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Effect of Sperm Selection Techniques in Frozen/Thawed Cat Spermatozoa on Sperm Motility Analyzed by CASA System

Efecto de Técnicas de Selección Espermática en Espermatozoides Congelados/Descongelados de Gato Sobre la Motilidad Espermática Analizada Mediante sistema CASA

C. Cheuquemán[1]; R. Sánchez[1,2] & J. Risopatrón[1,3]

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SUMMARY: Freeze/thawing process reduces sperm survival and fertilizing ability of cat spermatozoa, with sperm motility being the most sensitive sperm parameter altered, due to cryo-damage. In this context, swim-up and density gradient processing methods can help to recover high motile and normal spermatozoa. Maximizing the use of frozen semen sample is essential, especially in endangered felids or high value cats in which sample size, number of samples or access to semen collection is reduced. To our knowledge, there is no previous report describing an in depth analysis of sperm motility improvement, after sperm selection techniques in frozen cat semen. Accordingly, we evaluated the effect of percoll gradient (PG) and swim up (SU) sperm selection techniques on sperm motility parameters and sperm recovery rate in frozen/thawed spermatozoa of domestic cat. Next, we evaluated the individual effect of the cat over sperm motility after PG sperm selection of frozen/thawed spermatozoa. SU and PG improved significantly all sperm motility parameters of frozen/thawed cat spermatozoa compared to simple washing. However, PG allows better sperm recovery from the original frozen sample and works mostly homogeneously among individual cats. This new information could help to maximize the use of frozen semen in endangered felids or high value domestic cats for its subsequent application on *in vitro* fertilization and artificial insemination.

KEY WORDS: Motility; Percoll gradient; Swim up; Frozen semen; Cat spermatozoa.

INTRODUCTION

Sperm cryopreservation allows for long-term storage of genetics in germplasm banks, facilitating offspring propagation using ARTs like artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer (ET) and intracytoplasmic sperm injection (ICSI) (Chatdarong, 2017). However, freeze/thawing process reduces sperm survival and fertilizing ability of cat spermatozoa (Luvoni, 2006; Thiangtum *et al*., 2009; Terrell *et al*., 2012), being sperm motility the most sensitive sperm parameter altered due to cryo-damage (Filliers *et al*., 2008).

In that sense, loss of sperm function can be mitigated by post-thaw swim-up or density gradient processing methods that selectively recover motile or structurally-normal spermatozoa (Terrell *et al*.). Sperm preparation methods have an important role in determining the subsequent blastocyst quality during *in vitro* embryo production (Samardzija *et al*., 2006) by elimination of epithelial and red blood cells, leukocytes, bacteria or cell debris from contaminated semen, improving sperm quality (Crosier *et al*., 2009; Chatdarong *et al*., 2010).

To our knowledge, there is no previous report describing a deep analysis of sperm motility improvement after sperm selection techniques in frozen cat semen. Accordingly, our first aim was to evaluate the effect of percoll gradient and swim up sperm selection techniques on sperm motility parameters, and sperm recovery in frozen/thawed spermatozoa of domestic cat. Next, we evaluated the individual effect of the cat over sperm motility after percoll gradient sperm selection of frozen/thawed spermatozoa.

[1] Center of Biotechnology on Reproduction (BIOREN-CEBIOR), Faculty of Medicine, University of La Frontera, Temuco, Chile.
[2] Department of Preclinical Sciences, Faculty of Medicine, University of La Frontera, Temuco, Chile.
[3] Department of Basic Sciences, Faculty of Medicine, University of La Frontera, Temuco, Chile.
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MATERIAL AND METHOD

Experimental design

Experiment 1: We evaluated the best sperm selection technique for frozen semen according to sperm recovery rate and sperm motility. A pool of frozen cat semen was divided in three subsamples and processed as follow: 1) Simple washing (control), 2) Percoll gradient (PG) and 3) Swim up technique (SU). For all groups sperm concentration and sperm motility was assessed.

Experiment 2: We evaluated the individual effect on sperm motility after percoll gradient selection for each cat. Frozen semen of each cat were thawed and divided in two subsamples that were processed as follow: 1) Simple washing (control) and 2) by PG. For all groups sperm motility was assessed.

Ethical approval: All experimental protocols received institutional review board approval by the Scientific Ethics Committee from the Universidad de La Frontera. According to the three R's of animal welfare, the ethical committee allowed us to use only three feline males to develop this study. All procedures were conducted according to Chilean Law No. 20.380 for Animal Protection. Animal care, housing and experimentation complied with the international guiding principles for biomedical research involving animals.

Animals. Sperm samples were obtained from three clinically healthy mix breed domestic tomcats of 4.1± 0.3 kg weight and 2-3 years old. All animals were housed in a controlled environment at the Reproductive Biotechnology Center (CEBIOR), Faculty of Medicine, Universidad de La Frontera, Temuco, Chile. The cats were individually housed in cages (size 2.25 x 1.2 x 1.2 m), fed with dry commercial cat food (Proplan, Purina, Chile), drinking water *ad libitum* and exercised daily throughout the experiments, using environment enrichment with toys and plants, maintaining a friendly environment for cats to cover their behavior requirements (Rochlitz, 2005; Rodan, 2010; Gouveia *et al*., 2011). A veterinarian periodically checked the animals' health status and sanitary management (deworming and vaccinations). The study was performed from June to October of 2016, under natural photoperiod, in a ventilated room at 15-26 °C temperature.

Semen collection and evaluation. In each experimental trial, semen was collected once a week by electroejaculation into a prewarmed test tube. Electro-ejaculations were performed using a previously described protocol by Howard *et al*. (1990), with some modifications. After 12 hours of fasting, each male was clinically examined and pre-medicated with a subcutaneous injection of 0.02 mg/kg of atropine sulfate (Laboratorios Erma, Chile) and then anaesthetized by intramuscular injection of 10 mg/kg of Zoletil 50 (Zolazepam-Tiletamine hydrochloride) (Tebet *et al*., 2006). We used a custom probe electro-ejaculator model AC-1 (Beltron Instruments, Longmont, USA), with a diameter of 0.9 cm and length of 6 cm. The custom probe was lubricated with water-based lubricant and inserted 7-9 cm into the rectum, with the electrodes positioned ventrally. A 1.6 ml plastic tube was place over the penis. A total of 80 electrical stimuli divided in three series were applied: 1 (10 stimulation at 2, 3 and 4 Volts), 2 (10 stimulation at 3, 4 and 5 Volts) and 3 (10 stimulation at 4 and 5 Volts). Semen samples were immediately transported to the laboratory for sperm quality evaluations.

Volume of semen samples was evaluated using an adjustable micropipette. Sperm concentration was determined using a Neubauer counting chamber. Semen samples were centrifuged at 300 g for 3 min. Next, the pellet with spermatozoa was subject to freezing protocol.

S**perm motility evaluation by CASA.** Analysis was performed using an integrated sperm analysis system V1.0 (ISAS, PROISER®, Valencia, Spain). Samples were maintained at 37 °C on a heated plate (ISAS heat system, PROISER®, Valencia, Spain) 5 µl of sperm solution was mounted in a slide and coverslip. This system analyzed 25 consecutive and digitized photographic images obtained from a single field at 10x magnification and negative phase-contrast field in a microscope UB203i (PROISER®, Valencia, Spain). The set up parameters previously described for cats by Filliers *et al*. were considered for the analyzes. Five separate fields were taken from each sample for total motility (TMOT, %) and progressive motility (PMOT, %); average path (VAP, µm/s), curvilinear (VCL, µm/s), straight line (VSL, µm/s) velocities; linearity (LIN, %), straightness (STR, %), wobbler coefficient (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat/ cross-frequency (BCF, Hz).

Semen freezing. Slow freezing was performed according to Tsutsui *et al*. (2000), with some modifications. Briefly, the pellets of spermatozoa were re-suspended in EYT-FC 1 [1.3 g citric acid, 2.4 g TRIS, 1 g fructose, 100,000U penicillin, 0.1 g streptomycin, 20 % hen egg yolk in distillated water to 100 ml final volume] at 20 °C to obtain a concentration of 10 x 10^6/ml at room temperature. Semen samples were cooled in a water bath at 4 °C for one hour. After equilibration, samples were re-diluted (1:1v/v) with the YET-FC 2 [EYT-FC 1 supplemented with 14 % glycerol and 1 % Equex], reaching a final concentration of 7 %

glycerol, 0.5 % Equex and 5 x 10^6 spermatozoa/ml in a final volume of 100 µl per straw. The extended semen was packaged in 0.25 ml plastic straws and sealed with heat pins. The straws were then cryopreserved using a one-step freezing method described by Thiangtum *et al*. (2009). Briefly, the straws were stored horizontally on a metal rack in a Styrofoam box, 7 cm above the liquid nitrogen surface, for 10 min and then plunged into liquid nitrogen. Frozen straws were transferred into liquid nitrogen tank for storage until thawing. Samples remained in liquid nitrogen for at least 1 month before post-thaw analysis.

Experiment 1. Comparison of sperm selection techniques. In each experimental trial (N=8) six straws of cat semen (pool of 3 cats) were thawed in a water bath at 37 °C for 30 s and then divided in 3 subsamples of 200 µl that were processed as follow. For control samples freezing extenders were removed by simple washing in 400 µl Hepes-SOF (Zambelli *et al*., 2010), 300 xg during 3 min. Then, sperm pellet was analyzed for sperm concentration and sperm motility.

PG was prepared according to Filliers *et al*. with some modifications. Briefly, in an Eppendorf tube 100 µl of 90 % Percoll (P-4937, Sigma-Aldrich, Darmstadt, Germany) was added in the bottom of the tube. Next, a 100 µl upper layer of 45 % Percoll (90 % Percoll diluted 1:2 in Hepes-SOF) was added. Then, 200 µl of frozen/thawed semen was disposed over the two-percoll layers and the tube was centrifuged at 1000 xg during 20 min at room temperature (Fig. 1A). After that, 50µl containing the sperm pellet was washed in 300 µl of Hepes-SOF at 300 xg during 3 min. Then, sperm pellet was analyzed for sperm concentration and sperm motility.

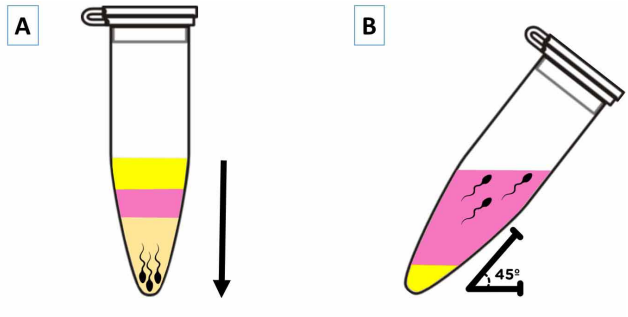

Fig. 1. Sperm selection techniques in frozen/thawed cat semen. A) Percoll gradient (PG): frozen semen (yellow); 45 % percoll layer (pink); 90 % percoll layer (orange); direction of sperm selection by centrifugation (black arrow). B) Swim up (SU): spermatozoa pellet (yellow); supernatant with selected spermatozoa by swimming (pink).

SU was prepared according to Spindler & Wildt (1999), with some modifications. Briefly, 200 µl of frozen/thawed semen was washed in 400 µl of Hepes-SOF at 300 xg during 3 min. Subsequently, the supernatant was removed and the sperm pellet was gently overlaid with 300 µl of Hepes-SOF in 45° inclination angle. These samples were incubated at 37 °C during 1 hour to allow motile spermatozoa to enter the supernatant. (Fig. 1B). After that, 250 µl of the supernatant with spermatozoa was recovered and concentrated by centrifugation at 300xg during 3 min; sperm pellet was then analyzed for sperm concentration and sperm motility.

Sperm recovery rate was calculated as a percentage of the total number of sperm recovered from each selection, divided by the initial number of sperm in the sample prior to sperm selection (control).

Experiment 2. Individual effect of PG on sperm motility. In each experimental trial (N=8) four straws of each cat were thawed in a water bath at 42 °C for 15 s and divided in 2 subsamples of 200 µl that were processed as follow: 1) Simple washing in Hepes-SOF (control) and 2) processed by PG, as in experiment 1. For all groups sperm motility was assessed by CASA.

Statistics. Data are provided as mean ± standard deviation (SD). D´Agostino and Pearson normality test was used to evaluate data normality.

Experiment 1: Sperm concentration and motility among groups were analyzed by One-way ANOVA and Tukey multiple comparison test. For comparison of sperm recovery rates between percoll and swim up techniques we used Student T-test. All statistical analyses were performed in GraphPad Prism 6.0 version software (San Diego, CA, USA). The level of significance was set at $P < 0.05$.

Experiment 2: Individual differences among cats after percoll gradient selection were measured using two- way ANOVA and Fisher's test (LSD). Data of sperm motility are presented as Media LS ± sigma LS. The level of significance was set at $P < 0.05$. All analyses were performed with STATGRAPHICS Centurion XVI.I 16.1.18 version software (Warrenton, VA, USA).

RESULTS

Experiment 1. Comparison of sperm selection techniques. Total sperm count recovered from each selection technique, were significantly lower in both sperm selection techniques compared to control samples. However, sperm recovery rate in GP were significantly higher than SU. Table I.

Table I. Effects of sperm selection methods on sperm recovery rate (Mean ± SD), in frozen/thawed cat spermatozoa processed by different sperm selection techniques (n = 8).

Method	Total sperm (x10⁶)	Recovery rate (%)
Simple washing	1.2 ± 0.2 [a]	100 [a]
Percoll	0.36 ± 0.06 [b]	29.9 ± 5 [b]
Swim up	0.16 ± 0.05 [c]	13.3 ± 4 [c]

[a, b, c] Different superscripts within columns indicate significant differences P < 0.05. The results are presented as mean ± standard deviation.

TMOT and PMOT were significantly higher in both sperm selection techniques compared to control samples. In addition, SU technique showed better PMOT than PG.

Sperm velocities (VAP, VCL, VSL) were significantly higher in both sperm selection techniques compared to control samples.

Linearity (LIN) was higher in SU than in PG and control samples. Straightness (STR) was similar among treatments.

Wobbler coefficient (WOB) was higher in SU than in control samples. Beat tail frequency (BCF) was lower in SU than in control samples. Lateral head displacement (ALH) was simmilar among treatments. All sperm motility parameters are summarized in Table II.

Experiment 2. PG selection among individual cats. PG selection compared to control samples, significantly increased TMOT (C: 64.4 ± 3.2 % and PG: 77.1 ± 3.2 %) and PMOT (C: 25.4± 4 % and PG: 41.4 ± 4 %) after semen thawing in all cats. The individual cat did not affect these sperm parameters.

PG selection significantly increased VSL (C: 48± 4.6 μm/s and PG: 67 ± 4.6 μm/s) and VAP (C: 57± 4.5 μm/s and PG: 77 ± 4.5 μm/s) after semen thawing in all cats. The individual cat did not change VSL or VAP. Similarly, the cat or the sperm selection technique did not affect VCL.

STR was affected by the cat and by the sperm selection technique. PG selection significantly increased STR (C: 83± 1 % y PG: 87 ± 1 %) after semen thawing in all cats. In addition, STR was significantly higher in B (86 ± 1.2 %) than in cat A (83 ± 1.2 %) (Fig. 2A).

LIN was affected by the cat and by the sperm selection technique. PG selection significantly increased LIN (C: 55± 1.6 % y PG: 66 ± 1.6 %) after semen thawing in all cats. In addition, LIN was significantly higher in C (65 ± 1.9 %) than in cat B (57 ± 1.9 %) (Fig. 2B).

WOB was affected by the cat and by the sperm selection technique. PG selection significantly increased WOB (C: 66± 1.4 % y PG: 77 ± 1.4 %) after semen thawing in all cats. In addition, WOB was significantly higher in cat A (73± 1.8 %) and C (77± 1.8 %) than in cat B (65± 1.8 %) (Fig. 2C).

The cat or the sperm selection technique did not affect BCF or ALH.

Table II. Sperm motility parameters in frozen/thawed cat spermatozoa processed by different sperm selection techniques.

Sperm parameter	Control (n=8)	Percoll (n=8)	Swim up (n=8)
TMOT (%)	40 ± 8 [a]	64 ±12 [b]	75 ±14 [b]
PMOT (%)	11 ± 4 [a]	31 ± 8 [b]	46 ± 4 [c]
VCL (μm/s)	86 ± 11 [a]	106 ± 8 [b]	106 ±12 [b]
VSL (μm/s)	51 ± 10 [a]	61 ±1 [b]	69 ± 9 [b]
VAP (μm/s)	60 ± 13 [a]	76 ± 5 [b]	82 ±14 [b]
LIN (%)	58± 5 [a]	58 ± 4 [a]	64 ± 4 [b]
STR (%)	84 ± 1 [a]	81± 5 [a]	84 ± 3 [a]
WOB (%)	69 ± 6 [a]	71 ± 2 [a, b]	76 ± 5 [b]
ALH (μm)	3.8 ± 0.7 [a]	3.6 ± 0.6 [a]	3.3 ± 0.3 [a]
BCF (Hz)	14 ± 3 [a]	14 ± 2 [a, b]	12 ± 1 [b]

[a, b] Different superscripts within rows indicate significant differences P < 0.05. The results are presented as mean ± standard deviation. Abbreviations: TMOT, motile spermatozoa; PMOT, spermatozoa with a progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobbler coefficient; ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.

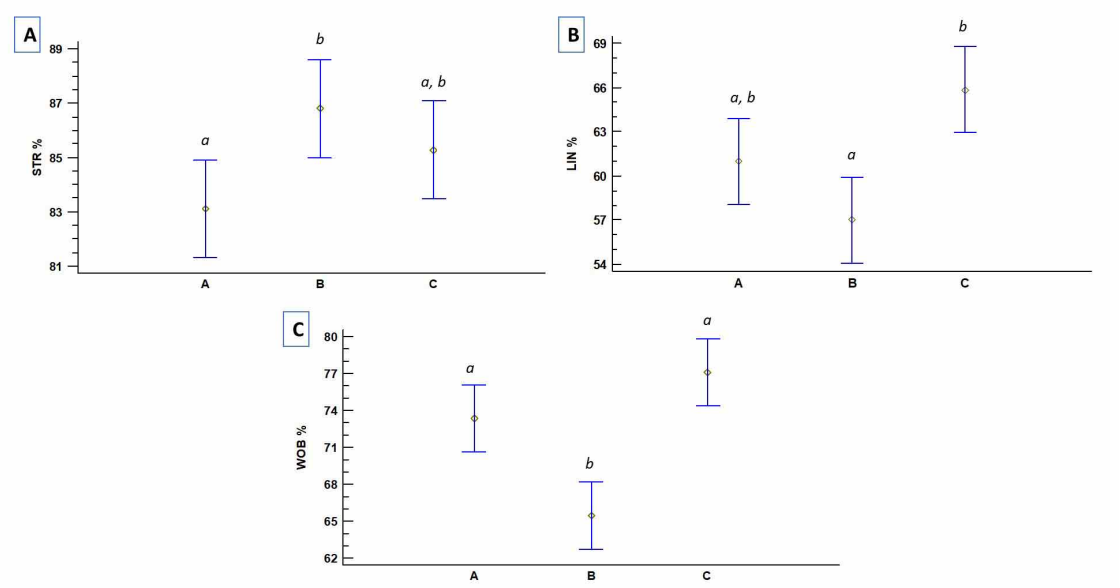

Fig. 2. Individual effect (cats A, B and C) of percoll gradient (PG) technique for sperm selection in frozen/thawed cat semen over motility parameters. A) Straightness (STR, %); B) Linearity (LIN, %) and C) Wobbler coefficient (WOB, %). a, b, c Different superscripts among cats indicate significant differences $P < 0.05$.

DISCUSSION

Sperm selection techniques are important to separate high quality spermatozoa from a semen sample, eliminating seminal plasma, debris, pathogens and sperm death, due to freezing (Crosier *et al*.; Chatdarong *et al*.). On the other hand, motility is the most sensitive sperm parameter damaged after cryopreservation (Filliers *et al*.). To our knowledge, there is no previous report describing a deep analysis of sperm motility improvement after sperm selection techniques in frozen semen of cats.

In the first experiment, we evaluated the effect of sperm selection techniques on sperm recovery rate and sperm motility parameters in frozen/thawed spermatozoa of domestic cat. PG and SU allows recovering lower sperm concentration than control samples. However, sperm recovery in PG was double than SU. Our sperm recovery in PG (30 %) was better than previous reports (10 %) using one layer centrifugation colloids (Androcoll) for frozen/thawed semen both in the domestic cat (Chatdarong *et al*.) and in clouded leopard (Tipkantha *et al*., 2016). In contrast, Terrell *et al*. described significantly higher sperm recovery (90 %) using Accudenz gradient for sperm selection. Similarly, Filliers *et al*. described 50 % of sperm recovery using PG in fresh epididymal sperm, significantly improving sperm function after selection. Fresh spermatozoa has better quality than frozen samples because freeze/thawing process significantly reduces sperm function (Luvoni; Thiangtum *et al*.; Terrell *et al*.). Therefore, sperm recovery could be influenced by the quality of the original semen sample as we obtained 30 % of high quality spermatozoa from frozen/thawed semen. In that sense, similar results had been obtained in frozen samples after gradient selection (Accudenz) in cheetah spermatozoa (27 %) (Crosier *et al*.). These sperm recovery results indicate that PG method applied in our research is efficient for sperm selection in frozen semen to maximize the use of a semen sample in cats.

SU sperm recovery rate (13 %) was higher than previous studies in frozen/thawed semen (2 %) in the domestic cat (Chatdarong *et al*.), but is similar to sperm recovery obtained in cheetah frozen samples (10 %) (Crosier *et al*.). In our experiments as well as Crosier *et al*., a nutrient-rich media was used (Hepes-SOF and Ham-F10, respectively) compared to the saline solution (TRIS-buffer) used by Chatdarong *et al*. The biochemical composition of the media that could be beneficial for sperm motility activation during SU incubation, could explain improved efficiency obtained in our study.

It is worthwhile noting that there are no previous reports comparing sperm motility parameters by CASA system after sperm selection techniques in the cat. Sperm selection studies only described subjective evaluations of sperm motility.

We observed that PG and SU improved sperm motility, velocity and LIN compared to simple washing. Both techniques increased total sperm motility by approximately 20 % and 30 %, respectively. Our results are better than those obtained by Terrell *et al*. that obtained a total sperm motility increment near to 20 % after SU and 10 % motility increment after Accudenz gradient for frozen semen in cat and cheetah. In the contrary, Crosier *et al*. described that Accudenz gradient worked better than SU, improving in 10 % total sperm motility of cheetah frozen samples. On the other hand, Chatdarong *et al*. did not improve total sperm motility after SU or Androcoll gradient: they obtained lower sperm motility than simple washing, which indicates that sperm selection methodology in that research did not work efficiently.

SU increased significantly WOB and PMOT compared to simple washing. SU selects the sperm for its ability to swim up in the supernatant (Parrish & Foote, 1987; Spindler & Wildt). Activation of motility in SU is due to a capacitating effect over spermatozoa during incubation (Esteves *et al*., 2000). SU induces high rates of metabolic function in cat spermatozoa compared to Accudenz centrifugation, which is related to high sperm motility (Terrell *et al*.). SU process resulted in better sperm motility compared to simple washing, but as a disadvantage, lower sperm recovery was obtained, as previously described Chatdarong *et al*.

In conclusion, both sperm selection techniques improved sperm motility, but PG allowed better sperm recovery from the original sample than SU. In addition, PG method was less time consuming than SU (20 min centrifugation compared to one-hour incubation, respectively), being more practical for semen processing. For that reason, we used PG in the second experiment.

Maximizing the use of a frozen semen sample in felines is essential, especially in endangered felids or high value cats in which sample size, number of samples or access to semen collection is reduced (Swanson, 2006; Gañán *et al*., 2009). Therefore, individual efficiency of sperm selection technique in frozen semen could be relevant for ARTs in felids. Accordingly, in the second experiment we evaluated the individual effect of the cat over sperm motility after PG selection of frozen/thawed spermatozoa.

PG increased sperm motility (TMOT and PMOT), velocities, LIN, STR and WOB in all cats compared to simple washing. Motility is crucial for achieving fertilization (Palomo *et al*., 2016), but in the cat, it decreased significantly due to cryopreservation (Filliers *et al*.; Choi *et al*., 2010). Our results support that PG was efficient for improving sperm motility parameters in frozen samples for all analyzed cats, similar to previous reports for fresh semen (Villaverde *et al*., 2009, 2014).

Individual differences were observed among cats only in LIN, STR and WOB after PG selection. For the other sperm motility parameters, PG worked homogeneously among cats. Individual variation of sperm quality was previously described for genetically identical male cats, but without significant differences on *in vitro* embryo production (Choi *et al*.). Individual differences on sperm quality may explain that variation over LIN, STR and WOB after PG selection in frozen semen of cat. In this context, semen samples containing high LIN, STR and WOB values are related to successful ZP binding (Ferraz *et al*., 2014), which may influence subsequent fertility. Further analyzes are needed to know the effect of sperm kinetic characteristics over fertilization in cats.

CONCLUSIONS

As a final remark, our results support that both sperm selection techniques (SU and PG) significantly improved sperm motility parameters in frozen/thawed cat spermatozoa compared to simple washing. In addition, PG allows recovering about 30 % of the sperm from the original frozen sample, working mostly homogeneously among individual cats.

This new information could help to maximize the use of frozen semen in endangered felids or high value domestic cats for its subsequent application on *in vitro* fertilization and artificial insemination.

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CHEUQUEMÁN, C.; SÁNCHEZ, R. & RISOPATRÓN, J. Efecto de técnicas de selección espermática en espermatozoides congelados/descongelados de gato sobre la motilidad espermática analizada mediante sistema CASA. *Int. J. Morphol., 35(4)*:1495-1501, 2017.

RESUMEN: El proceso de congelación/descongelación reduce la sobrevivencia espermática y la habilidad para fertilizar en los espermatozoides de gato, siendo la motilidad espermática el parámetro más sensiblemente alterado debido al daño por frío. En este contexto, los métodos de procesamiento de swim-up y gradiente de densidad pueden ayudar a recuperar los espermatozoides normales y de alta motilidad. Maximizar el uso de una muestra de semen congelado es esencial, especialmente en felinos amenazados o en gatos de alto valor en los cuales el tamaño de muestra, número de muestras o el acceso a la colecta de semen son reducidos. Para nuestro conocimiento, no hay reportes previos que describan un análisis profundo del mejoramiento de la motilidad luego de técnicas de se-

lección espermática en semen congelado de gato. De acuerdo a esto, evaluamos el efecto de las técnicas de selección espermática gradiente de percoll (PG) y swim up (SU) sobre los parámetros de motilidad y porcentaje de recuperación de espermatozoides congelados/descongelados de gato doméstico. Luego, evaluamos el efecto individual del gato sobre la motilidad espermática luego de la selección espermática con PG en espermatozoides congelados/descongelados. SU y PG mejoraron significativamente todos los parámetros de motilidad espermática de los espermatozoides congelados/descongelados comparado con el lavado simple. Sin embargo, PG permitió una mejor recuperación de espermatozoides desde la muestra congelada original y funcionó en su mayoría de manera homogénea entre los gatos individualmente. Esta nueva información puede ayudar a maximizar el uso del semen congelado en felinos amenazados o en gatos de alto valor para su posterior aplicación en fecundación *in vitro* e inseminación artificial.

PALABRAS CLAVE: Motilidad; Gradiente de Percoll; Natación; Semen congelado; Espermatozoides de gato.

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Corresponding author:
Prof. M.Sc. Jennie Risopatrón
Center of Biotechnology on Reproduction (BIOREN-CEBIOR)
Faculty of Medicine
Universidad de La Frontera
Montevideo 0870
Temuco
CHILE

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E-mail: jennie.risopatron@ufrontera.cl