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ACTIVITY OF Chinchilla laniger SPERMATOZOA COLLECTED BY ELECTROEJACULATION AND CRYOPRESERVED

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ABSTRACT

Because reproductive studies and the application of assisted reproductive techniques are relevant issues for an endangered species such as Chinchilla laniger, the availability of a source of viable spermatozoa becomes of utmost importance. In this paper, we evaluate several functional parameters (motility, viability, response to hypoosmotic swelling test and acrosomal integrity) of fresh or frozen-thawed spermatozoa. Electro-ejaculation trials (50-cyc/sec sinusoidal wave was applied for 5 of every 10 sec) were successful in all unanesthetized animals. After volume (108.3 ± 12.0 µL, n = 15) and concentration (421.8 ± 34.4 x 10^6 cells/mL, n = 15) measurements, the above mentioned parameters were determined. In frozen-thawed semen samples sperm motility, viability, hypoosmotic swelling test and acrosomal integrity were significantly lower than in fresh semen samples. The results clearly indicated that electro-ejaculation is a useful method for evaluating spermatozoa for genetic analyses or for used in AI in this species. In addition, the cryopreservation procedure in this study preserved adequate levels of functional sperm activity.

Key words: spermatozoa, Chinchilla laniger, electroejaculation, reproduction, cryopreservation.

INTRODUCTION

A source of viable spermatozoa is essential for andrological and reproductive studies. Moreover, assisted fertilization techniques require the collection of spermatozoa. Because of technical difficulties, most studies on sperm function in small animals have been performed using epididymal gametes (11). Obtaining gametes from ejaculates offers advantages such as not needing to euthanize animals and being able to obtain numerous samples from one individual.

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Scott and Dziuk (34) reported success when employing bipolar electrodes for electroejaculation in rats, mice and guinea pigs. Dalziel and Phillips (6) applied electroejaculation techniques in guinea pigs and chinchillas. Healey and Weir (17 ,18) published a detailed technique for electroejaculation in Chinchilla laniger and they studied the spermatozoal ultrastructure in this species.

A permanent source of readily available gametes requires the use of cryopreservation techniques, which should ensure the optimal functional activity of frozen-thawed spermatozoa. One of the major factors affecting cryopreservation of spermatozoa is the composition of the cryoprotective medium (1, 10, 13, 26, 27, 31). In a previous work, we cryopreserved epididymal spermatozoa from Chinchilla laniger and we compared the efficacy of several cryoprotectans. The best results were obtained in the presence of sodium citrate, TES, Tris, egg yolk, fructose and glycerol (30).

Chinchilla laniger possess a valuable fur, for which the species has been heavily exploited in South America. Indeed, this species has reached endangered status and has become nearly extinct in the wild (Appendix 1 of the Convention on International Trade in Endangered Species - CITES).

The present study was undertaken to develop a practical technique for adequate cryopreservation and storage of ejaculated chinchilla semen and to compare the activity of fresh and frozen-thawed gametes.

MATERIALS AND METHODS

Sexually mature Chinchilla laniger males (n = 6) weighing 498.49 ± 6.81 g were used in the study. The animals were maintained in individual cages on 12L:12D (light: dark) schedule at 21 to 22°C, with food and water available ad libitum. Semen was collected from April to October.

Electroejaculation

The unanesthetized chinchilla were placed in a box that covered the cephalic half of the body while the rest laid on a metallic grid support. After cleaning the genital area, the prepuce was retracted, and the penis was introduced into in a 2-mL Eppendorf plastic tube. The rectal probe was lubricated with glycerin, and the bronze bipolar electrode (length ≅ 40 mm, diameter ≅ 4.2 mm) was inserted into the rectum to a depth of 20 to 30 mm and held by the technician.

The alternative current (220 V, sinusoidal wave, 50 cycles/sec) was applied for 5 of every 10 sec. The current was controlled by a rheostat and was calculated from an oscilloscope reading of the voltage drop across a resister placed in the circuit. A series of 1 to 6 pulses was normally given at the 6 to 6.8 V setting.

Immediately after electrostimulation, a white viscous plug was obtained, which was discarded. The remaining portion of the ejaculate was collected and diluted with 150 µL

Tyrode's buffer medium without BSA (pH 7.4, 280 mOsm/Kg) in order to avoid coagulation. After measuring the volume, functional parameters of spermatozoa were determined within 5 to 10 min by subjective evaluation in fresh (immediately after electroejaculation) and in frozen-thawed semen. Two experienced technicians applied the following methods standard in our laboratory.

Sperm Concentration and Motility

Sperm concentration and motility were measured at 23 ± 2°C in a Makler counting chamber under an inverted microscope (Olympus CK2, Tokyo, Japan) at x 200 magnification. The results are expressed as the percentage of motile cells (progressive plus nonprogressive spermatozoa). No less than 200 gametes were examined (23).

Viability

The viability parameter was evaluated by supravital staining (39) with Hoechst 33258 (H258) (Calbiochem, La Jolla, CA, USA). Using the appropriate ultraviolet fluorescence optics (Axiolab, Zeiss, Jena, Germany), spermatozoa showing brightly fluorescent nuclei were scored as dead and sperm cells which excluded the H258 and were not fluorescent were scored as viable. The viability of 200 to 250 cells was assessed.

Acrosomal Integrity

Acrosomal status was determined by staining with Pisum sativum agglutinin labeled with fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, MO, USA) as described by Hinsch et al. (20) with slight modifications. The H258 (stock solution: 1 mg/mL) was dissolved in DPBS and added to a final concentration of 1 µg/mL and then co-incubated with spermatozoa for 10 min. Samples were washed free of unbound stain by centrifugation twice at 400 g for 10 min through 6 mL of 2% (w/v) PVP-DPBS; the supernatant was then carefully removed, and the final sperm pellet was resuspended in 50 to 70 µL DPBS. Cells were mounted as smears on glass slides and air dried in an incubator at 36°C. Subsequently, they were fixed with methanol for 30 sec. The slides were washed with a stream of distilled water for 2 min. After drying, spermatozoa were incubated 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 10 min. Finally, in order to avoid fading of fluorescence, the slides were mounted in mounting medium containing 1 mg/mL sodium azide and 100 mg/mL 1,4-diazabicyclo (2.2.2) octane (DABCO) in 90% (v/v) glycerol and 10% (v/v) DPBS (pH 9). The acrosomal status and viability were evaluated under a epifluorescence microscope (x 1000; Axiolab, Zeiss, Jena, Germany) equipped with an epi-illumination module and a mercury vapor UV source using a Zeiss FITC- (no. 09) and an H258 filter set (no. 02). The acrosomal status of FITC-PSA-labeled cells (n = 200 to 250) was assessed from 5 to 12 different fields, and the counts were scored blind and counted in duplicate slides. The following staining patterns were distinguished: a) viable acrosome intact (H258 negative with an intense green-turquoise blue fluorescent acrosome indicating acrosome-intact spermatozoa), b) viable acrosome-

eacted (H258 negative with no green/yellow PSA-FITC fluorescence over the whole head or a thin equatorial band), c) dead acrosome-intact (H258 positive with intensely PSA-FITC fluorescent acrosomal regions and a weak blue stain on the post-acrosomal region) and d) dead acrosome-reacted (H258 positive with almost no PSA-FITC fluorescence in the head region indicating degenerated acrosomes, also called "false" acrosome reaction).

Response to Hypoosmotic Shock

The hypoosmotic swelling test (HOS) evaluates whether an intact membrane is biochemically active (4). The procedure used for the HOS test was similar to the one described by Jeyendran et al. (21) and adapted by Ruiz et al. (32). The sperm suspension (0.1 mL) was mixed with the hypoosmotic solution 100 mOsm/L (Fiske Osmometer G-52, Bethel, CT, USA) and pH 7.4 for 45 min (37°C). Evaluations were made by phase-contrast microscopy at a magnification of x 400; one hundred or more cells were observed, and the percentage of spermatozoa that showed tail swelling was determined by dividing the number of reacted cells (x 100) by the total number of spermatozoa counted in the same area.

Preparation of Cryoprotective Media

All chemicals were purchased from Sigma (St. Louis, MO, USA) and were dissolved in double-distilled water. Eggs were purchased farm-fresh. Sterile glassware, cryostraws and pipettes were used, and care was taken to avoid contamination of media.

The procedure for cryopreservation of spermatozoa was the same as that reported by Ponce et al. (30). The cryoprotective buffer was made as described by Weidel et al. (38) with slight modifications as follows: Cryo-buffer: a zwitter ionic buffer system was prepared by titrating 325 mOsm TES with 325 mOsm Tris to make the TEST solution: 48% (vol/vol) TEST, 30% sodium citrate, 20% egg yolk, and 2% fructose. To extract the yolk, a fresh chicken egg was cracked into a sterile Petri dish and the yolk aspirated with a sterile 10-mL syringe. The mixture was centrifuged at x 10,000 g for 10 min, and the supernatant was filtered through filter paper (Whatman I). Glycerol was added to a final concentration of 6%. Penicillin 0.15 mg/mL and streptomycin 0.25 mg/mL were added. The pH and osmolality of extenders were measured before the addition of glycerol. Aliquots of cryo-buffer medium were stored at -20°C for later use.

Spermatozoa Freezing

The cryoprotective medium was added, drop by drop, to the remaining portion of the ejaculate (1:1 dilution), then it was gently shaken for 10 to 15 min to achieve complete semen-medium mixture, and finally it was pipetted into 500-μL cryostraws (3, 30). The straws were filled at room temperature, sealed by heat, colored, and identified with the animal number and date. The straws were immersed in a water bath (400 mL) and slowly cooled to 4°C at a rate of approximately 1.9 to 2.1°C/min; after 10 min, the cryostraws were exposed to -20°C for 15 min, and the samples were additionally cooled by exposure to a liquid nitrogen gas layer (10 cm above the liquid nitrogen level) for 10

min. The cryostraws were then immersed directly into liquid nitrogen (Air Liquide GT-11, Lyon, France), where they were stored for 3 to 6 mo before thawing. To avoid excessive temperature fluctuations, fast transfer was performed during all steps.

Thawing Procedure

Each cryostraw was immersed in water at 37°C for 60 sec and wiped dry; the contents were released in Tyrode's buffered medium. The final concentration of sperm suspension was adjusted to ~ 5 to 10 x 10^6 cells/mL.

Statistical Analysis

To compare the activity of recently ejaculated and frozen-thawed spermatozoa, the two-sample Student's t-test was applied (CSS: Statistica, StatSoft Inc, Tulsa, OK, USA). The variables analyzed were motility, viability, response to hypoosmotic shock and acrosomal status. Percentage data were analyzed after angular transformation by the formula y = arcsen [sqrt(x/100)]. Values were expressed as mean ± standard error of the mean (SEM). All P values less than 0.05 were considered statistically significant. Pearson's correlation coefficient (CSS: Statistica, StatSoft Inc) was used to evaluate the relationship between HOS and other sperm function parameters (36, 21).

RESULTS

Electrostimulation was efficacious for inducing ejaculation in 100% of the animals. Semen was usually obtained during the first or second "on period". The volume of the remaining portion of the ejaculate was 108.3 ± 12.0 µL (n = 15 ejaculates) and ranged between 50 and 200 µL. Sperm concentration was 421.8 ± 34.4 x 10^6/mL and ranged between 172 and 540 x 10^6/mL.

Table 1 shows the parameters reflecting sperm activity in fresh and in frozen-thawed semen. The percentages of motile, viable and swollen cells were significantly higher in the fresh samples. More than 95% of motile spermatozoa exhibited forwerd progressive motility in both fresh and frozen-thawed samples. After thawing, 46% of motile (progressive plus nonprogressive) and swollen (positive response to HOS) gametes were recovered.

Correlations between the percentage of swollen, motile and viable gametes were determined; the results are shown in Table 2. It is evident that in the post-thaw samples there was a clear positive correlation between results of responses to hypoosmotic media and the percentage of motile or viable cells. Moreover, in these samples, a significant correlation between motile and viable spermatozoa and between motile or viable vs viable acrosome-intact cells is observed.

Table 1. Parameters reflecting sperm functional activity of Chinchilla laniger spermatozoa immediately after electroejaculation (fresh) and after freezing-thawing

	Mean ± SEM	
Type of sample	Fresh semen	Frozen-thawed semen
No. of samples	15	16
Motility (% of motile cells)	97.4 ± 0.3	44.6 ± 2.2[a]
Viability (% of living cells)	93.5 ± 0.5	38.8 ± 2.2[a]
Hypoosmotic swelling test (% of swollen cells)	68.5 ± 1.0	31.3 ± 1.6[a]

[a] $P<0.05$ vs fresh semen.

Table 2. Correlation between functional parameters from Chinchilla laniger spermatozoa immediately after electroejaculation (fresh) or after freeze-thaw

Type of sample	Fresh semen		Frozen-thawed semen		
Variables	r	No. of Samples	r	P	No. of Samples
Swollen vs motile	-0.04707	15	0.69570	0.05	16
Swollen vs viable	0.27294	15	0.87476	0.05	16
Motile vs viable	0.47197	15	0.87106	0.05	16
Motile vs viable acrosome-intact	- 0.03245	8	0.83959	0.05	8
Viable vs viable acrosome-intact	0.19034	8	0.87064	0.05	8

r = Pearson's correlation coefficients. Swollen spermatozoa = positive response hypoosmotic swelling test.

With respect to acrosomal status, it is remarkable that the quality of the staining was similar for fresh and cryopreserved sperm samples. The percentage of viable acrosome-intact spermatozoa decreased significantly after cryopreservation (83.5 ± 2.5% in fresh samples and 40.3 ± 1.8% in frozen-thawed samples; $n = 8$; $P< 0.05$). The "false" acrosome reaction (dead cells acrosome-reacted) increased more than 5 times in cryopreserved gametes ($P< 0.05$ vs fresh semen samples).

Table 3. Viability and acrosomal status of fresh and cryopreserved Chinchilla laniger spermatozoa

	Mean ± SEM			
	Dead acrosome intact (%)	Dead acrosome reacted (%)	Viable acrosome intact (%)	Viable acrosome reacted (%)
Fresh spermatozoa (8 samples)	1.2 ± 0.5	10.2 ± 1.8	83.5 ± 2.5	5.1 ± 1.6
Cryopreserved spermatozoa (8 samples)	2.2 ± 0.5	53.1 ± 1.8[a]	40.3 ± 1.8[a]	4.4 ± 0.7

[a] Significantly different with respect to fresh semen values.

DISCUSSION

The main objectives of this study were to cryopreserve electroejaculated spermatozoa from Chinchilla laniger and to compare functional activity of fresh and frozen-thawed spermatozoa.

When the electroejaculation procedure employed here was compared with previous reports (6, 12, 17), notable differences arise; for instance, we employed a sinusoidal wave while the other workers utilized a square wave. In addition, we employed lower voltage and amperage of current in our experiments than those used by Healey and Weir (17) for the same purpose.

The effectiveness of the methodology employed in our study is evidenced by the fact that we obtained an ejaculation in 100% of the trials, while Dalziel and Phillips (6) and Healey and Weir (17) obtained 67 and 92%, respectively, in the same species.

With respect to the volume of semen collected, the above mentioned authors reported values lower than those in our present study. This difference is likewise important when the procedure is performed to obtain specimens for use in assisted reproductive techniques or for experimental purposes.

Additional practical aspects of our procedure were that the animals were not restrained in an unphysiological position, thus avoiding another stressor stimuli like immobilization, and that the method requires only 1 well–trained technician.

In our study we investigated whether fresh or frozen-thawed semen samples of Chinchilla laniger were suitable for assessment of viability and acrosomal status, respectively, using the bisbenzimide H258 fluorochrome and FICT-PSA staining

method. The staining procedure has been successfully employed for spermatozoa from various species (5, 8, 25, 37).

Our results demonstrate that, indeed, either fresh or frozen-thawed Chinchilla laniger spermatozoa are suitable for the above mentioned dye. Using this staining method, viability and acrosomal status can be properly assessed and evaluated simultaneously by changing the filter sets.

It is well known that sperm cryopreservation deleteriously affects cellular functional activity in all species studied. The effects vary according to various factors, including the composition of the cryoprotectant and the applied freezing-thawing procedure (9, 15, 28). Some critical properties must be taken into account when a cryoprotectant is chosen. These include low molecular weight, ability to permeate living cells, high solubility in aqueous electrolyte solutions, and absence of toxic effects (14, 19, 22).

According to our previous report, the medium employed adequately satisfies these conditions in the epididymal gametes of the chinchilla. Indeed, we demonstrated that a sufficient population of viable (49.1 ± 2.9%, n = 9), motile (56.6 ± 3.9%, n = 15) and viable acrosome-intact (38.0 ± 6.7%, n = 8) spermatozoa were found after thawing, similar to the results obtained with freshly ejaculated sperm cells (30).

Healey (16) recovered 25% of motile gametes after thawing Chinchilla laniger electroejaculated samples. From our assays, we obtained higher values. Healey (16) used a cryoprotectant composed of sodium citrate, egg yolk, glycerol and DMSO, and his freezing procedure was highly different. After thawing, semen was kept at 23 to 25°C. It has been shown that longevity is greater when semen is kept below 37°C and ideally at/or above 21°C (4, 24).

Our results demonstrate, as has previously been described in other species (2, 21, 32), that ejaculated Chinchilla laniger spermatozoa, whether fresh or frozen-thawed, undergo swelling when incubated in a hypoosmotic solution. The reacting spermatozoa showed alterations varying from a shortened and thickened tail to a swollen area that could be right at the tip of the tail, covering part or all of the tail.

When the results of HOS in fresh and frozen-thawed samples are compared, it is evident that in a fraction of the population, membrane integrity is disrupted by cryopreservation. We previously reported a similar finding for epididymal gametes (30).

Since the percentage of dead acrosome-reacted cells is higher than that of dead acrosome-intact cells in fresh or cryopreserved samples, it becomes evident that, in this species, cellular death also induces a "false" acrosome reaction. In frozen-thawed samples there were also more dead spermatozoa without acrosomes than in fresh samples. The cryopreservation procedure significantly decreased the percentage of viable acrosome-intact gametes but not the viable acrosome-reacted ones; this difference could be attributed to cellular death (20).

When values from various species are considered, correlation results between HOS and the percentage of motile or viable spermatozoa are controversial. In epididymal mouse spermatozoa, Ruiz et al. (32) reported a positive correlation between HOS and motility but not between HOS and viability, while in Chinchilla laniger species, a lack of correlation between HOS and motility was detected in fresh spermatozoa. In humans, Esteves et al. (7) found a high correlation between HOS and viability of fresh spermatozoa, but this correlation disappeared after sperm cryopreservation. These findings were not observed in any of the parameters studied for fresh chinchilla ejaculates.

Our results show a positive significant correlation between HOS and motility or viability in frozen-thawed samples. Correa and Zavos (2) reported similar findings in bull frozen-thawed spermatozoa.

Post-thaw sperm motility seems to be particularly well correlated with frozen-thawed viable acrosome-intact gametes. These results closely resemble those of Serafini et al. (35), who found a good correlation between sperm motility and the percentage of spermatozoa with intact acrosomes in human gametes.

Our results indicate that acrosomal integrity (a condition required for successful interaction with the female gamete) is also correlated with other parameters which reflect sperm function such as motility or viability.

Changes in the plasma membrane properties due to thermal shock can induce and/or enhance human sperm capacitation (33), probably because during cooling, freezing and thawing, the spermatozoa are exposed to drastic changes in their physical and chemical environment (19).

Our modified electroejaculation method allowed for reliable collection of semen from the Chinchilla laniger. Glycerol appears to be an effective cryoprotectant for ejaculated spermatozoa, and post-thaw motility was similar to that previously reported for epididymal spermatozoa. These findings may be especially important for the collection and long-term storage of valuable gametes from rare chinchillas because euthanasia of the animals is not required, and multiple samples can be collected from an individual animal.

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