



## Frozen dog spermatozoa are negatively affected during storage at -80, -21 and -8 °C



Antonio González<sup>a</sup>, Ander Arando<sup>b</sup>, Alberto Acosta<sup>a</sup>, Carlos J. Alcalá<sup>a</sup>,  
Francisco A. Arrebola<sup>c</sup>, Carlos C. Pérez-Marín<sup>a,\*</sup>

<sup>a</sup> Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain

<sup>b</sup> Department of Genetics, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain

<sup>c</sup> Instituto de Investigación y Formación Agraria y Pesquera (IFAPA) Hinojosa del Duque, Cordoba, Spain

### ARTICLE INFO

#### Keywords:

Dog  
Cryopreservation  
Liquid nitrogen  
Spermatozoa  
Temperature  
Viability

### ABSTRACT

Cryopreservation in liquid nitrogen (LN2) allows for semen to be stored for long periods of time while there is sustaining of sperm viability. In this study, there was assessment of effects induced by different storage temperatures on cryopreserved dog spermatozoa. After cryopreservation at -196 °C, sperm samples were transferred to storage conditions of -80, 21 or -8 °C. Sperm motility, morphology, viability, acrosome integrity, mitochondrial membrane potential and DNA fragmentation were determined in samples stored at -196 °C (evaluation time = 0 h), and then after 12 h and 1, 4, 7 and 15 d of storage at 80, -21 and -8 °C. In samples stored at -80 °C, sperm morphology, viability, acrosome integrity, mitochondrial membrane potential and DNA fragmentation did not differ at successive evaluation times. Progressive motility was less ( $P < 0.05$ ) after 12 h and total motility after 4 d of storage at -80 °C as compared with that of the 0 h sample. With storage at the other temperatures (-21 and -8 °C), there was a reduction of mean values for sperm total and progressive motility, viability and mitochondrial membrane potential after 12 h of storage at these temperatures. Results, therefore, indicate the use of ultra-freezers at -80 °C to store frozen dog semen allows for maintenance of sperm characteristics for at least 15 d but motility is sustained for only 1 d. Neither of the -21 or -8 °C storage temperatures were effective for storing of frozen dog sperm and retaining viability.

### 1. Introduction

Conventional freezing using liquid nitrogen (LN2) is the most common protocol for the cryopreservation of dog sperm (Farstad, 2010). In this species, semen is generally frozen in 0.5 mL straws (Nöthling and Shuttleworth, 2005) or as pellets (Ivanova-Kicheva et al., 1997). After cryopreservation, frozen semen needs to be immersed in LN2 or LN2-vapor for shipping or for storage until the samples are used.

When spermatozoa undergo freezing-thawing conditions, significant cellular damage may be induced by the formation of intracellular ice crystals, cellular dehydration, shifts in solute concentrations (as sugars, salts or proteins) and osmotic variations resulting from conditions that are imposed (Morris et al., 2012). Even though cryoprotectants are added to semen to reduce these negative effects on sperm structure and function so that there are conditions where there are gradual temperature decreases and increases in an effort to maintain cell structures and functions, cell damage cannot be entirely avoided. Frozen sperm samples are

\* Corresponding author at: Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain.  
E-mail address: [pv2pemac@uco.es](mailto:pv2pemac@uco.es) (C.C. Pérez-Marín).

immersed in LN2 for long-term storage and thermal injuries can occur when these cells undergo a temperature increase (due to failure in the maintenance of the LN2 in storage tanks or temperature oscillations due to incorrect manipulation of canisters and/or straws). Polge and Rowson (1952) suggested that frozen semen, properly stored in LN2, would have an indefinite retention of fertilizing potential and recent studies have confirmed that frozen sperm samples maintained at  $-196^{\circ}\text{C}$  have little or no damage during extended liquid nitrogen storage in humans (Feldschuh et al., 2005; Szell et al., 2013) and farm animals (Salamon et al., 2004; Aros et al., 2016).

Various studies have been conducted to evaluate other options for sperm cryopreservation using different cryopreservation resources, including dry ice and ultra-freezers (Alamo et al., 2005; Batista et al., 2006; Salinas et al., 2013; Pezo et al., 2017). To evaluate the effectiveness of ultra-freezers, which maintain temperatures of  $-152^{\circ}\text{C}$ , for the freezing and storage of dog spermatozoa, studies were conducted from which there were encouraging results when there were assessments of sperm motility, viability, acrosome integrity and morphology (Alamo et al., 2005; Batista et al., 2006). Storing frozen spermatozoa at temperatures greater than the glass transition temperature, however, is thought to lead to deterioration in quality (Mazur, 1984), and there have been some studies conducted to assess this issue. One of the first studies was conducted to assess the effect of different sub-zero storage temperatures on sperm motility in bulls (Miller and Vandemark, 1954). In this study, bull spermatozoa had maintenance of post-thaw motility values for more than 5 d when the samples were stored at  $-79$  and  $-65^{\circ}\text{C}$ ; however, motility decreased to zero in about 5 d when samples were maintained at  $-51^{\circ}\text{C}$ , and after about 24 h when there was transfer to temperatures of  $-37$  and  $-23^{\circ}\text{C}$ . Results from studies conducted to assess dog spermatozoa indicate post-thaw sperm quality was impaired when samples were stored at  $-80^{\circ}\text{C}$  (Salinas et al., 2013; Pezo et al., 2017) but *in vivo* fertility did not differ when semen stored at  $-80$  or  $-196^{\circ}\text{C}$  was used for insemination, although only a small number of bitches were inseminated (Pezo et al., 2017).

Relatively greater storage temperatures, such as those resulting from the use of domestic freezers, are supposed to induce more severe cell damage, and therefore, are assumed not to be useful for the storage of cryopreserved semen. This assumption, however, has never been investigated with dog spermatozoa and the extent of cellular damage induced by storage temperatures of greater than  $-80^{\circ}\text{C}$  is not known. The aim of the present study was to compare the quality of dog sperm conventionally frozen and stored using LN2 with the same samples stored for 15 d at  $-80$ ,  $-21$  or  $-8^{\circ}\text{C}$ .

## 2. Materials and methods

### 2.1. Animals

A total of six male dogs (three Andalusian Podencos, a Berger Blanc Suisse, a Dachshund and a Weimaraner), aged between 2 and 7 years and weighing between 8 and 38 kg, were used for this study. The animals were privately owned, fed commercial dry diets and provided water *ad libitum*. The dogs were housed in kennels with inside/outside areas. The animals were vaccinated and de-wormed in accordance with a standard health schedule. The owners gave informed consent for this research.

The present study was conducted using standards consistent with European Union legislation (2010/63/EU) as transposed into Spanish law (RD 53/2013). Authorisation from the Bioethical and Biosafety Committee of the University of Cordoba (Spain) was obtained to conduct this study (no. 2018PI/30).

### 2.2. Semen collection, dilution and thawing

Semen was collected once a week for 8 weeks using digital manipulation procedures in the presence of a bitch in oestrus. Only the sperm-rich fraction was recovered. Volume (graduated tubes) and concentration (Thoma chamber, Brand, Germany) were immediately determined in each ejaculate after collection, and then samples were diluted with a commercial tris extender (CaniPlus Enhance, Minitube Iberica, Tarragona, Spain). Diluted sperm samples were maintained at  $37^{\circ}\text{C}$  for 5 min for determining the sperm motility (CX21 microscope, Olympus, Germany) during the initial semen evaluation. Inclusion criteria for ejaculates were a sperm rich fraction volume of  $> 2\text{ mL}$ , concentration of greater than  $150 \times 10^6$  sperm/mL and a subjective total motility of greater than 85%. Ejaculates from the six dogs were pooled to minimise the individual effect. Samples were then centrifuged ( $700\text{ g} \times 15\text{ min}$ ) and the resulting pellet was re-suspended with a commercial glycerol-tris extender (CaniPlus Freeze, Minitube Iberica, Tarragona, Spain) supplemented with 20% egg yolk so that there was a final concentration of  $200 \times 10^6$  sperm/mL. Diluted semen was loaded into 0.5 mL straws at room temperature and cooled to  $+5^{\circ}\text{C}$  during a 2 h period (Cell Incubator, Mycom Control System 200, Welson, Korea). The freezing was performed placing the straws 4 cm over LN2 vapours for 10 min before immersion into LN2.

For thawing, straws were submerged in a water bath at  $37^{\circ}\text{C}$  for 30 s. The samples were maintained at  $37^{\circ}\text{C}$  for 10 min before sperm assessment occurred.

### 2.3. Experimental design

The straws were stored at  $-196^{\circ}\text{C}$  (in LN2) for 1 month before being transferred into different freezers to maintain the temperatures at  $-80^{\circ}\text{C}$  (Biomemory, Froilabo, Meyzieu, France),  $-21^{\circ}\text{C}$  (Liebherr IN IGN-3556-20, Liebherr International AG, Switzerland) or  $-8^{\circ}\text{C}$  (Corberó CF2P2600W, Electrolux, Sweden). Straws were subsequently thawed at 0 h (the time of transfer from  $-196^{\circ}\text{C}$  to the other temperatures) and at 12 h, 1 d, 4 d, 7 d and 15 d after placing the samples in the refrigeration units allowing for storage of samples at the varying temperatures. There were a total of eight replicates for conducting this study.

#### 2.4. Sperm motility assessment

The sperm samples were diluted to a final concentration of  $25 \times 10^6$  sperm/mL with CaniPlus Enhance. After 10 min at 37 °C, the samples were loaded onto a warmed disposable slide (ISASD4C20, Proiser, Valencia, Spain) and ISAS software v.1.2 (Integrated Semen Analyser System, Proiser, Valencia, Spain) was used for the assessment of the sample. A total of 1000 spermatozoa were evaluated in total from four different fields and these samples were analysed using the following criteria: head size 15 to 80  $\mu\text{m}^2$ , motile spermatozoa when VAP was greater than 10  $\mu\text{m}/\text{sec}$ , and straight trajectory when STR was greater than 75%. The percentages of total motility (TM) and progressive motility (PM) were assessed.

#### 2.5. Sperm morphology assessment

Sperm samples were diluted with CaniPlus Enhance so that the concentration was  $50 \times 10^6$  sperm/mL and incubated at 37 °C for 10 min. A 10  $\mu\text{L}$ -aliquot was subsequently smeared on a slide and air-dried before staining. Hemacolor rapid stain (EMD Millipore, USA) was used in accordance with the manufacturer's instructions. A total of 200 sperm cells were assessed using light microscopy (X1000; Olympus CHK2-F-GS, Taiwan). The most common sperm abnormalities observed were detached heads, abnormal heads, proximal and distal cytoplasmic droplets, bent midpieces, bent tails and coiled tails (Kolster, 2018). The percentage of normal spermatozoa was subsequently calculated.

#### 2.6. Flow cytometer assessment

Flow cytometry analysis was performed using the recommendations of the International Society for Advancement of Cytometry (Lee et al., 2008). Sperm viability, acrosome integrity, mitochondrial membrane potential and DNA integrity were assessed using a FACScalibur cytometer (Becton Dickinson Immunochemistry, San Jose, USA) equipped with an argon blue laser (488 nm). Green fluorescence from SYBR-14, fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) and acridine orange (AO) was assessed using a FL-1 photodetector (530/30 nm band-pass filter). Red fluorescence from propidium iodide (PI) and Mitotracker Red CMXRos was assessed using a FL-2 photodetector (585/42 nm band-pass filter). The FL-3 photodetector (630 nm long-pass filter) was used to distinguish red fluorescence from AO. Signals were logarithmically amplified, except when conducting the sperm chromatin structure assay (SCSA) where signals were used on the linear scale. Photomultiplier settings were adjusted to particular staining methods. About 10,000 events of a gated population were counted per sample for sperm viability, acrosome integrity and mitochondrial membrane potential. For DNA integrity, a total of 5000 events were counted per sample.

The sheath flow rate was set at  $12.0 \pm 3 \mu\text{L}/\text{min}$  for all analyses (LOW mode), and forward scatter (FSC) and side scatter (SSC) were recorded in a linear mode (in FSC vs. SSC dot plots). Data were acquired as list-mode data (LMD) files using BD Cell Quest Pro v. 6.0 (Becton Dickinson Immunochemistry, San Jose, USA). These data were analysed using FlowJo software v. 7.6.2 (FlowJo LLC, Ashland, OR, USA) utilising dot plots with the relative cell size (FSC), the internal complexity (SSC) and the specific fluorescence intensity for each probe.

A LIVE/DEAD kit (Molecular Probe Europe, Leiden, The Netherlands) was used to determine sperm viability. A volume of 100  $\mu\text{L}$  of diluted sperm was mixed with 150  $\mu\text{L}$  cytometer buffer (final concentration  $3 \times 10^6$  sperm/mL) and incubated in darkness for 15 min at room temperature with 2.5  $\mu\text{L}$  SYBR-14 (20 nM final concentration) and 5  $\mu\text{L}$  PI (10  $\mu\text{M}$  final concentration) (Pérez-Marín et al., 2018). Live spermatozoa were stained with SYBR-14 (emitting green fluorescence) and dead spermatozoa were stained with PI (red fluorescence). Unstained and single-stained samples were used for calibrating the FSC gain, FL-1 and FL-2 PMT voltages and for compensation of SYBR-14 spill over into the PI channel (9.8%). Non-sperm particles (also called "alien events") were located in the SYBR-14<sup>-</sup>/PI<sup>-</sup> quadrant and these did not contain DNA. Spermatozoa with an intact plasma membrane were located in the SYBR-14<sup>+</sup>/PI<sup>-</sup> quadrant.

For acrosome integrity evaluation, a volume of 5  $\mu\text{L}$  fluorescein isothiocyanate-conjugated peanut agglutinin (100  $\mu\text{g}/\text{mL}$  final concentration; Sigma-Aldrich, St. Louis, USA) and 5  $\mu\text{L}$  PI (6  $\mu\text{M}$  final concentration) were incubated with 100  $\mu\text{L}$  sperm for 5 min at room temperature in darkness. After the addition of 400  $\mu\text{L}$  of cytometer buffer, the samples were assessed. Unstained and single-stained samples were used for calibrating the FSC gain, FL-1 and FL-2 PMT voltages and for compensation of PNA-FITC spill over into the PI channel (9.8%). The cells with the PNA-FITC<sup>-</sup>/PI<sup>-</sup> staining pattern were considered to be viable sperm with an intact acrosome. The percentages of "alien" particles ( $f$ ) determined by SYBR-14/PI staining were used to correct the percentages of non-stained spermatozoa ( $q_1$ ) in each sample to obtain the corrected percentage of non-stained spermatozoa ( $q_1'$ ), as previously described by Petrunkina and Harrison (2010):  $q_1' = [(q_1 - f) / (100 - f)] \times 100$ .

To determine mitochondrial membrane potential, a modified protocol was used (Hallap et al., 2005; Santiani et al., 2016). A volume of 50  $\mu\text{L}$  of sperm (final concentration  $100 \times 10^6$  sperm/mL) was mixed with 350  $\mu\text{L}$  of cytometer buffer and then incubated for 10 min at 37 °C in darkness with 2  $\mu\text{L}$  SYBR-14 (2  $\mu\text{M}$  final concentration; Molecular Probes Europe, Leiden, The Netherlands) and 2  $\mu\text{L}$  Mitotracker Red CMXRos (10  $\mu\text{M}$  final concentration; Molecular Probes Europe, Leiden, The Netherlands). Spermatozoa with a relatively greater mitochondrial membrane potential (HMMP) were recorded. Unstained and single-stained samples were used for calibrating the FSC gain, FL-1 and FL-2 PMT voltages. Data were not compensated.

Chromatin stability in spermatozoa was determined using SCSA utilising AO (Sigma Aldrich, Darmstadt, Germany). This technique is based on the capacity of AO to emit green fluorescence when it is combined with double helix DNA (i.e., intact DNA, FL-1 photodetector), whereas it emits in red when it is intercalated within denaturalized DNA (FL-3 photodetector). Spermatozoa diluted (at a final concentration of  $2 \times 10^6$  sperm/mL) in 200  $\mu\text{L}$  TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, EDTA 1 mM; pH: 7.4) were

flash frozen in LN2 and stored at  $-80^{\circ}\text{C}$  until analysis. For the evaluation, samples were thawed and mixed with 400  $\mu\text{L}$  acid solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton X 100; pH 1.4) to induce the DNA denaturalization. After 30 s, 1.2 mL AO (6 mg/mL) was added and incubated in darkness for 3 min at room temperature (Pérez-Marín et al., 2018). The percentage of spermatozoa with fragmented DNA was determined. Unstained and single-stained samples were used for setting the FSC gain, FL-1 and FL-3 PMT voltages. Data were not compensated.

### 2.7. Statistical analysis

The SPSS 15.0 statistical software (Chicago, IL, USA) was used for data analyses. For verification of normality and homoscedasticity of data the Kolmogorov-Smirnov test and Levene test were conducted, respectively. Data had a non-normal distribution and unequal variances. To conduct parametric tests, TM, PM, morphology, sperm viability, acrosome integrity, mitochondrial membrane potential and SCSA were arcsine-transformed. A General Linear Model for repeated measures was used for comparing sperm values between thawing times in samples that were stored at the same temperature. To compare differences between storage temperatures, a one-way ANOVA for each time was conducted. Differences were considered significant when  $P \leq 0.05$ , in which case the Bonferroni *post-hoc* test was conducted. Data are expressed as means  $\pm$  SEM.

## 3. Results

Data in Table 1 are the mean values of the seminal characteristics from the ejaculates used in the present study.

### 3.1. Sperm motility assessment

After thawing, the percentage of total and progressive motility of sperm samples maintained at  $-196^{\circ}\text{C}$  was  $50.01 \pm 1.87\%$  and  $20.99 \pm 2.49\%$ , respectively. In comparison, sperm samples stored at  $-80^{\circ}\text{C}$  had a lesser total motility after 4 d, and in progressive motility after 12 h of storage as compared with the values for these variables in the 0 h sample. There were differences in values for sperm quality variables samples stored at  $-21$  or  $-8^{\circ}\text{C}$  compared to samples frozen at  $-196^{\circ}\text{C}$  as early as 12 h after being transferred to the  $-21$  or  $-8^{\circ}\text{C}$  temperature conditions. Total and progressive sperm motility in samples stored at  $-80^{\circ}\text{C}$  were greater compared than those maintained at  $-21$  or  $-8^{\circ}\text{C}$  during the study period, but there were no significant differences between samples stored at  $-21$  and  $-8^{\circ}\text{C}$ . In sperm samples maintained at  $-80^{\circ}\text{C}$ , total motility was less after the first day of storage than in the 0 h sample, although there were no significant differences until day 4. When straws were maintained at  $-21$  or  $-8^{\circ}\text{C}$ , there was a marked sperm motility reduction by 12 h after the transfer from the refrigeration unit that maintained the  $-196^{\circ}\text{C}$  temperature (Table 2).

### 3.2. Sperm morphology assessment

The percentage of morphologically normal spermatozoa did not vary during the experiment when there was imposing of any of the temperatures at which evaluations occurred (Table 2).

### 3.3. Sperm viability assessment

Sperm in straws stored at  $-21$  and  $-8^{\circ}\text{C}$  had a reduced viability ( $P \leq 0.05$ ) after 12 h in storage at this temperature as compared with the viability of thawed samples stored continuously at  $-196^{\circ}\text{C}$  (0 h sample). Frozen samples stored at  $-80^{\circ}\text{C}$  also had a lesser sperm viability after thawing when there was storage for 15 days at this temperature as compared with samples stored continuously at  $-196^{\circ}\text{C}$ , although there were no significant differences at this storage temperature as compared with continuous storage at  $-196^{\circ}\text{C}$  (Table 2).

### 3.4. Acrosome integrity assessment

When sperm were stored at  $-80$  or  $-21^{\circ}\text{C}$  and assessed subsequent to thawing, there were no acrosomal changes, whereas with sperm stored at  $-8^{\circ}\text{C}$  acrosomes there were markedly damaged acrosomes after 7 d of storage at this temperature as compared with that of thawed samples that had been continuously stored at  $-196^{\circ}\text{C}$  (Table 2).

### 3.5. Mitochondrial membrane potential assessment

**Table 1**

Characteristics of ejaculates ( $n = 48$ ) included in the present study (mean  $\pm$  SEM).

Volume (mL)	Concentration (x $10^6$ sperm/mL)	Total motility (%)	Progressive motility (%)	Normal morphology (%)	Live spermatozoa (%)	Intact acrosome (%)	HMMP (%)	DNA fragmented (%)
$2.61 \pm 0.63$	$226.08 \pm 65.96$	$87.30 \pm 7.26$	$51.88 \pm 11.43$	$93.38 \pm 5.22$	$80.27 \pm 6.74$	$75.03 \pm 9.71$	$62.38 \pm 22.42$	$2.91 \pm 1.52$

**Table 2**

Sperm quality assessment (expressed as percentage) in frozen straws stored at different temperatures (-8, -21 and -80 °C) during a 15 day-period; Time 0 h is where sperm quality of samples was determined in samples stored at -196 °C and thawed at the same time samples were transferred to other freezers; Data are expressed as mean  $\pm$  SEM.

Variable	Storage temperatures (°C)	Storage times					
		0 h (= -196 °C)	12 h	1 d	4 d	7 d	15 d
Total motility (%)	-80	50.01 $\pm$ 1.87 <sup>a</sup>	38.63 $\pm$ 2.99 <sup>a B</sup>	31.77 $\pm$ 11.28 <sup>a B</sup>	29.74 $\pm$ 3.73 <sup>b C</sup>	27.22 $\pm$ 3.55 <sup>b B</sup>	24.60 $\pm$ 1.61 <sup>b B</sup>
	-21	50.01 $\pm$ 1.87 <sup>a</sup>	12.48 $\pm$ 3.51 <sup>b A</sup>	5.93 $\pm$ 2.20 <sup>b, c A</sup>	2.47 $\pm$ 1.29 <sup>c B</sup>	0.81 $\pm$ 0.81 <sup>c A</sup>	0 <sup>c A</sup>
	-8	50.01 $\pm$ 1.87 <sup>a</sup>	11.34 $\pm$ 3.71 <sup>b A</sup>	5.71 $\pm$ 2.83 <sup>b, c A</sup>	0.90 $\pm$ 0.70 <sup>b, c A</sup>	0.87 $\pm$ 0.64 <sup>b, c A</sup>	0 <sup>c A</sup>
Progressive motility (%)	-80	20.99 $\pm$ 2.49 <sup>a</sup>	14.55 $\pm$ 2.12 <sup>b B</sup>	13.37 $\pm$ 2.91 <sup>b B</sup>	11.79 $\pm$ 2.15 <sup>b B</sup>	11.48 $\pm$ 2.14 <sup>b B</sup>	9.92 $\pm$ 2.22 <sup>b B</sup>
	-21	20.99 $\pm$ 2.49 <sup>a</sup>	2.73 $\pm$ 1.09 <sup>b A</sup>	1.84 $\pm$ 1.34 <sup>bA</sup>	0.08 $\pm$ 0.05 <sup>b A</sup>	0 <sup>b A</sup>	0 <sup>b A</sup>
	-8	20.99 $\pm$ 2.49 <sup>a</sup>	2.84 $\pm$ 1.80 <sup>b A</sup>	0.88 $\pm$ 0.78 <sup>bA</sup>	0.33 $\pm$ 0.24 <sup>b A</sup>	0.05 $\pm$ 0.05 <sup>b A</sup>	0 <sup>b A</sup>
Morphology (%)	-80	78.45 $\pm$ 8.17	72.69 $\pm$ 8.01	82.33 $\pm$ 4.57	79.05 $\pm$ 6.39	82.35 $\pm$ 3.70	79.78 $\pm$ 4.36
	-21	78.45 $\pm$ 8.17	71.79 $\pm$ 7.93	74.95 $\pm$ 6.31	81.89 $\pm$ 6.53	81.79 $\pm$ 5.41	72.32 $\pm$ 7.04
	-8	78.45 $\pm$ 8.17	68.72 $\pm$ 5.44	77.58 $\pm$ 3.94	73.71 $\pm$ 4.28	79.35 $\pm$ 4.73	75.11 $\pm$ 5.51
Live spermatozoa (%)	-80	39.44 $\pm$ 2.09	30.02 $\pm$ 3.41 <sup>B</sup>	31.76 $\pm$ 5.54 <sup>B</sup>	21.53 $\pm$ 5.15 <sup>B</sup>	21.87 $\pm$ 2.96 <sup>B</sup>	23.81 $\pm$ 5.04 <sup>B</sup>
	-21	39.44 $\pm$ 2.09 <sup>a</sup>	11.38 $\pm$ 2.61 <sup>b A</sup>	8.64 $\pm$ 3.79 <sup>b, c A</sup>	1.74 $\pm$ 0.41 <sup>b, c A</sup>	2.23 $\pm$ 0.72 <sup>b, c A</sup>	1.02 $\pm$ 0.31 <sup>c A</sup>
	-8	39.44 $\pm$ 2.09 <sup>a</sup>	13.03 $\pm$ 2.91 <sup>b A</sup>	6.67 $\pm$ 1.97 <sup>b A</sup>	2.95 $\pm$ 1.03 <sup>bA</sup>	3.49 $\pm$ 1.47 <sup>b A</sup>	1.77 $\pm$ 0.78 <sup>b A</sup>
Intact acrosome	-80	86.89 $\pm$ 1.11	86.87 $\pm$ 2.11	82.68 $\pm$ 2.47	81.05 $\pm$ 2.30	85.73 $\pm$ 1.63	82.61 $\pm$ 3.12
	-21	86.89 $\pm$ 1.11	83.36 $\pm$ 1.38	84.62 $\pm$ 1.79	81.13 $\pm$ 1.00	81.02 $\pm$ 2.86	78.50 $\pm$ 2.45
	-8	86.89 $\pm$ 1.11 <sup>a, b</sup>	82.51 $\pm$ 2.61 <sup>a, b</sup>	78.03 $\pm$ 1.85 <sup>a, b</sup>	76.81 $\pm$ 2.83 <sup>a, b</sup>	75.97 $\pm$ 2.94 <sup>a</sup>	73.33 $\pm$ 2.94 <sup>b</sup>
HMMP (%)	-80	52.17 $\pm$ 4.53	40.05 $\pm$ 3.01 <sup>B</sup>	36.13 $\pm$ 4.02 <sup>B</sup>	24.78 $\pm$ 2.88 <sup>B</sup>	25.45 $\pm$ 3.3 <sup>B</sup>	24.32 $\pm$ 3.30 <sup>B</sup>
	-21	52.17 $\pm$ 4.53 <sup>a</sup>	20.18 $\pm$ 2.70 <sup>b, c A</sup>	14.41 $\pm$ 2.70 <sup>b, c</sup>	6.36 $\pm$ 0.73 <sup>b, c A</sup>	6.29 $\pm$ 0.98 <sup>b A</sup>	2.36 $\pm$ 0.39 <sup>c A</sup>
	-8	52.17 $\pm$ 4.53 <sup>a</sup>	13.84 $\pm$ 1.84 <sup>b A</sup>	11.48 $\pm$ 1.45 <sup>a, b A</sup>	5.98 $\pm$ 1.29 <sup>b A</sup>	6.21 $\pm$ 0.44 <sup>b A</sup>	3.53 $\pm$ 0.29 <sup>b A</sup>
DNA fragmented (%)	-80	3.05 $\pm$ 0.25	3.55 $\pm$ 0.53	5.42 $\pm$ 1.09	3.83 $\pm$ 0.35	2.87 $\pm$ 0.37	3.68 $\pm$ 0.43 <sup>B</sup>
	-21	3.05 $\pm$ 0.25	4.26 $\pm$ 0.42	5.73 $\pm$ 1.31	3.47 $\pm$ 0.38	2.56 $\pm$ 0.37	3.43 $\pm$ 0.32 <sup>B</sup>
	-8	3.05 $\pm$ 0.25	4.78 $\pm$ 0.97	5.85 $\pm$ 1.26	3.68 $\pm$ 0.57	5.52 $\pm$ 2.15	7.61 $\pm$ 1.66 <sup>A</sup>

<sup>a, b, c</sup>Different lowercase letters between columns indicate differences ( $P < 0.05$ ) between times within storage temperature.

<sup>A, B, C</sup>Different uppercase letters between rows for each variable indicate differences ( $P < 0.05$ ) between temperatures at the same thawing time.

The HMMP in samples immediately after thawing when temperatures of -196 °C (0 h) was imposed was 52.17  $\pm$  4.53%, and this percentage was not different from samples maintained at -80 °C, whereas there was a reduction after 12 h for of storage in samples stored at -21° or -8 °C compared with samples continuously stored at -196 °C (0 h sample; Table 2).

### 3.6. Sperm chromatin structure assay

The results with evaluation of DNA structure were not different among the different groups when there was imposing of the different temperature storage regimens (Table 2).

## 4. Discussion

The LN2-free alternative methods for freezing dog sperm have been evaluated in various studies and promising results have been reported using ultra-freezers (-152 °C) for freezing and storing sperm samples (Alamo et al., 2005; Batista et al., 2006). There have been few studies in which there was analysis of frozen dog sperm samples (at -196 °C in LN2) after transfer and storage in refrigeration units maintained at greater temperatures (Salinas et al., 2013; Pezo et al., 2017). There are no thermally-induced reactions in cells maintained at -196 °C, a temperature at which the biological processes effectively cease to occur (Mazur, 1984). The glass transition temperature of sperm diluent is between -75 and -196 °C (Katkov and Levine, 2004), depending on the cryoprotectant used. When temperatures are greater than the glass transition temperature, there is a part of the solution in a non-frozen status and the biological activity of spermatozoa consequently continues albeit at lesser rates as compared when there are typical biological temperatures (Mazur, 1984). This situation leads to the occurrence of cellular damage that may affect the fertilizing potential of spermatozoa (Lieberman et al., 2016). Rapatz (1966) previously suggested that spermatozoa are stable at -100 °C and become vulnerable to cryopreservation damage when the temperature is greater than -80 °C. Results of studies conducted with frozen mice spermatozoa, however, indicate the transfer of these cells to a -80 °C freezer enables for the maintenance of fertility when *in vitro* procedures are used after the sperm have been stored for as long as 2 years (Raspa et al., 2017, 2018). The results of the present study indicate quality of frozen dog sperm decreases as indicated by total and progressive motility when semen was stored at -80 °C. The fact that

sperm do not entirely cease metabolic activity at this temperature leads to the decrease in quality of sperm as duration of storage increases. Furthermore, there was an earlier detection of harmful effects when frozen dog sperm were stored at -21 or -8 °C. In the present study, the frozen sperm samples underwent a stepwise warming process, i.e., firstly the temperature of the samples was slowly reduced to the experimental storage temperatures (-80, -21 or -8 °C) and these samples were thawed rapidly to 37 °C (for assessment). During the transfer to the storage temperature (-80, -21 or -8 °C), there was an increase of temperature in the straws at an extremely slow warming rate (less than 1 °C/min), which induced osmotic stress in spermatozoa stored at -21 and -8 °C, after reaching the glass transition temperature. As reported by Morris et al. (2012), spermatozoa maintained at temperatures of greater than -30 °C undergo devitrification of the intracellular compartment and the outer surface of the plasmalemma is injured as a consequence of the osmotic shock and recrystallization.

Progressive motility was markedly reduced 12 h after frozen sperm samples were stored at -80 °C as compared with samples continuously stored at -196 °C, but values were subsequently maintained with a minimum subsequent decrease during the 15 d storage period. Salinas et al. (2013) reported that there was a marked motility reduction when dog sperm samples were stored for 30 d at -80 °C. For bull semen, with the storage at -79 °C there was a greater percentage of progressively motile spermatozoa (Abdussamad et al., 2015), but a lesser percentage when samples were stored at -30 °C (Buranaamnuay et al., 2016), which is consistent with the results from the present study at -21 and -8 °C. The osmotic shock resulting from the transfer of sperm straws from conditions of -196 to -21 and -8 °C could be a primary and initial cause for the lesser sperm quality, rather than ice crystal formation. Sperm samples stored at -80 °C had a less marked reduction of motility, perhaps as a consequence of the physico-chemical properties of being maintained at -80 °C.

Sperm mitochondrial function is highly associated with sperm motility (O'Connell et al., 2002) and is affected by the freezing process. In the present study, there was a reduction in the percentage of dog spermatozoa with a relatively greater mitochondrial membrane potential after freezing, which became more marked as storage time increased and at greater storage temperatures. In frozen dog sperm stored at -21 and -8 °C, there was a reduction in HMMP spermatozoa in the present study that was also accompanied by a reduction of sperm motility. Samples stored at -80 °C also had variations in the sperm HMMP, although no significant differences were noted, a finding consistent with that reported by Salinas et al. (2013). In contrast, Pezo et al. (2017) reported differences in sperm quality of dog samples stored at -80 °C when the storage period was extended to 45 d. Cryopreservation conditions lead to damages in the sperm plasma and mitochondrial membranes, and also alters the production of energy and the availability of ATP in these cells (O'Connell et al., 2002). The variations observed at different storage temperatures in the present study should be further studied to discern how greater storage temperatures affect the structure and function of mitochondria, to explain the marked motility reduction observed in the present study.

There were no differences as storage time increased in dog sperm viability when there was assessment of frozen samples stored at -80 °C. These findings are consistent with those in previous studies where tomcat and bull spermatozoa were stored at -75 and -80 °C, respectively (Buranaamnuay et al., 2016; Buranaamnuay, 2018). The frozen dog sperm samples in the present study had a marked decrease in viability as early as 12 h after being stored at -21 and -8 °C. A negative effect on sperm viability 1 week after storage at -30 °C has been reported for bull sperm (Buranaamnuay et al., 2016). This result is consistent with those of Mazur (1984) in which the primary damage to membranes did not occur during cell storage in LN2, but there is damage when there is storage at -15 to -60 °C during the freezing and thawing process.

The results of present study indicate the freezing conditions when there transfer of dog semen frozen in straws from LN2 (-196 °C) to a freezer at -80 °C resulted in a reduction in sperm quality during a 15 d period of storage at -80 °C. When the frozen samples were transferred to refrigeration units in which the temperatures were -21 and -8 °C, the affect was more harmful on sperm survival, and detrimental effects were evident 12 h after placing the straws in freezers where the temperatures were greater than the glass transition temperature. Sperm cells transition slowly through the critical or lethal zones when freezing conditions are imposed (-15 to -60 °C) (Mazur, 1984) and osmotic changes occur inversely as compared to during freezing. In brief, when ice is melted, the solute concentration in the extracellular media is reduced and the cells swell to offset the osmotic pressure. In the present study, the frozen spermatozoa transferred from the -196 °C to -21 and -8 °C refrigeration units probably underwent volume shrinkage as a result of changes in solute concentrations during the long warming period, resulting in damage to the sperm cell that is detrimental to sperm functions.

In conclusion, the storage of frozen dog spermatozoa at -80 °C preserves many sperm characteristics, but not total or progressive motility, therefore, use of the refrigeration unit that maintains sperm at this temperature is not a viable alternative for storing the semen. Storing frozen dog semen at -21 or -8 °C induced a rapid deterioration in sperm quality when these storage conditions were imposed, therefore, refrigeration units that maintain these temperatures are also not viable alternatives for storage of dog semen.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Declaration of Competing Interest

The authors have no conflict of interest to declare with regard to these results.

## References

- Abdussamad, A.M., Gauly, M., Holtz, W., 2015. Temporary storage of bovine semen cryopreserved in liquid nitrogen on dry ice and refreezing of frozen-thawed semen. *CryoLetters* 36, 278–284.
- Alamo, D., Batista, M., González, F., Rodríguez, N., Cruz, M.G., Cabrera, F., Gracia, A., 2005. Cryopreservation of semen in the dog: use of ultra-freezers of -152 °C as a viable alternative to liquid nitrogen. *Theriogenology* 63, 72–82. <https://doi.org/10.1016/j.theriogenology.2004.03.016>.
- Aros, P., Hernández Díaz, A., Ramírez-Reveco, A., 2016. Freezability analysis of black Friesian bull semen stored to long-term at -196 °C. *Cryobiology* 73, 434–435. <https://doi.org/10.1016/j.cryobiol.2016.09.138>.
- Batista, M., Álamo, D., González, F., Cruz, M.G., Gracia, A., 2006. Influence of 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.* 41, 423–428. <https://doi.org/10.1111/j.1439-0531.2006.00687.x>.
- Buranaamnuay, K., 2018. Cryopreservation and storage of cat epididymal sperm using -75 °C freezer vs liquid nitrogen. *Anim. Reprod. Sci.* 191, 56–63. <https://doi.org/10.1016/j.anireprosci.2018.02.008>.
- Buranaamnuay, K., Seesuan, K., Saikhun, K., 2016. Preliminary study on effects of bovine frozen semen storage using a liquid nitrogen-independent method on the quality of post-thaw spermatozoa. *Anim. Reprod. Sci.* 172, 32–38. <https://doi.org/10.1016/j.anireprosci.2016.06.011>.
- Farstad, W.K., 2010. Artificial insemination in dogs. In: England, G.C.W., von Heimendahl, A. (Eds.), *BSAVA Manual of Canine and Feline Reproduction and Neonatology*. British Small Animal Veterinary Association, Gloucester, UK, pp. 80–88.
- Feldschuh, J., Brassel, J., Durso, N., Levine, A., 2005. Successful sperm storage for 28 years. *Fertil. Steril.* 84 (1017), e3–e4. <https://doi.org/10.1016/j.fertnstert.2005.05.015>.
- Hallap, T., Nagy, S., Jaakma, U., Johannisson, A., Rodríguez-Martínez, H., 2005. Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker Deep Red 633. *Theriogenology* 63, 2311–2322. <https://doi.org/10.1016/j.theriogenology.2004.10.010>.
- Ivanova-Kicheva, M.G., Bobadov, N., Somlev, B., 1997. Cryopreservation of canine semen in pellets and in 5-mL aluminum tubes using three extenders. *Theriogenology* 48, 1343–1349. [https://doi.org/10.1016/S0093-691X\(97\)00375-0](https://doi.org/10.1016/S0093-691X(97)00375-0).
- Katkov, I.L., Levine, F., 2004. Prediction of the glass transition temperature of water solutions: comparison of different models. *Cryobiology* 49, 62–82. <https://doi.org/10.1016/j.cryobiol.2004.05.004>.
- Kolster, K.A., 2018. Evaluation of canine sperm and management of semen disorders. *Vet. Clin. North Am. Small Anim. Pract.* 48, 533–545. <https://doi.org/10.1016/j.cvs.2018.02.003>.
- Lee, J.A., Spidlen, J., Boyce, K., Cai, J., Crosbie, N., Dalphin, M., Furlong, J., Gasparetto, M., Goldberg, M., Goralczyk, E.M., Hyun, B., Jansen, K., Kollmann, T., Kong, M., Leif, R., McWeeny, S., Moloshok, T.D., Moore, W., Nolan, G., Nolan, J., Nikolich-Zugich, J., Parrish, D., Purcell, B., Qian, Y., Selvaraj, B., Smith, C., Tchuvatkina, O., Wertheimer, A., Wilkinson, P., Wilson, C., Wood, J., Zigon, R., International Society for Advancement of Cytometry Data Standards Task Force, Scheuermann, R.H., Brinkman, R.R., 2008. MIFlowCyt: the minimum information about a flow cytometry experiment. *Cytometry A* 73, 926–930. <https://doi.org/10.1002/cyto.a.20623>.
- Lieberman, D., McClure, E., Harston, S., Madan, D., 2016. Maintaining semen quality by improving cold chain equipment used in cattle artificial insemination. *Sci. Rep.* 6, 28108. <https://doi.org/10.1038/srep28108>.
- Mazur, P., 1984. Freezing of living cells: mechanisms and implications. *Am. J. Physiol.* 247, 125–142. <https://doi.org/10.1152/ajpcell.1984.247.3.c125>.
- Miller, W.J., Vandemark, N.L., 1954. The influence of glycerol level, various temperature aspects and certain other factors on the survival of bull spermatozoa at sub-zero temperatures. *J. Dairy Sci.* 37, 45–51. [https://doi.org/10.3168/jds.S0022-0302\(54\)91230-8](https://doi.org/10.3168/jds.S0022-0302(54)91230-8).
- Morris, G.J., Acton, E., Murray, B.J., Fonseca, F., 2012. Freezing injury: the special case of the sperm cell. *Cryobiology* 64, 71–80. <https://doi.org/10.1016/j.cryobiol.2011.12.002>.
- Nöthling, J.O., Shuttleworth, R., 2005. The effect of straw size, freezing rate and thawing rate upon post-thaw quality of dog semen. *Theriogenology* 63, 1469–1480. <https://doi.org/10.1016/j.theriogenology.2004.07.012>.
- O'Connell, M., McClure, N., Lewis, S.E.M., 2002. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum. Reprod.* 17, 704–709. <https://doi.org/10.1093/humrep/17.3.704>.
- Pérez-Marín, C.C., Requena, F.D., Arando, A., Ortiz-Villalón, S., Requena, F., Agüera, E.I., 2018. Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: preliminary results. *Cryobiology* 80, 62–69. <https://doi.org/10.1016/j.cryobiol.2017.12.002>.
- Petrunkina, A.M., Harrison, R.A.P., 2010. Systematic misestimation of cell subpopulations by flow cytometry: a mathematical analysis. *Theriogenology* 73, 839–847. <https://doi.org/10.1016/j.theriogenology.2009.09.007>.
- Pezo, F., Cheuquemán, C., Salinas, P., Risopatrón, J., 2017. Freezing dog semen using -80 °C ultra-freezer: sperm function and in vivo fertility. *Theriogenology* 99, 36–40. <https://doi.org/10.1016/j.theriogenology.2017.05.007>.
- Polge, C., Rowson, L.E.A., 1952. Fertilizing capacity of bull spermatozoa after freezing at -79 °C. *Nature* 169, 626–627. <https://doi.org/10.1038/169626b0>.
- Rapatz, G.L., 1966. What Happens When Semen Is Frozen? *Proc 1st Tech. Conf. on Artif. Insem. and Reprod.* pp. 45–56.
- Raspa, M., Guan, M., Paoletti, R., Montoliu, L., Ayadi, A., Marschall, S., Fray, M., Scavizzi, F., 2017. Dry ice is a reliable substrate for the distribution of frozen mouse spermatozoa: a multi-centric study. *Theriogenology* 96, 49–57. <https://doi.org/10.1016/j.theriogenology.2017.04.003>.
- Raspa, M., Fray, M., Paoletti, R., Montoliu, L., Giuliani, A., Scavizzi, F., 2018. Long term maintenance of frozen mouse spermatozoa at -80 °C. *Theriogenology* 107, 41–49. <https://doi.org/10.1016/j.theriogenology.2017.10.036>.
- Salamon, S., Gillan, L., Evans, G., Maxwell, W.M.C., 2004. Fertility of ram semen after 35 years of frozen storage. In: *Proc 15th Int Congr Anim Reprod.* 2004. Porto Seguro. pp. 476.
- Salinas, P., Sánchez, R., Risopatrón, J., 2013. Criopreservación de espermatozoides caninos a -80 °C. *Int. J. Morphol.* 31, 217–224.
- Santiani, A., Ugarelli, A., Evangelista-Vargas, S., 2016. Characterization of functional variables in epididymal alpaca (*Vicugna pacos*) sperm using imaging flow cytometry. *Anim. Reprod. Sci.* 173, 49–55. <https://doi.org/10.1016/j.anireprosci.2016.08.010>.
- Szell, A.Z., Bierbaum, R.C., Hazelrigg, W.B., Chetkowski, R.J., 2013. Live births from frozen human semen stored for 40 years. *J. Assist. Reprod. Genet.* 30, 743–744. <https://doi.org/10.1007/s10815-013-9998-9>.