. Even so, G1 was able to keep the *PRDX1* expression similar to FG.

G1 also presented a reduced mitochondrial mass compared to FG. *SIRT1* promotes mitochondrial biogenesis in conditions of energy deficiency and has a role in triggering the dead or replacing the damaged mitochondria [48,49] and its expression was similar in G1 compared to GF. Considering the damage caused by the vitrification process, the reduced mass found could be a consequence of an oocyte effort to replace the damaged mitochondria, which can be endorsed by the mitochondrial better-preserved aspect in G1 compared to the other vitrified groups in TEM. Despite this reduction in mitochondrial mass, G1 presented a higher morphological quality, actin integrity, and maturation rates than other vitrified groups, indicating that this decline did not impact morphological aspects of oocytes and their ability to resume meiosis that is related to mitochondrial activity [43].

Although G1 presented a lower mitochondrial mass and a higher level of oxidative stress; its positive results in the above-mentioned parameters suggest that the mitochondria population from this group was able to maintain their activity even reducing their damaged mass, leading to the increased ROS, to recover from the metabolic challenges and damages caused by the vitrification process.

5. Conclusion

The AFP I supplementation in the vitrification solution of immature cat oocytes (G0.5 and G1) improved their morphological quality and maturation rates even though it did not impact the GSH levels. In the highest concentration tested (G1), AFP I also maintained mitochondrial activity, reduced mitochondrial mass, increased ROS levels, and kept the gene expression more similar to fresh oocytes, suggesting that 1 μg/mL AFP I supplementation during vitrification seems to be the most suitable concentration to mitigate some of the negative impacts of cryopreservation improving the integrity and cryosurvival of oocytes in the domestic cat model.

CRediT authorship contribution statement

Gabriela R. Leal: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Lúcia Prellwitz:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lucas F.L. Correia:** Writing – review & editing, Investigation, Formal analysis. **Thais A.**

Oliveira: Writing – review & editing, Visualization, Investigation, Data curation. **Mariana P.P. Guimarães:** Writing – review & editing, Investigation, Data curation. **Bruna R. Xavier-Getirana:** Writing – review & editing, Data curation. **Ângelo José B. Dias:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Ribrio Ivan T.P. Batista:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Joanna M.G. Souza-Fabjan:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest. Parts of the figures were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

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