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Cryopreservation of dog epididymal spermatozoa by conventional freezing or ultra-rapid freezing with nonpermeable cryoprotectant

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ABSTRACT

This study was aimed to assess the effectiveness of two methods for cryopreservation of dog epididymal spermatozoa, one by conventional freezing (CF) with shortening both equilibration and cooling times, and the other by ultra-rapid freezing (URF) with nonpermeable cryoprotectant. Sixty epididymides were recovered from thirty orchiectomized adult dogs and the sperm samples were retrieved by retrograde flushing using TCG-EY (tris, citric acid, glucose + 20% egg yolk) extender and then 20 pools were conformed. Each pool was divided into 2 aliquots and then cryopreserved by CF and URF methods respectively. The CF method maintained the cooled-pool samples for 2h (1h without and 1h with 5% glycerol) and then were frozen by liquid nitrogen (LN_2) vapors for 2 min. The URF method cryopreserved the cooled-pool samples using TCG-EY+250 mM sucrose, equilibrating during 30 min (5 °C) and submerging 30-μL drops directly in LN_2. The results showed that the URF method produced a lower percentage of total and progressive motilities and acrosome integrity ($P < 0.05$) than the CF method. However, the kinetic variables (curvilinear and straight-line velocities, straightness, linearity, wobble, amplitude of lateral head displacement, and beat-cross frequency) and plasma membrane integrity did not differ ($P > 0.05$) between both cryopreservation methods. Unlike the URF method, the width, area and perimeter of sperm head were reduced after the CF method ($P < 0.05$). In conclusion, despite the low motility achieved after the ultra-rapid freezing method, the similar values of kinetic, viability and head morphometric dimensions to those obtained after conventional freezing, suggest that ultra-rapid freezing with sucrose may be a useful alternative for the cryopreservation of canine epididymal sperm.

1. Introduction

Cryopreservation protocols enable canine sperm to be stored for a long time. Different cryopreservation protocols have been designed to successfully preserve dog spermatozoa from semen ejaculates [1]. However, several studies on dog sperm cryopreservation are aimed at preserving ejaculates sperm, and only few use epididymal sperm. It has been demonstrated that cryopreservation of epididymal canine sperm can be performed using methods similar to those established for ejaculates of the same species [2]. Nevertheless, the exposition of some major proteins of seminal plasma (e.g., BSP in bulls) from ejaculated semen directly influence sperm cryoresistance. These proteins determine an efflux of cholesterol and phospholipids from sperm membrane, causing major cryosensitivity to the freezing process [3]. In fact, it has previously been reported that epididymal sperm appear to be more cryoresistance than those from ejaculated sperm, with differences in membrane composition [4,5] that influence the equilibrium time required during cryopreservation [6]. Thus, cryopreservation of dog epididymal sperm could be a suitable technique to conserve both domestic and non-domestic canines that have died unexpectedly. Successful management of these cryopreserved gametes could be used successfully in assisted reproductive techniques (ART) [1,2] as

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demonstrated by Marks et al. (1994) [7] when they used cryopreserved dog epididymal semen and obtained a successful conception rate after artificial insemination (AI).

Dog sperm cells are susceptible to various stresses during cryopreservation and ultrastructural, biochemical and functional damage occurs in many sperm cells during the freezing-thawing process [8,9]. As result of cryoinjury, a reduction of motility, viability and fertilizing ability of cryopreserved dog sperm occur [10]. Unlike sperm from ejaculates, epididymal spermatozoa of dogs have been scarcely studied. The management conditions of testes and/or its epididymis (e.g., time and temperature of storage and recovery method) before cryopreservation [2,11,12] can influence cell survival and their motility, viability and fertilizing capacity. Dog epididymal sperm can be successfully recovered for up to 8 days of epididymis storage at 4 °C. In fact, it has been determined that the long-term storage of epididymis under chilled conditions has allowed recovery of motile and membrane-intact dog spermatozoa and fertilizing ability [13].

Conventional freezing protocols of dog sperm have showed variable cryosurvival results [14,15,16]. The conventional freezing protocols more used for cryopreservation dog semen use tris-based extender plus glycerol and straws exposed to liquid nitrogen (LN_2) vapors contained in Styrofoam cryo-box [17]. Concentrations of glycerol in the extenders (e.g., Tris-egg yolk, Pipes-egg yolk) ranged from 4 to 11% (v/v) [18,19,20]. The addition of 5 or 7% of glycerol (v/v) to sperm samples (cooled or at room temperature) diluted in tris-egg yolk (20%, v/v) extender for 1 h before freezing appears to be useful to cryopreserve ejaculated dog sperm [9,21]. Three hours is the standard equilibration time for freezing dog ejaculated sperm [22]. Epididymal sperm usually requires shorter equilibration times with cryoprotectants than ejaculated sperm [23], and thus a better response than longer equilibration times should also be expected in epididymal sperm from dogs. Moreover, a freezing procedure without cooling equilibration appears to be a successful alternative to conventional freezing of dog spermatozoa [21].

Ultra-rapid freezing and kinetic vitrification are cryopreservation methods that allow glass transition of extra- and intra-cellular liquid phase, through very rapid freezing rates [24,25]. Currently, ultra-rapid freezing has become an alternative technique to conventional freezing protocols showing encouraging results for the use of dog frozen-thawed sperm in ART (e.g., *in vitro* fertilization or intra-cytoplasmatic sperm injection) [26]. Ultra-rapid freezing and vitrification use disaccharides (sucrose or trehalose) as non-penetrating cryoprotectant agents (CPA) in high concentrations. Some attempts have been carried out on ultra-rapid freezing or vitrifying dog sperm with promising results [22,21,27]. Sucrose, alone or combined with bovine serum albumin (BSA) has a positive effect on sperm vitrification. This effect has already been observed in sperm of other species such as rabbit [28] and cat [29]. In human species, sperm vitrification using trehalose, sucrose or its combination has shown greater advantages than slow freezing regarding motility rates, integrity membranes (plasma and mitochondrial), morphological integrity, DNA integrity and lower alterations in sperm chromatin [30,31,32].

Caturla-Sánchez et al. (2018) [22] obtained low motility and progressivity of vitrified-warmed dog sperm when they used TCG (plus 20% egg yolk)-based extender supplemented with 250 mM sucrose and compared it to the conventional freezing method. However, its kinetic parameters and integrities of plasma and acrosome membranes were similar (although low) between both methods. On the other hand, Cerdeira et al. (2020) [33] suggested that the variations in the sperm head area depended on the cryopreservation procedure. They found that conventional freezing tended to decrease the head dimensions whereas vitrification led to an overall increase in the sperm head size. Therefore, setting up a canine sperm cryopreservation method that maintains head dimensions after thawing or warming remains a major drawback.

All these findings have been made using canine ejaculated semen. To our best knowledge, there are no studies on ultra-rapid freezing and conventional slow freezing method of dog epididymal sperm with shortening of glycerol exposure during equilibration time and shortening the cooling time during freezing procedure. Based on the aforementioned, we hypothesized that shortening the equilibration time before the slow freezing method or using the ultra-rapid freezing method with nonpermeable cryoprotectant through spheres of 30 μL directly plunged in LN_2 could yield desirable results of cryosurvival of dog epididymal spermatozoa. Thus, this experiment was conducted to assess the effectiveness of two cryopreservation protocols of dog epididymal sperm recovered from testes cold-stored for 96 h, one by conventional freezing with shortening both equilibration and cooling times, and the other by ultra-rapid freezing on cell cryosurvival.

2. Materials and methods

All diluents and media were prepared in the Animal Reproduction Biotechnology Research Laboratory using reagent-grade chemicals purchased from Sigma Chemical Co.

2.1. Animal and sperm collection

All animals were handled according to procedures approved by the Honorable Board of Directors of the Faculty of Agricultural Sciences from the University of Cuenca, and this research was performed in accordance with the chapter 7.8 of the Terrestrial Animal Health Code-2019© OIE (07/8/2019), regarding the protection of animals used in scientific experiments.

Testes and their epididymides were obtained from thirty healthy adult dogs of various breeds aged from 1 to 6 years after routine bilateral orchiectomy at a sterilization campaign of dogs and cats undertaken by the Faculty of Agricultural Sciences of the University of Cuenca, Cuenca, Ecuador. The testes and their intact epididymides were placed into sterile Ziploc® bags (properly labeled: left or right). Samples were kept in ringer lactate solution at room temperature and transported at Animal Reproduction Biotechnology Research Laboratory housed in 'Irquis farm' owned by the University of Cuenca (3°04′48.1″S 79°04′31.0″W). All tissues were stored at 5 °C for 96 h.

Cauda epididymis and vas deferens were dissected from each testis and placed in a dry pre-cooled petri dish. Epididymal sperm samples were collected by retrograde flushing administering 1 mL of cooled (5 °C) TCG-EY extender (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose, 0.54 mM Streptomycin, 2.14 mM Penicillin, + 20% egg yolk [v/v]; pH: 7.16 and osmolality: 320 mOsm/kg) through vas deferens and then the cauda epididymides were sliced [34]. The content was recovered by pipetting and then placed into a 1.5 mL Eppendorf tube. This procedure was developed within a cold-chamber (5 °C approximately). The percentage of motile sperm and the quality of motility were initially evaluated via phase contrast microscope (Nikon Eclipse, Nikon Instruments Inc., New York, USA). Only those epididymal sperm samples with a sperm motility value of >50%, and a score of >2 on a motility scale of 0 (lowest) to 5 (highest), were used in the subsequent experimental work.

Afterward, three epididymal samples (regardless, left or right) from different dogs were randomly allocated and then pooled into one final sample. A total of twenty epididymal pools were used in this experiment. The volume of each pooled sample was measured by pipetting content using a graduate micropipette (Boeco, Germany). The sperm concentration was evaluated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Each pool was divided into two aliquots which were used for conventional freezing and ultra-rapid freezing procedures, respectively.

2.2. Conventional freezing (CF)

The conventional freezing procedure was carried out in well-prepared two steps. The first step consisted in taking the first aliquot of each cooled-pool sample and diluted with TCG-EY extender (CPA

free) previously cooled at a concentration of 100 × 10^6 sperm/mL and then equilibrated for 1 h at 5 °C. The second step consisted in added a second cooled extender made of TCG-EY + 10% glycerol (v/v) (Sigma G9012, St. Louis, MO, USA) to the first aliquot sample in a volume equal (1:1) and equilibrated for 1 h more. Thereby, the final volume of these samples prior conventional freezing reached a final concentration of 50 × 10^6 sperm/mL and 5% glycerol (final osmolarity of freezing medium: 1116 mOsm/kg). Sperm samples were manually loaded into 0.25 mL IVM French straws (L'Aigle Cedex, France) and sealed with polyvinyl-alcohol (Sigma P8136, St. Lois, MO, USA). Straws were frozen using two ramps placed inside a Styrofoam cryo-box of 30 × 29 × 31 cm of length, width and height respectively, that contained 1.7 L of LN_2 (up to 2 cm of height). The LN_2 was previously placed 30 min before freezing process to equilibrium the LN_2 vapors inside the cryo-box. Straws were exposed to LN_2 vapors and placed in the first ramp at 24 cm above the LN_2 surface for 1 min, and then placed in a second lower ramp at 10 cm for 1 min more above the LN_2. Finally, straws were plunged in LN_2 to cool to −196 °C, and kept for three months.

In summary, each pooled sample for cryopreservation by CF method had a total equilibration time of 2 h (glycerol exposition time: 1 h) during the pre-freezing process; and a cooling time (with LN_2 vapors) of 2 min during the freezing process. Frozen straws (n = 60) were thawed by placing the straws in a water bath at 37 °C for 30 s. The contents were poured into dry 1.5 mL Eppendorf tubes and incubated for 5 min at 37 °C. Sperm motility, status of sperm membranes, and sperm head morphometry were subsequently evaluated (see below).

2.3. Ultra-rapid freezing (URF)

The ultra-rapid freezing procedure was also carried out in two steps. The first step consisted in taking the second aliquot of each pool sample and diluted with TCG-EY extender (CPA free) previously cooled at a concentration of 100 × 10^6 sperm/mL. Immediately, the second step consisted in added gently a second extender made of TCG-EY + 500 mM sucrose (w/v) (Sigma S1888, St. Lois, MO, USA) in aliquots of 100 μL each 2 min to the first sample up to reach a volume equal (1:1). These mixed samples were equilibrated for 30 min at 5 °C. Thereby, the final volume of these samples prior ultra-rapid freezing process reached a final concentration of 50 × 10^6 sperm/mL and 250 mM sucrose (final osmolarity of medium: 722 mOsm/kg). The diluted sperm samples were pipetted and plunged drop by drop (30 μL per drop) directly into liquid nitrogen from a height of 15 cm. The pellets formed were stored in Falcon® Conical Centrifuge Tubes (15 mL) (Life Sciences 352,099, Glendale, Arizona, USA) in a liquid nitrogen tank. Cryogenic tubes (n = 20) were stored in a liquid nitrogen tank for three months.

Sperm pellets were thawed using a warming system that consisted of a 20 mL sterilized glass beaker floating in water bath at 65 °C. The sperm spheres were placed into the glass beaker for 5 s and then all the liquid content was immediately transferred to 1.5 mL Eppendorf tubes. After thawing, the sperm was centrifuged for 5 min at 300×*g*. The supernatant was removed and 100 μL of a HEPES-medium (20 mM Hepes salt, 197 mM NaCl, KOH 2.5 mM and 10 M glucose) was added to reconstitute the samples. After thawing the sperm motility, status of sperm membranes and sperm head morphometry were subsequently evaluated (see below).

2.4. Sperm evaluation

Motility characteristics, plasma and acrosome membranes integrity, and morphometric head measures were analyzed from fresh-extended and frozen-thawed sperm samples.

The motility analysis was objectively assessed using a CASA system (Sperm Class Analyzer, SCA-Evolution® 2018, v.6.4.0.99 software. Microptic S.L., Barcelona, Spain) coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast). Sperm samples (5 μL) were placed on slides warmed at 37 °C and covered with a coverslip. A minimum of three fields and 200 sperm tracks were examined at 100 × (image acquisition rate 25 frames/s). The percentage of total sperm motility (TM), the percentage of progressive sperm motility (PSM), the curvilinear velocity (VCL, μm/s), average-path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz) were assessed as described by Galarza et al. (2020) [35]. Plasma and acrosome membrane status were assessed using a double association of fluorescent probes – propidium iodide (PI, Sigma P4170) and fluorescein isothiocyanate conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381) - according to Galarza et al. (2018) [36]. A total of 200 sperm cells per slide were examined using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (40 × magnification with an excitation: 450–490 nm, and emission: 520 nm) and four subpopulations of cells were quantified, i.e., those showing in percentages: (1) intact plasma membrane/intact acrosome (IPIA); (2) intact plasma membrane/damaged acrosome (IPDA); (3) damaged plasma membrane/intact acrosome (DPIA); and (4) damaged plasma membrane/damaged acrosome (DPDA). In addition, the total percentage of cells presenting an intact plasma membrane equivalent to live sperm (IPM: IPIA + IPDA) and intact acrosomal membrane (IAM: IPIA + DPIA) were calculated. Sperm head morphometry analysis was performed automatically using ASMA (*Automated sperm morphology analysis*) module of the same CASA system (SCA-Evolution® 2018, v.6.4.0.99 software. Microptic S.L., Barcelona, Spain): microscopic slides were prepared for each sample, placing 5 mL of epididymal sperm pool sample at the end of the slide and dragging the drop across with another. Smears were air-dried and stained using a SpermBlue® staining technique as previously described by Van Der Horst and Maree (2009) [37]. The head morphometric dimensions of fresh, frozen-thawed, and vitrified-warmed samples were acquired for 100 images: length (L, μm), width (W, μm), area (A, μm^2), and perimeter (P, μm).

2.5. Statistical analysis

The results are presented as mean ± SEM. All statistical analyses were performed using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA). Sperm variables that showed non-normal distributions, as determined by the Shapiro-Wilk test, were transformed to *arcsine* (percentages values) or *log10* (numeric values) before analysis. The significant differences in epididymal sperm quality between the CF and URF methods before and after cryopreservation were compared by one-way ANOVA using the General Linear Model procedure. When ANOVA revealed a significant effect, the values were compared by Tukey's multiple range test. Significance was set at $P < 0.05$.

3. Results

After freezing-thawing either by the CF or URF procedures, significant reductions ($P < 0.01$) were recorded for the most sperm quality variables (e.g., motilities, velocities, and integrity of plasmatic and acrosomal membranes) compared with fresh-extended sperm.

The results shown in Table 1 indicated that the URF method yielded lower ($P < 0.05$) motilities (TM and PSM) than the CF method. However, the post-thaw kinetic parameters based on velocities (VCL, VAP y VSL) and ALH of samples frozen by the URF method were similar ($P > 0.05$) to those sperm samples frozen by the CF method, and even the numerical trend of velocities were higher with ultra-rapid freezing. The post-thaw BCF value was reduced ($P < 0.05$) only in epididymal sperm samples frozen by the CF method. The BCF value of ultra-rapid frozen-thawed samples was similar to fresh-extended samples ($P > 0.05$).

The status of sperm membranes (assessed with fluorescent markers PI/PNA-FITC) in different categories according to their integrity or damage is depicted in Fig. 1. Sperm head dimensions (recorded by CASA-system) are shows in Fig. 2. The percentage of sperm with intact

Table 1
Sperm variable values (mean ± SEM) for dog spermatozoa before and after subjected to conventional freezing-thawing or ultra-rapid freezing-thawing procedures.

Parameters	Fresh-extended (n = 20)	Cryopreservation method	
		Conventional freezing-thawing (n = 60)	Ultra-rapid freezing-thawing (n = 20)
TM (%)	75.5 ± 3.63[a]	37.7 ± 4.43[b]	21.5 ± 3.02[c]
PSM (%)	24.1 ± 3.51[a]	8.3 ± 1.64[b]	4.3 ± 0.76[c]
VCL (μm/s)	63.5 ± 3.84[a]	38.5 ± 2.56[b]	42.3 ± 3.94[b]
VAP (μm/s)	38.3 ± 2.61[a]	22.5 ± 1.60[b]	28.4 ± 3.02[b]
VSL (μm/s)	26.4 ± 2.48[a]	16.5 ± 1.32[b]	19.9 ± 2.34[ab]
STR (%)	62.5 ± 2.55	67.1 ± 1.85	62.1 ± 2.43
LIN (%)	41.1 ± 2.35	42.7 ± 3.00	40.3 ± 3.00
WOB (%)	59.8 ± 1.58	58.8 ± 2.51	59.5 ± 2.54
ALH (μm)	2.8 ± 0.16[a]	1.9 ± 0.13[b]	1.8 ± 0.11[b]
BCF (Hz)	6.4 ± 0.26[a]	5.2 ± 0.32[b]	5.4 ± 0.41a[b]
IPIA (%)	73.0 ± 2.76[a]	39.7 ± 1.88[b]	33.0 ± 2.38[b]
IPDA (%)	12.5 ± 2.06[a]	2.7 ± 0.62[b]	10.7 ± 1.54[a]
DPIA (%)	7.5 ± 0.64[c]	21.7 ± 2.35[a]	15.6 ± 1.48[b]
DPDA (%)	7.0 ± 0.86[c]	35.9 ± 2.98[a]	40.7 ± 2.44[a]
IPM - viability (%)	85.5 ± 1.41[a]	42.3 ± 1.94[c]	43.7 ± 2.65[c]
IAM (%)	80.4 ± 2.41[a]	61.4 ± 3.03[b]	48.5 ± 2.51[c]

[a–c] Different superscripts within a same row differ significantly ([a–b–c] $P < 0.05$ and [a–c] $P < 0.01$). TM: total motility; PSM: progressive sperm motility; VCL: curvilinear velocity; VAP: average path velocity; VSL: straight line velocity; STR: straightness; LIN: linearity; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; IPIA: intact plasma membrane/intact acrosome; IPDA: intact plasma membrane/damaged acrosome; DPIA: damaged plasma membrane/intact acrosome; DPDA: damaged plasma membrane/damaged acrosome; IPM: intact plasma membrane or viability; and IAM: intact acrosomal membrane.

plasma and acrosome (IPIA) and total intact plasma membrane (IPM or viability) were similar ($P > 0.05$) among cryopreservation procedures. Nevertheless, after the ultra-rapid freezing-thawing procedure, the percentage of sperm with intact acrosome membrane (IAM) was lower than the conventional freezing-thawing procedure ($P < 0.05$) and non-cryopreserved samples ($P < 0.01$). In contrast, after the conventional freezing-thawing procedure, the percentage (undesirable) of sperm with the damaged plasma membrane and intact acrosome (DPIA) was greater than after the ultra-rapid freezing-thawing procedure ($P < 0.05$) and fresh samples ($P < 0.01$) (Table 1). Both width (W) and area (A) of dog epididymal sperm were affected only by conventional freezing-thawing procedure, increasing these values ($P < 0.05$) compared with fresh-extended sperm samples. Efficiently, the sperm head measurements value was not altered ($P > 0.05$) after the ultra-rapid freezing protocol (Table 2).

4. Discussion

The present findings show that the conventional freezing method yielded higher values of total and progressive motilities and acrosome membrane integrity, while the ultra-rapid freezing method maintained the kinetic variables and head dimensions of dog epididymal sperm after cryopreservation. Few studies have been conducted to preserve epididymal sperm by conventional freezing or ultra-rapid freezing. The results of the present study demonstrated a successful cryosurvival of dog epididymal sperm recovered from testes cold-stored for 96 h after castration and then frozen by both CF or URF procedures, and could be

Table 2
Mean sperm head measurements (mean ± SEM) of length (L), width (W), area (A) and perimeter (P) for fresh and cryopreserved sperm samples either by conventional freezing-thawing or ultra-rapid freezing-thawing methods.

Variable	Fresh-extended (n = 20)	Cryopreservation method	
		Conventional freezing-thawing (n = 60)	Ultra-rapid freezing-thawing (n = 20)
L (μm)	5.7 ± 0.11	5.9 ± 0.05	5.7 ± 0.04
W (μm)	3.4 ± 0.06[b]	3.5 ± 0.02[a]	3.4 ± 0.03[b]
A (μm^2)	19.6 ± 0.39[b]	20.7 ± 0.26[a]	19.9 ± 0.26[ab]
P (μm)	13.1 ± 0.24[b]	13.7 ± 0.10[a]	13.3 ± 0.11[ab]

[a–b] Different superscripts within a same row differ significantly ($P < 0.05$).

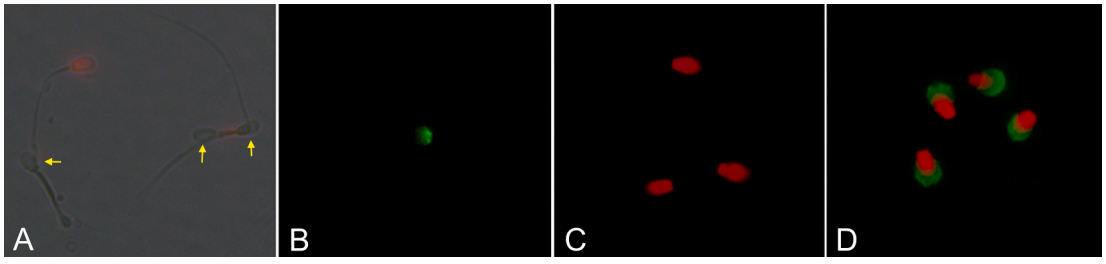

Fig. 1. Sperm stained with an association of *propidium iodide* and *fluorescein isothiocyanate-conjugated peanut agglutinin* (x400). (A) Three viable sperm cells with intact plasma membrane and intact acrosome (IPIA, yellow arrows), unstained cells; (B) viable sperm cell with intact plasma membrane and damaged acrosome (green) (IPDA); (C) three dead sperm cells with damaged plasma membrane (red) and intact acrosome (DPIA); and (D) four dead sperm cells with damaged plasma membrane and acrosome (red/green) (DPDA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

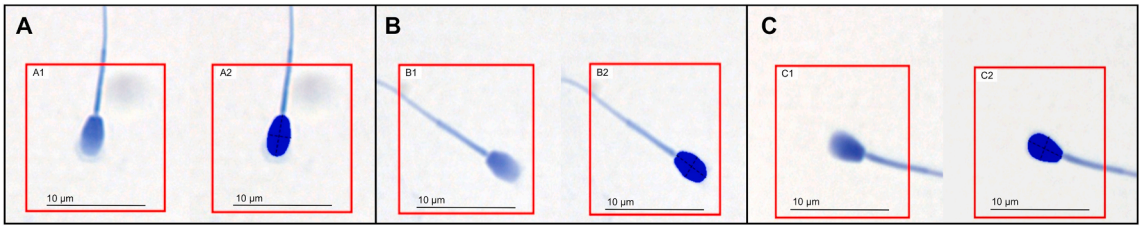

Fig. 2. Morphometric analysis of head of dog epididymal sperm (x600). Fresh (A), frozen-thawed (B), and ultra-rapid frozen-thawed (C) sperm samples stained with Sperm Blue® stain (A1, A2, and A3, respectively) and analyzed the length and width of the head by CASA system (A2, B2, and C2, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

used in ART such as ICSI [38,26]. Indeed, it has been previously reported that cryopreservation of epididymal canine sperm can be performed using methods similar to those established for ejaculates of the same species and that despite some damage, sperm retain their functional ability [2,13]. These CF and URF methods might be useful to apply to genetic resource banking projects in both domestic and non-domestic canine species, which have died under unexpected situations, or even endangered canine species from the Ecuadorian Andean region. To our knowledge, this is one of the few studies that the kinetic, membranes integrity, and head morphometry of dog epididymal sperm were analyzed before and after cryopreservation either by conventional freezing or ultra-rapid freezing.

Although sperm parameters were reduced after both cryopreservation methods, it can be seen that the URF method strongly affected the motilities of dog epidydimal sperm. However, the kinetic characteristics, as well as plasma membrane integrity were similar in URF and CF samples. Consequently, these findings make ultra-rapid freezing an attractive protocol to be easily applied in the field. In fact, ultra-rapid freezing is a quick method and does not require the use of expensive equipment for cryopreservation.

The causes for which ultra-rapid freezing significantly reduces motility are unclear or unknown. Adverse effects of ultra-rapid freezing or vitrification have previously been demonstrated in human sperm due changes in the composition of membrane lipid that increasing membrane damage, inducing acrosome reaction and apoptosis [39]. In fact, Le et al. (2019) [40] obtained lower motility and viability of human sperm after vitrification than conventional freezing method. However, spermatozoa undergoing vitrification were healthier regarding morphology with less defects than conventional freezing. Our results are consistent with the above findings. Other authors suggest that the disaccharides (e.g., sucrose or trehalose) employed to ultra-rapid freezing or vitrification of canine sperm reduce their motility more quickly during post-thawing incubation than monosaccharides (e.g., glucose or fructose), likely because monosaccharides are more readily metabolized by sperm. The inclusion of osmotically active, non-permeating compounds into the ultra-rapid freezing solution leads to additional rehydration of cells and, as a result, decreases toxic effects of the permeable cryoprotectants [41,27]. This experiment used a TCG-EY-based extender, which although contained glucose (easily metabolized), the motility was compromised when combined with sucrose as demonstrated by Parche et al. (2006) [42]. This negative effect of sucrose on motility has previously been demonstrated in both ejaculates and epididymal sperm from other domestic and wild species [43,44,45,46,27,47].

Another causes of reduction of motility after the ultra-rapid freezing-thawing process could be related to changes in the mitochondrial membrane potential [31]. Celeghini et al. (2007) [48] reported that ultra-rapid cryopreservation disrupts the mitochondrial function and consequently, reduces sperm motility post-thaw. The low mitochondrial function of frozen-thawed sperm occurs due to the reduction of both oxidative phosphorylation or ATP synthesis, thus the sperm kinetic variables also drop [49]. Bóveda et al. (2020) [44] demonstrated that after ultra-rapid cryopreservation using TCG-EY + 100 mM sucrose produced a lower percentage of sperm with intact plasmalemma, intact acrosome, and with intact mitochondrial membrane than after conventional freezing cryopreservation using TCG-EY + 5% glycerol. In this study, they used the cryo-scanning electron microscopy (Cryo-SEM) technique and evidenced ultrastructure cell damage in some sperms such as wrinkled or swollen acrosome ridge membrane, rolled tails, and broken midpieces. Tongdee et al. (2015) [50] demonstrated that sperm motility decreased more by ultra-rapid freezing than slow programmable freezing of human sperm.

On the other hand, equilibration time and temperature exposition of sucrose before vitrification may be influenced in sperm motility [27]. It has suggested that 400 mM sucrose supplemented 5 min before cryopreservation to TCF-EY-BSA (tris-citric acid-fructose, 20% egg yolk, and 2% bovine serum albumin) diluent and storing at 5 °C was linked to improved sperm motility following ram sperm vitrification [51]. The period of equilibration with vitrification solution, at 5 °C during 30 min, may also contribute to negative effects on motility as demonstrated by Caturla-Sánchez et al. (2018) [22]. The results of motility of the present study were lower after ultra-rapid freezing than conventional freezing, and are consistent with those obtained by Caturla-Sánchez et al. (2018) [22]. In the present study, we shortened the equilibration (to 2 h) and cooling (to 2 min) times during the CF method, whereas in the URF procedure, the equilibration time was 30 min. Thus, the time of exposure of epididymal sperm samples to sucrose during the ultra-rapid freezing was lower than conventional freezing even after the warming procedure (removed by centrifugation).

In dogs, it has been reported that the addition of BSA to the vitrification media is shown to give higher motility values in ejaculated samples [27]. BSA adsorbs on the membranes of sperm cells and coats the surface area of sperm [52], providing more resistant to the oxidative stress and cold shock during cryopreservation [53]. Although there are no studies in dog epididymal sperm, it is expected that the use of BSA could also counteract certain negative effects of vitrification or ultra-rapid freezing processes on kinetic activity.

Despite that motility parameters were strongly affected, the URF method returned similar results than the CF protocol for sperm kinetic based on velocities and viability. This can be explained by the ultra-rapid freezing characteristic of vitrification which passes rapidly over certain temperature ranges (between 20 and 5 °C), at which canine sperm membrane is particularly sensitive [27] and by the protective action that disaccharides may play at certain concentrations (250 mM of sucrose). Indeed, sperm viability, determined by plasma membrane integrity, obtained after ultra-rapid cryopreservation or vitrification has been similar to those frozen-thawed samples [43]. Controversial results were found in Iberian ibex sperm, with a lower percentage of plasma membrane integrity after the ultra-rapid freezing method than the conventional freezing method [44]. The percentages of viable sperm, similar to those obtained after conventional freezing, suggest that ultra-rapid frozen sperm might be successfully used in particular ART.

In the present study, acrosome integrity was more affected by the ultra-rapid freezing method than by conventional freezing protocol. Morphological changes occur during epididymal transit that includes alterations in the shape of the head due to a reduction in the size of the acrosomal cap among others [54]. Previous reports have demonstrated that vitrification of dog sperm using disaccharides such as sucrose and trehalose, better protects acrosome membrane than conventional freezing [22,27]. However, other studies carried out by the Cryo-SEM technique have determined that the ultra-rapid freezing method using TCG-EY + 100 mM sucrose produced damage acrosome both plasma and acrosome membranes of Iberian ibex sperm recovered by electroejaculation [44]. This effect was not evidenced in ultra-rapid frozen epididymal mouflon sperm when compared to the conventional freezing method with similar results [43]. The results of the present study demonstrated that although the URF method provoked more cryoinjury of acrosome, protected is also the plasma membrane as well as the CF protocol. Unlike Caturla-Sánchez et al. (2018) [22], these findings suggest that the physio-chemistry of ultra-rapid freezing in the presence of sucrose is beneficial for plasma membrane integrity, but not for the acrosome integrity.

On the other hand, the findings of this study showed that the ultra-rapid freezing-thawing process did not alter the sperm head dimensions, while the conventional freezing-thawing process reduced the width, area, and perimeter of the head. Both freezing-thawing procedures provoke disruption of sperm membranes and microtubules influenced by osmotic stress. It is known that osmotic stress determines changes in morphometric head dimensions [55]. In addition, it is known that the shape and size of the sperm head may influence their susceptibility to osmotic damage during the freeze-thaw process [56], especially to sperm more heterogeneous populations [57]. Previous studies

have determined that cryopreserved epididymal sperm suffer a reduction of the size of the head compared to before cryopreservation values [43,57,58]. It has been suggested that over-condensation of sperm chromatin, sperm with damaged plasma and acrosome, as well as acrosome loss and damage in the cell cytoskeleton could cause a reduction in the head size [59,60,61]. In fact, it has recently been determined by Cryo-SEM that the variations in the dog sperm head area depended on the cryopreservation procedure (conventional freezing or ultra-rapid freezing). Conventional freezing tended to decrease the head dimensions, and ultra-rapid freezing led to an overall increase in the sperm head size due to likely decondensation of chromatin and plasma membrane blebbing in the head region [33]. However, the study aforementioned was developed with dog sperm from semen ejaculated. Thus, the results of the present study suggest that the presence of 250 mM sucrose help maintain morphometric head dimensions after the ultra-rapid freezing-thawing process of the dog epididymal sperm. Nevertheless, the CF with glycerol (5%) does not maintain the dimensions of the sperm head, causing a reduction in its values.

In conclusion, ultra-rapid freezing with nonpermeable cryoprotectant provoked more motility decrease than conventional freezing with shortening both equilibration and cooling times. Despite this, achievements obtained after ultra-rapid freezing, with similar values of kinetic, viability and head morphometric dimensions than those obtained after conventional freezing, suggest that ultra-rapid freezing may be a useful alternative for the cryopreservation of canine epididymal sperm.

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Declaration of competing interest

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

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