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Effect of refrigeration at -1°C on spermatozoa quality of domestic cats[1]

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ABSTRACT.- Souza A.K., Trautwein L.G.C., Paranzini C.S., Schnitzer J.F., Perencin F.M., Cardoso G.S. & Martins M.I.M. 2020. **Effect of refrigeration at -1°C on spermatozoa quality of domestic cats.** *Pesquisa Veterinária Brasileira 40(4):306-314*. Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Campus Universitário, Cx. Postal 10.011, Londrina, PR 86057-970, Brazil. E-mail: imartins@uel.br

The objective of this study was to evaluate the sperm quality obtained of domestic cats by electroejaculation and recovery of the tail of the epididymis after cooling at -1°C and 4°C for 24 and 48 hours. Twenty-nine adult cats (2 to 6kg) were used. Sperm collection was performed by electroejaculation (EEJ), and after 48 hours, the cats were orchiectomized, and sperm sample was obtained from the vas deferens and epididymis tail (EPD). The samples were diluted in ACP-117® extender, and the sperm characteristics were evaluated at three different moments: when still fresh, 24 and 48 hours after cooling. In order to compare the two refrigeration temperatures, the first stage was to analyze if there was a difference between the harvesting techniques. After this, two experiments were conducted: in the first, sperm sample from 14 cats were used and the cooling was performed at -1°C; and in the second, sample from 15 cats were used and the sperm were refrigerated at 4°C. Sperm kinetics were evaluated by computerized analysis (CASA) and concentration by Neubauer chamber, spermatic morphology was evaluated by modified Karras staining, and membrane integrity was evaluated by eosin nigrosine. The results obtained were analyzed in R software, version 3.2.5 using the Mann-Whitney test for variables with abnormal distributions, considering significance at the level of 5%. In ejaculate samples, higher values of total morphological defects were observed after 24 and 48 hours of refrigeration at 4°C ($P<0.022$) compared to refrigeration at -1°C, using Friedman test. To quantify the decrease in sperm quality, parameter reductions were calculated among time points (F-24h/F-48h/24h-48h). In EPD samples, a greater reduction in sperm quality was detected after 24 hours of refrigeration at 4°C, both in motility and sperm kinetics and in the movement and velocity indices, compared to refrigeration at -1°C. Based on the results, it can be concluded that cooling of feline spermatozoa at -1°C for up to 48 hours was efficient in maintaining spermatic quality collected by EEJ and EPD, and it could be an alternative to spermatozoa cryopreservation in domestic felines.

INDEX TERMS: Refrigeration, spermatozoa, domestic cats, cryopreservation, coconut water extender, electroejaculation, epididymis, cats.

RESUMO.- [**Efeito da refrigeração a -1°C na qualidade de espermatozoides de gatos domésticos.**] O objetivo deste trabalho foi avaliar a qualidade espermática de gatos domésticos obtidos por eletroejaculação e recuperação da cauda do epidídimo após a refrigeração a -1°C e a 4°C por 24 e 48 horas. Vinte e nove gatos adultos (2 a 6kg) foram utilizados. A colheita de espermatozoides foi realizada por eletroejaculação (EEJ) e, após 48 horas, os gatos foram orquiectomizados, e as amostras espermáticas foram obtidas a partir do ducto deferente e da cauda do epidídimo (EPD). As amostras foram diluídas em ACP-117® e as características espermáticas foram avaliadas em três momentos distintos:

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fresco, 24 e 48 horas após a refrigeração. Para ser possível comparar as duas temperaturas de refrigeração, a primeira etapa foi analisar se havia diferença entre as técnicas de colheita. Após isto, dois experimentos foram conduzidos: no primeiro, espermatozoides de 14 gatos foram utilizados e a refrigeração foi realizada a -1°C; e no segundo, amostras de 15 gatos foram utilizados e os espermatozoides foram refrigerados a 4°C. A cinética espermática foi avaliada por análise computadorizada (CASA), a concentração por câmara de Neubauer, a morfologia espermática foi avaliada pela coloração de Karras modificada, e a integridade da membrana foi avaliada por eosina nigrosina. Os resultados obtidos foram analisados no software R, versão 3.2.5, utilizando o teste de Mann-Whitney para variáveis com distribuições anormais, considerando significância ao nível de 5%. No ejaculado, maiores valores de defeitos morfológicos totais foram observados após 24 e 48 horas de refrigeração a 4°C (P<0,022) em comparação com refrigeração a -1°C, usando o teste de Friedman. Para quantificar a diminuição na qualidade espermática, as reduções dos parâmetros foram calculadas entre os pontos de tempo (F-24h/F-48h/24h-48h). Na EPD, uma maior redução na qualidade espermática foi detectada após 24 horas de refrigeração a 4°C, tanto na motilidade e na cinética espermática quanto nos índices de movimento e velocidade, em comparação com a refrigeração a -1°C. Com base nos resultados, pode concluir-se que a refrigeração dos espermatozoides felino a -1°C, até 48 horas, foi eficaz na manutenção da qualidade espermático colhidos por EEJ e EPD, e pode ser uma alternativa para a criopreservação de espermatozoides em felinos domésticos.

TERMOS DE INDEXAÇÃO: Refrigeração, espermatozoides, gatos domésticos, criopreservação, diluente de água de coco, eletroejaculação, epidídimo.

INTRODUCTION

The survival and maintenance of species are facilitated by assisted reproduction, however, there are restrictions on genetic material preservation. One important tool is the cryopreservation of gametes (Farstad 2000), which enables the conservation of genetic material of domestic species, including those threatened with extinction. Semen refrigeration is used to allow the genetic material to be transported over long distances because with decreased temperature, the metabolic activity of sperm decreases, thus avoiding the formation of free radicals (Buranaamnuay 2017).

The domestic cat is the experimental model for wild feline species, so understanding of factors that interfere with sperm viability is necessary for the use of reproductive biotechniques in felids, such as the study of sperm cryopreservation conditions (Bronson 1985, Meyer 2013, Martins et al. 2014, Martins & Justino 2015).

The viability of cryopreserved spermatozoa is affected by several factors, such as the composition and concentration of extender, temperature and velocity of cooling processes and, mainly, fresh sperm quality (Buranaamnuay 2017). Although there have been promising results with refrigerated feline semen (Luvoni et al. 2003), there is still no definition of a cooling temperature with high success rates. There is evidence that a large proportion of spermatozoa that undergo the cryopreservation process acquire acrosomal damage, with compromise fertilization capacity (Tebet et al. 2006).

Only one study of artificial insemination in cats (Tsutsui 2006) reported the use of semen at a lower refrigeration temperature of 0°C, in which a conception rate of 57% was obtained. Garcia-Olivares et al. (2016) a boar spermatozoa refrigeration study comparing temperatures less than zero (-3°C, -5°C, -7°C), and they found that negative temperatures allowed the sperm plasma membrane to adapt better to changes in fluidity without loss of selective permeability, thus improving cell quality.

Based on the assumption that physiological spermatozoa characteristics are species specific, it is necessary to study refrigeration methodologies for cat semen, the cells of which are more resistant to low temperatures compared to other species and which can maintain their quality throughout three weeks (Harris et al. 2001, Chatdarong et al. 2009).

Alternative extenders for sperm cooling, such as coconut water, have achieved promising results both because it is a sterile, natural solution and because it presents longevity in powdered form (Barros & Toniolli 2011, Lima et al. 2016). It has been used in a standardized manner (ACP-117® - Biotechnology, Fortaleza, Brazil), being free of variability of biological origin, and because it does not contain egg in its composition, it reduces sanitary barriers.

The objective of this work was to evaluate the sperm quality of domestic felines, comparing cooling temperatures of -1°C and 4°C and the storage times of 24 and 48 hours for spermatozoa obtained from ejaculate and recovery from the epididymis tail.

MATERIALS AND METHODS

Ethical and local aspects. This study was approved by the Ethical Commission on the Use of Institutional Animals (CEUA), number 7099.2015.53.

Animals. Spermatozoa samples of twenty-nine domestic cats adult, mongrel, semi-domiciled domestic cats in good nutritional status, weighing from 2 to 6 kg and clinically healthy, were used from November 2015 to February 2016.

Experimental design. In order to compare the two cooling temperatures, the first step was to analyze if there was a difference between the sperm harvesting techniques of the 29 animals. After this, two experiments were conducted: in the first, sperm samples of 14 cats were used, and sperm cooling was performed at -1°C; and in the second, samples of 15 cats were used, and the spermatozoa were refrigerated at 4°C. Both were refrigerated in a domestic refrigerator which the thermostat was set to proper temperatures and checked daily.

In both experiments the harvesting and processing of sperm was performed in the same way. Initially, electroejaculation was performed and than, 48 hours later, elective orchiectomy was done and spermatozoa were recovered from the epididymis tail. The samples were diluted in ACP-117® extender (Biotechnology, Fortaleza, Brazil) and spermatozoa characteristics were evaluated at three different moments: when still fresh; after 24 and 48 hours of cooling.

Materials collected. By electroejaculation (EEJ): an anesthetic protocol with ketamine (12mg/kg - Dopalen®, Ceva, Brazil) associated with medetomidine (30µg/kg - Domitor®, Pfizer, France) was used. After anesthesia, urethral catheterization was performed with a 24G catheter coupled to a 1.5mL polypropylene microtube. The electrical impulse was produced by a transrectal probe, with two

longitudinal electrodes (0.9cm in diameter and 11cm in total length, inserted 2cm into the rectum) positioned above the prostate. The electroejaculation protocol was based on that described by Howard et al. (1990).

By epididymal sperm recovery (EPD): after elective orchiectomy, the vascularization of the duct deferens was isolated and with the aid of a hemostatic clamp, the epididymal tail was compressed to obtain the epididymal fluid in a Petri dish containing 100µL of 0.9% NaCl solution for each epididymis, with a final volume of 200µL per animal.

Sperm processing. The spermatic evaluation was performed at three different moments (when still fresh; after 24 and 48 hours of cooling. After fresh analysis, the samples were centrifuged at 300g for 10 minutes (MiniSpin Plus® Microcentrifuge - Eppendorf, São Paulo/SP, Brazil). The supernatant was discarded and the pellet resuspended with ACP-117 extender (pH 7.43, 297mOsm/kg H_2O, PL = 3.43g), prepared according to the manufacturer's recommendations, to the final concentration of 40 x 10^6 sperm/mL. The samples were aliquoted into 1.5 mL plastic tubes, which were packed in a plastic container containing 200 mL of water at room temperature so that the contents of tubes were submerged and cooled to -1°C (Experiment 1) or 4°C (Experiment 2). It is important to note that at the temperature at -1°C, only a thin layer of ice crystals is formed on the surface of the liquid solution, not enough to freeze the spermatozoid.

Computed Sperm Analysis: the parameters of spermatic kinetics were analyzed by the CASA system (HTR-IVOS, version 14.0), according to feline setup (Table 1). Total motility (% - MT) were obtained; progressive motility (% - MP); average path velocity (µm/s - VAP); straight-line velocity (µm/s - VSL); curvilinear velocity (µm/s - VCL); amplitude of lateral head displacement (µm - ALH); straightness (% - STR); and linearity (% - LIN) (Niżański et al. 2016). Subsequently, the sperm movement index (SMI = (VSL x 0.59) + (VAP x 0.37) + (LIN x 0.95) + (STR x 0.89) + (WOB x 0.83)) and sperm velocity index (SVI = (VCL x 0.87) + (VSL x 0.76) + (VAP x 0.90) + (ALH x 0.92)) were calculated according to Agarwal et al. (2003), modified by Núñez-Martinez et al. (2006), and the oscillation of spermatic movement, wobble (WOB) was calculated by the index VAP/VCL x 100.

Table 1. Setup used in HTR-IVOS 14.0 for the evaluation of feline spermatozoa samples

Parameters	Values
Chamber type	Cell-Vu®
Temperature, set (°C)	37
Frames acquired	30
Frame rate (hz)	60
Number of frames	5
Minimum contrast	40
Minimum cell size (pixels)	4
Straightness (STR), Threshold (%)	80
Vap cutoff (µm/s)	30
Prog. min VAP (µm/s)	70
VSL cutoff (µm/s)	20
Cell intensity	50
Magnification	1.89

STR = straightness, VAP = average path velocity, VSL = straight-line velocity

Concentration by the Neubauer Chamber: a 1:200 dilution of the semen sample was used in distilled water and was read in a Neubauer chamber under a phase contrast optical microscope at 200x magnification. The result was expressed as sperm count x 10^6/mL.

Spermatic Morphology: for sperm morphology evaluations, smears of sperm samples were performed on glass slides and stained with modified Karras (Papa 1988). A total of 100 sperm cells were evaluated using a 1000x magnification optical microscope. The morphological alterations were classified into major and minor defects and divided into defects of the head, intermediate piece, tail and acrosome. The results were expressed as a percentage.

Membrane Integrity: for the evaluation of membrane integrity, smears were performed with 3µL of sample plus 3µL of eosin nigrosine dye. A total of 100 cells per slide were counted under light microscopy (1000x). Sperm cells were classified as having an intact membrane when translucent or damaged when they had heads with pink coloration. The results were expressed as percentages of spermatozoa.

Statistical analysis. The results obtained were analyzed in R software, version 3.2.5. The non-parametric Mann-Whitney test was used to compare the refrigeration temperatures of -1°C and 4°C and the collection techniques used (EEJ and EPD), and Friedman's test was used to compare cooling times (when still fresh, at 24h and at 48h). The level of significance considered for all of the tests was 5%.

RESULTS

For the two experiments the ejaculate volume obtained was on average 50µL (ranging from 10 to 90µL) and the volume for the spermatic samples collected by EPD was predetermined at 200µL.

First stage: comparison with sperm harvesting techniques. The ejaculate (EEJ) and epididymal tail (EPD) sperm samples of both temperatures were compared. There was no difference in motility with the use of ACP-117®, as presented in Table 2. While fresh, there was a significant difference in the sperm between EEJ and EPD regarding the mean movement of cells in ALH, STR, LIN and SMI, as well as in the acrosome integrity evaluation. After 24 hours of refrigeration, significant differences were detected in BCF and STR, while after 48 hours, in addition to significant differences in BCF and STR, differences in slow and static sperm motility, SVI and total morphological defects were identified.

Second stage: comparison between the cooling temperatures of the two experiments. Regarding the kinetic parameters evaluated, after refrigeration at -1 and 4°C the spermatozoa collected by EEJ showed no difference. In the sample EPD harvest, spermatozoa showed a more progressive and linear movement characteristic at 4°C (Table 3), compared to cooling at -1°C (Table 4). However, when the morphology was evaluated, a significant difference was observed in the percentages of normal cells ($P<0.026$), major defects ($P<0.022$) and total defects ($P<0.026$) between the two refrigeration temperatures, and the results at -1°C above 4°C.

To identify the decrease in sperm quality, the reduction calculation of parameters among the moments (F-24h/F-48h/24 h-48h) at each (-1°C and 4°C) cooling temperatures (Tables 5 and 6) was performed. In samples from ejaculate, the percentage of minor defects showed a more pronounced reduction in the group refrigerated at 4°C. In epididymis spermatozoa, the parameters of MT, MP, velocity, ALH, SVI and SMI were difference between fresh and 24 hours of cooling,

and the sperm quality reduction was superior in the group refrigerated at 4°C in all of the parameters.

DISCUSSION

First stage: comparison with sperm harvesting techniques. Concerning the spermatozoa collection method, in the ejaculate, it was possible to obtain better results regarding rectilinearity (STR) at three different moments (when still fresh; at 24h and at 48h). Epididymal spermatozoa were significantly fewer than those in ejaculate, likely because they were recovered in physiological solution and initially presented lower sperm quality. It was necessary for a period to equilibrate the solution, and although it did not alter the pH or osmotic pressure, because no membrane changes were observed, the solution decreased the motility in a non-deleterious manner, and after addition of the diluent, it stabilized.

The higher percentage of damaged cells in the membrane integrity assessment of the ejaculate in the fresh samples could be associated with the time that the spermatozoa were in contact with seminal plasma since it is already known that there are proteins of higher molecular weight that can promote spermatic membrane lesions (Martins et al. 2004), in contrast to what occurred with epididymal spermatozoa, which were recovered in a shorter period of time and in physiological solution (Prochowska et al. 2015).

Second stage: comparison between the cooling temperatures of the two experiments. The results of the evaluations of kinetic parameters after cooling of samples feline spermatozoa for up to 48 hours at -1°C or 4°C suggest that the sperm viability is maintained at both cooling temperatures (-1 and 4°C). Authors recommend cooling to 4°C (Pukazhenthi et al. 1999, Zambelli et al. 2002, Tebet et al. 2006, Nunes et al. 2008, Nunes 2010, Buranaamnuay 2017). However the present article has shown that cooling at -1°C is an option especially when there are reproducers with the need to preserve the spermatic morphology after cooling. A greater percentage of total sperm defects was detected when the sperm samples were cooled at 4°C ($P<0.026$), showing that the temperature at -1°C caused less cryolysis, which could be explained by the rapid adaptability of the cells to lower temperatures (Rijsselaere et al. 2007, Buranaamnuay 2015).

The results of the reduction in the percentage of sperm parameters showed a decrease after cooling at both temperatures. It was observed that there was a significant reduction when comparing fresh/24 hours of refrigeration and fresh/48 hours of refrigeration, but there was no difference between 24 and 48 hours of refrigeration, suggesting cell stabilization at low temperature after the first few hours of refrigeration. Similar results were obtained by VillaVerde et al. (2006),

Table 2. Comparison of samples spermatozoa kinetics cooled using coconut water extender (ACP-117®) obtained by electroejaculation (EEJ) and retrieved from the epididymis tail (EPD) at three moments (fresh, 24 and 48 hours)

Sperm characteristics	Refrigeration time					
	Fresh		24 hours		48 hours	
	EE	EPD	EE	EPD	EE	EPD
MT (%)	67.6 ± 17.7	69 ± 15.3	33.9 ± 22	34.1 ± 21.7	30.3 ± 22.0	22.9 ± 20.4
MP (%)	43.5 ± 17.5	32.2 ± 13.5	21.1 ± 16.4	15.1 ± 12.6	11.9 ± 11.7	9.3 ± 11.9
VAP (µm/s)	139.2 ± 29.6	121.7 ± 31.8	98.7 ± 18.6	97.5 ± 25.8	75.8 ± 18.6	81.9 ± 36.4
VSL (µm/s)	120 ± 30.1	99.6 ± 28.9	85 ± 15.9	77.6 ± 21.8	62.2 ± 13.9	63.2 ± 30.4
VCL (µm/s)	184.5 ± 61.7	190.5 ± 34.6	162 ± 26	162.2 ± 28.9	138.4 ± 29.9	142.8 ± 50.2
ALH (µm)	6.1 ± 0.6*	6.6 ± 0.6*	5.2 ± 1.1	5.2 ± 1.4	5.2 ± 0.7	4.2 ± 2.6
BCF (Hz)	34.3 ± 4.9	32.9 ± 4.3	41.5 ± 4.1*	36.6 ± 5.4*	37.8 ± 3.6*	31.7 ± 10*
STR (%)	83.7 ± 5.3*	79.9 ± 3.9*	85.1 ± 3.7*	79.1 ± 7.9*	81.8 ± 4.8*	71.9 ± 21.4*
LIN (%)	59.2 ± 10.1*	51.6 ± 8.1*	53.3 ± 8.8	48.9 ± 10.6	46.6 ± 5.2	43 ± 17.2
AREA	6.8 ± 0.7	6.9 ± 1.4	5.6 ± 0.6	5.6 ± 0.7	5.9 ± 1	5 ± 1.5
WOB	69.3 ± 9.2	63.2 ± 7.9	61 ± 7.45	59.9 ± 10.7	54.6 ± 3.6	57.1 ± 13.9
SVI	395.6 ± 65.1	357 ± 78.5	299.2 ± 48.4	292.6 ± 59.4	240.6 ± 52.4*	292.6 ± 59.4*
SMI	310.5 ± 48.1*	276.3 ± 48.1*	263.7 ± 28.3	276.3 ± 43.4	227.1 ± 19.3	232.3 ± 47.8
Morphology						
Normal cells	51.5 ± 26.1	54.2 ± 15.9	43.8 ± 23.9	42.9 ± 18.7	39 ± 20.5	39.5 ± 20.8
Major defects	31.1 ± 23.1	19.7 ± 11.7	36 ± 20.1	28.7 ± 14.4	37 ± 20.1	32.2 ± 20
Minor defects	17.4 ± 11	27.2 ± 14.6	20.2 ± 8.9	28.3 ± 14.3	47 ± 28.7	28.1 ± 11,9
Total defects	48.7 ± 26.1	44.9 ± 17.7	56.2 ± 23.9	57.1 ± 18.7	38 ± 19.8*	60.4 ± 20.8*
Integrity membrane						
Injured	10.7 ± 6.8*	5.9 ± 3.4*	13.9 ± 7.7	8.9 ± 5.1	13.9 ± 8.5	11.1 ± 6.1

* Significant difference between the corresponding columns ($P<0.05$); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN =linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

who observed decreases in total and progressive motility 24 hours after refrigeration.

The SVI and SMI indices showed greater reductions in refrigerated spermatozoa at 4°C. Considering that the best results with cryopreservation are related to higher rates of movement and velocity, since they represent the biological status of spermatozoa (Núñez-Martínez et al. 2006, Niżański et al. 2016) it can be inferred that the results obtained in the cooled epididymal spermatozoa at 4°C could compromise the results of feline breeding biotechniques. Spermatozoa cooled to -1°C presented acceptable results according to standards CBRA (2013) in both sample sperm kinetics and normal cell percentage, demonstrating superior quality in analyzed parameters.

It is believed that the results obtained from cooling at -1°C were due to the physiological characteristics of the feline spermatozoa, the plasma membrane of which is better adapted and provides membrane fluidity at lower temperatures (Garcia-

Table 3. Results of the median (minimum-maximum) analyses of samples domestic feline spermatozoa collected by electroejaculation (EEJ) and from the epididymal tail (EPD), fresh and after refrigeration at 4°C for 24 hours and 48 hours

Sperm characteristics	Refrigeration 4°C					
	Electroejaculation			Epididymis		
	Fresh	24 h	48h	Fresh	24 h	48 h
MT (%)	61.5 (34 - 72)	17 (8 - 40)	17 (6 - 20)	71 (46 - 92)	23 (2 - 68)	10 (0 - 61)
MP (%)	34.5 (16 - 44)	8.5 (5 - 21)	4.5 (2 - 8)	38 (22 - 59)*	8 (1 - 41)	2 (0 -39)
VAP (µm/s)	121.3 (99.8 - 148.7)	98.3 (82.7 - 126)	70.7 (56.9 - 100.4)	142.4 (109.5 - 170.3)*	101.1 (61.4 - 151.4)	76.4 (0 - 132.4)
VSL (µm/s)	104.8 (82.2 - 123)	89 (68.8 - 101.5)	59.2 (50.1 - 73.1)	120.1 (89 - 144.5)*	76 (46.4 - 127.6)	60.6 (0 - 112.5)
VCL (µm/s)	186.3 (16.5 - 203.9)	151 (137.9 - 205.3)	128.3 (100.9 - 184.9)	217.1 (171.1 - 253.8)*	162.1 (106.4 - 193.6)	148.7 (0 - 191.6)
ALH (µm)	6 (5.6 - 6.4)	5.2 (2.7 - 5.9)	4.8 (4.2 - 5.4)	6.7 (5.6 - 7.7)	4.8 (3.4 - 6.5)	4 (0 - 7,2)*
BCF (Hz)	37.4 (26.7 - 40.2)	42.1 (37.7 - 47.4)	38.9 (37.1 - 41.1)	32.5 (21.8 - 39.7)	38 (28.9 - 50.3)	30.8 (0 - 41.2)
STR (%)	82 (79 - 86)	84.5 (80 - 91)	82.5 (73 - 88)	80 (76 - 87)	83 (68 - 92)	77 (0 - 95)
LIN (%)	56 (50 - 58)	57.5 (43 - 68)	48.5 (41 - 53)	55 (45 - 66)*	56 (36 - 71)*	44 (0 - 75)
Area	6.9 (6.5 - 8.2)	17 (8 - 40)	17 (6 - 20)	6.3 (5.2 - 7.9)	5.1 (4.4 - 6.8)	5 (0 - 6,5)*
WOB	61.5 (34 - 72)	65.8 (51.3 - 70.7)	55.1 (54.2 - 56.3)	67.3 (58.1 - 79.3)*	62.8 (46.1 - 85.1)	68.1 (39.2 - 85.5)*
SVI	357.9 (302.2 - 407.8)	280.8 (272.1 - 374.5)	224.7 (181.2 - 311.4)	402.7 (325.4 - 482.7)*	296.4 (198.9-392.2)	288 (97.2 - 346.4)
SMI	288.8 (254.7 - 314.4)	280.3 (227.6 - 295)	226.3 (226 - 229.2)	312.9 (258.8 - 350.9)*	246.4 (185.4-343.3)	236.7 (170.6 - 332.7)
Morphology						
Normal cells	30 (14 - 55)*	26.5 (13 - 37)*	28 (7 - 36)*	55 (36 - 73)	46 (14 - 66)	46 (12 - 71)
Major defects	55.5 (22 - 72)*	52.5 (36 - 65)*	46.5 (37 - 77)	25 (9 - 48)	29 (13- 60)	24 (7 - 60)
Minor defects	19 (4 - 24)	22 (14 - 33)	22 (16 - 34)	18 (9 - 29)*	25 (17 - 44)	28 (12 - 53)
Total defects	70 (45 - 86)*	73.5 (63 - 87)*	72 (64 - 93)*	45 (27 - 64)	54 (34 - 86)	54 (29 - 88)
Integrity membrane						
Injured	8 (3 - 16)	8 (5 - 16)	5 (3 - 18)	4 (1 - 13)	8 (1 - 21)	12 (1 - 30)

* Significant difference between the corresponding columns of Table 3 and 4 (P<0.05); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN = linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

Olivares et al. 2016), because during cryopreservation, the plasma membrane of the spermatozoon undergoes a series of changes related to temperature changes, progressively changing from the crystalline liquid phase to the gel phase, thus increasing the cell protection (Hazel 1995).

The superior results obtained with cooling at -1°C suggested that feline spermatozoa can be resistant to lower temperatures. This resistance of sperm cells to thermal stress could be attributed to the lower damage of domestic cats' spermatozoa, as well as that identified in dogs, likely caused by small proportions of polyunsaturated and saturated phospholipid fatty acids (White 1993, Rodenas et al. 2014). Lower cooling temperatures reduce the cells' metabolism, reaching near basal, allowing for greater survival of the more cold-resistant cells. In addition, considering that the

Table 4. Results of median (minimum-maximum) analyses of samples domestic cats spermatozoa collected by electroejaculation (EEJ) and from the epididymal tail (EPD), fresh and after refrigeration evaluations at -1°C for 24 hours and 48 hours

Sperm characteristics	Refrigeration -1°C					
	Electroejaculation			Epididymis		
	Fresh	24 h	48h	Fresh	24 h	48 h
MT (%)	77.5 (50 - 91)	46 (10 - 75)	43.5 (4 - 64)	66 (32 - 92)	44 (7 - 77)	24 (6 - 68)
MP (%)	49.5 (27 - 75)	26 (2 - 56)	14.5 (1 - 36)	29.5 (6 - 60)*	17.5 (0 - 37)	5 (34 - 0)
VAP (µm/s)	160.6 (109.8 - 185.2)	106.6 (60.1 - 115.1)	67.1 (60.1 - 109.6)	96.7 (68.6 - 152)*	95.1 (60.2 - 126.4)	83.7 (53.8 - 163.6)
VSL (µm/s)	140.5 (81.1 - 171.8)	91.9 (52.7 - 99.3)	54.5 (49.4 - 91.6)	78.3 (52.4 - 124.2)*	71.9 (44.3 - 106.4)	59 (40.3 - 136.4)
VCL (µm/s)	208.5 (178 - 234.7)	167.5 (115.2 - 189.3)	133.4 (107.9 - 175.3)	160.7 (125.1 - 222.2)*	176.2 (111.1 - 204.6)	166.7 (108.2 - 217.5)
ALH (µm)	5.9 (5.5 - 7.6)	5.3 (4.5 - 6.8)	5.6 (4.2 - 6.1)	6.6 (5.6 - 7.5)	6.1 (0 - 7.3)	5.7 (0 - 7.3)*
BCF (Hz)	33.8 (26.9 - 40.6)	40.2 (36.4 - 46.7)	37.2 (31.4 - 42.9)	33.9 (31 - 41.3)	37.5 (23.9 - 41.2)	35.4 (27.6 - 41.1)
STR (%)	86 (72 - 91)	84.5 (81 - 90)	82.5 (75 - 87)	77.5 (74 - 88)	77.5 (63 - 90)	78.5 (63 - 89)
LIN (%)	66 (39 - 72)	51 (43 - 61)	45 (37 - 53)	45 (38 - 60)*	44 (33 - 56)*	42 (28 - 61)
Area	6.5 (6 - 7.6)	46 (10 - 75)	43.5 (4 - 64)	7 (5.5 - 10.7)	6 (4.4 - 6.7)	5.4 (4.7 - 6.3)*
WOB	76.1 (52.7 - 80.7)	59.7 (52.1 - 66.4)	53.1 (48.2 - 62.5)	55.5 (50.9 - 69.7)*	54.8 (44.1 - 65.8)	50.5 (41.7 - 75.2)*
SVI	436.2 (342.3 - 506.8)	315.4 (198.5 - 337.6)	222.7 (194 - 325.5)	296 (216.9 - 426.1)*	299.1 (198.3 - 368.3)	277.3 (178.6 - 445)
SMI	344.3 (233.3 - 384.7)	267.1 (217.7 - 289.1)	222.5 (198.1 - 270.7)	246.7 (204.4 - 313.6)*	232 (182.2 - 290.9)	211.7 (171.3 - 333.4)
Morphology						
Normal cells	63.5 (38 - 92)*	58 (29 - 84)*	59 (20 - 65)*	58.5 (16 - 84)	38.5 (9 - 81)	28.5 (5 - 78)
Major defects	17.5 (4 - 32)*	16 (9 - 45)*	19 (12 - 41)	12 (0 - 36)	25.5 (8 - 63)	37.5 (10 - 82)
Minor defects	18 (4 - 37)	17 (7 - 31)	20 (17 - 39)	40.5 (5 - 60)*	22 (10 - 71)	29 (10 -56)
Total defects	36.5 (8 - 62)*	42 (16 - 71)*	41 (35 - 80)*	46.5 (6 - 84)	61.5 (19 - 91)	71.5 (22 - 95)
Integrity membrane						
Injured	10 (5 - 25)	15 (11 - 29)	17 (14 - 30)	6.5 (3 - 13)	7.5 (3 - 24)	9.5 (4- 19)

* Significant difference between the corresponding columns of Table 3 and 4 (P<0.05); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN =linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble

extender used caused membrane stabilization during the cooling process, it reduced the damage related to cold shock.

The present study demonstrated that the use of ACP-117® maintained the viability of the refrigerated spermatozoa obtained by electroejaculation, similar to those obtained from the epididymis tail, suggesting that it could be another low-cost and easy to obtain alternative for cooling feline semen since coconut water-based extender has been described only for the preservation of spermatozoa recovered from the epididymis tail or collected with an artificial vagina (Silva 2008, Lima et al. 2014, Lima et al 2016).

Cooling of feline semen at a temperature of -1°C resulted in lower cryolysis, mainly in total morphological defects, allowing for a smaller reduction in sperm quality after 48 hours of cooling, different from results available in the literature that determined 4°C as the ideal temperature for spermatic cooling (Goodrowe & Hay 1993, Pukazhenthi et al 1999, Harris et al. 2001).

Table 5. Percentage reduction in median values (min-maximum) among fresh (F), 24 hours and 48 hours of refrigeration at -1°C of samples domestic feline spermatozoa collected by electroejaculation (EEJ) and from the epidydimal tail (EPD)

Sperm characteristics refrigeration at -1°C	Electroejaculation			Epididymis		
	F - 24h	F - 48h	24h - 48h	F - 24h	F - 48h	24h - 48h
MT (%)	29.5 (7 - 56)	24.5 (14 - 62)	4.5 (-23 - 25)	26.5 (-1 - 59)*	33.5 (15 - 72)*	9.5 (-7 - 33)
MP (%)	23.5 (-3 - 50)	36 (-1 - 59)	35 (-1 - 30)	7 (-16 - 38)*	11 (-10 - 41)*	5 (-9 - 19)
VAP (µm/s)	61.6 (0.4 - 92.5)	75.9 (15.9 - 119.5)	20.2 (-7.6 - 46.6)	-1.1 (-30.9 - 44.3)*	4.8 (-32.3 - 58)*	11.3 (-51 - 31.2)
VSL (µm/s)	55.7 (3.6 - 81.1)	68.1 (3.6 - 115.9)	20.5 (-7.3 - 46.9)	3.8 (-29.7 - 38.2)*	7.2 (-34.2 - 49.4)*	9.8 (-46.6 - 29.7)
VCL (µm/s)	41 (2.2 - 88.1)	68.1 (33 - 109.9)	24 (10.5 - 53.9)	-5.9 (-60.9 - 69.9)*	-6.2 (-55.7 - 86.5)*	11.8 (-18.9 - 32.4)
ALH (µm)	1 (-0.6 - 1.4)	0.6 (-0.10 - 1.5)	-0.3 (-1.5 - 1.4)	0.8 (-1.3 - 7.5)*	0.6 (-0.7 - 7.5)*	0 (-1.1 - 4.8)
BCF (Hz)	-6.2 (-15.1 - -1)	-1.5 (-16 - 3.4)	2.9 (-4.3 - 15.3)	-2.3 (-7 - 9.4)	-0.8 (-7.7 - 8.2)	0.5 (-10 - 6.9)
STR (%)	1 (-9 - 4)	4 (-10 - 13)	3 (-1 - 9)	0.5 (-6 - 14)	1 (-8 - 13)	-0.5 (-4 - 11)
LIN (%)	13 (-4 - 20)	18 (-6 - 27)	4 (-5.0 - 18)	5.5 (-7 - 14)	7 (-11 - 13)	1.5 (-18 - 9)
AREA	0.9 (-0.3 - 1.2)	0.4 (-1.3 - 2.1)	-0.6 (-1.7 - 1.3)	1.2 (-0.6 - 4.6)	1.5 (0.1 - 5.5)	0.4 (-0.4 - 1.4)
WOB	15.1 (-0.6 - 22.9)	18 (-0.8 - 28.2)	4.6 (-5.9 - 17.2)	3.6 (-4.3 - 14.7)	6.4 (-8.5 - 16.3)	3.4 (-20.1 - 14.1)
SVI	127 (5.7 - 222.8)	180.98 (47.1 - 291.5)	54.2 (21.4 - 121.6)	-0.9 (-87.1 - 122.3)*	-4.1 (-81.5 - 166)	22.5 (-90.7 - 75.5)
SMI	89.1 (3.6 - 119.8)	101.8 (-7.3 - 165.3)	28.2 (-10.9 - 84.3)	17.1 (-44.2 - 63.9)*	23.1 (-56.8 - 70.4)*	16 (-83.7 - 48.1)
Morphology						
Normal cells	11.5 (-5 - 55)	20.5 (-3 - 55)	5.5 (-1 - 20)	3.5 (-22 - 72)	10 (-20 - 62)	10.5 (-15 - 25)
Major defects	- 5 (-20 - 27)	-7 (-16 - 27)	-0.5 (-10 - 4)	-10.5 (-55 - 3)	-20 (-65 - 11)*	-5.5 (-26 - 15)
Minor defects	3.5 (-13 - 18)	-6.5 (-16 - 18)	-5 (-13 - 2)*	11 (-63 - 93)*	14.5 (-48 - 32)*	0.5 (-23 - 31)
Total defects	-11.5 (-55 - 5)	-20.5 (-55 - 3)	-5.5 (-20 - 1)	-3.5 (-72 - 22)	-10 (-62 - 20)	-10.5 (-25 - 15)
Integrity membrane						
Injured	-4 (-14 - 15)	-6.5 (-12 - 15)	-2 (-6 - 7)	-3 (-20 - 8)	-3 (-13 - 8)*	0 (-7 - 7)*

* Significant difference between the corresponding columns of Table 5 and 6 (P<0.05); substituir por MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN = linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

Table 6. Percentage reduction in median values (minimum-maximum) among fresh, 24 hours and 48 hours of refrigeration at 4°C of samples domestic feline spermatozoa collected by electroejaculation and from epididymis tail (EPD)

Sperm characteristics refrigeration at 4°C	Electroejaculation			Epididymis		
	F - 24h	F - 48h	24h - 48h	F - 24h	F - 48h	24h - 48h
MT (%)	31.5 (26 - 58)	44 (28 - 53)	3.5 (-5 - 20)	51 (-20 - 68)*	60 (-15 - 76)*	9 (-1 - 35)
MP (%)	23.5 (11 - 28)	30.0 (14 - 36)	20 (3 - 13)	33 (-12 - 52)*	38 (-17 - 51)*	7 (-5 - 20)
VAP (µm/s)	17 (2.3 - 49.6)	38.8 (28.9 - 91.8)	26.4 (11.4 - 42.2)	56.6 (-32.2 - 108.7)*	76.9 (-22.9 - 168.9)*	15.2 (-12.4 - 73)
VSL (µm/s)	19.7 (-3.6 - 30.8)	38.6 (23.1 - 72.9)	27.5 (9.4 - 42.1)	42 (-34.3 - 91.1)*	60.1 (-22.9 - 144.3)*	14.8 (-7.3 - 64.2)
VCL (µm/s)	4.7 (-121.4 - 59.8)	31 (-112 - 99.8)	26.6 (9.4 - 40)	61.9 (-17.1 - 118.8)*	74.2 (17.7 - 212.8)*	21.9 (-55.6 - 106.7)
ALH (µm)	1 (0.1 - 2.9)	1.4 (0.2 - 1.9)	0.5 (-2.7 - 1.5)	1.6 (0.4 - 3.8)*	2.7 (-0.1 - 7.1)*	0.9 (-3 - 6.5)
BCF (Hz)	-6.6 (-11.0 - -3.3)	-0.35 (-14.4 - 0.9)	4.3 (-3.4 - 8.1)	-6.1 (-23.6 - 5.9)	0.7 (-16.1 - 33)	4.8 (-6.3 - 41.3)
STR (%)	-1.5 (-12 - 4)	1 (-9 - 10)	3.5 (0 - 7)	-2 (-10 - 14)	6 (-16 - 85)	5 (-7 - 92)
LIN (%)	-3.5 (-14 - 15)	7.5 (1 - 13)	12.5 (-5 - 15)	2 (-25 - 24)	8 (-21 - 66)	5 (-15 - 57)
AREA	1.4 (1.1 - 3.2)	1.65 (1.2 - 3.2)	0.05 (-0.1 - 0.6)	0.8 (-0.1 - 2.8)	0.9 (-0.3 - 5.8)	0.2 (-1.5 - 5.1)
WOB	2.9 (-10.7 - 14.6)	9.7 (5.3 - 17.6)	10.5 (-4.2 - 16)	5.5 (-26.8 - 30.1)	14 (-21.3 - 79.3)	6.1 (-12.1 - 61.3)
SVI	43.7 (8.6 - 121.5)	92.6 (71.7 - 226.5)	58.8 (47.4 - 105)	154.4 (-57.2 - 251.9)*	185.6 (-20.9 - 452.3)	36.2 (-55.1 - 200.3)
SMI	16.4 (-28.8 - 59.4)	61.1 (28.1 - 88.4)	52.4 (1.4 - 68.9)	36.6 (-84.4 - 138.1)*	79.6 (-64 - 350.9)*	19.2 (-27.2 - 246.4)
Morphology						
Normal cells	1.5 (-1 - 24)	3.5 (-5 - 28)	2.5 (-7 - 6)	12 (-48.0 - 45)	8 (-49 - 47)	1 (-31 - 31)
Major defects	-1.5 (-14 - 16)	-5 (-27 - 35)	-3.5 (-13 - 19)	-7 (-29 - 21)	1 (-38 - 31)*	0 (-19 - 27)
Minor defects	-4.5 (-18 - 2)	-3.5 (-28 -5)	-49.5 (-71 - -40)*	-9 (-37 - 5)*	-9 (-39 - 11)*	3 (-30 - 16)
Total defects	-1.5 (-24 - 1)	43 (21 - 70)	-2.5 (-6 - 7)	-12 (-45 - 48)	-8 (-47 - 49)	-1 (-31 - 31)
Integrity membrane						
Injured	0.5 (-13 - 10)	4.5 (-15 - 10)	1 (-2 - 6)	-3 (-16 - 5)	-4 (-25 - 1)*	-2 (-19 - 7)*

* Significant difference between the corresponding columns of Table 5 and 6 (P<0.05); MT = total motility, MP = progressive motility, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, STR = straightness, LIN =linearity, SMI = sperm movement index, SVI = sperm velocity index, WOB = wobble.

CONCLUSION

The refrigeration of cat spermatozoa samples at -1°C for up to 48 hours was efficient to maintain the quality of sperm cells obtained from ejaculate and the epididymis tail and could be an alternative temperature to the cooling of domestic feline spermatozoa.

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