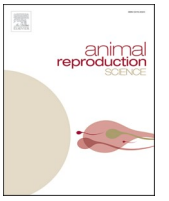
Animal Reproduction Science 235 (2021) 106887

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



Vitrification of Iberian wolf (*Canis lupus signatus*) sperm: A possible alternative to conventional cryopreservation

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ARTICLE INFO

Keywords:
Wolf
Cryopreservation
Vitrification
Sperm

ABSTRACT

Sperm vitrification is a simple, inexpensive method that allows the cryopreservation of sperm in the field and for endangered species is a useful alternative to conventional freezing. The study, therefore, is focused on the suitability of vitrification for cryopreserving Iberian wolf sperm and utilizing plasma testosterone concentration as a marker for procedure efficacy. Sperm and blood samples were collected from 17 wolves. There were 14 samples suitable for cryopreservation (12 ejaculated and two epididymal). Immediately after collection, these samples were proportioned into two aliquots for conventional freezing using a Tris-citric acid-glucose based extender (TCG) or vitrification utilizing an animal protein free extender (HTF®). Vitrification occurred by directly plunging a sperm suspension into liquid nitrogen. Sperm were assessed for motility, membrane integrity, acrosomal status and DNA integrity before and after cryopreservation. With both techniques, there were similar post-thaw/warming results ($P > 0.05$) with respect to progressive motility, kinetic variables VCL, VSL, VAP and BCF, DNA fragmentation, sperm membrane functionality and morphological abnormalities. Total motile sperm, progression ratios LIN, STR, and WOB, the ALH, sperm viability and sperm with intact membrane and acrosome were greater ($P < 0.05$) in the conventional frozen-thawed sperm than vitrified-warmed sperm. Plasma testosterone concentrations varied from 0.0 ng/mL to 7.7 ng/mL. For epididymal sperm, sperm motility and viability following thawing were greater in vitrified-warmed samples than conventionally-frozen samples; however, small sample numbers precluded statistical analysis. When considered together, these results indicate vitrification may be a possible alternative for wolf sperm cryopreservation.

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https://doi.org/10.1016/j.anireprosci.2021.106887
Received 1 April 2021; Received in revised form 4 November 2021; Accepted 5 November 2021
Available online 14 November 2021

1. Introduction

Results from most studies on wolf conservation substantiate the problem of inbreeding (Laikre, 1999; Lockyear et al., 2016). The Iberian wolf (*Canis lupus signatus*), a subspecies of the grey wolf, have less genetic diversity than other European populations (Pilot et al., 2014). The vulnerability of the Iberian wolf to extinction is further increased by susceptibility of these animals to environmental change, habitat loss and population fragmentation, decreasing prey populations, an ever increasing incidence of disease, and human interference with animals in their natural habitat (Oleaga et al., 2015). There are now fewer than 250 packs in the Iberian Peninsula where, conservation policies regarding the species are controversial among those with special interest in the wolves. With there having been a marked decrease in wolf numbers, conservation programs are now needed (Quevedo et al., 2019).

The mating period of wild Iberian wolves lasts from late January until April, with births mainly concentrated in late May (Vilà et al., 1990). Iberian wolf packs are usually include a mating pair with offspring of the pair also being members of the pack (Vilà et al., 1990). No information is available about seasonal reproductive changes (e.g. testicular and prostate sizes) and endocrine changes in Iberian wolves. There is a seasonal pattern of plasma testosterone concentrations and testicular size in the grey wolves housed in a zoological garden in Germany, with maximal values in winter (3.5–4.5 ng/mL) and basal values (< 2 ng/mL) during the rest of the year (Haase, 2000).

Conventional sperm freezing techniques have been utilized for male gamete cryopreservation in domestic dogs (*Canis lupus familiaris*), grey wolves (*Canis lupus*), Mexican grey wolves (*Canis lupus baileyi*) (Zindl et al., 2006; Christensen et al., 2011), and red wolves (*Canis rufus*) (Goodrowe et al., 1998; Lockyear et al., 2009; Franklin et al., 2018). Vitrification has been used for preserving embryos in canids, and in studies with oocytes from both domestic dogs and grey wolves there were promising post-warming viability results (Silber et al., 2013). Sperm cells are, however, the material most commonly preserved in germplasm banks, as a consequence of the more frequent collection of sperm as compared with oocytes (Prieto et al., 2014), but wolf sperm vitrification has not been attempted. Vitrification is an interesting alternative to conventional freezing. Vitrification is an efficacious method for cryopreserving human (Sanchez et al., 2012), horse (Consuegra et al., 2019), ibex (Pradiee et al., 2015), mouflon (Pradiee et al., 2017) and fish sperm (Merino et al., 2011) with viability of sperm being maintained post-thawing. Encouraging results, mainly related to sperm viability after vitrification-warming, have recently been reported for dog sperm (Sánchez et al., 2011; Caturla-Sánchez et al., 2018; Cerdeira et al., 2020), indicating vitrification is also an alternative method of cryopreserving Iberian wolf sperm.

Epididymal sperm provide a source of spermatozoa for cryopreservation in different endangered species (Garde et al., 2006) and might be a source of sperm when collected from recently deceased Iberian wolves. Ejaculated sperm have less cryo-damage than epididymal sperm due to capacitation-associated changes induced by the seminal plasma upon ejaculation (Martínez-Fresneda et al., 2019), plus changes in the characteristics of the sperm cell proteome (Martínez-Fresneda et al., 2019, 2021). Conception has resulted when there was use of conventionally frozen-thawed epididymal sperm of dogs for artificial insemination (AI) (Hori et al., 2004).

In non-domesticated ruminants, when there are relatively greater concentrations of plasma testosterone, there are negative effects on the freezability of sperm (Bóveda et al., 2021); while the effects of testosterone concentrations on canid sperm freezability has not been studied, there is need for evaluations of potential associations of testosterone concentrations and sperm viability subsequent to imposing freezing-thawing procedures on wolf semen. In the present study, therefore, there were assessments of sperm vitrification as an alternative to conventional freezing for efficacy of cryopreserving Iberian wolf sperm and examinations of effects of plasma testosterone concentrations on sperm cryoresistance to damage subsequent to conducting vitrification-thawing procedures.

Table 1
Characteristics of semen collected using electroejaculation and epididymal flushing ("e" after wolf no) procedures, scrotal circumference, hierarchical status and age of the different wolves, as well as plasma testosterone concentrations.

						Sperm sample		
Wolf *n*	Hierarchy/Group size	Age	Testosterone (ng/mL)	Date semen collection	SC, cm	Vol (μl)	Spz/mL	Total motility
1	*/1	8	1	13/03/18	16.9	4000	50×10^6	80%
2	Not alpha/3	4	0	09/05/18	14.5	1300	100×10^6	20%
3	Alpha/3	3	7.7	09/05/18	18	3170	42×10^6	60%
4**	Not Alpha /3	4	0	09/05/18	11	120	0	0
5**	Alpha /3	6	0.20	16/01/19	14.5	0	0	0
5e		6				5400	0	0
6	Not Alpha/6	5	4.7	16/01/19	16.5	4600	150×10^6	60%
7	Not alpha/6	3	3.1	16/01/19	14.5	3285	270×10^6	70%
8	Alpha/3	4	6.0	31/01/19	15.7	1700	26×10^6	70%
9	Not alpha/3	7	0.4	31/01/19	15.5	8580	7×10^6	70%
10**	*/1	8	0.0	07/02/19	16.7	0	0	0
11	*/1	6	0.0	07/02/19	16.5	4100	3×10^6	10%
12	*/1	7	1.8	07/02/19	16.7	127	80×10^6	65%
13**	Not alpha/2	6	0.0	12/05/18	13.1	0	0	0
14	Alpha/3	5	4.4	02/05/19	15	2500	300×10^6	75%
15**	Not Alpha/2	5	0.0	02/05/19	13.5	0	0	0
16	Alpha / 2	4	0.7	26/01/20	17	3000	6×10^6	70%
16e						1000	63×10^6	50%
17	Alpha /2	6	4.2	26/01/20	17.8	3400	170×10^6	55%
17e						850	400×10^6	55%

2. Material and methods

The media used in the present study were prepared at the Department of Animal Reproduction (INIA-CSIC), using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and Sigma Chemical Co. (St. Louis, Missouri, USA), unless otherwise stated.

2.1. Animals

The study was conducted from January to May during two consecutive years. Sperm and blood samples were collected from 17 Iberian wolves housed in different facilities in Spain in the: Principality of Asturias Council of Rural Development and Natural Resources ($n = 3$), Guadalajara Zoological Gardens ($n = 1$), Mundopark Wildlife Centre, Seville ($n = 5$), Cañada Real Nature Centre ($n = 2$), Zoo-Aquarium of Madrid ($n = 1$), Cordoba Zoological Gardens ($n = 3$), and Kuna Ibérica Wildlife Centre ($n = 2$). Four animals were housed alone (individuals 1, 10, 11 and 12, see Table 1), but had olfactory, visual, and auditory contact with females. Three of the four animals had been previously administered a 4.7 mg Suprelorin® slow release implant (Virbac, France) 6 months before the experiment started (Table 1). Although it was expected that the active compounds in these implants were depleted at the time of conducting the present study, because of the lesser and variable effectiveness of deslorelin in non-domesticated carnivores (Bertschinger et al., 2001), the possibility of some specific effects on values for fresh sperm variables should not be discounted (Lucas, 2014). These three wolves included in the experiment were not previously implanted. The males not housed with other wolves were members of stable groups of 3–6 animals. The ages of these animals ranged from 3 to 8 years. Information on the hierarchical dominance of the animals was subjectively reported by the biologists and veterinarians of the centers.

All procedures were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which conforms to European Union Directive 2010/63/UE regarding the protection of animals used in scientific experiments, and all the animal procedures utilized were reviewed by the Institutional Animal Care Committee of the different facilities involved.

2.2. Anaesthesia, reproductive tract evaluation and sperm collection

All animals were anesthetized using a projectile dart (TeleDart, GMBH & CO., KG, Westheim, Germany) fired by a CO_2-powered pistol, to provide 3.4 mg/kg intramuscular ketamine hydrochloride (Ketamidor®; Richter Pharma AG, Wels, Austria) and 0.02 mg/kg medetomidine (Domtor®; Ecuphar Veterinaria S.L.U, Barcelona, Spain). After anesthetization, experimental animals were intubated with an endotracheal tube (size 9.5), and anaesthesia was maintained using 1.5–2.5% isofluorane (IsoVet®; Braun, Barcelona, Spain) in oxygen (flow rate 2.5 L/min) supplemented with 1 mg/kg intravenous propofol (Propovet®; Zoetis Portugal, Porto Salvo, Portugal). After sample collection, anaesthesia was reversed utilizing an intramuscular administration of 0.11 mg/kg atipemazole (Antisedan®; Ecuphar Veterinaria S.L.U, Spain). All animals fully recovered from being anesthetized within 20 min. Meloxicam (0.2 mg/kg subcutaneously) (Metacam®; Boehringer Ingelheim Animal Health, Germany) was administered immediately before conducting the procedure to avoid any inflammation or pain.

With the animals in the dorsal recumbent position, scrotal circumference was measured (widest diameter) using a scrotal measuring tape (Ideal Instruments, Neogen Corporation, IL, USA). An ultrasonic examination of the prostate and testicles was performed using a 7.5 MHz Prosound 2 linear array probe (Aloka Co., Ltd, Tokyo, Japan) prior to semen collection. The area of prostate and the diameter of testes were determined using electronic calipers embedded in the ultrasonic device. The urinary bladder was examined and emptied using an urethral catheter for dogs (Buster, Langeskov, Denmark).

Ejaculates were collected using a transrectal ultrasonic-guided massage of the accessory sex glands (TUMASG) combined with electroejaculation (Santiago-Moreno et al., 2013) with minor modifications. Briefly, the penis was manually protruded from the prepuce. The protruded penis was subsequently cleaned and ejaculation was induced using a PTE Model 304 electroejaculator (P-T Electronics, Sandy, OR, USA). The probe was placed ventrally in the rectum above the prostate, and 10 electrical stimuli (1–8 V, lasting 5 s) were provided for as many as five cycles with there being periods of 30 s to massage the prostate with the probe. Stimulation was increased slowly until the hind-limbs extended and there was continued stimulation at an increasing voltage until ejaculation was induced. Semen was directly collected in a graduated glass vessel designed for the purpose.

Two wolves were orchiectomized after the electroejaculation procedure (as scheduled prior to all experimental procedures for managerial reasons) and, as a preliminary investigation, epididymal sperm was collected using the flushing method (Martínez-Fresneda et al., 2019).

2.3. Hormonal quantitation's in blood plasma

Blood samples from the cephalic vein were collected during anaesthesia into BD Vacutainer® lithium tubes that were heparinised (Becton Dickinson Co., Plymouth, UK). The contents in the tubes were then centrifuged at 1500*g* for 15 min and the separated plasma was stored at − 20 °C until testosterone quantification occurred using a radioimmunoassay with there being 100 μl aliquots assayed in duplicate with the assay having a sensitivity of 0.05 ng/mL (Santiago-Moreno et al., 2005).

2.4. Analysis of fresh semen samples

Fresh sperm samples were stored at 37 °C and immediately assessed with all assessments being were made by the same observer.

Ejaculated and epididymal sperm volumes were determined using a graduated micropipette (Gilson International, Villiers Le Bel, France). The percentage of motile sperm and the sperm motility were assessed subjectively using a phase contrast microscope (Zeiss, Oberkochen, Germany) and categorized using a scale from 0 to 5. Sperm concentration was determined using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany).

Sperm viability was assessed by nigrosin-eosin staining (Campbell et al., 1956). Plasma membrane functionality was assessed using the hypo-osmotic swelling test (Jeyendran et al., 1984). Sperm morphology and acrosome status (normal apical ridge) of glutaraldehyde fixed samples were assessed using a phase-contrast microscopy (Martínez-Fresneda et al., 2019).

The DNA fragmentation was analysed by TUNEL assay using the In Situ Cell Death Detection Kit (Roche Biochemicals, Basel, Switzerland) as previously described (Cardoso et al., 2020).

2.5. Sperm cryopreservation

Sperm samples were proportioned into two aliquots for freezing or vitrification.

2.5.1. Conventional freezing-thawing

Samples collected using electroejaculation procedures were transferred to 15 mL centrifuge tubes and TCG medium added in a proportion of 1:3 (v:v). Samples were then centrifuged for 10 min at 600 x *g* and the supernatant removed. The pellets were re-suspended with TCG and 20% egg yolk (TCG+20% EY). Epididymal sperm samples were flushed out with TCG supplemented with 20% egg yolk (TCG+20% EY) medium.

The diluted samples were then stored at 5 °C for 1 h before adding a second extender containing 10% glycerol (TCG+20%EY+10% GLY) at a 1:1 v:v ratio (final concentration of glycerol = 5%). Samples were adjusted to a concentration of 200 × 10^6 cells/mL. Samples were then maintained at 5 °C in a portable refrigerator for a further 2 h before being transferred into 0.25 mL straws (IMV, L'Aigle, France) as previously described (Cerdeira et al., 2020). The straws in which these samples were transferred were ultrasonically sealed with a handheld sealing device (Minitube Ultraseal 21™, Minitüb, Tiefenbach, Germany). All straws were placed 7.5 cm above the surface of the liquid nitrogen for 1 min (cooling rate 35 °C/min), were then positioned at 2.5 cm above the surface of the liquid nitrogen for 1 min (cooling rate 55 °C/min) until the sample temperature was − 86 °C. Samples were subsequently plunged into the liquid nitrogen.

At 6–9 months after samples were vitrified, straws were thawed by immersion in a water bath at 37 °C for 30 s. The response to cryopreservation was calculated by determining cryoresistance ratios (CR) (Esteso et al., 2015) for all these variables, with there being assessments using the same methodology before and after freezing (thus, motility variables determined using CASA were excluded because these samples were only assessed after thawing) using the following formula: CR = (Value after thawing (post)/value before thawing (pre)) × 100.

2.5.2. Vitrification-warming

Sperm samples obtained using the TUMASG-electroejaculation procedures were diluted 1:2 (v:v) with BoviPure Bottom® (Nidacon, Mölndal, Sweden) for sperm selection, and there was centrifugation for 20 min at 300×*g*, as previously described (Caturla-Sánchez et al., 2018) and Cerdeira et al. (Cerdeira et al., 2018) for improving the vitrification procedure for ejaculated samples. The supernatant was discarded and the pellet re-suspended at a final concentration of 25 × 10^6 cells/mL in HTF®, an animal protein-free extender (Irvine Scientific, Santa Ana, CA, USA), supplemented with 250 mM sucrose, 1% BSA and 0.6% HEPES (previously equilibrated at 5% CO_2 at maximum humidity and 37 °C).

Epididymal sperm were flushed from the lumen using HTF® supplemented with 250 mM sucrose 1% BSA and 0.6% HEPES. Epididymal sperm samples were adjusted to a final concentration of 25 × 10^6 cells/mL using the extender that was previously described.

Samples were treated as previously described (Caturla-Sánchez et al., 2018) with there being the following modifications. All samples were treated with a sucrose solution for 2–3 min prior to being plunged into the liquid nitrogen and the samples were subsequently pipetted and plunged drop by drop (30 μl per drop) directly into liquid nitrogen from a height of 11 cm. The pellets formed were stored in 2 mL cryogenic vials (Nuova Aptaca, Canelli, Italy) in liquid nitrogen.

Sperm pellets were warmed in HTF+ 1%BSA at 37 °C for 10 min (external atmosphere 5% CO_2, maximum humidity). The resulting sperm suspension was centrifuged for 5 min at 300×*g*, the supernatant removed, and 50 μl of HTF+BSA solution added to reconstitute the samples before evaluations occurred.

2.6. Analysis of frozen-thawed and vitrified-warmed samples

Sperm morphology, membrane integrity, acrosome status and DNA fragmentation were recorded as previously described in this manuscript. Sperm motility variables were also examined using a Sperm Class Analyzer (SCA) running version 4.0. software (Microptin S.L., Barcelona, Spain), coupled to a phase contrast microscope (Izasa S.A., Barcelona, Spain). Three progression ratios, expressed as percentages, were calculated from the three velocity measurements that were previously described in this manuscript: linearity (LIN = VSL/VCL × 100), straightness (STR=VSL/VAP × 100), and wobble (WOB=VAP/VCL × 100) (Santiago-Moreno et al., 2013). Briefly, semen samples were diluted 1:10–1:60 (v:v) in a TCG washing medium and placed on a pre-warmed (37 °C) 20 μm Leja 8 Chamber Slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). There was evaluation of samples in a minimum of three fields and there were assessments of 500 sperm tracks. The SCA settings were 25 frames per second, and there was a minimum sperm cell size of five

pixels. Sperm were considered to have progressive motility if cells had a VAP value of > 10 μm/s. Sperm viability and acrosomal status were simultaneously evaluated using a fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC). Live spermatozoa with an intact acrosome were not stained (PI-/PNA-FITC-) (Caturla-Sánchez et al., 2018).

2.7. Statistical analyses

The normality of data distributions was examined using the Kolmogorov-Smirnov (K-S) test and Shapiro-Wilk tests; non-normal values were arcsine transformed before analyses. The paired *t*-test was used to compare sperm variables between treatments (conventional freezing-thawing and vitrification-warming). Comparisons between values for epididymal and ejaculated sperm variables could not be made due to the small number of epididymal samples. Spearman correlation coefficients (r_s) were calculated to determine if there were any associations between the plasma testosterone concentration and the cryoresistance ratios for sperm viability, plasma membrane functionality and DNA fragmentation. The testosterone data were subjected to k-means cluster analysis to identify two populations: experimental animals with relatively greater and relatively lesser testosterone concentration. Differences in sperm cryoresistance ratios between populations were compared using the Mann-Whitney *U*-test. Ultrasonographic measures (testicular diameter, prostate area) were assessed using the *t*-test. There were considered to be mean differences when there was a $P < 0.05$. All data are reported as means ± SE. All calculations were made using Statistica for Windows v.13.3 software (TIBCO Software Inc., Palo Alto, CA USA).

3. Results

There were 12 ejaculates and two epididymal samples that were considered to be of a quality worthy for cryopreservation. Semen volume and sperm concentration varied widely among animals (Table 1). Testicular and prostate sizes, determined during ultrasonic examinations, were 2.16 ± 0.09 cm for testicular diameter, and 3.65 ± 0.32 cm^2 for prostate area. There were no differences ($P > 0.05$) between deslorelin-implanted animals and the other non-implanted experimental animals in terms of testicular diameter or the area of prostate. Experimental animals categorized as dominant individuals had larger ($P < 0.05$) prostate sizes (4.65 ± 0.20 cm^2) than the other animals (3.10 ± 0.30 cm^2).

Data for fresh ejaculated ($n = 15$) and epididymal ($n = 2$) sperm variables (means ± SE) are reported in Table 2. The percentage of viable sperm was greater than 77% and percentage of sperm with DNA damage less than 8% in both the ejaculated and epididymal samples. In contrast, there were large percentages of sperm abnormalities (> 30%) in both types of samples, with alterations of the tail being the most common abnormality.

With the vitrification-warming technique, results were very similar ($P > 0.05$) to those when there was imposing of the conventional freezing-thawing method in terms of values for ejaculated sperm progressive motility (PM), kinetic variables VCL, VSL, VAP and BCF, percentage of sperm with DNA fragmentation, sperm membrane functionality, and morphological abnormalities. Values for total motile sperm (TM), progression ratios LIN, STR and WOB, ALH, sperm viability as assessed using eosin-nigrosin staining, and percentage of live spermatozoa with intact acrosome (IP-/PNA-FITC-) were all greater ($P < 0.05$) for the frozen-thawed compared to vitrified-warmed samples (Table 3).

For the epididymal sperm, motility and viability were apparently greater in the vitrified-warmed samples than conventional frozen-thawed samples (Table 3). The small number of epididymal sperm samples ($n = 2$), however, precluded any statistical analysis.

Table 2
Fresh ejaculated and epididymal wolf sperm variables (means ± SE).

	Ejaculated ($n = 12$)	Epididymal ($n = 2$)
Volume (μl)	3313.5 ± 603.5	2416.8 ± 1492.3[a]
Concentration (Spzx10^6)	100.3 ± 29.5	154.3 ± 124.2
Subjective Total Motility (%)	58.7 ± 6.2	36.7 ± 15.9
Score (1–5)	2.5 ± 0.2	2.2 ± 0.8
Viable Sperm (%)	77.4 ± 3.6	87.0 ± 5.6
Membrane functionality (%)	66.8 ± 7.2	86.3 ± 4.9
Intact Acrosome (%)	82.4 ± 2.7	77.7 ± 22.3
Sperm DNA fragmentation (TUNEL+) (%)	5.2 ± 1.7	7.0 ± 4.0
Sperm morphological abnormalities (%)	54.3 ± 4.0	34.7 ± 2.8
Head abnormalities (%)	2.3 ± 1.4	0.3 ± 0.3
Decapitated (%)	2.5 ± 1.1	2.0 ± 1.1
Midpiece abnormalities (%)	6.0 ± 1.8	8.7 ± 6.7
Coiled tail (%)	20.7 ± 3.4	0 ± 0.0
Bent tail (%)	3.3 ± 2.6	12.0 ± 6.0
Cytoplasmic droplets (%)	12.7 ± 10.4	21.0 ± 6[b]

[a] Total volume of the fresh epididymal sperm after flushing with 2 mL of extender.
[b] Distal cytoplasmic droplets are not considered morphological abnormalities in epididymal sperm because these are commonly detected in epididymal sperm cells; Percentage of sperm with distal and proximal cytoplasmic droplets, however, were determined separately.

The plasma testosterone concentration varied among the wolves from 0.0 ng/mL to 7.7 ng/mL (Table 1). Cryoresistance ratios (for ejaculated sperm) were small ($P > 0.18$) with the correlation with testosterone concentration for all variables studied being the following: $r_s = -0.45$ for viability, $r_s = -0.34$ for plasma membrane functionality, $r_s = -0.06$ for DNA fragmentation in frozen-thawed samples; $r_s = -0.25$ for viability, $r_s = -0.33$ for plasma membrane functionality, $r_s = 0.21$ for DNA fragmentation in vitrified-warmed samples. There were no differences in the cryoresistance ratios for any sperm variables among wolves with relatively greater and relatively lesser plasma testosterone concentrations. Cluster analysis results indicated there was a total of six wolves in the relatively greater testosterone group, and the other eleven were in the relatively lesser testosterone group. Within the six wolves considered as dominant alpha, four (nos. 3, 8, 14, 17, Table 1) were included in the cluster sample with the relatively greater testosterone concentration, and two alpha wolves (no: 5, 16) were in the group with relatively lesser testosterone.

4. Discussion

With the present findings, the conventional slow freezing method resulted in greater values of total motile sperm, LIN, STR, WOB, ALH, sperm viability and sperm with intact membrane and acrosome. With the vitrification method, however, there were similar results as that for conventional freezing for progressive motility, kinetic variables VCL, VSL, VAP and BCF, DNA fragmentation, and morphological abnormalities. Consequently, vitrification of Iberian wolf sperm is an efficacious procedure for easy application in the field for preserving wolf spermatozoa. The results also indicate there are not detrimental effects on the freezing capacity that are associated with plasma testosterone concentration at the time of semen collection.

The motility of the frozen-thawed ejaculated sperm was very similar to that reported for red wolves (*Canis lupus rufus*) (Goodrowe et al., 1998). In this previous study the motility of the sperm of the fresh sample was much greater (71%). The conventional freezing method utilized in the present study involved the use of a Tris-based extender with egg yolk, which when used resulted in a greater viability (40.7%) and intact acrosome percentage (62.9%) than those reported for other wolf subspecies such as the Mexican grey wolf (10.3% and 57.3%, respectively) or grey wolf (2.4% and 40.7%, respectively) (Zindl et al., 2006; Christensen et al., 2011), therefore, this procedure would seem to be quite appropriate for use when conducting assisted reproduction technologies.

Sperm vitrification, however, is easier to perform (rendering it valuable for field use) and less economically expensive (Swanson et al., 2017; Consuegra et al., 2020). The results indicate there is a lesser total sperm motility, viability and intact acrosome percentage in vitrified-warmed samples compared to when the freezing-thawing technique is imposed, which could be indicative of a lesser fertilizing capacity with vitrified spermatozoa. Nevertheless, the present results indicate there are similar results with use of vitrification-warming procedure to that with conventional freezing-thawing in terms of ejaculated sperm plasma membrane functionality, progressive motility, and values for sperm kinetic variables, all of which are also positively correlated with fertility (Núñez-Martínez et al., 2006). Furthermore, the percentage of sperm with damaged DNA was similar with vitrified and samples after conducting the freezing-thawing procedures. Interestingly, with the frozen-thawed sperm, there were greater ALH and WOB values than for the vitrified-warmed samples, indicating conventionally cryopreserved sperm become hyperactive due to cryo-capacitation (Kuroda et al., 2007). Differences in the processing of sperm might have resulted in the differences in CASA motility values (Contri et al., 2010). Differences in sperm selection method (Cerdeira et al., 2018), the time required for thawing or warming, or in the use of centrifugation after warming (but not after thawing), can also affect the values for these motility variables (Contri et al., 2010). In dogs, sperm vitrification can damage the mitochondria and sperm tail (Cerdeira et al., 2020) which negatively affect values for motility variables (Caturla-Sánchez et al., 2018), however, in the present study for ejaculated vitrified-warmed sperm, values for total and progressive motility were greater than those reported in the previous study (Caturla-Sánchez et al., 2018). It may be that there is less

Table 3
Iberian wolf sperm variables (means ± SE) - ejaculated samples (EJ), epididymal samples (EP) - after conventional freezing-thawed and vitrification-warming.

	Post-thawing EJ ($n = 12$)	Post-warming EJ ($n = 12$)	Post-thawing EP ($n = 2$)	Post-warming EP ($n = 2$)
Total Motility (%)	26.1 ± 6.7[a]	11.2 ± 3.9[b]	37.9 ± 19.1	45.7 ± 12.8
Progressive Motility (%)	3.1 ± 0.9	2.7 ± 1.4	6.7 ± 3.5	8.2 ± 3.9
VCL (μm/s)	25.3 ± 4.1	15.7 ± 6.6	23.7 ± 11.9	38.5 ± 7.9
VSL (μm/s)	12.3 ± 3.9	8.2 ± 4.2	8.9 ± 4.5	25.1 ± 8.0
VAP (μm/s)	16.63 ± 4.0	10.2 ± 4.8	13.4 ± 6.7	29.2 ± 7.7
LIN (%)	36.5 ± 6.6[a]	19.3 ± 6.7[b]	21.7 ± 11.0	59.2 ± 9.8
STR (%)	55.88 ± 6.9[a]	31.2 ± 9.7[b]	37.9 ± 19.0	76.6 ± 8.9
WOB (%)	53.6 ± 6.9[a]	27.6 ± 8.5[b]	35.9 ± 18.0	72.7 ± 6.0
ALH (μm)	1.4 ± 0.2[a]	0.7 ± 0.3[b]	1.3 ± 0.6	1.4 ± 0.1
BCF (Hz)	3.0 ± 0.9	1.7 ± 0.8	2.7 ± 1.4	4.1 ± 0.7
Viable Sperm (%)	54.9 ± 3.8[a]	39.1 ± 6.9[b]	54.0 ± 12.2	55.0 ± 6.7
Membrane functionality (%)	62.9 ± 4.1	62.3 ± 4.2	58.7 ± 16.1	64.3 ± 3.5
Intact Membrane and acrosome (%)	40.7 ± 7.2[a]	10.4 ± 2.6[b]	45.3 ± 18.8	32.3 ± 9.5
DNA fragmentation (TUNEL+) (%)	3.9 ± 0.8	7.4 ± 1.9	5.0 ± 2.1	6.0 ± 2.5
Morphological Abnormalities (%)	32.6 ± 3.4	30.5 ± 6.3	60.1 ± 21.6	38.0 ± 5.5

Curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF).
Within rows, values with different superscripts differ ($P < 0.05$).

damage of the mitochondria and sperm tails in the wolf. Nevertheless, there were similar results in wolf sperm in terms of the values for kinetic variables. Species-specific sensitivity to cryopreservation may explain these differences in findings among studies.

The percentage of viable spermatozoa with an intact acrosome was greater for the frozen-thawed compared to vitrified-warmed samples. Conversely, results from previous studies with dog sperm indicated acrosome integrity was markedly greater in vitrified than frozen samples, using 250 mM of sucrose, but the acrosome damage was greater when there was vitrification utilizing larger concentrations of sucrose (Caturla-Sánchez et al., 2018). Furthermore, results from ultrastructural analysis did not indicate differences in the morphological damage of membrane and acrosome between samples that were conventionally frozen or vitrified (Cerdeira et al., 2020). Results indicate there was a greater sensitivity of the wolf sperm acrosome to vitrification-warming procedures that might be explained, among other factors, by having inadequate sucrose concentrations for protection of the acrosome when imposing these procedures. The experimental approaches used in the study were based on results from a previous study (Sánchez et al., 2011) where there was use of a vitrification media without inclusion of a permeable cryoprotectant. There should be future studies conducted utilizing lesser disaccharide concentrations combined with permeant cryoprotectants in non-domesticated species in attempts to improve the acrosome integrity after vitrification-warming procedures occur.

There have been efficacious regimens reported for the vitrification of epididymal and ejaculated sperm from other non-domesticated species (Bóveda et al., 2018). Although in the present study it was not feasible to conduct a statistical analysis, the present findings indicate that for epididymal sperm there is a greater retention of semen quality after vitrification-warming than occurs with ejaculated sperm. Certainly, epididymal sperm from bulls (Cunha et al., 2016), boars (Perez-Patiño et al., 2019) and stallions (Braun et al., 1994) have been reported to be more resistant to cryo-damage than ejaculated sperm. The epididymosomes (Nixon et al., 2019) and extracellular vesicles (Leahy et al., 2020) contribute to the replacement of sperm proteins during epididymal transit and ejaculation respectively, and the resulting proteome differences between ejaculated and epididymal sperm might have effects on sperm freezability (Martínez-Fresneda et al., 2021). While the vitrification of epididymal sperm has been recommended for the Mhorr gazelle (*Nanger dama mhorr*), giraffe (*Giraffa camelopardalis rothschildi*) and brown bear (*Ursus arctos*) (O'Brien et al., 2019), there is need for greater study to confirm whether utilization of this procedure should be recommended for wolves. Furthermore, other vitrification procedures such as cryoloops should be evaluated for wolf sperm (Isachenko et al., 2004), or applying the two-straw technique (Diaz-Jimenez et al., 2021). The addition of glycerol as a permeant cryoprotectant along with cholesterol, in the vitrification medium, could be also evaluated in future studies in wolf sperm (Hosseini et al., 2019).

The sperm quality after imposing vitrification-warming procedures provides some support for the use of the cryopreservation technique when conducting assisted reproductive techniques in wolves (i.e., artificial insemination, for Iberian wolf conservation). Artificial insemination with sperm of Iberian ibexes cryopreserved similarly to our study has resulted in production of offspring (Pradiee et al., 2015).

Testosterone has effects in modifying the morphological and functional properties of sperm cells, affecting sensitivity to freezing-thawing procedures (Flores-Gil et al., 2020). Certainly, in some non-domesticated ruminant species, the relatively greater testosterone concentrations of some individuals are associated with less spermatozoa freezing capacity (Bóveda et al., 2021). In this study, the relatively greater plasma testosterone concentrations in some individuals did not affect sperm cryoresistance to freezing, although there was a trend for a negative correlation between testosterone concentration and sperm viability values. These results should be considered with caution, however; until studies are conducted including larger numbers of samples. In addition, it is important to recognize that testosterone secretions occur in a pulsatile pattern (Cofey, 1988), with marked fluctuations throughout the day. Because only one blood sample was collected from each wolf in the present study, the reliability of these findings is limited. Blood samples need to be collected more frequently to effectively make endocrinological determinations, but while such procedures are conducted with domestic animals (Pelletier et al., 1982), it would be much more challenging for sequential blood samples of this type to be collected from wolves. The hierarchic social structure has been positively correlated with testosterone concentrations, testis size, and semen production in many mammalian species (Gomendio et al., 2007). Findings in the present study indicate that only four of the six wolves considered to be dominant were classified in the relatively greater testosterone concentration category ($>$ 4 ng/mL), whereas two of these individuals had the relatively lesser testosterone concentrations ($<$ 0.8 ng/mL). Similarly, there have been previous reports in other species, such as sheep, where testosterone concentrations did not vary with the social rank except at the onset of the breeding season (Ungerfeld and Lacuesta, 2015). I In baboons, the associations among social dominance and relatively greater testosterone concentrations appear to occur only in unstable social settings (Sapolsky, 1991). Results from the present study, however, should be considered with caution due to the small number of dominant wolves for the group with the relatively lesser testosterone concentrations which limits adequate statistical validation. The prostate size was larger in dominant wolves, similar to that reported for African wild dogs (*Lycaon pictus*) (Van den Berghe et al., 2019). It is widely recognized that quantity of secretions from the prostate are regulated by testosterone with there being greater secretions when there are greater testosterone concentrations in blood (Luke and Coffey, 1994).

The proportions of sperm morphological abnormalities in the present study were substantially greater than those reported for dogs (England, 1993) and red foxes (*Vulpes vulpes*) (Jalkanen, 1993). In dogs, Oettlé determined that if the percentage of normal spermatozoa was less than 60%, the fertility of the donor was less (Oettlé, 1993), and in the present study the values for ejaculated samples were less than this threshold (46%). Along with a possible negative effect on fertility, there was a greater percentage of morphologically abnormal sperm which might be indicative that cryopreservation of spermatozoa using either method might have marked detrimental effects on fertility when semen is used for artificial insemination. Results from studies with the clouded leopard (*Neofelis nebulosa*) indicate the cause of the large percentage of abnormal sperm was related to a lesser genetic diversity (Wildt et al., 1986), and certainly, extent of inbreeding cannot be discounted for the captive Iberian wolf populations in Spain's zoological gardens. Alterations of the sperm tail were a common abnormality detected in the present study. Tail defects are frequently detected in the clouded leopard

also (Tipkantha et al., 2017).

5. Conclusion

In conclusion, the conventional slow freezing procedures for preservation of Iberian wolf sperm were more efficacious than vitrification procedures when various aspects of sperm viability were considered. Sperm vitrification, however, can result in cryosurvival of Iberian wolf sperm and, therefore, there can be utilization in *in ex situ* conservation programs. Considering the practicality of conducting the vitrification procedures, and relatively lesser economical costs of the procedure, this technique may be efficacious as an alternative for conserving sperm of subspecies of wolves. More studies need to be conducted to refine the vitrification procedures so that values for some sperm variables such as sperm motility and acrosome integrity, after thawing can be improved, as well as to further ascertaining of whether the vitrification of epididymal sperm results in sperm with greater viability than sperm collected using electroejaculation procedures in wolves.

Conflicts of interest

None to declare.

Acknowledgments

This research was funded by MCINN/AEI/FEDER grant AGL2017-85753-R and by the *Fundación Parques Reunidos*—INIA agreement CC19-096. The authors thank to Oscar del Aguila for his help in the management of the wolves at the Cañada Real Nature Centre.

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