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Functional Activity of Frozen Thawed *Chinchilla lanigera* Spermatozoa Cryopreserved with Glycerol or Ethylene Glycol

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Contents

The cryopreservation of spermatozoa constitutes a valuable tool for the captive breeding management of valuable and/or threatened species. *Chinchilla lanigera* is a species almost extinct in the wild, and the domestic counterpart has one of the most valuable pelts in the world. The objectives of this study were to: (i) compare the functional activity of post-thawed chinchilla spermatozoa cryopreserved at −196°C either with glycerol (G) or ethylene glycol (EG) as cryoprotectants (1 M final concentration) and (ii) investigate the effects of incubating the gametes for 4 h in the presence or in the absence of the cryoprotectants; evaluations were performed taking into account motility, viability, response to hypo-osmotic shock and acrosome integrity of the cells. Parameters reflecting post-thaw (0 h) sperm functional activity were significantly lower than those of freshly ejaculated gametes. When comparing the cryoprotectant efficiency of G vs EG, neither cryoprotectant agent offered appreciable advantages. After 4 h of incubation, in the presence or absence of the cryoprotectant agent, a rapid and significant decrease was found in all functional parameters and remained at ~ 20–30% motile, viable and viable acrosome intact cells. Viability was significantly lower when the cryoprotectant was removed from the media (possibly due to the centrifugation process). With respect to the maintenance of sperm membrane integrity, only ~ 10% of cells showed membrane resistance to hypo-osmotic conditions after the 4 h incubation period. These results constitute new insights for cryopreservation protocols and the development of assisted reproductive techniques in this species.

Introduction

The chinchilla is a member of the rodent suborder Hystricomorpha and it bears the most valuable pelt in the world. Excessive hunting for fur greatly reduced the wild populations and today, both chinchilla species (*Chinchilla lanigera* and *Ch. brevicaudata*) are on the brink of extinction (Jimenez 1996).

On the other hand, a cross-breeding of the two taxas has been domesticated, bred and selected for more than 80 years; the ranch-raised chinchilla has vastly improved in fur quality over its wild counterpart, and is one of the most appreciated pelts by the fur industry (Grau 1986).

Because our knowledge of the reproductive processes in this species is extremely limited, the development of a database of basic reproductive physiology and improvements in the use of assisted reproductive techniques for captive breeding management have been hindered (Weir 1966, 1973). Among other studies (Ponzio et al. 2004; Busso et al. 2005a), we have improved a method for electroejaculation, and developed an adequate cryoprotectant media for the preservation of domestic chinchilla sperm at −196°C using glycerol (G) as cryoprotectant agent (CPA) (Ponce et al. 1998a,b).

Mammalian spermatozoa are typically cryopreserved, using the permeating cryoprotectant G (Medeiro et al. 2002). Conversely, besides its cryoprotective properties, G can induce alterations in the organization and viscosity of the sperm cytoplasm, and in the permeability and stability of the plasma membrane through the disruption of phospholipid and protein structural organization (Watson 1979; Amann and Pickett 1987). These phenomena would cause potential deleterious effects on the sperm fertilizing ability. Although the chemical structures of ethylene glycol (EG) and G are quite similar, EG has a smaller molecular weight (62.07 vs 92.10), a characteristic that may result in lower toxicity and higher cell permeability (Massip 2001). Consequently, EG is widely used for sperm freezing in various mammalian species, and showed the highest cell permeability in human (Gilmore et al. 1995), mouse (Phelps et al. 1999), boar (Gilmore et al. 1998), stallion (Henry et al. 2002; Mantovani et al. 2002) and ram (Molinia et al. 1994) spermatozoa.

In a previous work, we have reported the benefits of using EG in the cryoprotectant media, to store chinchilla sperm at 4°C for limited periods of time (Carrascosa et al. 2001).

However, the application of assisted reproductive techniques requires the availability of spermatozoa at any moment, which could only be achieved using a lower temperature for cyopreservation procedures.

Therefore, the objectives of this study were to: (i) compare the functional activity of the post-thawed *Ch. lanigera* spermatozoa cryopreserved at −196°C either with glycerol or ethylene glycol as cryoprotectants and (ii) investigate, after thawing, the effects of incubating the gametes for 4 h in either the presence or in the absence of the cryoprotectants.

Materials and Methods

Animals

Sexually mature domestic chinchilla (*Ch. lanigera*) males (n = 6; 500–600 g) were employed for this study, maintained in an indoor laboratory facility with exposure to natural fluctuations in photoperiod and controlled temperature (22–25°C) in Córdoba, Argentina. These animals were individually housed in stainless steel cages, fed with a pelleted chinchilla mixture (Cargill SACI, Pilar, Córdoba, Argentina) and water *ad libitum*. All procedures

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were conducted in conformity with international policies about animal care and use, and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, publication 85–23, revised 1996).

Semen collection

The semen was obtained by electroejaculation with a probe designed in our laboratory, and previously described by Ponce et al. (1998b). Briefly, a bronze bipolar electrode (length 40 mm, diameter 4.2 mm) was lubricated with glycerin, and inserted into the rectum to a depth of 20 to 30 mm and held in place by the technician. An alternating current (sinusoidal wave, 50 cycles/seconds; 6–6.5 V setting) was then applied for 5 s every 10 s of rest. One to 3 pulses were usually enough to produce the ejaculate, which was collected into a polyethylene Eppendorf tube containing 150 μl of Tyrode's medium. Together with the ejaculate, a white gel-like viscous plug was obtained, which was immediately discarded to prevent coagulation.

Incubation media

For all procedures, spermatozoa were suspended/incubated in modified Tyrode's buffer medium with the following composition (mM): $CaCl_2$: 1.8; $MgCl_2$: 0.929; KCl: 2.68; $NaHCO_3$: 11.903; NaCl: 136.86; NaH_2PO_4: 0.287; glucose: 5.56; penicillin 0.5 mg/ml and streptomycin 0.075 mg/ml. pH: 7.3–7.5; 270–290 mOsm (all reagents were purchased from Sigma Chemical Co., St Louis, MO, USA)

Preparation of cryoprotective media

The procedure and preparation of the cryoprotectant media was performed as previously reported by Ponce et al. (1998a,b); briefly, a zwitterionic buffer system was prepared by titrating 325 mOsm/l TES (N-tris[Hydroxymethyl] methyl-2-aminoethanesulfonic acid) with 325 mOsm/l Tris to make the TEST solution. To prepare the cryobuffer, 48% (vol/vol) TEST, 30% sodium citrate, 20% egg yolk, and 2% fructose were mixed. The mixture was centrifuged at 10 000 ***g*** for 10 min, and the supernatant was filtered through filter paper (Whatman I). Penicillin 0.15 mg/ml and streptomycin 0.25 mg/ml were then added and the pH (7.4–7.5) and osmolality of the extenders measured. The cryobuffers were finally added to glycerol (TESTY-G) or ethylene glycol (TESTY-EG) at a 2 M concentration. Aliquots of the cryoprotectants were stored at −20°C for later use.

Spermatozoa freezing

Ejaculates from six animals (one ejaculate/male) were obtained, considering that each individual raw ejaculate met minimal quality standards to allow use in the experiment (at least 92%motile and viable spermatozoa) (Busso et al. 2005a). In order to prevent individual male differences in the results, ejaculates were pooled; one half of the sperm suspension was added drop by drop with the glycerol-based cryoprotectant medium and the other half with the ethylene glycol-based medium (1 : 1 dilution; 1 M final concentration of the cryoprotectants). The suspensions were gently shaken to achieve a complete semen-medium mixture. Two plastic cryostraws (0.5 ml) were filled at room temperature, with each one of the obtained suspensions, and finally heat sealed. Then, they were slowly cooled at 4°C at a rate of approximately 2°C/min. (measured with an analogical thermometer). After 10 min, the cryostraws were exposed to −20°C for 15 min, and afterwards cooled by exposure to liquid nitrogen gas vapour (10 cm above the liquid nitrogen level) for 10 min; the cryostraws were finally immersed in liquid nitrogen (−196°C), and stored for 1 month before thawing. In order to avoid excessive temperature fluctuations, fast transfer was performed at all steps. The entire experimental procedure was repeated 10 times in different days of experimentation.

Thawing procedure

For thawing, straws were rapidly immersed in a water bath (37°C) for 60 s and wiped dry. One half of the samples were processed without removing the cryoprotectant media (the content of the straws was released directly into 500 μl of Tyrode's buffered medium) and in the other half, the cryoprotectant was removed by flushing the sperm suspension into an Eppendorf tube with 500 μl Dulbecco's phosphate-buffer saline-2% (w/v) polyvinylpyrrolidone-40 (DPBS-PVP, pH 7.4, Sigma Chemical Co.) per straw. The sperm suspension was washed twice by centrifugation (100 ***g***, 10 min). The precipitate was then resuspended in 1 ml Tyrode's medium and adjusted to a final sperm concentration of $\sim 5–10 \times 10^6$ cells/ml.

Sperm functional activity

Motility

In a Makler counting chamber (Makler 1980) (Sefi Medical Instruments, Haifa, Israel) that was placed over a thermostatized chamber to keep the sperm warm during the evaluation (37°C) (Carl Zeiss, Jena, Germany) under inverted microscope at 200 × magnification (Olympus CK2, Tokyo, Japan). Results were expressed as percentage of motile cells (progressive plus non-progressive sperm).

Viability

By supravital staining with Hoechst 33258 (H258) (1.5 μg/ml) (Yelian and Dukelow 1992) (Calbiochem, San Diego, CA, USA) viability was performed. Using the appropriate ultraviolet fluorescence optics, (Axiolab; Zeiss, Stuttgart, Germany) sperm having brightly blue fluorescent nuclei were scored as dead whereas those without fluorescence were scored as viable. No fewer than 200 cells were assessed. Results were expressed as percentage of viable cells.

Response to hypo-osmotic shock

The procedure used was adapted according to Ruiz et al. (1996). An aliquot of the sperm suspension

(100 μl) was mixed with a hypo-osmotic solution (100 mOsm; 1 ml, pH 7.4) of fructose and sodium citrate in distilled water. After 45 min incubation (37°C), evaluations were made in a phase-contrast microscope at 400 × magnification, and the percentage of spermatozoa that showed tail swelling was determined. No fewer than 100 cells were assessed. Results were expressed as percentage of swollen cells.

Acrosome reaction

Double staining with *Pisum sativum* agglutinin labelled with fluorescein isothiocyanate (FITC-PSA) as described by Cross et al. (1986) with slight modifications. Briefly, after supravital staining, samples were washed out of unbound stain by centrifuging twice at low speed for 10 min with 4–6 ml of isotonic solution; the supernatant was removed, and the final sperm pellet was resuspended in 100 μl isotonic solution. Smears were produced on glass slides and dried in an incubator at 40°C. Subsequently, they were fixed with methanol for 30 s. Slides were then washed with a stream of distilled water for 2 min. After drying, spermatozoa were stained with 30 μg/ml FITC-PSA (stock solution: 2 mg/ml) in DPBS for 30 min and washed again with a stream of distilled water for 2 min. Slides were evaluated at 1000 × magnification in an epifluorescence microscope and the viability and acrosomal status of at least 100 spermatozoa were assessed. Under blue wavelength, spermatozoa with brightly yellow fluorescents acrosomes were considered acrosome intact, whereas those with no fluorescence or only in the equatorial region were considered acrosome reacted. The filter was then changed to ultraviolet and each cell was additionally scored as viable or non-viable. The results were expressed as percentage of viable spermatozoa with intact acrosome (VIA), dead spermatozoa with intact acrosome (DIA), viable spermatozoa with reacted acrosome (VRA) or dead spermatozoa with reacted acrosome (DRA).

Statistical analysis

Values are expressed as mean ± standard error of mean (SEM). Normality and homogeneity of variances of the data were corroborated for all sperm functional activity parameters. Data of sperm motility, viability and response to hypo-osmotic swelling test were analysed using a paired Student's *t*-test, whereas the data on sperm acrosome integrity were analysed using the Wilcoxon matched pairs test (for paired samples). All *p* values less than 0.05 were considered statistically significant. Statistical procedures were performed with the software program Infostat [Infostat 1.1 version (2000), Grupo Infostat, National University of Córdoba, Argentina].

Results

Immediately after all semen samples were obtained and pooled (n = 10 ejaculate pools), the percentages of motile and viable cells were 97.4 ± 0.3 and 93.5 ± 0.5% respectively; 68.5 ± 1.5% of the cells were positive to the hypo-osmotic swelling test.

Parameters reflecting post-thaw (0 h) sperm functional activity of the samples cryopreserved with TESTY-G or TESTY-EG are illustrated in Fig. 1. As can be seen, there were no significant differences when comparing both treatments, and all values were significantly lower than those of freshly ejaculated gametes ($p < 0.05$).

The percentage of VIAs in fresh samples was 83.5 ± 2.5%. Table 1 shows the acrosomal status of post-thawed samples; here also, there were no significant differences either with TESTY-G or TESTY-EG and all values were significantly lower than those detected in fresh samples ($p < 0.05$).

The functional activity of post-thawed sperm incubated for 4 h in presence of the cryoprotectants, or washed and resuspended in Tyrode's medium (Fig. 2, panel A and B respectively) dramatically declined over time and exhibited reduced motility, viability and response to hypo-osmotic shock than values obtained immediately after thawing ($p < 0.05$).

The percentages of viable spermatozoa in samples incubated in the presence of TESTY-G or TESTY-EG, were significantly higher than in those samples incubated after removing the cryoprotectants (Fig. 2 panel B; $p < 0.05$).

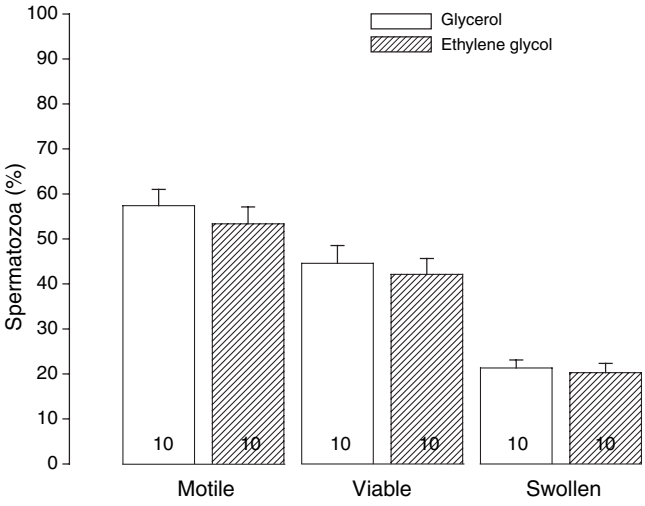

Fig. 1. Functional activity of post-thawed *Chinchilla lanigera* spermatozoa cryopreserved with TESTY-G (TES Tris egg yolk-glycerol) or TESTY-EG (TES Tris egg yolk-ethylene glycol). Determinations were performed immediately after thawing. Values are expressed as mean ± SEM. Number of experiments are indicated at the bottom of each column

Table 1. Viability and acrosomal status of cryopreserved *Chinchilla lanigera* spermatozoa

	VAI	DAI	VAR	DAR
TESTY-G (n = 9)	43.2 ± 3.7	3.0 ± 0.9	2.9 ± 1.2	50.7 ± 2.6
TESTY-EG (n = 10)	40.0 ± 4.1	5.0 ± 1.7	2.6 ± 0.8	52.3 ± 3.7

Values are expressed as mean ± SEM. In parentheses, number of straws evaluated.
TESTY-G, TES Tris egg yolk-glycerol; TESTY-EG, TES Tris egg yolk-ethylene glycol. Determinations were performed immediately after thawing. VAI, viable acrosome intact; DAI, dead acrosome intact; VAR, viable acrosome reacted; DAR, dead acrosome reacted.

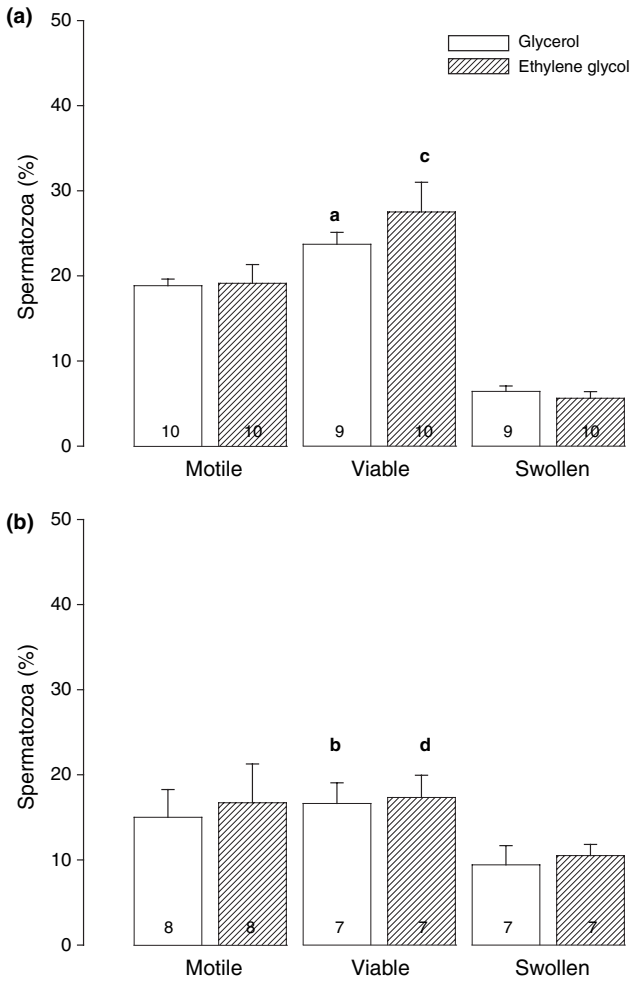

Fig. 2. Functional activity of post-thawed *Chinchilla lanigera* spermatozoa cryopreserved with TESTY-G (TES Tris egg yolk-glycerol) or TESTY-EG (TES Tris egg yolk-ethylene glycol), and incubated for 4 h with (panel A) or without the cryoprotectants (panel B). Values are expressed as mean ± SEM. Number of experiments are indicated at the bottom of each column. a vs b and c vs d: $p < 0.05$

As can be seen in Table 2, after 4 h of incubation, the percentage of viable cells with intact acrosome was similar, whether the cryoprotectant was present or not. However, in washed samples there was a significant decrease in the percentage of dead cells with reacted acrosomes. No other effects were evident in the evaluation of sperm acrosome membrane integrity.

Discussion

At present, assisted reproductive techniques are an important pillar for the optimization of domestic animal production and to aid in the conservation of critically endangered species. The long-term availability of good quality sperm is an essential task to fulfil for the application of those techniques.

In the study of sperm physiology of chinchillas, few results were reported. Healey (1969), employing a cryoprotectant composed of sodium citrate, egg yolk, glycerol and dimethylsulfoxide recovered only a 25% of motile gametes after thawing.

Table 2. Viability and acrosomal status of post-thawed *Chinchilla lanigera* spermatozoa incubated during 4 h in presence or absence of the cryoprotectant medium

	VAI	DAI	VAR	DAR
Incubated with the cryoprotectants				
TESTY-G (n = 9)	18.9 ± 3.9	16.6 ± 4.2	7.6 ± 2.2	56.8 ± 2.9a
TESTY-EG (n = 10)	21.6 ± 2.0	13.1 ± 1.7	6.8 ± 1.5	57.9 ± 3.4 c
Incubated without the cryoprotectants				
TESTY-G (n = 7)	25.4 ± 6.1	34.2 ± 8.2	7.7 ± 4.1	31.9 ± 9.1b
TESTY-EG (n = 7)	20.1 ± 4.8	31.9 ± 9.5	16.3 ± 9.2	31.2 ± 15.3d

Values are expressed as mean ± SEM. In parentheses, number of straws evaluated.
TESTY-G, TES Tris egg yolk-glycerol; TESTY-EG, TES Tris egg yolk-ethylene glycol; VAI, viable acrosome intact; DAI, dead acrosome intact; VAR, viable acrosome reacted; DAR, dead acrosome reacted.
a vs b; c vs d: $p < 0.05$.

As in most species, the post-thaw viability of cryopreserved sperm is reduced as a consequence of accumulated cellular injuries that arise throughout the cryopreservation process. A reasonable result for sperm cryopreservation was estimated as maintenance of sperm motility around 50% than that in the fresh samples (Medeiro et al. 2002). In other rodents, such as the mouse, protocols have resulted in 60% maintenance of motility in frozen-thawed sperm (Koshimoto et al. 2000). In the chinchilla, using a different cryoprotectant media and freezing procedures, we were able to recover much higher functional activity values than those obtained by Healey and similar to those obtained in other species (Ponce et al. 1998a,b; Carrascosa et al. 2001). In the present work, our immediate post-thaw values (0 h) reached similar percentages.

Glycerol is currently used as the cryoprotectant of choice to the cryopreserve mammalian sperm. The use of alternative permeating cryoprotectants with lower molecular weight and higher membrane permeability, such as ethylene glycol, might help to reduce the osmotic damage caused during their addition prior to freezing and their removal after warming (Guerrero 2006). This encouraged our laboratory to search for other less toxic and effective cryoprotectants for chinchilla sperm freezing. Carrascosa et al. (2001) have reported a significant difference in the cryoprotectant capacity of G vs EG when the chinchilla semen samples were preserved at 4°C for several days, being the media added with EG the one that better preserved chinchilla sperm functional activity.

However, the results here described indicate that, at lower temperatures of cryopreservation, neither CPA offers appreciable advantages.

The potential beneficial effects of EG for sperm cryopreservation could not be demonstrated in other species such as llama, Cynomolgus monkeys and horses (Alvarenga et al. 2000; Li et al. 2005; Santiani et al. 2005). Cell permeability to cryoprotectants is likely to be different among species, as it depends on the structure and composition of the membrane. Also, the length of time and temperature of exposure would be a relevant

issue in determining toxicity effects, and more investigation is needed to understand further the effects of EG on chinchilla spermatozoa.

After 4 h of incubation at 37°C, in presence or absence of the CPA, a rapid and significant decrease was found in all functional parameters here evaluated and remained at $\sim$ 20% motile and viable cells. Nevertheless, some of these detrimental effects can be lessened i.e. by increasing the number of sperm in the insemination dose. Considering that chinchilla semen samples had a remarkably high sperm concentration ($2145.9 \pm 365.3 \times 10^6$/ml, Busso et al. 2005a), these percentages of sperm recovery after cryopreservation and incubation can still allow their use for assisted reproductive techniques.

Results obtained when viability was quantified after 4 h incubation showed that keeping the cryoprotectants in the incubation media, may slightly reduce cellular death. It is well known that the centrifugation process itself can damage sperm cells (Sharma et al. 1997) and therefore, the results obtained may reflect this fact.

In conclusion, we did not find clearly significant differences in the cryoprotective efficiency of glycerol or ethylene glycol for preserving the functional activity of ejaculated *Ch. lanigera* spermatozoa at −196°C. Nevertheless, we corroborated that cryopreservation is a useful method for long-term storage of valuable gametes from rare chinchillas.

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