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Functional activity of epididymal *Chinchilla laniger* spermatozoa cryopreserved in different extenders

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SUMMARY

Chinchilla laniger is an endangered species and improved cryopreservation of spermatozoa would constitute a significant advance in the development of assisted reproductive techniques in this species. The functional activity of epididymal spermatozoa from adult males was studied immediately after extrusion and after 24 hours incubation, and the ability of five extenders to protect these gametes during cryopreservation was determined. A decrease in sperm motility, viability, acrosome intact cells and response to hypo-osmotic swelling test was detected 24 hours later. The extenders here assayed showed well-defined differences in their cryoprotective ability; however such differences could not be attributed to any one of their individual components. The presence of TES plus Tris, the proportion of the individual constituents and/or the differences in metabolic substrate content could explain the above-mentioned finding. The results indicate that cryo-buffer II (TES-Tris-egg yolk-fructose-glycerol) is the most powerful protector of sperm functional activity in this species.

SEMEN cryopreservation has been described in various species including monkey (Seier et al 1993), human (Bunge and Sherman 1953), bovine (Foulkes and Stewart 1977), mouse (Nakagata 1993), rabbit (Parrish and Foote 1986) and pig (Pursel et al 1978). Its application is of importance in assisted reproductive techniques, preservation of species and studies about sperm functional activity and its interaction with the female gamete.

Chinchilla laniger is a highly endangered species (Redford and Eisengerg 1992); what is more, several problems related to its natural mating have been described such as the fact that a male cannot usually mate with more than a few females during a season. Therefore the production of high quality animals is a very slow process. However, it has been claimed that artificial insemination in this species tends to eliminate genital diseases, fewer males would therefore need to be kept which represents an economy in feed and care. Seasonable variations might also be controlled (Parkinson 1987).

A technique for electro-ejaculation in chinchillas has been described. However, from a practical point of view, there are some problems with its employment in assisted reproductive techniques, such as: the ejaculate varied from a coagulum-free seminal emission through a coagulating material containing spermatozoa to a sperm-free one, but non-coagulating fluid and the ejaculation volume varied between 0·01 and 0·07 ml (Healey and Weir 1967).

One of the critical factors related to cryopreservation of spermatozoa is the composition of the cryoprotective medium. A variety of cryoprotective media have been used in mammals and different degrees of success have been achieved when storing sperm in these protective media and in the subsequent insemination (Jedrzejczak et al 1996). Glycerol was the first cryoprotectant employed to freeze sperm (Polge et al 1949) and it is still the most widely used agent. Recently, more complex cryoprotective buffer systems have been introduced and several seem to be capable of improving sperm functional activity recovery after thawing (Hinsch et al 1997).

The objective of the present study is to compare the effectiveness of five cryoprotective media currently applied in others species to preserve the epididymal *Chinchilla laniger* spermatozoa. We also describe the functional activity of epididymal male chinchilla gametes as reflected by the following parameters: motility, viability, responses to hypo-osmotic shock and acrosomal integrity.

MATERIALS AND METHODS

Animals

Sexually mature male chinchilla were employed. Animals were maintained on 12:12 (light:dark) basis at 21-22°C with food and water ad libitum.

Preparation of spermatozoa

After neck dislocation, the testis were removed, the distal portions of cauda epididymis were isolated and placed in 5 ml of Tyrode's medium (Fraser 1983). Spermatozoa were obtained by making incisions in the tissue and by allowing the sperm to extrude into the medium for 15 minutes. The spermatozoa were dispersed by shaking the dish at room temperature for about 30 seconds.

Pre-freezing evaluation

Immediately after extrusion and after 21-24 hours incubation at 37°C in Tyrode's medium, containing 0·3 per cent BSA (gassed with air: CO_2; 19:1) the functional parameters were assessed by at least two independent experienced observers.

Sperm concentration and motility were measured at 23±2°C in a counting Makler chamber under inverted

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microscope at 200 x magnification. Results are expressed as percentage of motile cells (Makler 1980).

Viability was evaluated by supravital staining with Hoechst 33258 (H258) (Yelian and Dukelow 1992). Using the appropriate ultraviolet fluorescence optics, sperm with brightly fluorescence nuclei were scored as dead and sperm which excluded the H258 and were not fluorescent were scored as viable. The viability of 200 cells was assessed.

Acrosomal integrity was determined by staining with *Pisum sativum* agglutinin labelled with fluorescein isothiocyanate as described by Cross et al (1986) and Liu and Baker (1988) with slight modifications described previously (Fiol de Cuneo et al 1994). Scoring was performed under oil immersion at 1000 x. The viability and acrosomal status of 200 sperm was assessed. Under blue wavelength fluorescein-appropriate excitation, sperm with brightly fluorescing acrosomes were considered acrosome intact, while the sperm with no fluorescence over 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. Only the viable cells were considered and the results are expressed as percentage of acrosome intact cells.

Response to hypoosmotic shock was evaluated according to Ruiz et al (1996). The sperm suspension (0·1 ml) was mixed with hypoosmotic solutions 100 mOsm (1 ml) for 45 minutes. Evaluations were made by phase-contrast microscopy at 400 x; 100 or more cells were observed and the percentage of spermatozoa that showed tail swelling was determined.

Preparation of cryoprotective media

The five cryoprotective buffers were made as described by Prins et al (1986) (buffers I, II, III and V) and Seier et al (1993) (buffer IV) with slight modifications, and their composition are summarised in Table 1.

To extract the yolk, a fresh chicken egg was cracked into a sterile Petri dish and the yolk was aspirated with a sterile 10-ml syringe.

Spermatozoa freezing

Each cryoprotective medium was added drop by drop to a portion of pool sample to a 1:1 dilution and introduced into the straws (450 µl) at 23°C±2°C. The samples were immersed in a water bath (400 ml) and then slowly cooled to 4°C at a rate of approximately 0·8°C min^{-1}, the straws were exposed to –20°C by placing the water bath in a freezer for 15 minutes. After that, the samples were additionally cooled by exposure to liquid nitrogen gas layer (10 cm above the liquid nitrogen level) for 10 minutes. The straws were then immersed directly into liquid nitrogen, where they were stored for three to six months before thawing. Final concentration of each sample was adjusted to ~5-10 x 10^6 cells ml^{-1}.

TABLE 1: Cryo-buffers composition

	Cryo-buffer				
	I*	II*	III*	IV**	V*
Sodium citrate (325 mOsm, v/v)	80%	30%	12·5%	–	–
TES (325 mOsm)	–	24% (v/v)	70% (v/v)	43 mg ml^{-1}	–
Tris (325 mOsm)	–	24% (v/v)	–	13 mg ml^{-1}	–
HEPES (325 mOsm)	–	–	–	–	20% (v/v)
KOH (325 mOsm)	–	–	–	–	20% (v/v)
Egg Yolk (v/v)	20%	20%	17·5%	30%	20%
Fructose (w/v)	2%	2%	–	–	–
Dextrose	–	–	–	10 mg ml^{-1}	–
Glucose	–	–	–	–	4% (w/v)
Glycerol (v/v)	7·5%	6%	10%	4%	6%

* The mixture was centrifuged at 10,000 x *g* for 10 minutes, and the supernatant was filtered through filter paper (Whatman I), before glycerol addition.
** The buffers and dextrose were made up to 100 ml in double distilled water. Egg yolk was next added.
In all buffers, penicillin 0·15 mg ml^{-1} and streptomycin 0·25 mg ml^{-1} were added. The pH (7·2-7·4) and osmolality of each batch of extenders were measured before the addition of glycerol, and then they were stored in a deep freezer at –20°C

TABLE 2: Some functional parameters of epididymal *Chinchilla laniger* spermatozoa

	Fresh[a]	24 hours[b]
Motility (% of motile cells)	95·0±1·5 (9)	32·8±9·6 (7)
Viability (% of living cells)	92·6±2·4 (9)	66·7±3·5 (8)
HOST (% of swollen cells)	78·5±4·0 (9)	17·5±1·6 (7)
Acrosome integrity (% of intact cells)	85·9±4·3 (8)	63·0±5·9 (7)

Values are expressed as mean±SEM. In parentheses: number of experiments. HOST: hypoosmotic swelling test. Determinations were performed immediately after extrusion (fresh) or after incubation in Tyrode's medium during 24 hours. a vs b: P<0·05 in each row

Post-thawing evaluation

Each straw was thawed in a water bath at 37°C for 60 seconds. The cryoprotective media was removed by washing twice with Dulbecco's phosphate buffered saline (by centrifugation at 400 *g* for 10 minutes at room temperature). In order to evaluate sperm functional activity, the spermatozoa were resuspended in Tyrode's promoting-capacitation medium and incubated as previously described. The final concentration of sperm suspension was adjusted to ~3-5 x 10^6 cells ml^{-1}.

Immediately after thawing and after four hours incubation the parameters above mentioned were assessed.

Statistical analysis

The values were expressed as means ± standard error of the mean (SEM). Data were analysed by Student's *t* test. Percentages were converted to a Gaussian distribution by arcsin transformation of square root. All P values less than 0·05 were considered statistically significant.

RESULTS

Some parameters which reflect functional activity of epididymal *Chinchilla laniger* spermatozoa are displayed in Table 2. After 24 hours incubation in Tyrode's medium, percentage or motile, viable, swollen and acrosome intact spermatozoa dropped significantly with respect to the values obtained immediately after extrusion.

After liquid nitrogen preservation with the cryo-buffers here employed, a significant reduction of motility, viability and response to hypo-osmotic shock of sperm was detected when compared to the fresh samples. Fig 1 shows the percentage of motile spermatozoa after thawing. Evaluations were performed at zero and four hours after thawing (panel [a] and [b] respectively).

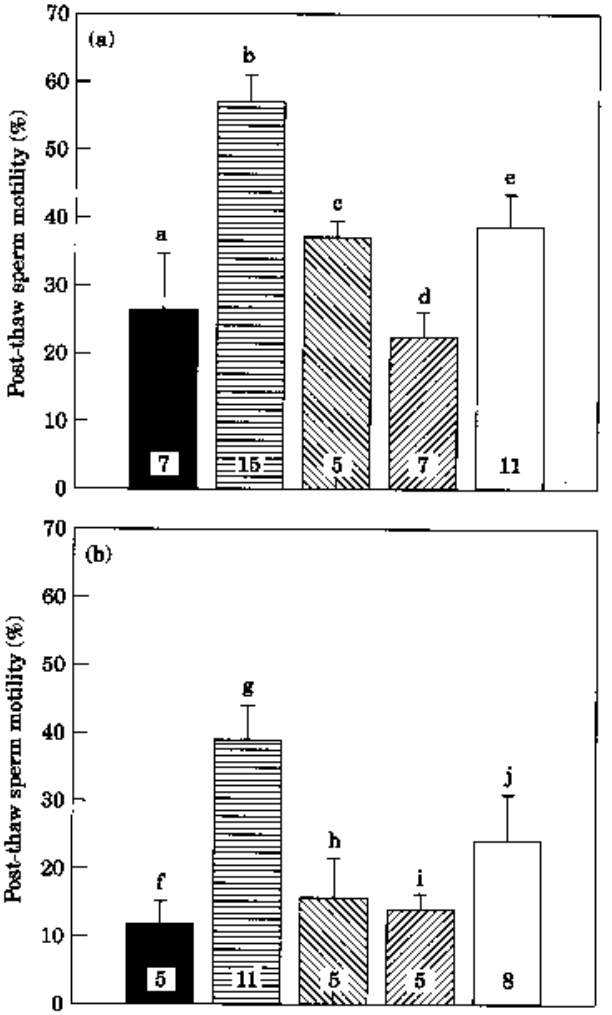

FIG 1: Post-thaw motility of *Chinchilla laniger* spermatozoa cryopreserved in five cryo-buffers. Determinations were performed immediately after thawing (a) or after incubation in Tyrode's medium during four hours (b). Results are expressed as mean±SEM. Number of experiments are indicated on the bottom of each column. (a) b versus a, c, d, e; c versus d: P<0·05; (b) g versus f, h, i; f versus h: P<0·05. Key: ■, cryo-buffer I; ▤, cryo-buffer II; ▧, cryo-buffer III; ▨, cryo-buffer IV; □ cryo-buffer V

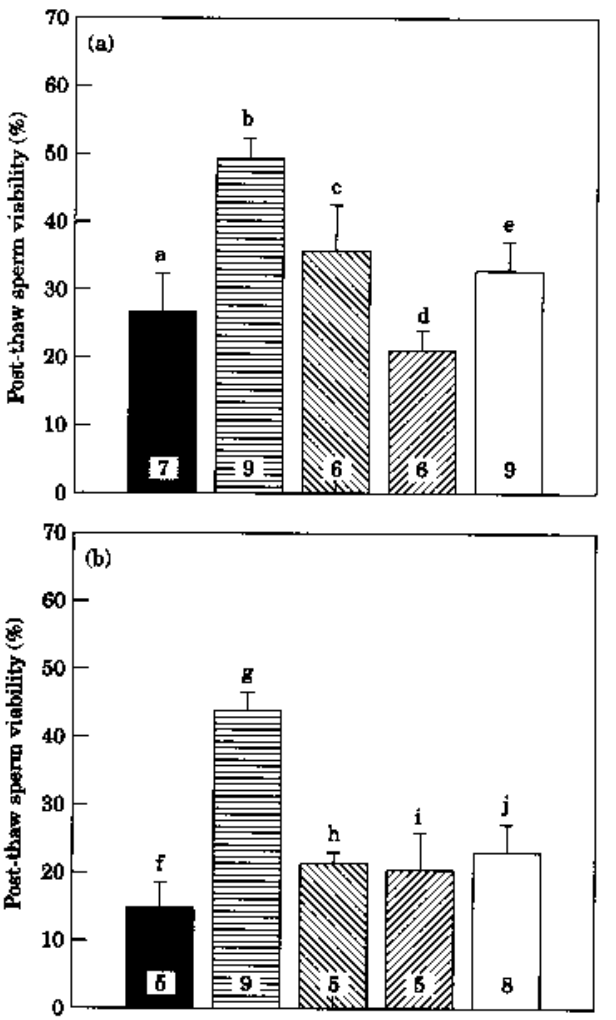

FIG 2: Post-thaw viability of *Chinchilla laniger* spermatozoa cryopreserved in five cryo-buffers. Determinations were performed immediately after thawing (a) or after incubation in Tyrode's medium during four hours (b). Results are expressed as mean±SEM. Number of experiments are indicated on the bottom of each column. (a) b versus a, d, e; d versus e: P<0·05; (b) g versus f, h, i, j: P<0·05. Key: ■, cryo-buffer I; ▤, cryo-buffer II; ▧, cryo-buffer III; ▨, cryo-buffer IV; □ cryo-buffer V

Immediately after thawing (Fig 1a), the percentage of motile cells was 56·6±3·9 per cent (n = 15) in the samples cryopreserved in buffer II, this value being significantly higher than those obtained with all other cryo-buffers. After four hours post-thaw (Fig 1b), the sperm motility decreased in all samples, remaining this parameter significantly higher in that preserved with buffer II (38·7±5·2 per cent, n = 11).

The results of the evaluation of viability after liquid nitrogen storage with five different protective media are presented in Fig 2. Cryo-buffer II was again the most effective one to maintain this parameter (P<0·05 versus cryo-buffers I, IV and V). Values obtained four hours after thawing (Fig 1b) did not differ significantly from those obtained at zero hours in all media.

Responses to hypo-osmotic shock after sperm preservation in the five cryoprotectants are displayed in Table 3. Immediately after thawing, the highest response was obtained with cryo-buffer II (30·8±2·7 per cent of swollen spermatozoa; n = 12). After four hours incubation in Tyrode's medium, the percentage of swollen gametes dropped, values obtained with cryo-buffer II being significantly different with respect to zero hours.

Acrosomal integrity was also evaluated after thawing; its results are expressed in Table 4. Percentage of acrosome intact cells decreased after four hours post-thawing incubation, the values being significantly higher in samples cryopreserved in buffers II, IV and V.

DISCUSSION AND CONCLUSIONS

The results reported here suggest clearly that functional activity of epididymal *Chinchilla laniger* spermatozoa, as reflected by determinations of motility, viability, hypo-osmotic swelling test and acrosome integrity varies significantly after 24 hours incubation in Tyrode's medium. The decrease in sperm motility during this period could be attributed to modifications in cellular metabolic patterns that occur during incubation (Fraser and Ahuja 1988). Similar modifications were previously described in epididymal mouse spermatozoa after 240 minutes incubation (Fiol de Cuneo et al 1994).

It is known that during capacitation, there occur modifications of mammalian sperm membrane fluidity, mainly

TABLE 3: Response to hypo-osmotic swelling test of frozen-thawed *Chinchilla laniger* spermatozoa cryopreserved in different extenders

	0 hours	4 hours
Cryo-buffer I	23·7±5·2 (7)	14·9±3·1 (5)
Cryo-buffer II	30·8±2·7[a] (12)	20·2±3·3[b] (10)
Cryo-buffer III	27·6±3·9 (6)	20·2±5·8 (5)
Cryo-buffer IV	23·8±4·1 (6)	16·7±2·9 (5)
Cryo-buffer V	26·4±3·3 (10)	16·9±2·6 (8)

Values indicate percentage of swollen spermatozoa and are expressed as mean±SEM. In parentheses: number of experiments. Determinations were performed immediately after thawing (0 hours) or after incubation in Tyrode's medium during 4 hours. a vs b: P<0·05 (for further details, see text)

TABLE 4: Acrosome integrity of post-thawed epididymal *Chinchilla laniger* spermatozoa cryopreserved in different extenders

	0 hours	4 hours
Cryo-buffer I	75·3±9·7 (6)	69·4±4·1 (6)
Cryo-buffer II	84·4±2·7* (11)	60·6±8·2 (8)
Cryo-buffer III	70·0±9·3 (7)	58·7±6·0 (6)
Cryo-buffer IV	91·3±2·8* (6)	57·8±14·5 (5)
Cryo-buffer V	85·7±5·0* (5)	57·7±8·4 (7)

Values indicate percentage of acrosome intact cells and are expressed as mean±SEM. In parentheses: number of experiments. Determinations were performed immediately after thawing (0 hours) or after incubation in Tyrode's medium during 4 hours. * P<0·05 vs 4 hours (for further details, see text)

due to the removal of cholesterol by albumin, (Langlais and Roberts 1985). These phenomena could be an explanation of the significant decrease in the percentage of swollen cells detected after 24 hours.

Since the physiological role of spontaneous acrosome reaction in mammals is unknown at present, it has been postulated that in order to achieve fertilisation, a basal level of acrosome reacted spermatozoa is necessary (Zaneveld et al 1991, Klemm and Engel 1991). This statement seems to be confirmed in *Chinchilla laniger*, since the acrosome intact gametes decreased significantly during incubation, eg in the absence of any physiological stimuli such as follicular fluid, zona pellucida or progesterone.

The ability of five extenders to protect the *Chinchilla laniger* sperm was studied in this work. Functional parameters were measured immediately after thawing or four hours later. Well-defined differences exist between the cryoprotective effects of medium II with respect to the others assayed here. Thus, post-thawed sperm motility and viability were significantly higher in the samples cryopreserved in this extender. Similar results employing this cryo-buffer were obtained by Prins and Weidel (1986) in human gametes.

With respect to the other parameters considered, responses to hypo-osmotic swelling test suggest that these cryo-buffers did not preserve membrane integrity in optimal conditions. Nevertheless, immediately after thawing, the percentage of acrosome intact spermatozoa was similar to that obtained in fresh samples, indicating that all cryo-buffers were able to preserve acrosome integrity.

After thawing and during four hours incubation, the percentage of motile cells dropped in a similar way to that described in fresh samples after 24 hours incubation. On the other hand, viability of post-thawed gametes was not significantly modified after the same period. In this context, it is known that sperm motility depends not only on membrane integrity; other biochemical reactions such as energy supply and the microtubular action of the tail fibers are involved. These latter processes could be responsible for motility impairment (Yanagimachi 1994).

At present, effects of thawing on membrane properties are not clearly understood. In this context, Watson et al (1992) claimed that the freezing-thawing process could exert a direct effect on the sperm plasma membrane, which results in a loss of some intracellular components that would affect viability. Besides, Carreras et al (1992) reported that in human sperm, it is possible to find living cells with no response to hypo-osmotic shock and vice versa. These observations could explain differences between the percentage of swollen and viable cells reported here. Previously, we found similar phenomena in epididymal mouse gametes (Ruiz et al 1996).

Although differences in sperm recovery with the different extenders here employed could not be attributed to any of the individual components, certain considerations could be taken into account, ie the presence of TES plus Tris in cryo-buffer II could enhance the capacity to pick up hydrogen ions from the surrounding media, thereby aiding the dehydration process. Zwitter ionic buffers have shown to differ in protective capacity for bovine sperm as well (Graham et al 1972).

The proportion of the individual components could be another critical factor. Hammitt et al (1988) postulated that the concentration of glycerol required by cryopreserved human sperm for optimal survival and for in vitro fertilising capacity is dependent on the type of medium used for freezing; Wilmut and Polge (1977) reported that when boar sperm were frozen in a medium with more than 6 per cent glycerol, the extent of osmotic damage to sperm was so severe that a complete loss of fertilising capacity occurred; our results would indicate that the optimal concentration of glycerol in this species is about 6 per cent.

The differences in metabolic substrate content would constitute another relevant factor to consider. However, in this context, Silva et al (1996) conclude that the inclusion of sodium citrate and/or fructose to the cryopreservation medium does not alter human sperm motility or viability.

Finally, from the above discussed results, we claim that an adequate cryopreservation method for epididymal *Chinchilla laniger* spermatozoa can be achieved using buffer II containing sodium citrate, TES, Tris, egg yolk, fructose and glycerol and that this constitutes a significant advance in order to achieve successful assisted reproductive techniques in this species.

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