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Cryoprotective effect of different glycerol concentrations on domestic cat spermatozoa

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ABSTRACT

Cryopreservation of spermatozoa is a pivotal tool in assisted reproduction, and studies aiming to establish optimal freezing/thawing protocols are essential to enhance sperm survival. The objectives of the present study were to (1) compare the cryoprotective efficiency of three different glycerol concentrations (3%, 5%, and 7%) on the basis of post-thaw sperm quality and (2) investigate whether the incidence of morphologically abnormal sperm in fresh samples is related to cryodamage sensitivity. Semen was collected from six tomcats using an artificial vagina (total 18 ejaculates). Each ejaculate was diluted using Tris-egg yolk–based extender (TEY), evaluated, equally divided into three aliquots, and rediluted using TEY with and without glycerol to achieve final concentrations of 3%, 5%, and 7%. Samples were loaded into 0.25 mL straws, equilibrated for 60 minutes at 5 °C, frozen, and then thawed at 46 °C for 12 seconds. Fresh and frozen-thawed samples were evaluated for sperm motion parameters (computer-assisted sperm analysis), plasma membrane integrity (PMI; propidium iodide and carboxyfluorescein diacetate), and DNA integrity (acridine orange). Plasma and acrosomal membrane integrity were assessed by flow cytometry (propidium iodide and fluorescein isothiocyanate–conjugated pea (*Pisum sativum*) agglutinin) immediately after thawing. Sperm motion parameters were also evaluated at 30 and 60 minutes of postincubation. For all treatment groups, cryopreservation significantly impaired the PMI and sperm motion parameters, except for straightness and amplitude of lateral head displacement. DNA integrity showed a slight reduction ($P < 0.05$) when 3% glycerol was used. The percentage of total motility, progressive motility, and rapid spermatozoa were significantly lower immediately after thawing and up to 60 minutes of incubation for the 3% glycerol group when compared with 5% and 7%. No difference ($P > 0.05$) was found for PMI, acrosome integrity, and DNA integrity among post-thaw groups. However, higher ($P < 0.05$) incidence of viable cells with reacted acrosome and dead cells with intact acrosome were observed with 7% and 3% glycerol, respectively. Percentage of morphologically abnormal spermatozoa in fresh sample was positively correlated with PMI only in the 3% glycerol group and negatively correlated with sperm motility in the 5% and 7% groups. In conclusion, the final concentration of 5% glycerol offered better cryoprotective effect for ejaculated cat sperm, and the relationship found between prefreezing sperm morphology and post-thaw sperm quality showed to be dependent on final glycerol concentration.

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1. Introduction

Nowadays, with the exception of the domestic cat, all felids species are classified as threatened, vulnerable, or endangered [1]. Aiming to improve the reproductive performance of these species, the domestic cat has been used as an important research model in the development and improvement of assisted reproductive techniques, which could be applied to its wild relatives as well as to some valuable domestic cat breeds [2].

Among the available reproductive biotechnologies, cryopreservation of gametes is considered an essential tool when establishing a conservation program. This technique allows long-term storage and transportation of germplasm, which can be used in artificial insemination and/or IVF procedures [3]. Concerning the domestic cats, positive results following artificial insemination [4–7], IVF [8–10], and intracytoplasmic sperm injection [11,12] have been achieved when both ejaculated and epididymal frozen-thawed spermatozoa were used. Nevertheless, it is worth stating that several critical steps in the freezing/thawing procedure of cat spermatozoa still need to be adjusted in order to enhance post-thaw sperm quality and, consequently, their fertilizing potential.

To achieve higher cell survival after freezing and thawing procedures, compounds generically named cryoprotectants are included in sperm extenders. Interestingly, glycerol is the most widely used cryoprotectant for sperm preservation in both livestock and wild species [13,14]. This cell permeable cryoprotectant has been found to effectively lower the freezing point of intracellular water and to induce greater dehydration at lower temperatures, reducing intracellular ice crystal formation, and thereby protecting sperm cells from cryogenic injury [14,15]. However, glycerol has also shown the potential to cause osmotic damage and toxic effects on spermatozoa, leading to plasma membrane rupture, acrosomal damage, and alterations in cell metabolism and cytoskeleton actin [15–18]. Sperm tolerance to the negative effects exerted by glycerol has been found to vary among species and in a dose-dependent manner within them [16–19]. Thus, it seems reasonable to affirm that the optimal glycerol concentration in a given species would be the point at which one can obtain the maximum beneficial effects whilst with the least toxicity.

In domestic cats, besides one report addressing the use of DMSO [20], all studies on sperm-freezing protocols have utilized glycerol as their cryoprotectant and in a final concentration ranging from 3% [11,21] to 7% [5,6,22], with 4% [7,9,10,23,24] and 5% [25–28] being the most commonly used. Regarding optimal final glycerol concentration, because different extenders and freezing/thawing methods were used in most studies, a comparison among them is very complicated and offers reduced reliability. Nonetheless, two previous reports comparing different glycerol concentrations have shown that in cats although 3% and 4% glycerol resulted in similar post-thaw sperm motility and percentage of total abnormal sperm morphology [29], 4% glycerol was more efficient in preserving sperm motility compared with 8% [20]. To the authors' best knowledge, no previous studies have been conducted in cats to determine the effect of different glycerol concentrations on plasma membrane, acrosome, and DNA integrity after sperm cryopreservation.

Therefore, the aim of the present study was to evaluate the cryoprotective effect of three different glycerol concentrations (3%, 5%, and 7%) during freezing/thawing procedure of ejaculated domestic cat sperm on the basis of post-thaw results for sperm motion and integrity of plasma membrane, acrosome, and DNA. Furthermore, we investigated whether the percentage of morphologically abnormal sperm in fresh samples is related to cryodamage sensitivity in cats and whether this relationship is dependent on glycerol concentration.

2. Material and methods

2.1. Animals

Animal use was approved by the Ethical Committee for Experimental Animal Uses of the College of Veterinary Medicine and Animal Science, UNESP, Botucatu, Brazil. Six adult mixed-breed male cats, aged between 4 and 7 years, were maintained under artificial lighting for more than 12 hours per day and provided with dry commercial cat food (FIT 32, Royal Canin, Descalvado, Brazil) and water *ad libitum*.

2.2. Sample collection and processing

Semen samples were collected three times from each tomcat ($n = 18$ ejaculates) using an artificial vagina and with at least a 2-day interval. To achieve higher sperm quantity, each male was collected twice consecutively with an interval of ~10 minutes, changing the artificial vagina and, thereafter, pooling the first and second ejaculates from the same animal. Immediately after collection, the sperm samples were diluted with 50 to 100 μL of a Tris-egg yolk–based extender (TEY) (2.4% (wt/vol) Tris, 1.4% (wt/vol) citric acid, 0.8% (wt/vol) glucose, 0.02% (wt/vol) amikacin sulfate, 1% (vol/vol) Orvus ES paste (OEP), 20% (vol/vol) egg yolk in distilled water; pH = 6.6; 309 mOsm/L) and sperm concentration was determined using a hemocytometer. Samples were submitted to sperm motility, plasma membrane integrity (PMI), and DNA integrity evaluation. Sperm sample from each male was then divided into three equal parts. Different proportions of TEY nonsupplemented and supplemented with 10% glycerol were used to dilute each aliquot by dropwise and at room temperature. Therefore, all three glycerol concentrations (3%, 5%, and 7%) were tested in each ejaculate. In addition, all aliquots presented a final concentration of 50×10^6 to 60×10^6 spermatozoa/mL.

Samples were then loaded into 0.25-mL straws, equilibrated for 60 minutes in a programmable refrigerator at 5 °C (Minitüb, Minitüb do Brasil, Brazil) (average cooling rate 3.4 °C/min), and placed at 6 cm above liquid nitrogen for 15 minutes (average freezing rate; 0–5 minutes at 14 °C/min, 5–10 minutes at 4.6 °C/min, 10–15 minutes at 2 °C/min). Straws were plunged into liquid nitrogen and stored at −196 °C for at least 1 week before evaluation. Thawing was performed in a water bath at 46 °C for 12 seconds. Sperm samples were maintained for 5 minutes at 38 °C and then

sperm motility, acrosome integrity, PMI, and DNA integrity were determined. Sperm motion parameters were also assessed after 30 and 60 minutes of incubation at 38 °C.

2.3. Fresh and thawed sperm evaluation

2.3.1. Sperm morphology and motility

The mean percentage of morphologically abnormal sperm production was determined for all tomcats around 1 month previously to sperm cryopreservation trials. Three ejaculates were collected from each male as described above, and sperm morphology of 200 cells per sample was evaluated using the 1% Fast green FCF/1% Rose bengal single-step staining technique as described by Pope et al. [30]. Thereafter, the mean percentage of total abnormal sperm morphology was calculated for each male.

Sperm motion parameters were analyzed by computer-assisted sperm analysis (HTM, IVOS 12; Hamilton Thorne Research, Beverly, MA, USA) using domestic cat setup [7]. Briefly, a 10-μL drop of each sample was placed on a preheated Makler counting chamber (10 μm depth) at 38 °C, and at least three randomly selected fields were scanned.

2.3.2. DNA integrity

Acridine orange (AO) stain was used to assess sperm DNA integrity as previously described in cats [26]. In brief, 10 μL of the sperm sample was smeared on a glass slide, allowed to air-dry for 20 minutes, and then fixed overnight in freshly made Carnoy's solution (1:3 glacial acetic acid and methanol) at room temperature. The slide was removed from Carnoy's solution, air-dried, and stained with AO solution (10 mL of AO (Sigma-Aldrich Co., St Louis, MO, USA) diluted in distilled water (10 mg/mL), 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M $Na_2HPO_4 \cdot 7H_2O$) for 5 minutes in darkness. The slide was gently rinsed by a stream of distilled water, covered with a cover slip, and 200 cells were analyzed using an epifluorescence microscope (NIKON, Episcopic Fluorescence Attachment "EFA" HalogenLamp Set) within 1 hour after staining. Sperm heads with normal DNA (double-stranded) presented green fluorescence, whereas those presenting denatured DNA (single-stranded) showed orange, yellow, or red fluorescence.

2.3.3. Plasma membrane and acrosome integrity

In fresh sperm samples, evaluation of PMI was performed by mixing 10 μL of semen to 40 μL of a 3% sodium citrate solution containing propidium iodide (PI) (5 μg/mL; Sigma-Aldrich Co.) and carboxyfluorescein diacetate (9.2 μg/mL; Sigma-Aldrich Co.). For each sample, 200 cells were assessed using an epifluorescence microscope. Live spermatozoa stained green, whereas dead spermatozoa stained red or exhibited dual staining (both red and green).

For post-thaw samples, live acrosome-intact spermatozoa were determined using a double-labeling method with PI and fluorescein isothiocyanate–conjugated pea (*Pisum sativum*) agglutinin (FITC-PSA). An aliquot of 5 μL of frozen-thawed semen was diluted in 500 μL PBS, and 0.5 μL of PI (500 μg/mL; Sigma-Aldrich Co.) and 1.5 μL of FITC-PSA (100 μg/mL; Sigma-Aldrich Co.) were added. Final concentration of spermatozoa was approximately 5×10^5 to 6×10^5 cells/mL. After a 5 minute-incubation period at 37 °C, the stained sperm suspensions were run through a flow cytometer FACSCalibur (BD). A total of 20,000 events per sample were acquired using CellQuest Pro (BD) software. The fluorophores were excited using a 488-nm argon ion laser. The red fluorescent signal from PI-positive cells, differentiating live/dead cells, was detected in the FL3 sensor through a 670-nm bandpass filter, whereas the green fluorescent signal from FITC-PSA–positive cells, representing damaged acrosomes, was analyzed using the FL1 fluorescence detector through a 530-nm bandpass filter. Flow cytometer data were analyzed using CellQuest Pro (BD) and Paint-A-Gate (BD) software. Forward and side scatter parameters were analyzed, and non-sperm events were gated out from the analyses based on the scatter properties. Two-dimensional dot plots of FITC-PSA versus PI fluorescence events were drawn (FL1 × FL3, respectively). Four subpopulations of spermatozoa were observed in the scattergram and then divided by quadrants to calculate their frequency. The observed cell subpopulations were as follows (Fig. 1): (P1) sperm with damaged plasma membrane and intact acrosome, stained only in red (PI); (P2) sperm with damaged plasma and acrosomal membranes, stained both in red (PI) and green (FITC-PSA); (P3) sperm with intact plasma membrane and damaged acrosome, stained only in green (FITC-PSA); and (P4) sperm with intact plasma and acrosomal membranes, showing no staining.

To confirm the accuracy of flow cytometry data for PMI, frozen-thawed sperm samples were also analyzed under epifluorescence microscope using PI and carboxyfluorescein diacetate dyes as described above for fresh samples.

2.4. Statistical analysis

Data for sperm motion parameters, acrosome integrity, DNA integrity, and PMI were compared between fresh and

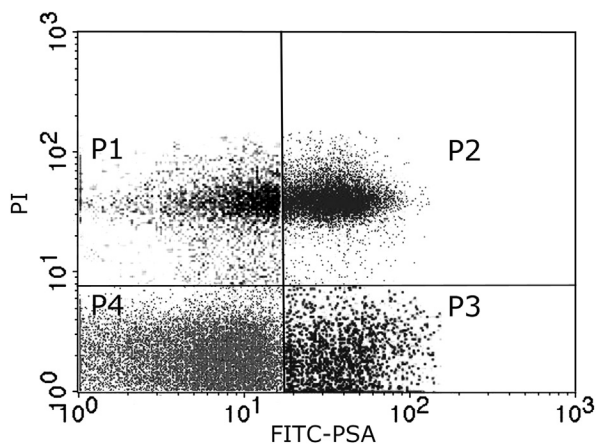

Fig. 1. Representative example of flow cytometry analysis of frozen-thawed cat sperm after dual staining with PI and FITC-PSA. Four subpopulations were detected: (P1) dead cells with intact acrosome; (P2) dead cells with reacted acrosome; (P3) viable cells with reacted acrosome; and (P4) viable cells with intact acrosome.

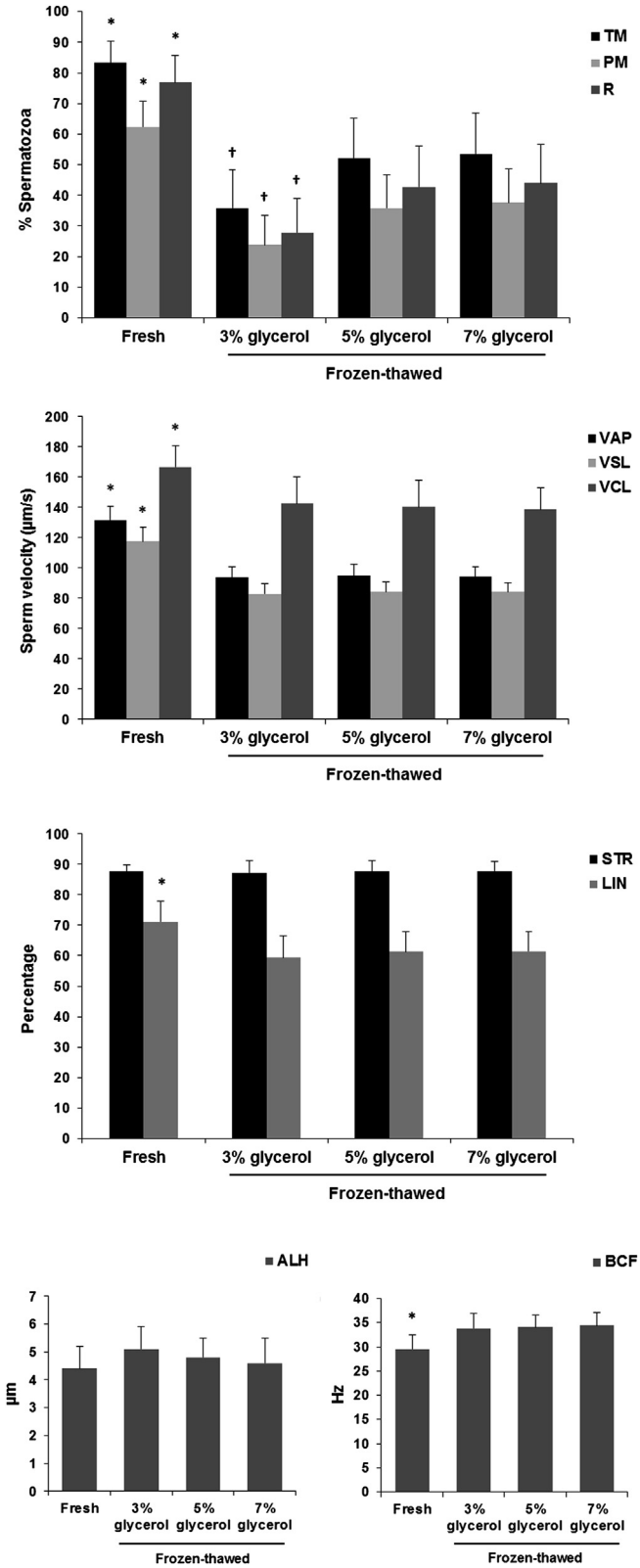

Fig. 2. Motion parameters of fresh and frozen-thawed cat sperm assessed by computer-assisted sperm analysis. Sperm samples were frozen using three different glycerol concentrations (3%, 5%, and 7%). TM, total motility; PM, progressive motility; R, percentage of rapid spermatozoa; VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; STR, straightness; LIN, linearity; ALH, amplitude of lateral head displacement; and BCF, beat cross frequency. Within each sperm motion parameter, significant differences ($P < 0.05$) among groups are indicated with distinct symbols.

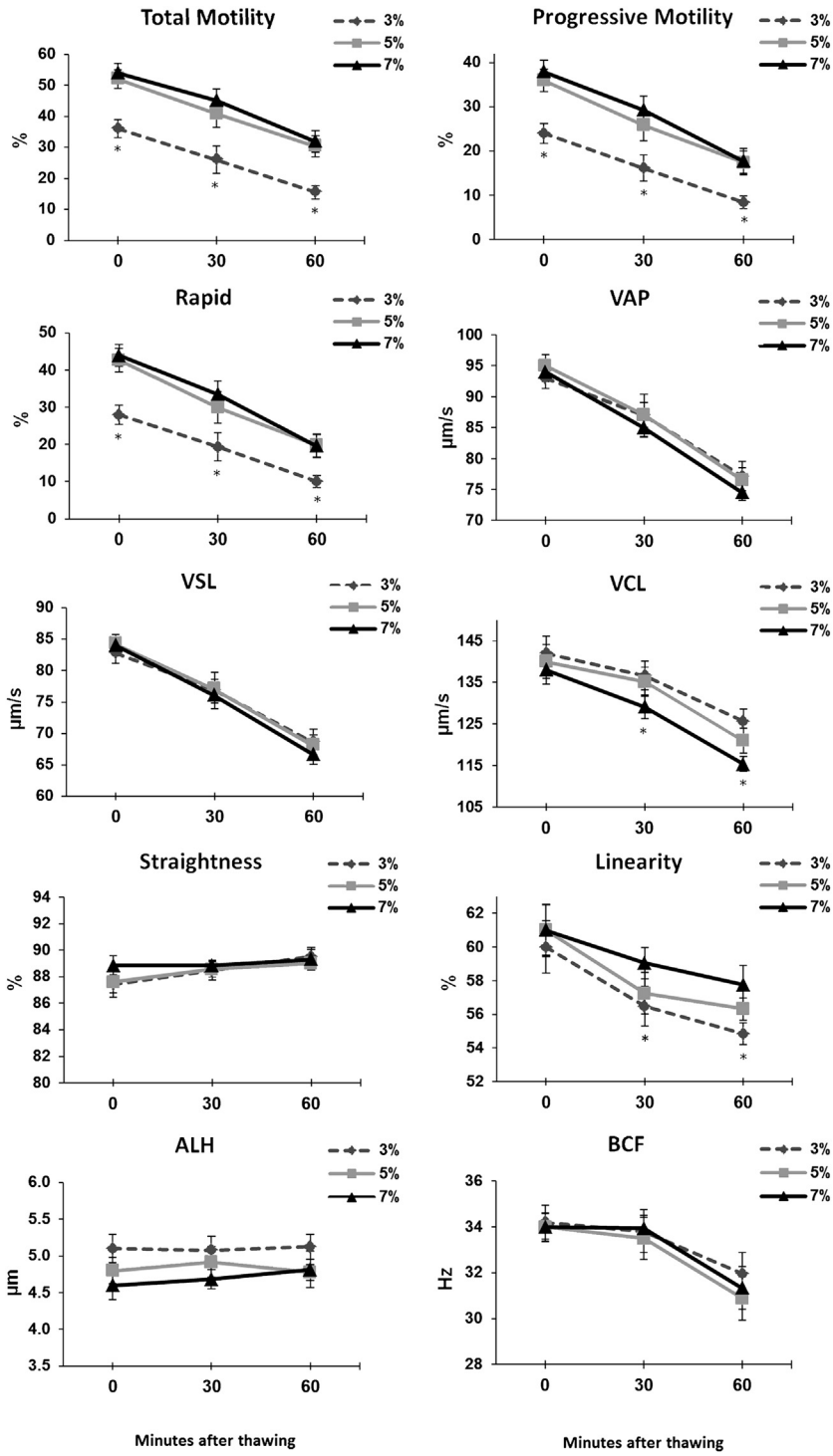

Fig. 3. Sperm kinematics determined by computer-assisted sperm analysis during post-thaw incubation (38 °C) of cat spermatozoa previously frozen with three different glycerol concentrations. TM, total motility; PM, progressive motility; R, percentage of rapid spermatozoa; VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; and BCF, beat cross frequency. At a given incubation moment, an asterisk indicates significant difference ($P < 0.05$). Values are means ± SEM.

frozen-thawed groups (3%, 5%, and 7% of glycerol) and among all three frozen-thawed groups using one-way ANOVA and Tukey test. Comparison of sperm motion values among frozen-thawed groups (3%, 5%, and 7% of glycerol) was performed within each incubation moment (0, 30, and 60 minutes after thawing) using the Friedman

test. Data obtained for PMI using both methods of evaluation (epifluorescence microscopy vs. flow cytometry) were compared by paired Student's *t*-test. Pearson's correlation coefficient (r) test was applied to verify the existence of a relationship between the percentage of morphologically abnormal sperm and post-thaw sperm quality (total sperm motility, progressive sperm motility, PMI, acrosome integrity, and DNA integrity). Differences were considered to be significant at $P < 0.05$. Data are expressed as means ± SD, unless otherwise stated.

3. Results

The mean value for total sperm amount (first and second ejaculates) was 51.9 ± 18.0 million spermatozoa. The average percentage of total abnormal sperm morphology was 48.8 ± 22.3%, with a mean value of 92.9 ± 3.4% for normal acrosome.

3.1. Motility

Data for sperm motion parameters in fresh and post-thaw samples are shown in Figure 2. Regardless the glycerol concentration used, all post-thaw groups showed a reduction ($P < 0.05$) in total motility (TM), progressive motility (PM), percentage of rapid spermatozoa (R), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), and linearity (LIN) when compared with fresh samples. On the contrary, no difference ($P > 0.05$) was found between fresh and frozen-thaw samples for straightness (STR) and amplitude of lateral head displacement (ALH) values.

Considering post-thaw results, although no difference ($P > 0.05$) was observed among the three treatment groups for VAP, VSL, VCL, ALH, BCF, STR, and LIN, the 3% glycerol group exhibited significantly lower values for TM, PM, and R compared with both 5% and 7% glycerol groups (Fig. 2). These lower ($P < 0.05$) values of TM, PM, and R in the 3% glycerol group persisted during all post-thaw incubation period (Fig. 3). In addition, after 30 and 60 minutes of post-thaw incubation, the 3% glycerol group showed slightly higher ($P < 0.05$) VCL and lower ($P < 0.05$) LIN when compared with the 7% glycerol group.

3.2. DNA integrity

Regarding the results obtained for DNA integrity, there was no significant difference among the post-thaw groups (3% glycerol, 92.8 ± 3.3%; 5% glycerol, 94.6 ± 1.9%; 7% glycerol, 95.2 ± 2.7%). However, when compared with fresh samples (97.1 ± 2.1%), only the 3% glycerol group showed a slight reduction ($P < 0.05$) in the percentage of nuclear integrity.

3.3. Plasma membrane and acrosome integrity

Data for PMI and acrosome integrity assessed by flow cytometry are presented in Table 1. After thawing, all groups exhibited a significant reduction in PMI about half of the value found in fresh samples (81.0 ± 5.9%). The mean value of PMI was not different ($P > 0.05$) among post-thaw groups. However, the percentage of dead cells with intact acrosome was slightly higher ($P < 0.05$) for the 3% glycerol group when compared with both 5% and 7% groups. In addition, although the percentage of total intact acrosomes was not different ($P > 0.05$) among post-thaw groups, the percentage of viable cells presenting a reacted acrosome was higher ($P < 0.05$) in the 7% glycerol group when compared with 3% glycerol. No difference was found ($P > 0.05$) between the techniques of epifluorescence microscopy and flow cytometry to assess PMI.

3.4. Correlation between sperm morphology and post-thaw sperm quality

Significant relationship between the mean percentage of morphologically abnormal sperm in fresh samples and post-thaw sperm quality was only found for total motility in the 7% glycerol group ($r = -0.87$; $P = 0.0226$), progressive motility in the 5% ($r = -0.88$; $P = 0.0203$) and 7% glycerol groups ($r = -0.92$; $P = 0.0087$), and PMI in the 3% glycerol group ($r = 0.95$; $P = 0.0044$). Post-thaw results for intact acrosome and DNA integrity were not correlated with sperm morphology in fresh samples, regardless of the glycerol concentration used.

4. Discussion

Freezing and thawing procedures impose extreme survival conditions to sperm cells, leading to structural and metabolic cell damage which results in loss of motility and integrity of membranes [13–17]. To minimize these cryogenic-induced injuries, the addition of cryoprotectant agents to freezing extenders plays a vital role. Nonetheless, optimal concentration of cryoprotectants needs to be defined for each species. In the present study, using three different glycerol concentrations (3%, 5%, and 7%) to cryopreserve ejaculated domestic cat spermatozoa we observed

Table 1
Percentage of spermatozoa presenting PMI, acrosomal membrane integrity (AcrI), damaged plasma membrane and intact acrosome (P1), damaged plasma and acrosomal membranes (P2), intact plasma membrane and damaged acrosomal membrane (P3), and intact plasma and acrosomal membranes (P4) in frozen-thawed domestic cat sperm samples evaluated by flow cytometry.

Group	PMI (%)	AcrI (%)	P1 (%)	P2 (%)	P3 (%)	P4 (%)
3% Glycerol	39.2 ± 9.5	68.6 ± 8.1	31.8 ± 7.1*	29.0 ± 7.9	2.4 ± 0.8*	36.8 ± 9.5
5% Glycerol	47.3 ± 11.3	68.7 ± 8.4	24.3 ± 6.7	28.5 ± 8.2	2.9 ± 0.9*†	44.4 ± 11.1
7% Glycerol	41.4 ± 12.7	62.0 ± 8.7	24.1 ± 8.0	34.5 ± 8.9	3.5 ± 1.1†	37.9 ± 12.2

Values are means ± SD, $N = 18$ ejaculates.
Within columns, means with different superscripts differ significantly ($P < 0.05$).

that, although a concentration of 3% glycerol was less capable of preserving post-thaw sperm motility, the use of 7% glycerol resulted in higher acrosomal membrane damage in viable cells after thawing. We also observed that sperm morphology in fresh samples is related to post-thaw sperm motility and PMI depending on final glycerol concentration.

In this study, a decrease in sperm motility and velocities after thawing was not precluded, regardless of the final glycerol concentration tested. In addition, all frozen-thawed groups showed a decrease in LIN and BCF values when compared with fresh samples, whereas STR and ALH remained unaffected. Likewise, epididymal cat spermatozoa cooled at 4 °C for 10 days showed no alteration in STR and ALH values [31].

Interestingly, we observed that both post-thaw percentages of motile and rapid spermatozoa increased when higher glycerol concentrations were used (5% and 7%), albeit not altering other motion parameters. These higher values of TM, PM, and R in the 5% and 7% groups persisted up to 60 minutes after thawing. Improvement in post-thaw motility as a result of increasing glycerol concentration has also been demonstrated in other species, even after a 2-hour post-thaw incubation [16,32,33]. Furthermore, a negative relationship between the percentage of morphologically abnormal spermatozoa in fresh samples and post-thaw sperm motility was found in the 5% and 7% glycerol groups. Thus, it seems that the beneficial effect of glycerol on post-thaw sperm motility is more pronounced on sperm cells presenting normal morphology.

In a previous report, using 4% glycerol and a freezing protocol similar to this study, we obtained results for total and progressive motility (52.8 ± 6.9% and 32.9 ± 5.4%, respectively) closer to the ones found in the present study with 5% and 7% glycerol [7]. Nevertheless, using concentrations of 3% and 4% glycerol to freeze ejaculated cat spermatozoa, Baran et al. [29] found no difference in subjective motility after thawing. However, besides the fact that different protocols were used in these studies, sperm motion parameters were objectively assessed by computer-assisted sperm analysis in the present study, which may have improved the comparison among treatment groups. For this reason, we can speculate that glycerol may exert a dose-dependent beneficial effect on frozen-thawed cat sperm motility up to a concentration of 4% to 5%, reaching a plateau. It is important to note that the use of glycerol at a final concentration of 8% has shown to impair post-thaw motility of epididymal cat spermatozoa [20]. Therefore, probably due to the toxic effects of glycerol, post-thaw motility may return to decrease if concentrations above 7% are used, as already shown to occur in red deer spermatozoa [34].

Sperm plasma membrane is considered as one of the main sites susceptible to cryodamage [15]. Regarding all treatment groups in this study, around 40% to 50% of the viable spermatozoa in fresh samples underwent plasma membrane disruption after cryopreservation. Although the 5% glycerol group resulted in higher overall mean PMI than 3% glycerol, no statistical difference was found between these groups probably due to a high individual variation on freezing/thawing sensitivity.

Of interest, using lower glycerol concentration (3%), we observed that the protection provided by glycerol against plasma membrane damage was positively related to the percentage of morphologically abnormal sperm in fresh samples. In contrast, this relationship was not found in the 5% and 7% glycerol groups, being in accordance with a previous report in cats using 5% glycerol [25]. The meaning of this finding is uncertain, but it is worth mentioning that susceptibility to variations in osmolarity has already been demonstrated to be related to sperm morphology in domestic cats [35].

Similar to the plasma membrane, the acrosome membrane is also highly susceptible to cryodamage during freezing/thawing procedures [14,36]. In domestic cats, reduction on acrosomal integrity following sperm cryopreservation is a common finding [22,23,25]. Furthermore, although the inclusion of antioxidants to freezing extenders showed to be unable to minimize acrosome injury in cat spermatozoa [26–28], the addition of detergents such as Equex STM paste and OEP remains controversial in this matter [22,24,25].

In the present study, neither reduction nor increase in final glycerol concentration significantly improved the overall percentage of intact acrosomal membranes. Nonetheless, the percentage of viable spermatozoa presenting reacted acrosomes showed to rise as glycerol concentration increased, possibly as a consequence of capacitation-like changes. In other species, cooling and freezing/thawing procedures have already shown to induce membrane changes resembling the ones associated with capacitation, probably ascribed to increased membrane permeability and calcium influx [36]. Additionally, 3% glycerol resulted in higher percentage of intact acrosomes in dead cells compared with 5% and 7% glycerol. Thus, a detrimental effect on acrosome integrity due to high glycerol concentrations seems to exist in domestic cat spermatozoa as already reported in other species [16,34,37].

In this study, post-thaw DNA integrity was not significantly different among the three treatment groups. However, the use of 3% glycerol was less able to preserve DNA structure during cryopreservation, because a slight increase in DNA denaturation was observed in this group after thawing. Conversely, DNA integrity was not adversely affected following cryopreservation in both 5% and 7% glycerol groups. Likewise, storage of cat spermatozoa at 4 °C has been demonstrated not to alter DNA fragmentation index [31].

In conclusion, comparing three different glycerol concentrations to cryopreserve ejaculated domestic cat spermatozoa, we observed that (1) although glycerol concentrations above 3% had a beneficial effect on sperm motility immediately after thawing and up to 60 minutes of post-thaw incubation, increasing glycerol concentration above 5% showed no significant improvement; (2) overall PMI was not affected when varying glycerol concentration within the range of 3% to 7%; and (3) glycerol at a high concentration (7%) showed to increase the incidence of reacted acrosome in viable cells after thawing. It is worth stating that the ability of spermatozoa to withstand glycerol toxicity may not only depend on final concentration but also on the moment and temperature of exposure to

this chemical compound combined with cooling/freezing rates and extender composition. Therefore, it seems plausible to assume that 5% glycerol may be an optimal final concentration to cryopreserve domestic cat spermatozoa when considering the freezing/thawing conditions established in this work.

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