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Effect of cryoprotectant and equilibration temperature on cryopreservation of *Lama glama* spermatozoa

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Summary

The aim of this study was to determine the effect of two equilibration temperatures (5 °C and room temperature) and two cryoprotectants (glycerol and dimethylformamide, both at 7%) on llama sperm cryopreservation. Llama ejaculates were divided into four aliquots. A lactose-EDTA-egg yolk (LEEY) extender with either 7% glycerol (LEEY-G) or 7% dimethylformamide (LEEY-DMF) was added to two of the aliquots, which were equilibrated for 20 min at room temperature and subsequently frozen. The other two aliquots were extended in LEEY, cooled to 5 °C, then LEEY-G or LEEY-DMF was added, equilibrated for 20 min at 5 °C and frozen. No significant differences ($P > 0.05$) were observed in membrane function and chromatin condensation between any of the freeze–thawing protocols. Post-thaw motility was greater ($P < 0.05$) in LEEY-DMF than LEEY-G. DNA fragmentation was not different between raw and frozen semen with LEEY-DMF but was high in all samples with glycerol. Our results indicate that 7% glycerol would be detrimental for llama spermatozoa, but further studies are needed to evaluate effectiveness if used at lower concentrations. Dimethylformamide preserved motility and DNA integrity of frozen–thawed llama spermatozoa and could be used to replace glycerol at the concentrations used in this study.

Introduction

South American Camelids (SACs) are internationally valued because of their special fine fibres and meat with low cholesterol content; therefore, a rising interest in SAC production has developed over the last years. The increase in commercialisation of SAC-derived products makes it necessary to use reproductive strategies to improve herd production and the genetic pool of future reproductive individuals. Among the limitations to SAC production and genetic improvement is the lack of extensive artificial insemination (AI) campaigns using cryopreserved semen. This is a result of the very low pregnancy rates obtained when using preserved semen in these species (Bravo *et al.*, 2000; Aller *et al.*, 2003; Vaughan *et al.*, 2003; Giuliano *et al.*, 2012).

It is well accepted that permeable cryoprotectants, such as glycerol, do have toxic effects (as distinguished from osmotic effects) (Fahy, 1986; Holt, 2000; Watson, 2000). There is a wide variation in glycerol tolerance between species: from 3% in porcines up to 10–20% in marsupials, with sperm tolerance showing a complex interaction between freezing rates and the percentage of cryoprotectant used (Holt, 2000). With regard to SAC semen cryopreservation protocols, 7% glycerol has practically been the only cryoprotectant used in these species, obtaining pregnancy rates of 0–26% (Bravo *et al.*, 2000; Aller *et al.*, 2003; Vaughan *et al.*, 2003). It is noteworthy that even when inseminations are carried out using equivalent amounts of motile and viable cryopreserved or raw spermatozoa, pregnancy rates are lower after AI with cryopreserved semen (0–26%) than with raw semen (50–80%) (Bravo *et al.*, 2000; Aller *et al.*, 2003; Vaughan *et al.*, 2003; Tibary & Vaughan, 2006; Huanca *et al.*, 2007; Maxwell *et al.*, 2008; Giuliano *et al.*, 2012). As a result, it becomes necessary to increase our knowledge on cryoprotectant toxicity towards llama spermatozoa to understand and significantly reduce freezing injury during cryopreservation. Thus, the study of

glycerol and its toxic effects on different llama sperm parameters may provide understanding as to the reasons for the lack of success with this cryoprotectant in these species and allow us to improve current cryopreservation protocols. Besides, the current tendency is to replace glycerol with other cryoprotectants. For this reason, in recent years, the use of amides as cryoprotectants has increased, obtaining good results in stallions (Alvarenga *et al.*, 2005), rabbits (Okuda *et al.*, 2007), turkeys (Blanco *et al.*, 2011) and fish (Varela Junior *et al.*, 2012). Furthermore, the efficacy of cryoprotectant agents is almost always based on comparing the pre-freeze sperm motility with the immediate post-thaw motility; however, this approach does not take into account damage that may have been caused by the intrinsic toxicity of the cryoprotectant (Macías García *et al.*, 2012).

Another variable to be taken into account when implementing cryopreservation protocols is the temperature of equilibration used after adding the cryoprotectant to the sperm cells. In SAC, the only equilibration temperature used has been 4–5 °C (Bravo *et al.*, 2000; Aller *et al.*, 2003; Vaughan *et al.*, 2003).

Currently, there are no reports in SAC that have studied, in the same ejaculate and under the same experimental conditions, an alternative equilibration temperature and cryoprotectant, on different parameters of frozen–thawed spermatozoa. Therefore, the aim of this study was to determine the effect of two equilibration temperatures (5 °C and room temperature) and two cryoprotectants (glycerol and dimethylformamide, both at 7%) on *Lama glama* sperm motility, membrane function, membrane integrity and on chromatin structure, after cryopreservation.

Materials and methods

Reagents

The reagents for the SCD test and the TB stain were purchased from Sigma Chemicals and from Global Lab S.A., Buenos Aires, Argentina. Collagenase and the reagents of the H-TALP-BSA medium were purchased from Sigma Chemicals, Buenos Aires, Argentina. Type I collagenase (Clostridium peptidase A from *Clostridium histolyticum*) was used. TALP medium (Parrish *et al.*, 1986) was supplemented with 15 mM HEPES and 3 mg ml^{-1} BSA (H-TALP-BSA).

Animals and location

The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, in Buenos Aires, Argentina. The city is situated at sea level, latitude 34° 36′ and longitude 58° 26′.

For the study, seven male llamas ranging between 6 and 10 years of age and weighing 139.83 ± 14.31 kg (mean ± SD) were used. Animals were kept out at pasture in pens and supplemented with bales of alfalfa; they also had free access to fresh water throughout the study. To minimise heat stress, all males were shorn during the month of November.

Semen collection

Semen collections were carried out using electroejaculation (EE) under general anaesthesia, according to the technique described by Director *et al.* (2007). The frequency of collection for each male was determined randomly. As EE requires general anaesthesia, this method was not used on the same male at an interval of <15 days and this was taken into account. All procedures were approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2010/24).

Freeze–thawing of llama semen

A total of 15 ejaculates were collected, and one was discarded due to urine contamination. Therefore, for the study, 14 ejaculates were obtained from seven male llamas ($n = 7$, $r = 2$). Each ejaculate was diluted 4 : 1 in 0.1% collagenase in H-TALP-BSA medium (Parrish *et al.*, 1986) and incubated 4 min at 37 °C according Giuliano *et al.* (2010) with the objective of decreasing thread formation and facilitating manipulation of the samples. Afterwards, the ejaculates were divided into four aliquots. A lactose-EDTA-egg yolk (LEEY) extender (based on Martin *et al.*, 1979) with either glycerol (LEEY-G) or dimethylformamide (LEEY-DMF) as cryoprotectant was added to two of the aliquots, which were then equilibrated for 20 min at room temperature (RT). The other two aliquots were extended in LEEY, cooled to 5 °C (over 2.5 h), after which LEEY-G or LEEY-DMF (also at 5 °C) was added, followed by equilibration for 20 min at 5 °C. After each equilibration period, the semen samples were placed in 0.50-ml straws (40×10^6 spermatozoa per straw). Freezing was carried out according to the manual method described by Miragaya *et al.* (2001). Briefly, temperature descent was carried out in three phases by placing the straws submerged in a mixture of ethanol: 2-propanone (1 : 1), in a bronze canister with a graduated handle and holding over liquid nitrogen vapours. Temperature phases were as follows: (i) from equilibration temperature (either RT or 5 °C) until −15 °C (temperature descent at a rate of 10–12 °C min^{-1}); (ii) until −120 °C at a rate of 25–40 °C min^{-1}; and (iii) finally,

the straws were plunged into the liquid nitrogen at −196 °C. In all cases, the final concentration of the cryoprotectants (either DMF or G) was 7%. After a month of storage in liquid nitrogen, the samples were thawed in a 37 °C water bath for 60 s. The experimental design is shown in Fig. 1.

Routine evaluation of seminal characteristics

The following seminal characteristics were evaluated: ejaculate volume, total sperm motility (oscillatory and progressive), concentration, membrane function and integrity. Sperm motility was evaluated, using a phase-contrast microscope (100×) and a warm stage (37 °C), in the following samples: (i) raw semen; (ii) after collagenase incubation; (iii) after equilibration; and (iv) after freeze–thawing. Sperm numbers were calculated using a Neubauer haemocytometer. The hypo-osmotic swelling (HOS) test was used for assessing membrane function, and the fluorochromes 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) were used for assessing membrane integrity (viability). Both these techniques were carried out according to Giuliano *et al.* (2008) to evaluate raw and frozen–thawed semen samples. Briefly, for the HOS test, semen was incubated (37 °C) in a hypo-osmotic solution containing fructose and sodium citrate (50 mOsm). After incubation, a minimum of 200 spermatozoa were evaluated using a phase-contrast microscope. Osmolarity of the solutions was measured using an automatic cryoscopic osmometer (Osmomat® 030, Gonotec, Berlin, Germany). For assessing membrane integrity, samples of semen were incubated (37 °C) with CFDA and PI in an isotonic saline solution. A minimum of 200 spermatozoa were evaluated per sample using an epifluorescence microscope with a rhodamine and standard fluorescein filter set.

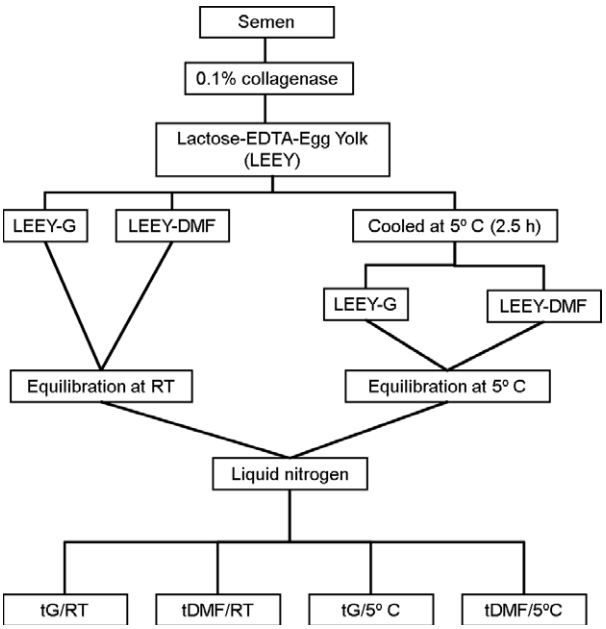

Fig. 1 Diagram showing the experimental design used to evaluate the different combinations of equilibration temperature and cryoprotectant in a freeze–thaw protocol in llama spermatozoa ($n = 7$, $r = 2$). LEEY-G: semen extended with lactose-EDTA-egg yolk and glycerol. LEEY-DMF: semen extended with lactose-EDTA-egg yolk and dimethylformamide. tG/RT: thawed semen, cryopreserved with glycerol and equilibrated at room temperature. tDMF/RT: thawed semen, cryopreserved with dimethylformamide and equilibrated at room temperature. tG/5 °C: thawed semen, cryopreserved with glycerol and equilibrated at 5 °C. tDMF/5 °C: thawed semen, cryopreserved with dimethylformamide and equilibrated at 5 °C.

DNA evaluation

Extended semen, equilibrated at RT, was evaluated only with toluidine blue stain (TB). The following samples were evaluated using both the sperm chromatin dispersion assay (SCD) and TB: (i) raw semen; (ii) cooled semen extended with LEEY (without cryoprotectant); (iii) cooled semen after equilibration at 5 °C with either DMF or G; and (iv) thawed semen (tG/RT, tDMF/RT, tG/5 °C and tDMF/5 °C).

DNA condensation

The TB stain was carried out according Carretero *et al.* (2009) to evaluate the degree of chromatin condensation. Briefly, each sample was smeared on clean, nongreasy slides and once dry, fixed with ethanol 96°C and stained with a working solution of 0.02% TB. Preparations were observed directly under immersion oil (1000×) evaluating a minimum of 200 spermatozoa per smear. Spermatozoa were classified into three groups according to the degree of chromatin condensation: light blue (negative, no chromatin decondensation), light violet (intermediate, some degree of decondensation) and dark blue-violet (positive, high degree of decondensation). Dithiothreitol (DTT) 1% in distilled water was used as a positive control for the TB stain.

DNA fragmentation

The SCD assay was carried out according to Carretero *et al.* (2012a) to evaluate the degree of DNA fragmentation. Briefly, each sperm suspension was mixed with low-melting-point aqueous agarose and pipetted onto a glass slide. Each slide was incubated in different lysing solutions, dehydrated in sequential ethanol baths and stained with Giemsa. Images of sperm heads were captured by a

Leica DC180 camera (Leica Microsystems Co., Wetzlar, Germany), obtaining 200 images per sample. Spermatozoa were classified into four patterns according to the size of the halo: (i) nuclei with large DNA dispersion halos; (ii) nuclei with medium halos; (iii) nuclei with small halos; and (iv) nuclei with no halo. The first two patterns (i and ii) were considered spermatozoa without DNA fragmentation and the other two (iii and iv) spermatozoa with DNA fragmentation. Very occasionally, it may be difficult to discriminate between spermatozoa with medium- and large-sized halos. In these cases, if the halo width is similar to or larger than the minor diameter of the core of the nucleoid, it was considered a sperm cell with a large halo. Semen incubation at 100 °C during 30 min was used as a positive control of sperm DNA fragmentation. To avoid possible error due to differences in solutions or times of incubation, duplicates of the post-thaw semen samples were assessed for DNA fragmentation in such a way that all protocols (tG/RT, tDMF/RT, tG/5 °C and tDMF/5 °C) were evaluated on the same slide; hence, they were subjected to exactly the same steps of the DNA assay. This also permitted simultaneous evaluation of the different protocols assayed.

Statistical analysis

For routine seminal characteristics, an analysis of variance (factorial design) was used to compare raw semen and all freeze–thawing protocols, using the male as a blocking factor. A Kruskal–Wallis test was used to evaluate sperm motility in raw semen, after collagenase incubation, after equilibration and after freeze–thawing. For DNA condensation, three factorial designs were carried out, using the male as a blocking factor. The first factorial design (5 levels) was carried out to compare raw semen to extended equilibrated semen (5 levels: raw semen, extended semen with G and equilibrated at RT, extended semen with DMF and equilibrated at RT, extended semen with G and equilibrated at 5 °C, and extended semen with DMF and equilibrated at 5 °C). The second factorial design (5 levels) was carried out to compare raw semen to frozen–thawed semen (5 levels: raw semen, frozen–thawed semen cryopreserved with G and equilibrated at RT, frozen–thawed semen cryopreserved with DMF and equilibrated at RT, frozen–thawed semen cryopreserved with G and equilibrated at 5 °C, and frozen–thawed semen cryopreserved with DMF and equilibrated at 5 °C). Finally, the third factorial design compared equilibrated extended semen and frozen–thawed semen, having three factors with two levels each (time: with levels before and after freeze–thawing; cryoprotectants: with levels DMF and G; and equilibration temperature: with levels RT and 5 °C). A Kruskal–Wallis test was carried out to compare DNA fragmentation between raw semen, cooled semen, and all semen-freezing protocols. All statistical analyses were performed using the R 2.2.1. Program (2005).

Results

Routine seminal characteristics in raw and thawed semen

The volume of raw ejaculates was 2.46 ± 0.83 ml, and the concentration of raw semen was $88.0 \pm 38 \times 10^6$ spermatozoa/ml (mean + SD). No interaction was observed between the different equilibration temperatures and the cryoprotectants for any of the routine seminal characteristics evaluated.

Motility of thawed semen cryopreserved with DMF was not significantly different to that of raw semen ($P > 0.05$). In addition, total sperm motility tended to be greater in the samples frozen with DMF than raw semen samples, whereas in those frozen with G, motility was significantly lower than raw semen and semen frozen with DMF ($P \leq 0.05$). Thawed semen, cryopreserved with DMF, retained a 55.3% (22.1/40.0) of the total motility seen in extended samples when equilibrated at RT and a 60.9% (22.5/36.9) of the motility seen in extended samples when equilibrated at 5 °C. In contrast, thawed semen cryopreserved with glycerol retained a 5.4% (0.8/14.7) of the total motility seen in extended samples when equilibrated at RT and a 13.8% (3.2/23.2) of the total motility seen in extended samples when equilibrated at 5 °C.

The percentages of spermatozoa with functional membranes and the percentages of live spermatozoa were significantly greater ($P \leq 0.05$) in raw semen than in frozen–thawed semen for all protocols tested. However, a high proportion of the initial sperm membrane function was retained in all cryopreservation protocols. Accordingly, DMF retained a 76.0% (23.1/30.4) when equilibrated at RT and a 79.6% (24.2/30.4) when equilibrated at 5 °C, while glycerol retained a 66.8% (20.3/30.4) when equilibrated at RT and an 84.2% (25.6/30.4) when equilibrated at 5 °C. The percentage of live thawed spermatozoa after equilibration at 5 °C was significantly greater ($P \leq 0.05$) than that obtained after equilibration at room temperature. Descriptive analysis of the variables motility, membrane function and membrane integrity is summarised in Table 1.

Sperm DNA condensation

All cryopreservation protocols showed a significant increase ($P < 0.05$) in the percentage of thawed spermatozoa with a high degree of chromatin decondensation (TB positive) with respect to raw semen. No significant differences ($P \geq 0.05$) in TB patterns were observed

Table 1 Routine seminal characteristics (motility, membrane function and membrane integrity) observed in llama spermatozoa from raw semen, after incubation with collagenase, after equilibration and after freeze–thawing

	Routine seminal characteristics		
Cryopreservation stage	Motility (%)	Membrane function (% swelling)	Membrane integrity (% viability)
Raw semen	13.7 ± 8.7^{a}	30.4 ± 12.8^{a}	48.8 ± 10.8^{a}
Collagenase	22.7 ± 10.5^{ab}		
Equilibrated semen			
eG/RT	14.7 ± 15.5^{ab}	–	–
eDMF/RT	40.0 ± 13.5^{b}	–	–
eG/5 °C	23.2 ± 21.2^{ab}	–	–
eDMF/5 °C	36.9 ± 13.8^{b}	–	–
Thawed semen			
tG/RT	0.8 ± 1.8^{c}	20.3 ± 11.4^{b}	22.4 ± 10.0^{b}
tDMF/RT	22.1 ± 11.7^{ab}	23.1 ± 13.4^{b}	24.2 ± 13.0^{b}
tG/5 °C	3.2 ± 6.0^{c}	25.6 ± 6.7^{b}	26.5 ± 12.7^{c}
tDMF/5 °C	22.5 ± 11.7^{ab}	24.2 ± 10.9^{b}	29.3 ± 8.2^{c}

Cryopreserved semen was equilibrated at different temperatures (room temperature and 5 °C) with two different cryoprotectants (glycerol or dimethylformamide). The values are expressed as mean ± SD ($n = 7$, $r = 2$). Different letters within a column indicate significant differences ($P \leq 0.05$). *Equilibrated semen*: eG/RT, semen equilibrated with glycerol at room temperature; eDMF/RT, semen equilibrated with dimethylformamide at room temperature; eG/5 °C, semen equilibrated with glycerol at 5 °C; eDMF/5 °C, semen equilibrated with dimethylformamide at 5 °C. *Thawed semen*: tG/RT, thawed semen, cryopreserved with glycerol and equilibrated at room temperature; tDMF/RT, thawed semen, cryopreserved with dimethylformamide and equilibrated at room temperature; tG/5 °C, thawed semen, cryopreserved with glycerol and equilibrated at 5 °C; tDMF/5 °C, thawed semen, cryopreserved with dimethylformamide and equilibrated at 5 °C.

between all freeze–thawing protocols. Results are summarised in Table 2.

Sperm DNA fragmentation

A significant increase in the percentage of spermatozoa with DNA fragmentation (small halo + no halo) was observed in thawed semen cryopreserved with glycerol when compared to raw semen and to all other samples evaluated. No significant differences were observed ($P > 0.05$) between the rest of the samples studied. Results are shown in Table 3 and Fig. 2.

Discussion

The present study reports the first use of dimethylformamide and room temperature equilibration in a SAC semen cryopreservation protocol. It is also the first report in which the state of llama sperm chromatin (both condensation and integrity) was evaluated after implementing a freeze–thawing protocol.

When studying the influence of cryoprotectants on sperm variables after freeze–thawing, the samples extended with DMF were the only ones that maintained sperm motility after the process, regardless of the equilibration temperature used. The post-thaw motility observed with DMF was around 23%, while with glycerol, post-thaw motility was between 0 and 5%. This difference in motility observed between glycerol and DMF equilibrated at room temperature could be because addition of glycerol at this temperature might have a more harmful effect on llama spermatozoa as it permits a greater access of this cryoprotectant to the cells. It is known that optimal glycerol concentration to be used in extenders is limited by its possible toxicity, which in turn depends on species, rate of cooling, extender composition and method used to add it to the media (Holt, 2000). Therefore, addition of glycerol at room temperature could be increasing the intracellular toxicity of this cryoprotectant.

With regard to the samples equilibrated at 5 °C, our results with glycerol are similar to those of most studies found in the literature, where it has been reported that the use of 6–8% glycerol does not promote motility despite using different extenders (llama: von Baer & Helleman, 1999; Aller *et al.*, 2003; alpaca: Vaughan *et al.*, 2003; Santiani *et al.*, 2005; Santiani Acosta *et al.*, 2013). On the surface, the only report that differs is that of Bravo *et al.* (2000) who obtained 30–40% motility after thawing llama and alpaca spermatozoa frozen with an extender containing citrate, egg yolk and glycerol as cryoprotectant. Nevertheless, the initial motility of the samples (80%) used by these authors was considerably higher than that reported in the rest of the literature (including our present study), and despite this, the decrease in the post-thaw motility observed in Bravo's work (50%) was even higher than the decrease we observed in our study (23%).

On the other hand, amides such as DMF are permeable cryoprotectants with a lower molecular weight than glycerol; therefore, they can cross membranes faster, thus decreasing their osmotic toxicity (Squires *et al.*, 2004). Therefore, the difference in the post-thaw motility between both cryoprotectants could also be because the osmotic pressure assisted by DMF would be less deleterious to the sperm cells than that exerted by glycerol.

With regard to sperm membrane function, no differences were observed between any of the freeze–thawing protocols. There is only one other report in the literature (Santiani Acosta *et al.*, 2013) that evaluates this parameter in frozen–thawed SAC spermatozoa, and similar to our observations, these authors reported a decreased

Table 2 Percentages of TB patterns (positive, intermediate, positive + intermediate and negative) observed in llama spermatozoa from raw, equilibrated and thawed semen samples

Cryopreservation stage	TB patterns (%)			
	Positive	Intermediate	Positive + intermediate	Negative
Raw semen	2.0 ± 0.7^{a}	19.9 ± 5.4^{a}	21.9 ± 5.9^{a}	78.1 ± 5.9^{a}
Equilibrated semen				
eG/RT	2.5 ± 0.9^{abcd}	22.5 ± 3.8^{ac}	25.0 ± 4.6^{abcd}	75.0 ± 4.6^{abcd}
eDMF/RT	1.8 ± 0.5^{ac}	22.4 ± 4.4^{ac}	24.2 ± 4.4^{abc}	75.8 ± 4.4^{abc}
eG/5 °C	3.2 ± 0.9^{bde}	25.2 ± 6.7^{abcd}	28.4 ± 6.9^{abcde}	71.6 ± 6.9^{abcde}
eDMF/5 °C	3.3 ± 1.3^{bde}	27.4 ± 7.6^{bcd}	30.7 ± 7.9^{bcde}	69.3 ± 7.9^{bcde}
Thawed semen				
tG/RT	3.2 ± 0.9^{de}	27.2 ± 5.1^{cd}	30.4 ± 5.7^{cde}	69.6 ± 5.7^{cde}
tDMF/RT	4.5 ± 1.2^{e}	30.7 ± 6.2^{d}	35.2 ± 6.2^{e}	64.8 ± 6.2^{e}
tG/5 °C	4.0 ± 1.5^{e}	28.0 ± 5.8^{cd}	32.0 ± 6.2^{cde}	68.0 ± 6.2^{cde}
tDMF/5 °C	4.4 ± 1.7^{e}	28.6 ± 4.6^{cd}	33.0 ± 4.0^{de}	67.0 ± 4.0^{de}

Cryopreserved semen was equilibrated at different temperatures (room temperature and 5 °C) with two different cryoprotectants (glycerol or dimethylformamide). The values are expressed as mean ± SD (n = 7, r = 2). Different letters within a column indicate significant differences ($P \leq 0.05$). *Equilibrated semen*: eG/RT, semen equilibrated with glycerol at room temperature; eDMF/RT, semen equilibrated with dimethylformamide at room temperature; eG/5 °C, semen equilibrated with glycerol at 5 °C; eDMF/5 °C, semen equilibrated with dimethylformamide at 5 °C. *Thawed semen*: tG/RT, thawed semen, cryopreserved with glycerol and equilibrated at room temperature; tDMF/RT, thawed semen, cryopreserved with dimethylformamide and equilibrated at room temperature; tG/5 °C, thawed semen, cryopreserved with glycerol and equilibrated at 5 °C; tDMF/5 °C, thawed semen, cryopreserved with dimethylformamide and equilibrated at 5 °C.

Table 3 Percentages of llama spermatozoa with DNA fragmentation (small halo, no halo, small halo + no halo) and with intact DNA (large halo + medium halo) observed in raw, equilibrated and frozen–thawed semen

Cryopreservation stage	Sperm DNA fragmentation (%)			Sperm with intact DNA (%)
	Small halo	No halo	Small halo + no halo	Large halo + medium halo
Raw semen	1.6 ± 1.2^{a}	17.6 ± 12.9^{a}	19.2 ± 13.6^{a}	80.8 ± 13.6^{a}
Cooled semen (LEEY)	2.5 ± 1.7^{a}	24.5 ± 15.5^{ab}	27.0 ± 16.0^{a}	73.0 ± 16.0^{a}
Equilibrated semen				
eDMF/5 °C	3.3 ± 2.0^{a}	25.0 ± 18.7^{ab}	28.3 ± 20.1^{a}	71.7 ± 20.1^{a}
eG/5 °C	3.3 ± 2.8^{a}	35.4 ± 22.7^{ab}	38.7 ± 22.8^{a}	61.3 ± 22.8^{a}
Thawed semen				
tG/RT	63.9 ± 30.5^{b}	34.6 ± 28.5^{ab}	98.5 ± 3.3^{b}	1.5 ± 3.3^{b}
tDMF/RT	3.7 ± 2.3^{a}	32.6 ± 18.9^{ab}	36.3 ± 20.4^{a}	63.7 ± 20.4^{a}
tG/5 °C	47.2 ± 28.2^{b}	46.6 ± 25.1^{b}	93.8 ± 9.7^{b}	6.2 ± 9.7^{b}
tDMF/5 °C	3.3 ± 1.3^{a}	25.7 ± 1.3^{ab}	29.0 ± 16.8^{a}	71.0 ± 16.8^{a}

Cryopreserved semen was equilibrated at different temperatures (room temperature and 5 °C) with two different cryoprotectants (glycerol or dimethylformamide). The percentages are expressed as mean ± SD (n = 7, r = 2). Different letters within a column indicate significant differences ($P \leq 0.05$). LEEY, lactose-egg-yolk extender without cryoprotectants. *Equilibrated semen*: eG/5 °C, semen equilibrated with glycerol at 5 °C; eDMF/5 °C, semen equilibrated with dimethylformamide at 5 °C. *Thawed semen*: tG/RT, thawed semen, cryopreserved with glycerol and equilibrated at room temperature; tDMF/RT, thawed semen, cryopreserved with dimethylformamide and equilibrated at room temperature; tG/5 °C, thawed semen, cryopreserved with glycerol and equilibrated at 5 °C; tDMF/5 °C, thawed semen, cryopreserved with dimethylformamide and equilibrated at 5 °C.

membrane function after cryopreservation. With regard to sperm membrane integrity, all freeze–thawing protocols used in this study retained between 46 and 60% of sperm viability in the thawed samples. It is generally accepted that a substantial number (50%) of spermatozoa are damaged during cryopreservation (Watson, 2000), and this decrease in viability in cryopreserved semen has been observed in various species (horse: Neild *et al.*,

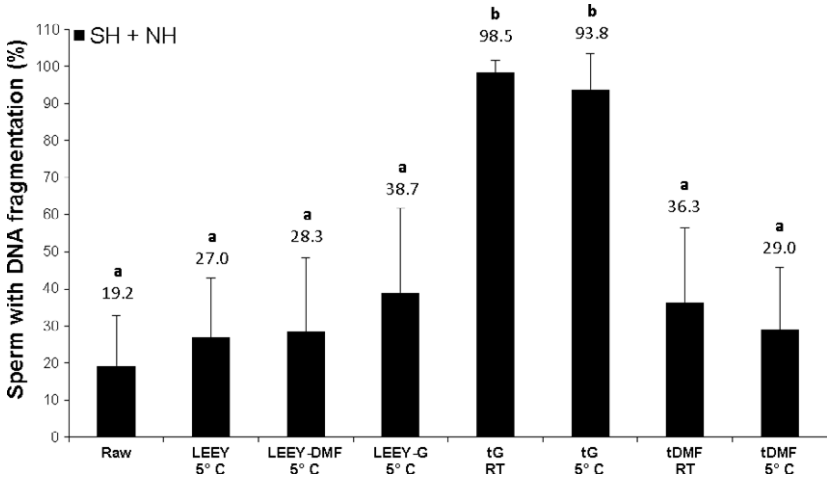

Fig. 2 Percentages of llama spermatozoa with DNA fragmentation (SH + NH) observed at different stages of a freeze–thawing protocol. SH + NH: spermatozoa with small halos + spermatozoa with no halo. [a,b]Different letters indicate significant differences ($P \leq 0.05$). LEEY 5 °C: extended semen cooled to 5 °C (without added cryoprotectants). LEEY-DMF/5 °C: extended semen with dimethylformamide and equilibrated at 5 °C. LEEY-G/5 °C: extended semen with glycerol and equilibrated at 5 °C. tG/RT: thawed semen, cryopreserved with glycerol and equilibrated at room temperature. tG/5 °C: thawed semen, cryopreserved with glycerol and equilibrated at 5 °C. tDMF/RT: thawed semen, cryopreserved with dimethylformamide and equilibrated at room temperature. tDMF/5 °C: thawed semen, cryopreserved with dimethylformamide and equilibrated at 5 °C.

2003; boar: Fraser & Strzeżek, 2007; dog: Kim *et al.*, 2010; human: Hammadeh *et al.*, 2001) and also in SAC (llama: Aller *et al.*, 2003; alpaca: Santiani *et al.*, 2005; Santiani Acosta *et al.*, 2013).

According to our results, sperm motility was more affected than either membrane function or membrane integrity in samples cryopreserved with glycerol. This could be because, as has been reported, motility appears to be more sensitive to osmotic stress than membrane integrity (Songsasen *et al.*, 2002). As mentioned before, the samples cryopreserved with DMF would seem to suffer less osmotic stress (due to their lower molecular weight) than those preserved with glycerol and therefore showed better motility.

With regard to chromatin decondensation, in our study, samples equilibrated at 5 °C during 20 min showed an increase in chromatin decondensation compared with values in raw semen and extended semen equilibrated at room temperature. It is important to note that this increase in chromatin decondensation was observed at a very early stage in the cryopreservation protocol, but only appeared in samples in which 5 °C was used as the equilibration temperature. Similar results have been observed in llama semen cooled to 5 °C and stored for 24 h (Carretero *et al.*, 2012b).

In the present study where the same extender and cryopreservation protocol were used to allow comparison of two different permeable cryoprotectants, DNA fragmentation was severely impaired by the use of 7% glycerol (87–100% fragmentation in all samples). This could be indicating that at a final concentration of 7%, glycerol would be detrimental for llama sperm DNA. The low pregnancy rates reported after artificial insemination with frozen–thawed semen in llama and alpaca (Bravo *et al.*, 2000; Aller *et al.*, 2003; Vaughan *et al.*, 2003) using 6–7% glycerol reinforce this hypothesis. In contrast, dimethylformamide preserved DNA integrity with values that did not differ significantly from raw semen.

In SAC, there is only one report in alpacas that evaluates sperm DNA fragmentation using TUNEL in frozen semen (Santiani Acosta *et al.*, 2013). They cryopreserved ejaculates with ethylene glycol and superoxide dismutase analogues, and their results showed a DNA fragmentation index that varied between 16.7 and 38.8%. Despite using a different protocol to the one in this study, these results are similar to the ones presented here with DMF (DNA fragmentation between 29.0% and 36.3%).

As mentioned previously, in SAC, the only temperature used to equilibrate spermatozoa with the cryoprotectants in the extender has been 4–5 °C (Bravo *et al.*, 2000; Aller *et al.*, 2003; Vaughan *et al.*, 2003; Santiani *et al.*, 2005; Morton *et al.*, 2010; Santiani Acosta *et al.*, 2013). This is probably due to basing the studies on work carried out in bovines and small ruminants; but in equines, room temperature has been shown to be effective for equilibration (Miragaya *et al.*, 2001). After comparing these two equilibration temperatures, our results show that equilibration at room temperature would be a possible alternative for using in cryopreservation protocols, because it would seem to avoid the early rise in chromatin decondensation

observed when equilibrating at 5 °C. Furthermore, it has the advantage of simplicity, making the process more agile.

In conclusion, the lower motility and the higher percentage of DNA fragmentation in spermatozoa cryopreserved with 7% glycerol indicate that glycerol used at this final concentration would seem to be detrimental for llama spermatozoa. In contrast, dimethylformamide preserved motility and DNA integrity of frozen–thawed llama spermatozoa and could be used to replace glycerol at the concentrations used in this study. However, glycerol could be more effective for cryopreserving llama spermatozoa if used at lower concentrations, and further studies are currently being carried out to evaluate this possibility. Also, equilibration at room temperature appeared to be effective for cryopreserving llama spermatozoa; thus, it could be used to simplify cryopreservation protocols.

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