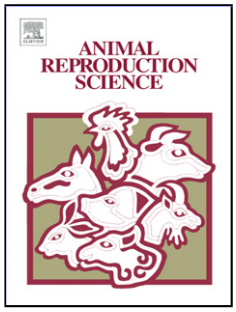

Title: Effect of cooling rate on sperm quality of cryopreserved Andalusian donkey spermatozoa

Authors: S. Demyda-Peyrás, M. Bottrel, D. Acha, I. Ortiz, M. Hidalgo, J.J. Carrasco, V. Gómez-Arrones, J. Gósalvez, J. Dorado

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Effect of cooling rate on sperm quality of cryopreserved Andalusian donkey spermatozoa

Short title: Cooling rates for freezing donkey spermatozoa

S Demyda-Peyrás[ad], M. Bottrel[a], D. Acha[a], I. Ortiz[a], M. Hidalgo[a], J.J. Carrasco[b], V. Gómez-Arrones[b], J. Gósalvez[c], J. Dorado[a,*]

[a]Veterinary Reproduction Group, Department of Animal Medicine and Surgery. Faculty of Veterinary Medicine, University of Cordoba, 14071 Cordoba, Spain

[b]Equine Reproduction Center, Centro de Selección y Reproducción Animal (CENSYRA-Extremadura Government), 06007 Badajoz, Spain

[c]Department of Biology, Genetics Unit, Autonomous University of Madrid, 20849 Madrid, Spain

[d]Current address: IGEVET – Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP - CONICET LA PLATA). Faculty of Veterinary Sciences, La Plata State University, 1900 La Plata, Argentina.

[*]**Corresponding author at**: Department of Medicine Animal and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Campus Universitario de Rabanales (Edif. Hospital Clínico Veterinario), Ctra. Madrid-Cádiz, km 396, 14071 Cordoba, Spain. Tel:

+0034 957 212136; fax: +0034 957 211093. E-mail address: jdorado@uco.es (J. Dorado).

Highlights

- Different cryopreservation protocols were compared.
- Andalusian donkey is particularly sensitive to pre- freezing cooling rate.
- Controlled pre-freeze cooling rate improve all sperm parameters evaluated.
- Rapid freezing reduces donkey spermatozoa quality after thawing.
- No effect on DNA integrity was observed.

ABSTRACT

The aim of this study was to evaluate the effect of different cooling rates on post-thaw quality of cryopreserved donkey spermatozoa. Eighteen ejaculates from six adult Andalusian donkeys (three ejaculates per donkey) were collected using an artificial vagina. Pooled semen samples (two ejaculates per pool) were divided into three aliquots, and frozen in Gent freezing extender using three different cryopreservation protocols (P): P1 (conventional slow freezing, as control): semen pre-cooled in an Equitainer for 2 h and frozen in liquid nitrogen (LN_2) vapour; P2 (controlled pre-freeze cooling rate): semen pre-cooled at a controlled rate for 73 min and frozen in LN_2 vapour; and P3 (rapid freezing) semen frozen immediately in LN_2 vapour. After thawing at 37 ºC for 30 s, semen samples were assessed for motility, morphology, acrosome and plasma membrane integrity; spermatozoa were also tested for DNA integrity. Significant ($P<0.01$) differences were found between the cryopreservation protocols for all sperm parameters evaluated, except for DNA integrity. Semen samples frozen using P2 showed significantly ($P<0.01$) higher values for sperm motility, morphology, sperm membrane integrity, and acrosome integrity. On the contrary, P3 reduced sperm motility ($P<0.01$) and increased the percentage of spermatozoa with damaged plasma membrane ($P<0.001$). In our study, we demonstrated that the sperm of Andalusian donkey is particularly sensitive to the cooling rate used before freezing. Furthermore, Andalusian donkey semen can be successfully cryopreserved using controlled cooling rates combined with freezing in LN_2 vapour.

Keywords: Equus asinus; Donkey; Spermatozoa; Cryopreservation; Cooling rate

1. Introduction

The Andalusian donkey (*Equus asinus*) is a Spanish donkey breed considered as endangered by the Spanish Government (Real Decreto 2129/2008, regulation of the National Catalogue of Endangered Species). According to the Food and Agricultural Organization (FAO, 2014), this breed is currently distributed in very small herds (average herd size: 4.8) with a very restricted number of reproductively active individuals. This situation has increased the crosses between related individuals with the subsequent increase in the inbreeding level of the breed (Aranguren-Mendez et al., 2001). One of the most important management actions to mitigate the increase of inbreeding coefficient is the flow of non-related males among herds which will increase the genetic pool and variability (Howard et al., 2017). Therefore, the use of cryopreserved semen become an extremely necessary practice since the movement of the individuals among the livestock farms is difficult (Trimeche et al., 1998). However, information on semen cryopreservation in Andalusian donkeys is still scarce (Ortiz et al., 2015a).

Assisted reproductive technologies (ARTs) developed in horses have been directly applied in donkeys for decades. Consequently, most of the procedures used for cryopreservation of donkey semen have derived from those reported in horses (Canisso et al., 2011; Madison et al., 2013; Trimeche et al., 1998). Despite sperm freezing protocols resulted in an excellent post-thawing semen quality, the use of artificial insemination (AI) with cryopreserved semen has given disappointing results in this species (Canisso et al., 2011; Rota et al., 2012; Serres et al., 2014; Vidament et al., 2009). To date, the best pregnancy rate (61.5%) was reported when jennies were

inseminated with donkey semen cryopreserved in INRA 96 extender supplemented with 2% egg yolk and 2.2% glycerol (Rota et al., 2012). However, a recent study performed in Andalusian donkeys showed that the use of an egg yolk freezing extender provided better post-thaw motility parameters than INRA 96 egg yolk glycerol (Acha et al., 2016).

The cooling rate at which sperm is refrigerated before freezing is one of the most important parameters involved in the effectiveness of a given cryopreservation procedure (Barbas and Mascarenhas, 2009). This is particularly important in equines, in which semen cryopreservation procedures are still under development (Devireddy et al., 2002b; Salazar et al., 2011). It is well known that the exposure of sperm to low temperatures results in morphologic, biochemical and functional damage to spermatozoa, thereby reducing fertility (Watson and Morris, 1987). Other authors have related this cold shock damage to fast rates of cooling (> −0.3 ºC/min) during the lipid phase transition in sperm plasma membranes (Moran et al., 1992; Varner et al., 1988). In addition, the range of cold shock sensitivy has been determined (19 ºC to 8 ºC) for stallion spermatozoa (Moran et al., 1992).

It is known that other steps of the cryopreservation process can affect sperm parameters. In this sense, the effect of freezing rate on the post-thaw semen quality of stallions (Neuhauser et al., 2014; Vidament et al., 2000) has been well established. It is known that if stallion spermatozoa are rapidly frozen the formation of intracellular ice crystals will be high producing the death of the cell due to damage in the plasma membrane. However, if the freezing rate is too slow, spermatozoa will be too dehydrated, also causing irreversible cell damage (Gao and Critser, 2000). In addition, recent research has demonstrated that stallion spermatozoa suffer osmotic stress during

the cryopreservation process (Peña et al., 2011). This effect has been identified with adding or removing permeable cryoprotective agents (CPAs), such as glycerol, which creates an anisotonic environment for the spermatozoa, thereby causing the cells to shrink or swell, respectively (Glazar et al., 2009). In a recent study performed in Brazilian donkeys (Oliveira et al., 2016) it was suggested that the freezing protocol employed (liquid nitrogen (LN_2) vapors: −10 °C/min from 5 °C to −60 °C and −8 °C/min from −60 °C to −100 °C; or automated freezer: −15 °C/min from 5 °C to −10 °C and −40 °C/min from −10 °C to −140 °C) did not produce differences in post-thaw sperm motility and viability. However, and despite the importance of the pre-freezing cooling rate in the success of cryopreservation of stallion spermatozoa, this procedure was not yet evaluated in donkey sperm.

Rapid freezing of spermatozoa has been recently proposed as an alternative to conventional slow freezing methods (Katkov et al., 2006). Hypothetically, this technique would avoid the intracellular formation of large ice crystals and the cytotoxic effect of high salt concentrations during cryopreservation. In addition, this procedure is simple and takes only a few minutes (Isachenko et al., 2004), which is extremely important in extensively raised species and breeds. However, conventional rapid freezing methods (i.e. ultrarapid freezing and vitrification) requires high concentrations of CPAs which could be toxic to spermatozoa (Gilmore et al., 1997). Consequently, new methodologies are being developed based on the use of higher cooling rates, which can be achieved by decreasing the volume of CPA and increasing the surface-to-volume ratio of the sample (Isachenko et al., 2004). These procedures have obtained quite satisfactory results in sperm quality and fertility in human (Isachenko et al., 2012),

canine (Kim et al., 2012; Sánchez et al., 2011) and wild ruminant (Pradiee et al., 2015). However, to our knowledge, there are no previous studies performed in donkeys.

Therefore, the aim of the present study was to compare three low-CPA-based donkey semen cryopreservation protocols: (i) conventional slow freezing (as control), (ii) controlled pre-freeze cooling rate; and (iii) rapid freezing. The efficiency of the protocols was assessed in terms of motility, morphology, plasma and acrosome membrane integrity, and DNA integrity of frozen-thawed donkey spermatozoa.

2. Materials and methods

This study was conducted in Badajoz (Spain) and all animal procedures were approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in accordance with Regional Government of Andalusia (no. 31/08/2017/105) and the Spanish laws for animal welfare and experimentation (Real Decreto 53/2013).

2.1. Animals and experimental design

Six healthy Andalusian jacks (4 to 15 years of age) with proven fertility were used. The animals were housed in individual paddocks at the Centro de Selección y Reproducción Animal (CENSYRA) and fed with a diet based on hay, grain, and water *ad libitum*.

A total of 18 ejaculates (three per jack) were collected to evaluate the effect of different cooling rates on the quality of frozen-thawed donkey semen. Sperm samples were collected using a Missouri model artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line gel filter (Minitüb GmbH, Tiefenbach, Germany) to permit collection of free-gel semen. Ejaculates from two alternative donkeys were pooled (nine

in total) on each collection day to avoid introduction of uncontrolled male-to-male variation (Dorado et al., 2014). Pooled semen samples were divided into three aliquots, and frozen in Gent freezing extender using three different cryopreservation protocols. Semen quality was evaluated before freezing and after thawing.

2.2. Sperm evaluation

After collection, gel-free volume was measured in a graduated tube (mL), and the sperm concentration was calculated with a photometer (SpermaCue, Minitüb GmbH, Tiefenbach, Germany) as described by Vidament et al. (2009). At the same time, a semen aliquot was diluted to 25 $x10^6$ spermatozoa/mL in a skim milk based extender (EquiPro, REF. 13570/0201, Minitüb GmbH, Tiefenbach, Germany) and equilibrated for 10 min at 37 ºC (Dorado et al., 2013). Aliquots of the diluted semen were subsequently taken as needed to conduct the appropriate analyses.

Sperm motility parameters were assessed using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer – SCA, Microptic S.L., Barcelona, Spain), following the methodology previously described by Miro et al. (2005). Prior to the assessment of movement, aliquots of diluted semen were incubated at 37 ºC for 5 (fresh semen) or 10 min (frozen-thawed samples). For each evaluation, three consecutive 5 µL drops of each diluted semen sample were evaluated using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a pre-warmed stage at 37 ºC at 100X magnification. Two microscopic fields per drop were filmed randomly, including a minimum of 200 spermatozoa. Objects incorrectly identified as spermatozoa were minimized by using the playback function. Regarding the setting variables of the program, spermatozoa with a mean average path velocity (VAP) < 10 µm/s were

considered immotile. Spermatozoa with VAP > 90 μm/s were considered as rapid, while spermatozoa deviating < 25% from a straight line were designated as linear motile. The measured variables of sperm motion were total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm/s), straight line velocity (VSL; μm/s); average path velocity (VAP; μm/s), linearity (LIN, as VSL/VCL; %), straightness (STR, as VSL/VAP; %), wobble (WOB, as VAP/VCL; %), beat cross frequency (BCF; Hz), and amplitude of lateral head displacement (ALH; μm). Definitions of these descriptors of sperm movement can be found in Dorado et al. (2007).

Spermatozoa morphology was examined in smears stained with Diff-Quick® (Medion Diagnostics AG, Düdingen, Switzerland) at 1000X magnification on an Olympus BH-2 light microscope (Olympus Optical Co., LTD, Tokyo, Japan) following the classification described by Brito (2007). At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology (ASM, %), and different types of sperm abnormalities were scored (head, midpiece, and tail abnormalities).

Sperm membrane integrity was assessed at 400X magnification by epi-fluorescence microscopy (Olympus BX40, Tokyo, Japan), following the procedure described by Dorado et al. (2014), and using the fluorescent probes propidium iodide (PI) and acridine orange (AO) from the Vitaltest® Kit (Halotech DNA SL, Madrid, Spain). Briefly, an aliquot (10 μL) of diluted semen (15 $x10^6$ spermatozoa/mL) was placed on a microscope slide at 37 ºC and mixed with 1 μL PI stock solution and 1 μL AO stock solution. At least 200 spermatozoa per slide were counted, considering green spermatozoa as membrane-intact spermatozoa – MIS (AO+; %).

To evaluate the sperm acrosomes, the PI/peanut agglutinin-fluorescein isothiocyanate (FITC-PNA) double stain (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was use as described by Dorado et al. (2014). In brief, a 10 µL aliquot of diluted semen (25 x10^6 spermatozoa/mL) was spread on a microscope slide, air dried, fixed, and permeabilized with 70% (v:v) ethanol for 30 s. A mixture of 10 µL PI (0.1 mg/mL in ultra-pure water) and 20 µL FITC-PNA (0.1 mg/mL in PBS), previously incubated at 38 ºC for 5 min in the dark, was then spread over each smear and the slides were incubated in a dark, moist chamber at 4 ºC for 30 min. The slides were subsequently rinsed with deionized water at 4 ºC, mounted with 10 µL of Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA), covered with a coverslip, and sealed with nail polish. At least 200 spermatozoa were evaluated on each slide under 1000X magnification by epi-fluoresence microscopy. Ethanol-permeabilized donkey spermatozoa could be classified into two groups: (1) acrosome-intact spermatozoa – AIS (the acrosomal region of the sperm head displayed bright green fluorescence; PI+/FICT-PNA+), and (2) acrosome-reacted spermatozoa – ARS (green fluorescent at the equatorial segment or no anterior acrosomal staining; PI+/FICT-PNA-). All spermatozoa showed red fluorescence due to counterstaining with PI. Values were expressed as percentages.

Sperm DNA fragmentation (sDF) was assessed post-thaw using the Halomax® Kit (Halotech DNA SL, Madrid, Spain) as described Ortiz et al. (2015b) for donkeys. A dynamic approach to the determination of sDF was also conducted by incubating aliquots of the original diluted sample for 24 h at 37 ºC as was described by Crespo et al. (2013). Consequently, sDF was assessed at T0 (baseline), T6 and T24 h. For each sample, a minimum of 300 spermatozoa were counted using a fluorescence microscope

at 400X magnification. The percentage of spermatozoa with fragmented DNA (large halos of chromatin dispersion, at least the double diameter than the core) was calculated and expressed as a percentage of the total sperm count (sDFI, %).

2.3. Sperm cryopreservation protocols

Prior to cryopreservation, aliquots of each ejaculate were pooled (two ejaculates per pool) and diluted in Equipro at a 1:1 ratio (semen:extender, v:v). Pooled semen samples were centrifuged at 400 x *g* for 7 min at room temperature and the sperm pellets were re-suspended in an egg yolk freezing extender with 5% glycerol (Gent, REF. 13571/1045; Minitüb GmbH, Tiefenbach, Germany) to a final concentration of 200 $x10^6$ spermatozoa/mL. Thereafter, the extended semen was split into three aliquots and frozen according to different protocols (Table 1): Protocol 1 (P1): Conventional slow freezing method (as control): spermatozoa were slowly cooled (–0.13 ºC/min) for 2 h in a container (Equitainer I^{TM}, Hamilton-Thorn Research, Danvers, MA) and then filled in 0.5 mL plastic straws (Minitüb GmbH, Tiefenbach, Germany) at 5 ºC. The straws were placed onto floating racks 2.5 cm above LN_2 surface for 5 min and then plunged directly into LN_2, as described by Ortiz et al. (2015a) in donkeys; Protocol 2 (P2): Controlled pre-freeze cooling rate: whereby spermatozoa were loaded in 0.5 mL straws at room temperature and then slowly cooled using a programmable bio-freezer (Kryo 10 Series III, Planer, Middlesex, UK) following a three steps temperature decreasing ramp (–2 ºC/min from 25 ºC to 22 ºC; –0.3 ºC/min from 22 ºC to 10 ºC; and –0.2 ºC/min from 10 ºC to 4 ºC) of 73 min approximately (Salazar et al., 2011). Semen-filled straws were then frozen in LN_2 vapour for 5 min and plunged into LN_2, as described in P1 above; and Protocol 3 (P3): Rapid freezing: spermatozoa were filled

into 0.5 mL capacity straws at room temperature and frozen immediately in LN_2 vapour as described before (Kim et al., 2012).

Straws were thawed at 37 ºC for 30 s (Ortiz et al., 2015a) and diluted to a concentration of 25 $x10^6$ spermatozoa/mL with Gent. Semen assessments were performed after recovery (in skim milk-glucose extender) and after thawing (in Gent extender). For assessment, aliquots of diluted semen were incubated at 37 ºC and evaluated as described previously.

2.4. Statistical analysis

All data were analyzed using the SAS statistical package (v9.0; SAS Institute Inc., Cary, NC, USA). Normality of data was assessed with the Kolmogorov-Smirnov test. Since data reported in this study were not normally distributed, percentages were subjected to arc sine transformation and absolute measures to logarithmic transformation. Sperm characteristics of different freezing protocols semen were compared using a general linear model (PROC MIXED), considering pools as a random effect. Differences between mean values were analyzed by the Duncan method. The rate of sDF was calculated from the slope of the linear regression equation determined from each semen sample. Results are expressed as mean ± SEM and $P<0.05$ was considered significant.

3. Results

Cryopreservation had a significant ($P<0.01$) effect on most of the sperm parameters evaluated (Tables 2 and 3), and was different depending on the cryopreservation protocol used. In general, P1 and P2 yielded similar ($P>0.05$) results

for sperm motility (TM and PM), whereas P3 showed the worst outcome (TM: 22.4%; PM: 16.3%; P<0.001). In addition, PM values obtained in P2 were comparable to that of fresh semen (54.1% *vs.* 62.7%; P>0.05). Sperm velocities (VCL, VAP, and VSL) were also higher (P<0.001) in P1 and P2, compared to P3 (Table 2). Similarly, mean values of LIN, STR, and WOB were significantly (P<0.01) higher in P1 and P2 than in P3. There were no differences (P>0.05) between cryopreservation protocols for ALH, whereas BCF was significantly (P<0.001) lower in P3, compared with P1, P2, and fresh semen.

As shown in Table 3, P2 resulted in a less damage produced to the spermatozoa (P<0.001) in comparison to the other protocols. Although no differences (P>0.05) were observed between P2 and P3 for acrosome integrity, semen samples frozen using P2 had higher (P<0.001) percentages of morphologically normal spermatozoa with intact acrosome and plasma membranes. Indeed, no differences (P>0.05) were observed between P2 and fresh semen for ASM (5.3% *vs.* 9%). The percentage of spermatozoa with abnormal tails was lower (P<0.01) in P2 compared to P1 and P3.

The comparison of the sDFI at different incubation times (0, 6, and 24 h) revealed a significant increase of the DNA damage (P<0.001) over incubation time in all protocols (Table 4). However, no significant (P>0.05) differences were found for sDFI among protocols, in any of the time-points evaluated. Regression analysis of the sDFI showed no difference (P>0.05) with respect to the slopes and the intercepts of the regression equations, indicating that sDFI increased over time at the same rate in all treatments.

4. Discussion

Effective protocols of semen cryopreservation are critical to create germplasm banks of endangered donkey breeds. Studies of stallion sperm cryopreservation have demonstrated that an optimal cooling rate is crucial to obtain an adequate post-thaw viability (Devireddy et al., 2002b; Moran et al., 1992; Oldenhof et al., 2012). However, in donkeys, the evaluation of sperm freezing protocols has been mainly focused on the use of different extenders (Acha et al., 2016), post-thawing supplementation (Sabatini et al., 2014), and different CPAs (Acha et al., 2016; Rota et al., 2012).

This study demonstrated, for the first time, the influence of cooling rates on post-thaw sperm characteristics of Andalusian donkeys. In general, total and progressive motility were higher when a controlled pre-freeze cooling rate (P2) was used. On the contrary, rapid freezing of the samples (P3) produced drops in TM and PM near 70% and 50%, respectively. Higher values of sperm velocities (VCL, VSL, and VAP) were observed when the sperm was carefully cooled (P2) or equilibrated for 120 min (P1). Furthermore, P2 showed the best results of morphology, plasma membrane and acrosome integrity, whereas P3 showed unacceptable results. These findings are in agreement with those reported for horses (Moran et al., 1992), in which they concluded that rapid cooling rates are suitable until 19 ºC but cannot be used during the transition phase (temperatures between 19 ºC and 8 ºC). In fact, the direct freezing of the sperm without cooling (rapid freezing) has shown disappointing results in ejaculated (Lorenzoni et al., 2011; Salazar et al., 2011) and epididymal stallion spermatozoa (Neuhauser et al., 2014). It has been recently suggested that rapid cooling rates applied during the transition phase produce cold-shock injuries (Aurich, 2008), which lead to a disruption in plasma and acrosome membrane functional status by increasing their permeability and decreasing the metabolic activity (Devireddy et al., 2002a). Our results

support the hypothesis that the cooling rate is a vital part of a successful cryopreservation protocol in donkeys.

Most cryopreservation protocols for equine sperm use an equilibration period with the CPA (i.e. glycerol) to protect membranes during cold-shock. However, glycerol may exert deleterious effect on stallion (Macías García et al., 2012) and donkey (Vidament et al., 2009) spermatozoa. Our data suggest that donkey spermatozoa should be cooled and equilibrated up to 4ºC for 73 min to obtain an acceptable post-thaw quality, which is in consonance with others (Moran et al., 1992; Varner et al., 1988). However, further studies using other extenders and protocols should be performed to improve the efficacy of rapid freezing of donkey spermatozoa.

It is interesting to note that the percentage of spermatozoa with abnormal tails increased when the sperm was not equilibrated prior to freezing (P3) or when the temperature ramp was controlled to a lesser extent (P1). Conversely, the percentage of head and mid-piece abnormalities was not affected by the protocol, which is in consonance with Holt et al. (1988). This previous study demonstrated that a percentage of spermatozoa underwent a sudden morphological transformation when temperature drops (between 12 ºC and 8 ºC) too fast, including tail bending and membrane swellings. This is also supported by the fact that P3 showed the highest rate of bent tails, since the immediate freezing of the sample produced the steepest drop in temperature, and P2, with a carefully controlled temperature drop, showed results statistically equal to the fresh semen. However, in consonance with Kim et al. (2012), conventional slow freezing (P1) increased the percentage of total sperm abnormalities compare to rapid freezing (P3), the cause of which remains unclear until now. Although P1 (conventional slow freezing method) maintained the cold chain during filling straws,

one possible reason is that a series of inadvertent thermal fluctuations within the straws could have occurred and consequently affect post-thaw sperm quality (Holt, 2000; Lieberman et al., 2016). Another possible reason is that cryopreservation sensitivity seen in donkey spermatozoa could be different from horses (19 ºC to 8 ºC), as has been previously proposed (Holt, 2000). To our knowledge, the specific range of temperatures at which donkey spermatozoa is damaged by rapid cooling has not been determined. Nevertheless, our findings suggest that the morphology of donkey spermatozoa is also sensitive to sharp drops in temperature during cooling, as was described in other species (Amann and Pickett, 1987; Mocé et al., 2014).

Stallion spermatozoa membranes have been described as highly sensitive to cooling and freezing procedures (Morris et al., 2007; Salazar et al., 2011). In our study, P3 showed the lower values of plasma membrane and acrosomal integrity, whereas the opposite was found for P2. Differences in plasma membrane integrity were higher than the observed among acrosomes, ranging 12% and 36% among P2 and P1 and P3, respectively. It was demonstrated that drastic temperature changes induce stresses on membranes, probably mediated by lipid phase changes (Parks and Lynch, 1992; Sieme et al., 2015). This process, as well as the described in sperm morphology, occurs mostly during spermatozoa passage through the 15 ºC and 5 ºC temperature range (Watson, 2000). This hypothesis was supported by our results, since P2 and P3, the most controlled and drastic cooling treatments, showed the highest differences. Acrosome intactness status was different between protocols but to a lower extent, in the range of 10% between P2 and P3, with an intermediate value in P1. In donkeys, Flores et al. (2008) observed a large reduction of post-thaw acrosome integrity in slowly cooled spermatozoa. Other authors reported, however, no differences (Acha et al., 2016; Contri

et al., 2012). It is well known that sperm capacitation is a physiological process which starts right after ejaculation. Although this process can be minimized by a decreased temperature, the fact that P1 and P2 have at least 70 extra min of incubation time prior to cryopreservation (120 min in the case of P1) compared to P3 can cause an increase in the percentage of capacitated spermatozoa. Therefore, even though P3 could suffer a major damage due to the cryopreservation process, the shorter incubation of this protocol can mitigate the differences observed in AIS.

Previous studies conducted on horses (Crespo et al., 2013) and donkeys (Cortes-Gutierrez et al., 2008; Ortiz et al., 2015b) have demonstrated that DNA fragmentation should be assessed as a dynamic parameter to evaluate the stability of DNA molecule after submitting semen samples to different stressors. In agreement with previous studies (Boe-Hansen et al., 2005; López-Urueña et al., 2014), we found no significant differences in sDFI among the studied protocols at each time point of the incubation period (T0, T6, and T24) as well as in the slope and intercepts of the regression equations. Although the cryopreservation of stallion spermatozoa increases the sDFI (Baumber et al., 2003), no differences were found among freezing treatments in that species (Serafini et al., 2017). Besides, it has been demonstrated that the chromatin package in donkey spermatozoa is different (Cortés-Gutiérrez et al., 2014), which could explain our results.

5. Conclusions

In this study, we determined that post-thaw quality of frozen Andalusian donkey spermatozoa is particularly sensitive to the cooling rate employed during conservation, in terms of sperm quality. In particular, we demonstrated that such decrease of post-

thaw sperm quality was mediated by a reduction of (total and progressive) motility and an increase in plasma membrane and acrosome damage, and tail abnormalities. Furthermore, we also determined that the use of a controlled pre-freeze cooling rate prior to freezing is crucial in order to obtain sperm samples with superior post-thaw quality.

Conflict of interest statement

There are no conflicts of interest to declare.

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Tables

Table 1

Freezing protocols used

Protocol 1	Protocol 2	Protocol 3
Conventional slow freezing	Controlled prefreeze cooling rate	Rapid freezing
Equilibration time 120 min 23.2 ºC (−0.13 ºC/min) 7.2 ºC	Equilibration time 73 min 25 ºC (−2 ºC/min) 22 ºC 22 ºC (−0.3 ºC/min) 10 ºC 10 ºC (−0.2 ºC/min) 4 ºC	Equilibration time -
Nitrogen vapour 2.5 cm above LN_2 for 5 min	Nitrogen vapour 2.5 cm above LN_2 for 5 min	Nitrogen vapour 2.5 cm above LN_2 for 5 min
LN_2 for at least 24 h	LN_2 for at least 24 h	LN_2 for at least 24 h
(Ortiz et al., 2015a)	(Salazar et al., 2011)	(Kim et al., 2012)

LN_2- liquid nitrogen; values in brackets are cooling rates

Table 2

Mean values (± SEM) of sperm motility evaluated by the Sperm Class Analyzer system, in fresh semen (n = 18) and frozen-thawed samples (n = 9)

Parameters	Fresh semen	Frozen-thawed samples		
		Protocol 1	Protocol 2	Protocol 3
TM (%)[a]	91.6±1.1[a]	49.4±5.2[b]	60.9±4.6[b]	22.4±6.1[c]
PM (%)[b]	62.7±2.2[a]	42.2±4.8[b]	54.1±4.3[ab]	16.3±4.7[c]
VCL (µm/s)	128.0±3.4[a]	108.5±4.8[b]	114.4±4.6[b]	87.9±5.1[c]
VSL (µm/s)	91.9±3.4[a]	91.8±4.5[a]	98.2±4.0[a]	69.3±5.0[b]
VAP (µm/s)	113.7±3.0[a]	102.3±5.2[a]	108.6±4.8[a]	81.3±5.2[b]
LIN (%)	71.3±2.5[b]	78.3±2.2[a]	81.1±1.3[a]	67.5±2.8[b]
STR (%)	79.0±2.0[b]	84.3±1.8[a]	86.5±0.8[a]	76.1±2.9[b]
WOB (%)	88.4±1.2[a]	89.9±1.6[a]	91.6±1.2[a]	83.5±2.0[b]
ALH (µm)	2.7±0.1[a]	1.9±0.1[b]	1.9±0.1[b]	1.7±0.0[b]
BCF (Hz)	7.4±0.1[a]	7.4±0.3[a]	7.5±0.1[a]	6.1±0.3[b]

[a]Total motility is defined as the percentage of spermatozoa with a mean velocity > 10 µm/s
[b]Progressive motility is defined as the percentage of spermatozoa with a mean velocity > 90 µm/s and straightness > 75%
TM- total motility; PM- progressive motility; VCL- curvilinear velocity; VSL- straight line velocity; VAP- average path velocity; LIN- linearity; STR- straightness; WOB- wobble; ALH- amplitude of lateral head displacement; BCF- beat cross frequency
Different superscript letters (a-c) in the same row indicate differences (P<0.05)

Table 3

Mean values (± SEM) of sperm morphology, acrosome integrity and membrane integrity in fresh semen (n = 18) and frozen-thawed samples (n = 9)

Parameters	Fresh semen	Frozen-thawed samples		
		Protocol 1	Protocol 2	Protocol 3
ASM (%)	5.3±0.5[a]	15.5±3.8[b]	9.0±1.4[a]	21.0±3.1[b]
Head abnormalities	0.1±0.1[a]	0.5±0.2[a]	0.1±0.1[a]	1.1±0.5[a]
Midpiece abnormalities	1.8±0.2[a]	3.1±0.8[a]	2.3±0.8[a]	4.5±0.7[a]
Tail abnormalities	3.4±0.5[a]	11.9±3.0[b]	6.6±1.0[a]	15.4±2.5[b]
AIS (%)	95.5±0.8[a]	75.2±4.9[c]	84.6±4.0[b]	76.6±5.6[bc]
MIS (%)	84.7±2.7[a]	37.9±4.1[c]	50.8±4.4[b]	14.3±2.7[d]

ASM- Abnormal sperm morphology; AIS- Acrosome-intact spermatozoa; MIS- Membrane-intact spermatozoa

Different superscript letters (a-d) in the same row indicate differences (P<0.05)

Table 4

Mean (± SEM) DNA fragmentation index (sDFI) values associated with the freezing protocols and regression analysis of sDFI *vs*. incubation time (immediately after thawing (T0) and after 6h (T6) and 24 h (T24) of incubation)

Freezing protocols	sDFI			Slope	*Y*-axis Intercept
	T0	T6	T24		
Protocol 1	22.8±2.5[a]	45.1±3.1[a]	53.8±1.8[a]	1.1±0.1[a]	29.5±2.5[a]
Protocol 2	20.5±1.6[a]	42.9±1.9[a]	57.8±3.5[a]	1.4±0.2[a]	26.5±1.3[a]
Protocol 3	18.8±3.0[a]	47.6±4.1[a]	58.9±4.8[a]	1.4±0.2[a]	27.5±3.1[a]
P-value	0.518	0.579	0.553	0.256	0.652

Values sharing *the same superscript within a column are* not statically different (*P>0.05).*