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Influence of glycerol addition on the quality of cat epididymal sperm during the freeze-thaw process

Influência da adição do glicerol sobre a qualidade dos espermatozoides epididimários de gato durante as etapas da congelação-descongelação

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Highlights:
Reduction in sperm quality after thawing occurred in both groups.
The sperm parameters were more compromised in glycerolization for 10 minutes.
Post-thaw sperm morphology did not differ between the groups.
The most critical step in the freeze-thaw process is thawing.
Glycerolization using a 5% glycerol solution is recommended for 5 minutes.

Abstract

Cryopreservation of epididymal sperm is a useful tool for preserving the genetic potential of valuable animal specimens. The domestic cat is used as a model to study and develop cryogenics for other felines. However, regulation of the entire cryopreservation process is essential for the success of this biotechnology. Thus, our aim was to evaluate the effects of glycerol equilibration time and freeze-thaw stages on the quality of epididymal sperm obtained from domestic cats. Epididymal sperm were recovered with TRIS and immediately evaluated for total motility (TM), vigor, viability, membrane functionality (HOST), and morphology. Then, TRIS-20% egg yolk was added to the samples, which were equally divided into two 1.5 mL tubes and refrigerated at 4 ºC for 1 hour. Subsequently, glycerol was added at a final concentration of 5%. The samples were incubated with glycerol (equilibration time) for either 5 or 10 minutes (groups G5 and G10, respectively) and then frozen. Thawing occurred at 37 ºC for 30 seconds. The samples were evaluated at all stages. A reduction in TM was observed only after thawing; however, it was higher in G5 (39.00 ± 4.07%) than in G10 (18.50 ± 4.54%). Vigor declined in both groups after thawing; however, they did not differ from each other. Sperm viability was maintained in G5 after glycerolization (53.60 ± 2.59%); in G10, sperm viability decreased in the glycerolized sample (48.80 ± 2.93%) when compared to that in the fresh sample (59.90 ± 1.74%). Post-thaw viability of G5 (33.80 ± 1.89%) was higher than that of G10 (18.80 ± 3.01%). In the HOST, a decrease in viability was only observed after thawing, with no difference between the groups (41.50 ± 2.84% for G5 and 40.20 ± 3.49% for G10). With regard to sperm morphology, normal sperm decreased while sperm with post-thaw secondary defects increased in both groups. In conclusion, a shorter equilibration time for glycerolization preserves epididymal sperm quality better and the freeze-thaw process is the most critical stage of thawing.

Key words: Cryo-injury. Cryopreservation. Sperm. Feline. Intracellular cryoprotectant.

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Resumo

A criopreservação dos espermatozoides epididimários é uma ferramenta útil para preservar o potencial genético de um animal valioso. Além disso, o gato doméstico é modelo eleito para o estudo e desenvolvimento da criogenia para os demais felinos. Contudo, para o sucesso dessa biotécnica é essencial o controle de todo o processo de criopreservação. Assim, objetivou-se avaliar o efeito do tempo de equilíbrio da glicerolização e das etapas da congelação-descongelação sobre a qualidade dos espermatozoides epididimários de gato doméstico. Para tanto, espermatozoides epididimários foram recuperados com TRIS e imediatamente avaliados quanto à motilidade total (MT), vigor, viabilidade, funcionalidade de membrana (HOST) e morfologia. Em seguida, as amostras foram adicionadas de TRIS-gema a 20%, fracionadas igualmente em dois tubos de 1,5 mL, refrigeradas a 4 ºC por 1 hora e, posteriormente, adicionadas de glicerol na concentração final de 5%. As amostras foram incubadas com glicerol (tempo de equilíbrio) por 5 ou 10 minutos (grupos G5 e G10, respectivamente) e depois congeladas. A descongelação ocorreu a 37 ºC por 30 segundos. As amostras foram avaliadas em todas as etapas. Uma redução na MT foi observada apenas na pós-descongelação, no entanto G5 (39,00 ± 4,07%) foi superior ao G10 (18,50 ± 4,54%). O vigor declinou pós-descongelação em ambos os grupos; contudo, não diferiram entre si. A viabilidade espermática foi mantida no G5 pós-glicerolização (53,60 ± 2,59%), diferentemente do observado em G10, em que a amostra glicerolizada (48,80 ± 2,93%) reduziu em relação à fresca (59,90 ± 1,74%). A viabilidade pós-descongelação de G5 (33,80 ± 1,89%) foi superior à de G10 (18,80 ± 3,01%). No HOST, uma redução da viabilidade só foi observada pós-descongelação, não havendo diferença entre os grupos (41,50 ± 2,84% para G5 e 40,20 ± 3,49% para G10). Em relação à morfologia espermática, os espermatozoides normais diminuíram, enquanto os espermatozoides com defeitos secundários pós-descongelação aumentaram em ambos os grupos. Conclui-se que um menor tempo de equilíbrio para a glicerolização preserva melhor a qualidade dos espermatozoides epididimários e a etapa mais crítica do processo de congelação-descongelação é a descongelação.

Palavras-chave: Criodanos. Criopreservação. Espermatozoide. Felino. Crioprotetor intracelular.

Introduction

Cryopreservation of feline epididymal sperm is a promising tool for the conservation of the genetic material of valuable animals that may suddenly die, or animals that are affected by some reproductive disorder that prematurely interrupts their reproductivity, but maintains their spermatic cells, as in case of alteration of the penile retractor muscle (Amstislavsky, Lindeberg, & Luvoni, 2012; Luvoni & Morselli, 2017). The study of gamete cryobiology using the domestic cat model contributes to the development or adjustment of cryopreservation protocols for other wild cats (Chatdarong, 2017).

In general, the cryopreservation process involves subjecting sperm cells to a gradual reduction in temperature, followed by cell dehydration and freezing; the cells then remain in a quiescent state until thawed (Hartwig, Papa, & Dell'Aqua, 2012).

Controlling each step of cryopreservation is crucial for the success of this biotechnology, because the sperm are subjected to adverse physical-chemical conditions, which, if not well adjusted, result in irreversible damage to gamete structure and function (Chatdarong, 2017; Cheuquemán, Faúndez, Sánchez, & Risopatrón, 2018). In this context, the time of exposure of the sperm cells to the glycerol may affect their post-thaw quality (Leite et al., 2010; Almeida et al., 2017).

Glycerol is the main cryoprotective substance used in the freezing of feline sperm, together with egg yolk, associated with TRIS, TES, or TES-T diluent (Buranaamnuay, 2017). The interaction between glycerol and the sperm cell occurs through specific bonds involving phospholipids and plasma membrane glycoproteins; in addition, this cryoprotectant is able to penetrate the cell, partially replacing its water and electrolyte contents, thereby

promoting dehydration and reducing the cryoscopic point of the cell content (E. C. B. Silva & Guerra, 2012; R. A. Oliveira, 2013; Di Domenico, Pedroso, & Teixeira, 2015).

Glycerol may be added at room temperature or at 4 or 5 °C, with final concentrations of 3% (Thuwanut, Arya, Comizzoli, & Chatdarong, 2015), 4% (Zambelli, Caneppele, Castagnetti, & Belluzzi, 2002; VillaVerde et al., 2009; Jiménez, Pérez-Marín, Vizuete, Millán, & Agüera, 2013), 5% (Thuwanut, Chatdarong, Techakumphu, & Axnér, 2008; Buranaamnuay, 2015, 2018), 6% (Prochowska, Niżański, & Partyka, 2016), 7% (Thuwanut & Chatdarong, 2009; Cheuquemán et al., 2018), or 8% (Bogliolo et al., 2004) for the sperm of domestic cats. Villaverde et al. (2013) confirmed that medium containing 5% glycerol led to a better balance between cryoprotection and toxicity to the sperm cell structure, standardizing the concentration of this cryoprotectant for feline cells.

However, studies on the effect of feline sperm cell exposure time on glycerol are scarce. Buranaamnuay (2015) recommends that glycerol addition be performed in two steps for freezing media that have concentrations above 3% of cryoprotectant. Feline sperm were subjected to 10 minutes of glycerolization (Buranaamnuay, 2015). Other protocols, however, use shorter glycerolization equilibrium times, 5 minutes exposure (Cocchia et al., 2010), or simply the addition of the second part of the extender followed by exposure to liquid nitrogen (Thuwanut et al., 2015).

Therefore, researches that specifically addresse the cryodanes suffered by sperm during the different stages of cryopreservation are necessary. In previous studies, glycerolization was identified as the most critical step of cryopreservation that affects post-thaw cell survival (T. F. P. Silva, 2008; Guerra et al., 2009; García et al., 2012; Almeida et al., 2017). Therefore, controlling this step is essential for the success of this biotechnology, because the effect of glycerol on sperm cells depends on a species-specific tolerance and is influenced by the chosen cooling rate (VillaVerde et al., 2009; Deco-Souza et al., 2013).

Thus, the present study aimed to evaluate the effects of glycerolization equilibration time and the steps of the freeze-thaw process on the quality of domestic cat epididymis sperm.

Material and Methods

Experimental design

Ten adult male cats, aged between 1 and 4 years and subjected to routine orchiectomy at the State University of Ceará (UECE), were included in this study. The animals' testicular-epididymal compartments (TECs) were kept in 50 mL tubes containing 0.9% saline solution prewarmed to 37 ºC until processing, which occurred within 1 hour.

The epididymides (n= 20) of each TEC (n=10) were transferred to Petri dishes containing TRIS (composed of 1.25g D-fructose, 1.78g citric acid monohydrate and 3.028g TRIS diluted in 100ml distilled water) diluent and preheated to 37 ºC for sperm recovery. The recovered sperm were evaluated for total motility, vigor, viability, membrane functionality, and morphology.

The collected sperm samples were diluted in TRIS-yolk (TRIS extender added with 20% egg yolk) and equally divided into two prewarmed 1.5 mL tubes. They were then incubated for one hour. After this period, another sperm evaluation and glycerol addition were performed and the samples were incubated for five (n= 10) or 10 (n=10) minutes, in groups named G5 and G10, respectively. The samples were evaluated and cryopreserved. Thawing was performed one week after freezing. The sperm were again evaluated for the same aforementioned parameters.

All procedures performed in this study were approved by the local Ethics Committee for the Use of Animals under protocol number 7438550/2017.

Collection, freezing, and thawing of epididymal sperm

The epididymal sperm were recovered by the slicing technique (Tittarelli et al., 2006). To this end, the TECs were dissected and the anatomical regions of the body and the tail of the epididymis were separated from the other regions and submitted to successive external washing with previously heated saline solution (37 ºC). Then, the tail of the epididymis was sectioned and incubated in 0.5 mL of TRIS diluent for five minutes at 37 ºC. The tissue fragments were removed, and the recovered epididymis was transferred to a 2 mL tube, prewarmed to 37 ºC, for further sperm evaluation.

The recovered sperm were diluted in TRIS-egg yolk (to adjust the sperm concentration to 200 x 10^6 cells/mL) and subjected to an equilibration curve of 4 ºC for 1 hour (cooling rate 1.2 ºC/min) (Cocchia et al., 2010). Subsequently, the second extender, i.e., the TRIS-egg yolk diluent containing 10% glycerol (5% final glycerol concentration), was added and the refrigerated sperm samples (final sperm concentration of 100 x 10^6 cells/mL) were kept for either 5 or 10 minutes at 4 ºC. The sperm were loaded into 0.25 mL straws, exposed to nitrogen vapors (-70 ºC) for 20 minutes, dipped in liquid nitrogen and stored in a cryogenic tank.

Thawing was performed at 37 ºC for 30 seconds in a water bath (Cocchia et al., 2010). The sperm samples were then transferred to 1.5 mL plastic tubes prewarmed to 37 ºC and submitted for sperm evaluation immediately.

Sperm evaluation

Total motility (%) and vigor (0 to 5) of sperm were assessed using 5 µL samples placed on a prewarmed slide (37ºC). Two to three different fields of the slide were examined under an optical microscope (100X) (Colégio Brasileiro de Reprodução Animal [CBRA], 2013). The values of motility and vigor were assessed by the same evaluator throughout the execution of the experiment.

Sperm viability was determined by bromophenol blue vital staining. On a prewarmed slide (37 ºC), 5 µL of sperm sample were added to 5 µL of dye, followed by smearing and random counting of 100 cells, including both viable (unstained) and non-viable (stained) cells, using an optical microscope (400X) (R. V. Oliveira et al., 2009).

Membrane functionality was evaluated using the hypo-osmotic test. For this purpose, a 5 µL aliquot of sperm sample was incubated in medium (150 mOsm/L) for 60 minutes at 37 ºC and 100 sperm with or without coiled tails were subsequently counted under an optical microscope (400X). The percentage of reactive cells was subtracted from the percentage of sperm with coiled tail after morphology evaluation, resulting in the corrected value of membrane functionality (Jeyendran, Van Der Ven, Perez-Pelaez, Crabo, & Zaneveld, 1984).

Sperm morphology was evaluated by preparing a slide smear of 5 µL of sperm sample diluted in 45 µL of 1.5% Rose Bengal dye. A total of 200 sperm were randomly counted using an optical microscope (1000 X) and classified as normal or abnormal. The morphological defects observed were classified as primary or secondary (Raskin & Meyer, 2011).

The morphological defects observed were classified as primary (for the presence of proximal cytoplasmic droplet, abnormalities in the acrosome and midpiece, macrocephaly, microcephaly and abnormal shape of the head, double head and double tail, abaxial insertion) or secondary (normal detached head and coiled or folded tail) (CBRA, 2013). The presence of cytoplasmic droplet was not accounted for as an abnormality, as determined by Axnér, Hermansson and Linde-Forsberg (2004).

Statistical analysis

The data obtained were expressed as mean and standard error of the mean (SEM) and analyzed using the statistical software R version 3.5.1. The Barttlet and Cramer von-Mises tests were

used to evaluate homoscedasticity and normality, respectively. Data transformation in arcsine was performed for sperm viability. Data regarding total motility, viability, membrane functionality, and morphology were subjected to analysis of variance (ANOVA) followed by the Student-Newman-Keuls test (SNK) to compare evaluation times and groups. Sperm vigor data were subjected to the Mann-Whitney test. The results were considered significant when $p < 0.05$.

Results and Discussion

The results regarding the evaluation of sperm in the freeze-thaw process with different times of glycerolization are shown in Table 1.

Table 1
Mean ± SEM of the parameters of fresh cat epididymis spermatozoa, refrigerated (4 °C/1 h), post-glycerolization for 5 minutes (G5) or 10 minutes (G10), and post-defrosting in the glycerolized group for 5 minutes (G5) or 10 minutes (G10)

Parameters	Fresh	Cooled	Post-glycerolization		Post-thaw	
			G5	G10	G5	G10
Motility (%)	86.50 ± 2.99[a]	79.00 ± 6.69[a]	76.50 ± 6,75[a]	71.00 ± 8.05[a]	39.00 ± 4.07[b**]	18.50 ± 4.54[b]
Vigor (0-5)	4.10 ± 0,23[a]	3.80 ± 0.80[ab]	3.60 ± 0.16[ab]	3.50 ± 0.17[ab]	3.20 ± 0.13[b]	2.80 ± 0.20[c]
Viability (%)	59.90 ± 1.74[a]	55.70 ± 2.27[ab]	53.60 ± 2.59[ab]	48.80 ± 2.93[b]	33.80 ± 1.89[c**]	18.80 ± 3.01[c]
Membrane functionality (%)	67.90 ± 2.04[a]	64.60 ± 2.75[a]	64.80 ± 3.27[a]	61.10 ± 2.50[a]	41.50 ± 2.84[b]	40.20 ± 3.49[b]
Normal morphology (%)	72.50 ± 2.46[a]	-	-	-	60.25 ± 1.23[b]	55.36 ± 1.89[b]
Primary defects (%)	7.41 ± 0.78[a]	-	-	-	9.67 ± 1.78[a]	9.57 ± 1.08[a]
Secondary defects (%)	19.57 ± 2.49[a]	-	-	-	29.50 ± 2.49[b]	35.07 ± 2.24[b]

Different lower-case letters indicate a statistical difference between freezing steps ($p < 0.05$).
* Indicates if there was a difference between G5 and G10 after glycerolization.
** Indicates if there was a difference between G5 and G10 after thawing.

Total motility remained constant during cooling in the pre- and post-glycerolization steps ($p > 0.05$), unlike the findings of T. F. P. Silva (2008) regarding the cryopreservation of cat semen, in which an immediate decline in total motility (from 92% to 70%) and vigor (from 4.6 to 4) was observed after glycerolization in relation to the initial dilution.

The reduction of motility observed in G10 after thawing was significantly higher (18.50 ± 4.54%) than that in G5 (39.00 ± 4.07%, $p < 0.05$).

The sperm exposed to glycerol for 5 minutes only showed a reduction in vigor after thawing ($p < 0.05$); in group G10, vigor decreased immediately after the addition of glycerol and was drastically reduced in cryopreserved samples, from 3.50 ± 0.17 to 2.80 ± 0.20, respectively ($p < 0.05$). There were no differences between the groups G5 and G10 in the post-glycerolization and post-thaw evaluations ($p > 0.05$).

Regardless of glycerol exposure time, vigor reduction was only observed after thawing (3.20 ± 0.13 for G5 and 2.80 ± 0.20 for G10) compared to fresh samples (4.10 ± 0.23) ($p < 0.05$). The G10 group presented lower post-thaw vigor than the refrigerated sample (3.80 ± 0.80) and post-glycerolization for the same group (3.50 ± 0.17) (p

<0.05). There was no difference between groups G5 and G10 in the post-glycerolization and post-thaw sperm vigor evaluations (p> 0.05).

Sperm motility is the main parameter affected in the freeze-thaw process and is even more critical for sperm originating from the epididymis, due to their greater susceptibility to osmotic stress (Cheuquemán, Sánchez, & Risopatrón, 2017; Kunkitti et al., 2017). Thus, loss of motility may result from the osmotic stress endured during freezing, which may be caused by an increase in osmolarity after the formation of ice crystals and the concentration of solutes in the environment surrounding the sperm or by the increase in the permeability of the plasma membrane and consequent loss of cellular osmoregulation mechanisms (Figueroa, Valdebenito, & Farias, 2016; Cheuquemán et al., 2018).

The exposure of feline sperm to glycerol for ten minutes was shown to be unfavorable for motility and sperm vigor. This is possibly due to the greater penetration of the cryoprotectant inside the cells and increased toxicity to sperm structures, especially mitochondria, as already reported in studies with stallion sperm (García et al., 2012). In addition, total post-thaw motility is reported to be 16.5% to 21.6% (Buranaamnuay, 2015; Prochowska et al., 2016) and 32% to 53% (Cocchia et al., 2010; Thuwanut et al., 2015) for feline epididymal sperm incubated with glycerol for ten and five minutes, respectively, which is in line with the present findings.

Sperm viability remained unchanged until the glycerolization stage for G5 (p> 0.05), unlike that observed for G10 (p <0.05), where there was a reduction of 59.90 ± 1.74%. to 48.80 ± 2.93%, between fresh and post-glycerolization samples. No differences were observed between G5 and G10 at refrigeration and after glycerolization evaluation times (p > 0.05). After thawing, there was a significant reduction in viability in both groups, which was greater in G5 than in G10 (p < 0.05).

The impairment observed in the samples subjected to longer exposure time to glycerol is probably caused by the toxicity of the cryoprotectant to the sperm cells, as a result of physical-osmotic changes in the intra- and/or extracellular environment, as well as biochemical changes in the cells (R. A. Oliveira, 2013; Kunkitti et al., 2017). According to Burg, Ferraris and Dmitrieva (2007), the toxic effect of glycerol is associated with the disorganization/redistribution of cytoskeletal protein filaments, which prevents this structure from protecting the cell from the deleterious effect of excessive shrinkage.

Reduced membrane functionality was only observed in thawed samples (p < 0.05), with no differences between the evaluated groups (p > 0.05). This result may be an indication that the toxicity of glycerol to the cells is not linked to osmotic effects, but rather to changes in the cytoskeleton, plasma membrane, and glycocalyx, and/or interference of this cryoprotectant with the actin filaments of the flagella (S. V. Silva & Guerra, 2011).

Our findings match those of Martins et al. (2009) who subjected feline sperm to 10 minutes of glycerolization and obtained values of motility and membrane integrity after thawing of 40% and 49.4%, respectively. Previous work using sperm dilution in one step exposing cells throughout the freezing curve to glycerol also showed good results (Tebet et al., 2006; Klaus, Eder, Franz, & Müller, 2016). We believe that the use of commercial freezing media in these studies may have more effectively aided sperm cell survival, since most of them are composed of several antioxidant substances and employ purified egg yolk lipoprotein. Incorporating additives into media, such as Equex STM paste, also contributes to improved freezing protocols, aiding in cell interaction with the cryoprotectant and protecting it from thermal shock (Costa, Castelo, Souza, Lima, & Silva, 2013).

The reduction in the percentage of morphologically normal sperm and increase in secondary defects after thawing was not influenced by the time of glycerolization (p > 0.05). No

differences were found in the number of primary defects between fresh and thawed samples ($p > 0.05$).

Sperm morphology is extremely important for the ability of sperm to fertilize, and is also an indication of the compatibility of the freezing process and/or medium required by the cells (Santos et al., 2015; García-Vazquez, Gadea, Matás, & Holt, 2016). The main defects accounted for in the study were coiled or bent tail, curved midpiece, and detached head; the tail defects were notoriously elevated after thawing, a fact that may be explained by the response of the sperm cell to osmotic stress and/or thermal shock caused by the freezing process and not necessarily by the exposure to glycerol (Ozkavukcu, Erdemli, Isik, Oztuna, & Karahuseyinoglu, 2008).

Conclusion

A shorter glycerolization equilibrium time provides better preservation of the quality of frozen epididymal sperm. The most critical stage of the freeze-thaw process is thawing, which is a reflection of the effect of glycerolization.

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