



Freezing dog semen using -80°C ultra-freezer: Sperm function and *in vivo* fertility



F. Pezo ^{a, b}, C. Cheuquemán ^a, P. Salinas ^c, J. Risopatrón ^{a, d, *}

^a Center of Biotechnology on Reproduction (BIOREN-CEBIOR), Faculty of Medicine, University of La Frontera, Temuco, Chile

^b Laboratory of Veterinary Anatomy, School of Veterinary Medicine, Faculty of Veterinary Medicine and Natural Resources, Universidad Santo Tomás, Chile

^c Institute of Biology, Pontificia Universidad Católica de Valparaíso, Chile

^d Department of Basic Sciences, University of La Frontera, Temuco, Chile

ARTICLE INFO

Article history:

Received 17 November 2016

Received in revised form

5 May 2017

Accepted 6 May 2017

Available online 7 May 2017

Keywords:

Dog semen

Sperm function

Freezing

Ultra-freezer

Artificial insemination

Pregnancy

ABSTRACT

Long term storage of canine frozen semen is conventionally performed in liquid nitrogen (LN_2). However, previous works in freezing canine semen using a -80°C ultra-freezer (-80°C -UF) showed no differences on sperm quality after thawing. The main objective of this study was to compare the effects of the freezing techniques using LN_2 or -80°C -UF on sperm function and *in vivo* fertility of frozen–thawed dog semen. The sperm-rich fraction of the ejaculate was collected separately from five Chihuahua breed, and each one divided into two aliquots, and frozen and stored in LN_2 or -80°C -UF. Sperm function was analyzed for motility and viability, acrosome integrity, mitochondrial function and phosphatidylserine translocation by flow cytometry before and after cryopreservation. A total of 10 bitches were intravaginal inseminated (IVAI); LN_2 frozen–thawed semen = 5 and -80°C -UF frozen–thawed semen = 5). Pregnancy status was confirmed 30 d after IVAI by transabdominal ultrasonography and live born puppies at term were recorded. Sperm function parameters were affected for both freezing protocols. Differences ($P < 0.05$) were found between freezing and storage methods in most of the parameters of sperm function analyzed, except in the phosphatidylserine translocation. The percentages of pregnancies were not different between the two freezing and storage protocols used. Semen freezing and storage using -80°C UF is an effective technique for long-term preservation of canine spermatozoa.

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Long term storage of canine frozen semen is conventionally performed in liquid nitrogen (LN_2), obtaining pregnancy rates near to 60% [1]. Cryopreservation processing, however, exposes cells to stress resulting in cellular damage compromising sperm function, these alterations decrease sperm life span, ability to interact with the female reproductive tract, and fertilizing potential [2–4].

The use of ultra-low temperature freezers for semen cryopreservation have been previously described [5–9]. These equipments allow the storage of a large number of straws, decrease the time between thermic equilibration and freezing, reduce the risk of accidents due to mishandling of LN_2 and the economic cost due to its

continuous reposition [5].

In canine, studies have confirmed that the use of ultrafreezers (UF) at -152°C for freezing and storing canine semen could represent a potential alternative to LN_2 [1,5]. Similarly, in goat semen has been validated the use of UF at -150°C for freezing and storing semen, this study showed that the *in vitro* seminal quality (2 months after freezing) was not significantly different compared with frozen and stored in LN_2 [9], at present, live birth goats using the same technique is described [6]. In a previous study [8], it was demonstrated the use at -80°C -UF for freezing and storing semen, this study showed that the sperm function (*in vitro* sperm quality) with respect to plasma membrane integrity, acrosome intactness, mitochondrial function and phosphatidylserine translocation, were not significantly different for semen frozen and stored in LN_2 when compared with those frozen and stored in the ultra-freezer at -80°C .

To our knowledge, no published data exist regarding *in vivo* fertilizing capacity of sperm frozen and stored by ultra-freezer at -80°C . Therefore, our aim was to compare the effects of the

* Corresponding author. Center of Biotechnology on Reproduction (BIOREN-CEBIOR), Faculty of Medicine, University of La Frontera, Montevideo 0870, Temuco, Chile.

E-mail address: jennie.risopatron@ufrontera.cl (J. Risopatrón).

freezing and storage of semen using LN₂ and -80°C-UF on sperm function and *in vivo* fertility of frozen–thawed dog semen following intravaginal insemination.

2. Materials and methods

All experimental protocols received institutional review board approval by the Scientific Ethics Committee from University of La Frontera and were conducted according to Chilean Law No. 20.380 for Animal Protection. The entire study was developed at the Center of Biotechnology on Reproduction (CEBIOR), Faculty of Medicine, University of La Frontera, Temuco, Chile.

All chemicals and reagents used in this study were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Experimental design

The study consisted of two experiments: the aim of experiment 1 was to test the effects of the storage of frozen semen in LN₂ (Control) or -80 °C UF on canine frozen–thawed semen quality with a special focus on sperm function parameters such as motility, viability, acrosome integrity, mitochondrial function and phosphatidylserine translocation. The aim of experiment 2 was to test the effects of the storage of frozen semen in LN₂ (Control) or -80°C-UF on *in vivo* fertilizing capacity of frozen–thawed spermatozoa. Ten bitches were inseminated IVAI (LN₂ frozen–thawed semen = 5; -80 °C UF frozen–thawed semen = 5).

2.2. Animals

Five healthy adult male dogs (weight: 2.5 ± 0.5 kg, 1–4 years old) of the Chihuahua breed, of proven fertility, were used as semen donors. Also, ten healthy adult bitches, weight: 3 ± 0.5 kg, 2–6 years old (Chihuahua breed) of proven fertility (with regular estrous cycles), were used in this study. All animals belong to a private dog kennel and were fed with dry commercial dog food (Purina Pro Plan puppy, San Luis, Missouri, USA), water *ad libitum* and exercised daily throughout the experiments. The animal health status was periodically checked by a veterinarian.

2.3. Semen collection and evaluation

The sperm rich fraction of the ejaculate (n = 6) from each dog was collected by manual manipulation after sexual/mating abstinence for at least a week, as previously described [2], into a pre-warmed sterile glass [10]. The semen quality of each ejaculate was assessed immediately after collection and the following parameters were determined: sperm concentration and total motility [10] and sperm function parameters were assessed by flow cytometry (see 2.6. Sperm function evaluation) [3,8]. Only semen samples with progressive sperm motility >70%, were included in this study.

2.4. Semen freezing

Canine semen freezing was performed as previously described [3], with some modifications. The collected ejaculates were centrifuged at 300 × g for 5 min and the seminal plasma was removed. Sperm pellets were diluted in Extender 1 [TRIS supplemented with 20% [v/v] egg yolk and 3% [v/v] glycerol] to obtain a sperm concentration of 200 × 10⁶/ml at room temperature. Semen samples were cooled at 4 °C for 1 h. After equilibration, they were rediluted (1:1 v/v) in Extender 2 [TRIS supplemented with 20% [v/v] egg yolk, 7% [v/v] glycerol and 1% [v/v] Equex STM paste (Nova Chemical Sales, Scituate, MA, USA)] to reach a final concentration of

100 × 10⁶/ml. After 10 min at 4 °C, 0.5 ml straws (Minitüb, Tiefenbach, Germany) were filled with the extended semen and sealed with heat pins.

Six semen freezes were performed during the study. In each experimental trial, two different protocols of freezing were tested. 1) LN₂ (Conventional technique): The straws (n = 20) were placed on a freezing rack (Minitüb, Tiefenbach, Germany) at 4 cm above the surface of the LN₂ in a polystyrene box and were frozen over the LN₂ vapour for 15 min; finally, straws were packaged into rack and plunged directly into LN₂ tank for storage until thawing, 2) -80°C-UF: the straws (n = 20) were packaged into rack and plunged directly into ultra-freezer at -80 °C (model Revco ULT1386-5V; Thermo®, Waltham, Massachusetts, USA) and stored until thawing [8]. The time elapsed to move the straws from the cooler to the ultra-freezer was approximately 10–15 s [5]. Before initiating the experiments, the cooling velocity of the ultra-low temperature was defined in the -80°C-UF technique, the freezing rate into straws was obtained using a Digi-Sense® Type-K thermocouple with a thermosensitive sonde. The sonde was placed inside the straws filled with sperm suspension and showed at -80°C-UF a fast freezing rate of -10 °C/min from 4 °C to -70 °C, and a slow freezing rate of -0.26°C/min from -70 °C to -80 °C, the data obtained for the freezing rates were expressed as the mean ± standard deviation (Fig. 1). In both freezing protocols, straws were stored at least for 45 days until sperm function evaluation or IVAI. Frozen semen was thawed in a water bath at 37 °C for 45 s.

2.5. Sperm function evaluation

For fresh and thawed semen samples, spermatozoa were

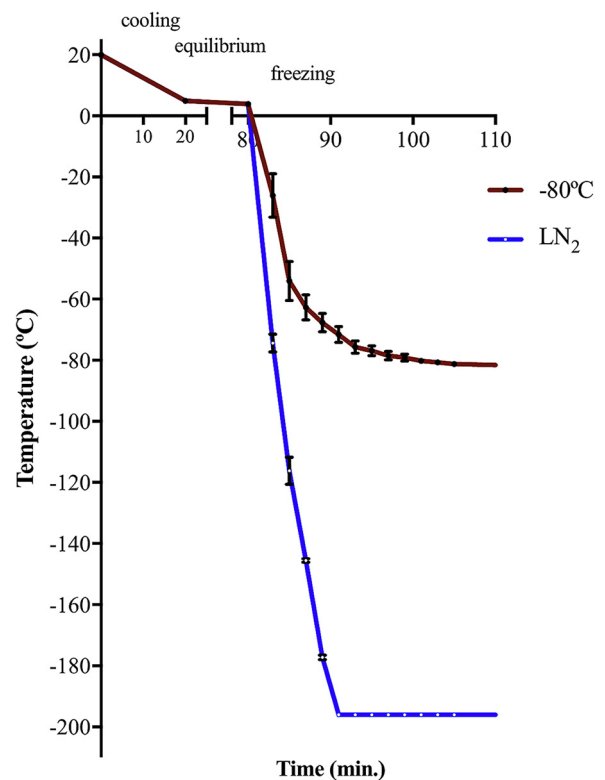


Fig. 1. Freezing curves of 0.5-ml straws obtained in different uncontrolled freezing devices a) Ultra-freezer (-80 °C), non-programmable model Revco ULT1386-5V (Thermo®, Waltham, Massachusetts, USA), b) Liquid nitrogen (LN₂). The data of five measurements obtained for the freezing rates are presented as mean ± standard deviation (SD).

centrifuged at $300 \times g$ for 5 min in PBS (P-4417; Sigma, St. Louis MO, USA). In each sperm quality test, 2×10^6 /ml were analyzed.

2.5.1. Sperm motility

The motility was determined as described [11,12,21] using a phase-contrast microscope (Carl Zeiss, Jena, Germany) at magnification X 400. Ten μ l of sperm suspension were placed in a warm glass slide with a cover slip and a minimum of 300 cells were examined in 6 different fields for total motility. All measurements were performed by two trained observers.

2.5.2. Viability

It was detected using SYBR-14/PI (LIVE/DEAD[®] Sperm Viability kit; Molecular Probes L-7011, Eugene, OR, USA) according to Cheuquemán et al. [12] with some modifications. 250 μ l of sperm solution in PBS (2×10^6 /ml) and 0.5 μ l of SYBR-14 (10 μ M) were incubated 7 min at 38 °C. After that, 1 μ l of propidium iodide (PI; 250 μ g/ml) was added and incubated 7 min at 38 °C. Finally, 1 ml of PBS was added and centrifuged at $300 \times g$ for 5 min. Supernatant was discarded and sperm cells were re-suspended in 400 μ l of PBS for analysis by flow cytometry.

2.5.3. Mitochondrial membrane potential

High mitochondrial membrane potential (HMP) was determined using the JC-1 fluorescent cationic dye according to Smiley et al. [13]. 250 μ l of sperm solution in PBS (2×10^6 /ml) and 0.6 μ l of JC-1 stock solution (1 μ l JC-1 in 500 μ l PBS) were incubated for 15 min at 38 °C. Finally, 1 ml of PBS was added and centrifuged at $300 \times g$ for 5 min. Supernatant was discarded and sperm cells were re-suspended in 400 μ l of PBS for analysis by flow cytometry.

2.5.4. Acrosome membrane integrity (AI)

It was assessed with the fluorescent dye FITC- conjugated lectin from *Arachis hypogaea* (peanut) (PNA-FITC) according to the manufacturer's instructions (L-7381; Sigma-Aldrich, Co. St. Louis MO, USA). 250 μ l of sperm solution in PBS (2×10^6 /ml), 0.5 μ l PNA/FITC and 2 μ l of PI (250 μ g/ml) were incubated for 15 min at 38 °C. Finally, 1 ml of PBS was added and centrifuged at $300 \times g$ for 5 min. Supernatant was discarded and sperm cells were re-suspended in 400 μ l of PBS for analysis by flow cytometry.

2.5.5. Phosphatidylserine (PS) translocation

Annexin-V-FITC/PI apoptosis detection kit APOPTEST-FITC (Nexins Research, Hoeven, The Netherlands) was used according to Cheuquemán et al. [12] with some modifications. Briefly, 100 μ l of sperm solution were added in 100 μ l of binding buffer at 4 °C (2×10^6 /ml). After the addition of 1 μ l of Annexin-V-FITC (25 μ g/ml) and 2.5 μ l of PI (250 μ g/ml) stock solution, the sample was incubated for 15 min at 4 °C in the dark. Finally, 1 ml of PBS was added and centrifuged at $300 \times g$ for 5 min. Supernatant was discarded and sperm cells were re-suspended in 400 μ l of PBS for analysis by flow cytometry.

2.5.6. Flow cytometry

It was performed in a BD FACS Canto II™ Flow Cytometer (BD Biosciences, San José, California, USA) (April 2010) SN: V96101286, (USA) using BD FACS DIVA™ software (updated for version 6.0). For each sample, 10,000 events were evaluated.

2.6. Intravaginal artificial insemination (IVAI)

Ten bitches were randomly separated into two groups for insemination with semen cryopreserved in LN₂ (n = 5) or -80 °C-UF (n = 5). Ovulation days were determined by behavior, exfoliative vaginal cytology [14] and clinical sign: swelling of the vulva, bloody discharge and acceptance of male (lordosis). Serial samples of the vagina were collected every two days. These cells were analyzed with Diff-Quik[®] dye (Hartman Leddon Co., Philadelphia, USA). Bitches were considered in estrus according to criteria such as: they demonstrated proactive receptivity to mounting by males and increased male-seeking behavior, initial wrinkling and crementation of the endoscopically viewed vaginal mucosa, in vaginal smear >80% of squamous superficial cells were found. Once the bitches presented all the criteria described then they were inseminated [14,15].

For IVAI procedure, bitches were positioned in dorsal recumbence with the pelvic limbs elevated. Sperm suspension was deposited immediately into the cranial vagina of the bitch [2] using a sterile 22 cm long canine vaginal AI catheter (Minitüb, Tiefenbach, Germany). After IVAI, bitches were maintained with their hind-quarters raised for 10 min to prevent the reflux of semen [2,16]. Three IVAI in 3 consecutive days were applied using 2 straws of 0.5 ml in each insemination (100×10^6 of total spermatozoa) [17]. Pregnancies were diagnosed using transabdominal ultrasonography (MINDRAY[®] mod. DP-6600 vet, 5–7.5 MHz, Shenzhen, China) at 30 d post-insemination.

2.7. Statistics

Data were expressed as mean \pm standard deviation (SD). The D'Agostino-Pearson test was used to evaluate data normality. One-way Anova and Tukey's post-test of multiple comparisons was used for to compare frozen-thawed semen sperm function between freezing protocols (LN₂ and -80 °C UF). The level of significance was set at $P < 0.05$ and analyzed using GraphPad Prism software for MAC OS X, version 6.0 (GraphPad Software, San Diego, CA, USA).

3. Results

The results of sperm function parameters in fresh and frozen-thawed semen using different freezing and storage protocols are presented in Table 1.

All sperm function parameters were affected for both freezing protocols. Significant differences were found between freezing methods in sperm function parameters such as: motility, viability,

Table 1
Parameters of sperm function in fresh and frozen-thawed semen stored in liquid nitrogen (LN₂) and an ultrafreezer (UF).

	Fresh (n = 6, N° of ejaculates per dog)	Freezing protocols	
		LN ₂ (n = 20 straws)	-80 °C-UF (n = 20 straw)
MOT	73.57 \pm 12	41 \pm 1.9% ^a	34 \pm 1.9% ^b
Viability	74.56 \pm 11	51 \pm 2.1% ^a	43.5 \pm 2.1 ^b
HMP	84.82 \pm 5.75	45.7 \pm 1.8% ^a	36.6 \pm 1.8% ^b
AI	63.06 \pm 10	59.1 \pm 2.1% ^a	50.1 \pm 2.1% ^b
PS	0.32 \pm 0.3	0.32 \pm 0.08%	0.34 \pm 0.08%

^{a,b}Different letter superscripts within rows indicate significant differences between both protocols, $P < 0.05$. The results are presented as mean \pm standard deviation. MOT: total motility, IA: intact acrosomes, HMP: high mitochondrial potential, PS: phosphatidylserine translocation.

acrosome membrane integrity, high mitochondrial membrane potential.

No differences were found between freezing protocol in the effects of the storage of frozen semen in LN₂ (Control) or –80°C-UF on *in vivo* fertilizing capacity, the rate of fertility was 60% in both freezing protocols.

4. Discussion

In the dog, semen quality before and after cryopreservation in ultra freezers have been analyzed in previous studies [5,8], however, the effects of the storage of frozen semen in non-programmable ultra-freezers at –80 °C on *in vivo* fertilizing capacity of frozen-thawed spermatozoa, have not been previously evaluated. To our knowledge, this is the first study reporting the birth of Chihuahua breed puppies obtained after the IVAI of spermatozoa frozen and stored in a –80 °C UF.

Sperm function parameters of fresh semen showed high quality and were according to previous description for dog semen with respect to concentration and volume [18,19], total motility [12,20,21], viability [20,22], AI [12,23] and HMP [12,20,21]. In contrast, the percentages of PS translocation were lower than those described in previous studies with pool of semen from different breeds [3,12]. This sperm function parameter is an apoptotic marker related to spermatogenesis or apoptosis like process in spermatozoa [24]. The low percentages of PS translocation obtained confirm the good quality of the semen used in this study.

Cryopreservation processing, exposes cells to stress resulting in cellular damage compromising sperm function [2–4], the evaluation of cryoinjury of dog spermatozoa is a key factor in achieving better cryopreservation results [25]. Our results show that all the parameters of sperm function analyzed in the thawed semen were affected for both freezing protocols. As expected, the freezing and thawing procedure of canine spermatozoa resulted in a dramatic decrease in motility, viability, PMM, AI and PS translocation increase [3,8,25]. These changes in spermatozoa may compromise the fertility of cryopreserved semen by rendering the cells less stable in the reproductive tract after artificial insemination and therefore relatively short-lived [25].

Comparing two cryopreservation protocols (LN₂ vs. –80 °C UF), motility, viability, AI and HMP for –80 °C UF frozen samples were lower than LN₂ frozen semen, suggesting that the cryodamage induced during the lowering of the temperature or during the storage period at –80°C-UF induced greater damage to sperm function. Various criteria have been used to evaluate the effects of freezing on sperm, the most common ones being sperm motility and membrane integrity [26]. The results of total motility obtained during the present study were consistent with those reported in previous studies with frozen–thawed pooled semen from different dog breeds [8]. However, our results were lower than described for Mongrel breed frozen in –152 °C UF [5]. Viability and plasma membrane integrity were similar to those reported in previous studies using LN₂ and –80°C-UF to store semen from different breeds of canines [8,27,28]. HMP was consistent with semen frozen and storage in LN₂, nevertheless, were higher than described for –80 °C UF [8]. AI is an essential step for fertilization, however, this cell structure is highly damaged during freezing/thawing process in dog spermatozoa [29]. AI, was lower than studies using pool of frozen semen by LN₂ and –80°C-UF techniques [8,27,30]. Differences comparing our results with previous researches using –80°C-UF [8] can be attributed to individual differences between animals, canine spermatozoa respond differently to cryopreservation than those of other domestic animals, often with high individual variability [26,31], usually the pool is used to eliminate variability between different semen samples [8,13,32,33]. In this

study, sperm PS translocation was not affected when frozen semen was stored using the –80°C-UF, our results indicate that the storage of semen in –80 °C UF preserved spermatozoa from PS translocation as with LN₂ technique [8]. This sperm function parameter is an apoptotic marker for somatic cells and is associated with apoptosis like process in spermatozoa and capacitation like changes, reducing life span of cryopreserved semen and sperm fertilizing potential [20].

Our results are consistent with fertility rates in canine after IVAI of frozen semen with LN₂ [2,34–37], and higher than reported at –152 °C [6]. Finally, in addition to the advantages over the sperm functional parameters the ultra freezer has additional practical advantages such as: storage of a large number of straws, reducing time for samples processing and economic cost, best organization in the storage of the straws that allows easy to find them and decreasing the risk of accidents for the operators, in addition, it is especially indicated in situations where LN₂ is difficult to obtain [1,6,7]. Further researches on alternatives for improving sperm function parameters in frozen/thawed spermatozoa are needed.

In conclusion, the fertility results confirmed that canine semen maintain *in vivo* fertilizing capacity after frozen in LN₂ and stored in an ultra-freezer at –80 °C.

Competing interest

None of the authors have any conflict of interest to declare.

Acknowledgements

This study was supported by Grand DI12-0089 Research Direction, University of La Frontera. The authors thank Monteblanco Dog Kennel for providing the studied animals.

References

- [1] Batista M, Alamo D, González F, Cruz MG, Gracia A. Influence of the freezing technique (nitrogen liquid vs ultrafreezer of ?152°C) and male-to-male variation over the semen quality in canarian mastiff breed dogs. *Reprod Domest Anim* 2006;41(5):423–8.
- [2] Rota A, Milani C, Romagnoli S, Zucchini P, Mollo A. Pregnancy and conception rate after two intravaginal inseminations with dog semen frozen either with 5% glycerol or 5% ethylene glycol. *Anim Reprod Sci* 2010;118(1):94–7.
- [3] Kim SH, Yu DH, Kim YJ. Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. *Theriogenology* 2010;73(3):282–92.
- [4] Sieme H, Oldenhof H, Wolkers WF. Mode of action of cryoprotectants for sperm preservation. *Anim Reprod Sci* 2016;169:2–5.
- [5] Alamo D, Batista M, González F, Rodríguez N, Cruz G, Cabrera F, et al. Cryopreservation of semen in the dog: use of ultra-freezers of –152 degrees C as a viable alternative to liquid nitrogen. *Theriogenology* 2005;63(1):72–82.
- [6] Batista M, Niño T, Alamo D, Castro N, Santana M, González F, et al. Successful artificial insemination using semen frozen and stored by an ultrafreezer in the Majorera goat breed. *Theriogenology* 2009;71(8):1307–15.
- [7] Sánchez R, Risopatrón J, Schulz M, Villegas JV, Isachenko V, Isachenko E. Vitriified sperm banks: the new aseptic technique for human spermatozoa allows cryopreservation at –86 °C. *Andrologia* 2012;44(6):433–5.
- [8] Salinas Paulo, Sánchez Raúl, Risopatrón Jennie. Criopreservación de Espermatozoides Caninos a –80C. *Int J Morphol* 2013;31(1):217–24.
- [9] Medrano A, Cabrera F, González F, Batista M, Gracia A. Is sperm cryopreservation at –150 degree C a feasible alternative? *Cryo Lett* 2002 May-Jun;23(3):167–72.
- [10] Kutzler MA. Semen collection in the dog. *Theriogenology* 2005;64(3):747–54.
- [11] Risopatrón J, Catalán S, Miska W, Schill WB, Sánchez R. Effect of albumin and polyvinyl alcohol on the vitality, motility and acrosomal integrity of canine spermatozoa incubated *in vitro*. *Reprod Domest Anim = Zuchthygiene* 2002;37(6):347–51.
- [12] Cheuquemán C, Bravo P, Treulén F, Gijalás L, Villegas J, Sánchez R, et al. Sperm membrane functionality in the dog assessed by flow cytometry. *Reprod Domest Anim = Zuchthygiene* 2012;47(1):39–43.
- [13] Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc Natl Acad Sci U S A* 1991;88(9):3671–5.
- [14] Linde C, Karlsson I. The correlation between the cytology of the vaginal smear

- and the time of ovulation in the bitch. *J Small Anim Pract* 1984;25:77–82.
- [15] Concannon PW. Reproductive cycles of the domestic bitch. *Anim Reprod Sci* 2011;124(3–4):200–10.
- [16] Pinto CR, Eilts BE, Paccamonti DL. The effect of reducing hindquarter elevation time after artificial insemination in bitches. *Theriogenology* 1998;50(2):301–5.
- [17] Hye JK, Hyun JO, Jang G, Min KK. Birth of puppies after intrauterine and intratubal insemination with frozen-thawed canine semen. *J Vet Sci* 2007;8:75–80.
- [18] Albrizio M, Siniscalchi M, Sasso R, Quaranta A. Effects of the environment on dog semen parameters and testosterone concentration. *Theriogenology* 2013;80(7):800–4.
- [19] Merlo B, Zambelli D, Cunto M, Iacono E, Nasi L, Giaretta E, et al. Sex-sorted canine sperm cryopreservation: limits and procedural considerations. *Theriogenology* 2015;83(7):1121–7.
- [20] Volpe S, Leoci R, Aiudi G, Lacalandra GM. Relationship between motility and mitochondrial functional status in canine spermatozoa. *Reprod Domest Anim = Zuchthygiene* 2009;44(Suppl 2):275–8.
- [21] Risopatrón J, Muñoz M, Sanchez R, Sepulveda N. Cryopreservation of canine semen with different protein fractions of seminal fluid. *Rev Científica Univ Zulia* 2012;22:145–53.
- [22] Goericke-Pesch S, Failing K. Retrospective analysis of canine semen evaluations with special emphasis on the use of the hypoosmotic swelling (HOS) test and acrosomal evaluation using Spermac[®]. *Reprod Domest Anim = Zuchthygiene* 2013;48(2):213–7.
- [23] Varesi S, Vernocchi V, Morselli MG, Luvoni GC. DNA integrity of fresh and frozen canine epididymal spermatozoa. *Reprod Biol* 2014;14(4):257–61.
- [24] Ortega-Ferrusola C, Anel-López L, Martín-Muñoz P, Ortiz-Rodríguez JM, Gil MC, Alvarez M, et al. Computational flow cytometry reveals that cryopreservation induces spermptosis but subpopulations of spermatozoa may experience capacitation like changes. *Reproduction* 2016;3:293–304.
- [25] Alhaider AK, Watson PF. Cryopreservation of dog semen: the effects of Equex STM paste on plasma membrane fluidity and the control of intracellular free calcium. *Anim Reprod Sci* 2009;110(1–2):147–61.
- [26] Yu I, Songsasen N, Godke RA, Leibo SP. Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. *Cryobiology* 2002;44(1):62–78.
- [27] Hermansson U, Linde Forsberg C. Freezing of stored, chilled dog spermatozoa. *Theriogenology* 2006;65(3):584–93.
- [28] Futino DO, Mendes MC, Matos WN, Mondadori RG, Lucci CM. Glycerol, methyl-formamide and dimethyl-formamide in canine semen cryopreservation. *Reprod Domest Anim = Zuchthygiene* 2010;45(2):214–20.
- [29] Oettlé EE. Changes in acrosome morphology during cooling and freezing of dog semen. *Anim Reprod Sci* 1986;12(2):145–50.
- [30] Bencharif D, Amirat L, Pascal O, Anton M, Schmitt E, Desherces S, et al. The advantages of combining low-density lipoproteins with glutamine for cryopreservation of canine semen. *Reprod Domest Anim = Zuchthygiene* 2010;45(2):189–200.
- [31] Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* 2000;53(1):47–58.
- [32] Rijsselaere T, Van Soom A, Maes D, Verberckmoes S, De Kruif A. Effect of blood admixture on *in vitro* survival of chilled and frozen-thawed canine spermatozoa. *Theriogenology* 2004;61:1589–602.
- [33] Versteegen J, Onclin K, Iguer-ouada M. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris-glucose extender: *in vitro* and *in vivo* studies. *Theriogenology* 64, 720–733.
- [34] He Huang, Ya-guang Tian, Peng Zheng, Gui-xue Zhang. Study on semen freezing preservation of German shepherd dogs. *J Northeast Agric Univ Engl Ed* 2012;19(4):53–7.
- [35] Mason SJ, Rous NR. Comparison of endoscopic-assisted transcervical and laparotomy insemination with frozen-thawed dog semen: a retrospective clinical study. *Theriogenology* 2014;82(6):844–50.
- [36] Thomassen R, Sanson G, Krogenaes A, Fougner JA, Berg KA, Farstad W. Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. *Theriogenology* 2006;66(6–7):1645–50.
- [37] Linde-Forsberg C, Ström Holst B, Govette G. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. *Theriogenology* 1999;52(1):11–23.