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BRIEF COMMUNICATION
Storage of *Chinchilla lanigera* Semen at 4°C for 24 or 72 h with Two Different Cryoprotectants

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The objectives of this study were to: (a) test the functional activity of *Chinchilla lanigera* spermatozoa suspended in either glycerol or ethylene glycol, cooled to 4°C, and stored for 24 or 72 h and (b) investigate, after these cooling periods, the effects of incubating sperm at 37°C (for 4 h) upon sperm functional activity. The ejaculate was mixed with the cryoprotectant medium (at 1 M final concentration) and cooled to 4°C. After warming, sperm motility, sperm viability, hypoosmotic swelling test results, and acrosomal integrity were significantly higher for samples containing ethylene glycol than for those in glycerol, stored for 24 or 72 h, and then assayed after 0 or 4 h incubation at 37°C. A significant reduction of sperm motility and viability was detected only when the glycerol cryoprotectant agent was employed, compared to the fresh samples. These results clearly indicate that under our experimental conditions, ethylene glycol is a better protectant for sperm storage than glycerol.

Key Words: 4°C storage; ethylene glycol; glycerol; sperm; *Chinchilla lanigera*.

Sperm from most species survive cryopreservation protocols very poorly and the best efforts usually result in recovery of only about half of the original sperm motility (3, 6, 9, 10). Sperm function is also impaired after thawing, as manifested by shorter longevity and reduced membrane stability (7).

Although glycerol is the most common cryoprotectant used, since it was described (8), several authors have observed detrimental effects of this cryoprotectant agent upon some aspects of sperm functional activity (3, 4). The present study was undertaken in order to: (a) test the functional activity of *Chinchilla lanigera* spermatozoa suspended in either glycerol or ethylene glycol, cooled to 4°C, and stored for 24 or 72 h and (b) investigate, after these cooling periods, the effects of incubating sperm at 37°C (for 4 h) upon sperm functional activity.

Sexually mature *C. lanigera* males (500–600 g body weight) maintained in individual cages with food and water *ad libitum* were used in the study.

The cryoprotective medium was prepared according to Ponce *et al.* (9). Briefly, a zwitterionic buffer system was prepared by titrating 325-mOsm TES with 325-mOsm Tris to make the TEST solution. The medium was prepared with 48% (v/v) TEST, 30% sodium citrate, 20% egg yolk, and 2% fructose. The mixture was centrifuged at 10,000*g* for 10 min, and the supernatant was filtered through filter paper (Whatman I). Glycerol or ethylene glycol was added to the cryoprotectant medium at 2 M concentration and the medium was supplemented with penicillin 0.5 mg/ml and streptomycin 0.25 mg/ml. The pH (7.4–7.5) and osmolarity of extenders were measured before the addition of glycerol or ethylene glycol. Aliquots of cryoprotectants were stored at −20°C until use.

The semen was obtained as described by Ponce *et al.* (10). The ejaculate was collected and diluted with 150–300 μl of modified Tyrode's medium (9) in order to avoid coagulation. The cryoprotective medium was added drop by drop to the ejaculate (1:1 dilution; 1 M cryopro-

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tectant final concentration) and then pipetted into 500-μl plastic straws (length ~133 mm, diameter ~2.8 mm) at room temperature (22–23°C), sealed by heat, and identified. The samples then were cooled to 4°C at a rate of approximately 2°C/min and stored horizontally for 24 or 72 h.

For the warming procedure, the straw contents were flushed into an Eppendorf tube with 150–250 μl Tyrode's medium per straw and warmed to 37°C (5% CO_2:95% air) for 0 h (5–10 min) and 4 h to evaluate sperm functional activity. For all measurements, the final concentration of sperm suspension was adjusted to ~5–10 $\times$ 10^6 cells/ml. The following parameters were measured:

- Sperm concentration and motility were assessed in a Makler counting chamber as previously described (9). Results were expressed as a percentage of motile cells (progressive plus nonprogressive sperm).
- Viability was assayed by Hoechst 33258 (H258) supravital staining, 1.5 μg/ml, as described (9). Sperm having brightly fluorescent nuclei were scored as dead and sperm which excluded the H258 (not fluorescent) were scored as viable.
- Acrosomal integrity was determined by staining with *Pisum sativum* agglutinin labeled with fluorescein isothiocyanate (FITC-PSA) as previously described (9). The acrosomal status of FITC-PSA-labeled cells (n = 100–150) was assessed. The following staining patterns were distinguished: (a) viable acrosome intact (H258 negative with an intense green-yellow fluorescent acrosome, indicating acrosome-intact spermatozoa), (b) viable acrosome reacted (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 (d) dead acrosome reacted (H258 positive with no PSA-FITC fluorescence in the head region, also called "false acrosome reaction").
- Response to hypoosmotic shock, for the evaluation of the functional integrity of sperm membrane, was measured according to Ponce *et al.* (9). After incubation for 45 min (100 mOsm; 37°C), the percentage of spermatozoa that showed tail swelling was determined.

The data were analyzed by a three-way ANOVA and LSD Fisher. Percentages were converted to a Gaussian distribution by angular transformation: y = arcsine [sq rt (x/100)]. The values were expressed as means ± SEM. All P values less than 0.05 were considered statistically significant.

Previously, we reported the cryopreservation of *C. lanigera* semen in liquid nitrogen using 6% glycerol as cryoprotectant agent (CPA) and found after thawing a decrease in sperm motility and viability higher than 50% in comparison to fresh samples (9, 10). On the other hand, Maurer *et al.* (6) found that rabbit semen extended with dimethyl sulfoxide and stored at 5°C for 5 h had a significantly higher motility, viability, and fertility than semen stored at 37 or −196°C for the same period of time. In previous assays, we tested the characteristics of semen storage for 24 and 72 h at 4°C with medium but without CPA. Under these experimental conditions, we observed that percentages of spermatozoa that reacted to the hypoosmotic shock were remarkably lower than those of freshly ejaculated samples (hypoosmotic swelling shock: 24 h, 41.0 ± 9.0, n = 5; 72 h, 16.1 ± 2.8, n = 5; fresh, 74.4 ± 7.2, n = 10; $P < 0.01$). Furthermore, the number of acrosome-reacted sperm increased (72 h, 76.0 ± 9.2, n = 6; fresh, 19.0 ± 6.9, n = 7; $P < 0.01$).

The hypoosmotic swelling test as well as the acrosomal status reflects membrane integrity and are strongly correlated to the sperm fertilization ability (1, 11). Taking into account the unsatisfactory results obtained in previous experiments on the parameters mentioned above, the purposes of this study were to preserve at 4°C *C. lanigera* semen for 24 and 72 h storage with a medium containing glycerol or ethylene glycol and to compare sperm functional activity.

In the present study, we found decreases in sperm motility of 30 and 38% (24 or 72 h, respectively) using glycerol as CPA in comparison to fresh samples (Table 1) ($P < 0.01$) and decreases of 5 and 7% (24 or 72 h, respectively) using ethylene glycol. Results obtained after

TABLE 1
Parameters Reflecting *Chinchilla lanigera* Sperm Functional Activity Immediately after Ejaculation

	Mean ± SEM
Motility (% of motile cells)	94.9 ± 1.6 ($n = 11$)
Viability (% of living cells)	95.2 ± 0.9 ($n = 10$)
Hypoosmotic swelling test (% of swollen cells)	74.4 ± 7.2 ($n = 10$)
Viable acrosome intact (% of cells)	80.3 ± 7.2 ($n = 7$)
Dead acrosome intact (% of cells)	0.7 ± 0.5 ($n = 7$)
Viable acrosome reacted (% of cells)	7.8 ± 2.6 ($n = 7$)
Dead acrosome reacted (% of cells)	11.2 ± 5.4 ($n = 7$)

Note. The number of animals used is in parentheses.

cooling to 4°C for 24 h are illustrated in Fig. 1. As can be seen in Fig. 1A (0 h), after samples were diluted in Tyrode's medium and warmed to 37°C, the percentages of motile and viable cells were significantly higher in those samples cooled in medium containing ethylene glycol than in glycerol ($P < 0.01$). After 4 h incubation (Fig. 1B), the functional parameters studied here were again best preserved with ethylene glycol. From the comparison of the results displayed in Figs. 1A and 1B, it clearly can be seen that although motility and viability remained with no significant changes during the 37°C incubation time, the population of swollen gametes significantly diminished after 4 h incubation with ethylene glycol ($P < 0.05$).

Similar results were obtained when the gametes were cooled for 72 h (Fig. 2). At 0 h incubation, the percentages of motile, viable, or swollen spermatozoa were significantly higher when ethylene glycol was employed in the medium than when glycerol was used (Fig. 2A) ($P < 0.01$). After 4 h incubation (Fig. 2B), similar results were obtained, but the percentage of swollen gametes diminished in similar manners with both cryoprotectants ($P < 0.01$). Comparing Figs. 2A and 2B, the percentage of swollen spermatozoa from the ethylene glycol-cryopreserved samples was higher at 0 h incubation than at 4 h incubation ($P < 0.01$).

From the results shown in Table 2, it became evident that in samples warmed for a short time (24 h, 0 h incubation), ethylene glycol was able to maintain higher percentages of viable cells with intact acrosome than glycerol, whereas the percentage of dead spermatozoa with reacted acrosome diminished ($P < 0.01$). When acrosomal status of 72 h samples was analyzed, it became evident that at 0 h as well as at 4 h incubation, glycerol diminished the number of viable spermatozoa with intact acrosome and increased the number of dead sperm with reacted acrosome ($P < 0.01$).

The results previously stated clearly indicate that under our experimental conditions, and at all the evaluation times, ethylene glycol is a better cryoprotectant agent than glycerol. In this respect, Jeyendran *et al.* (4) noted that spermatozoa that have been cryopreserved with glycerol develop a "glycerol dependence" after thawing that reduces both motility and the ability of spermatozoa to become capacitated and fuse with oocytes (3). However, in our experimental model, we did not wash the semen sample to remove the CPA; thus, the detrimental effects of glycerol that we found could not be explained by this glycerol dependency, suggesting a detrimental effect of this agent per se. Accordingly, it is important to note that in our experiments, the storage of chinchilla semen without CPA resulted at 24 h in a significantly higher viability and percentage of intact acrosome sperm than glycerol storage (viability 91.3 ± 1.9, $n = 6$, and 73.1 ± 5.7, $n = 12$, respectively, $P < 0.05$; intact acrosome sperm 83.8 ± 5.2, $n = 6$, and 51.8 ± 8.6, $n = 11$, respectively, $P < 0.05$).

Finally, it is important to highlight that in the present work, no significant differences were found comparing the functional parameters of spermatozoa cooled for 24 h (0 h incubation) vs 72 h (0 h incubation), neither with glycerol nor with ethylene glycol, suggesting that at 4°C storage for up to 3 days did not have detrimental effects per se. These findings do not agree with those of other authors, who found a significant decrease in sperm motility during 48 h storage of cooled spermatozoa (5). These discrepancies could be due to differences in experimental conditions, to the cryoprotectant medium composition, or to species sperm characteristics (2).

In conclusion, the results described here indicate that ethylene glycol is a better protectant

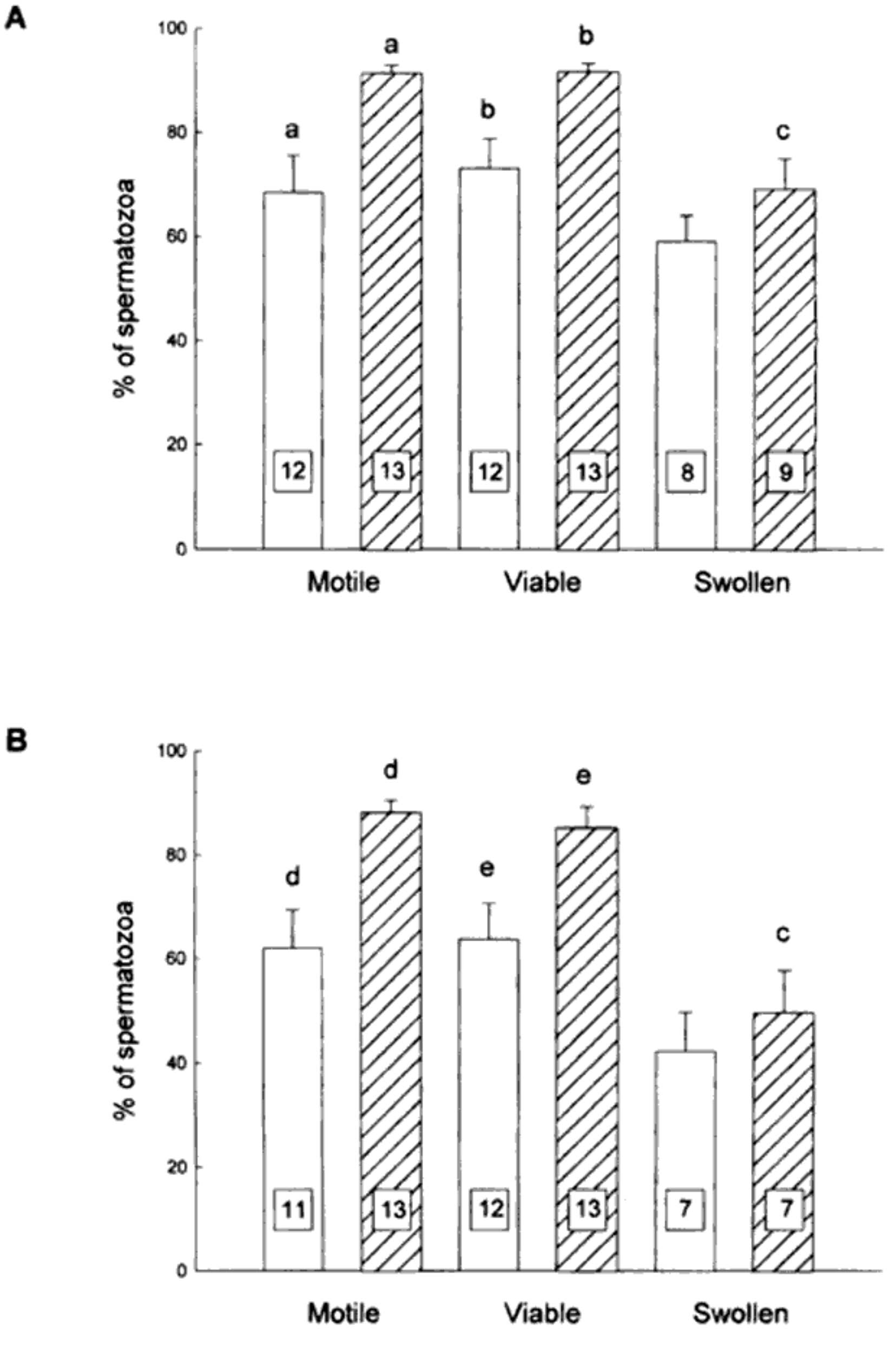
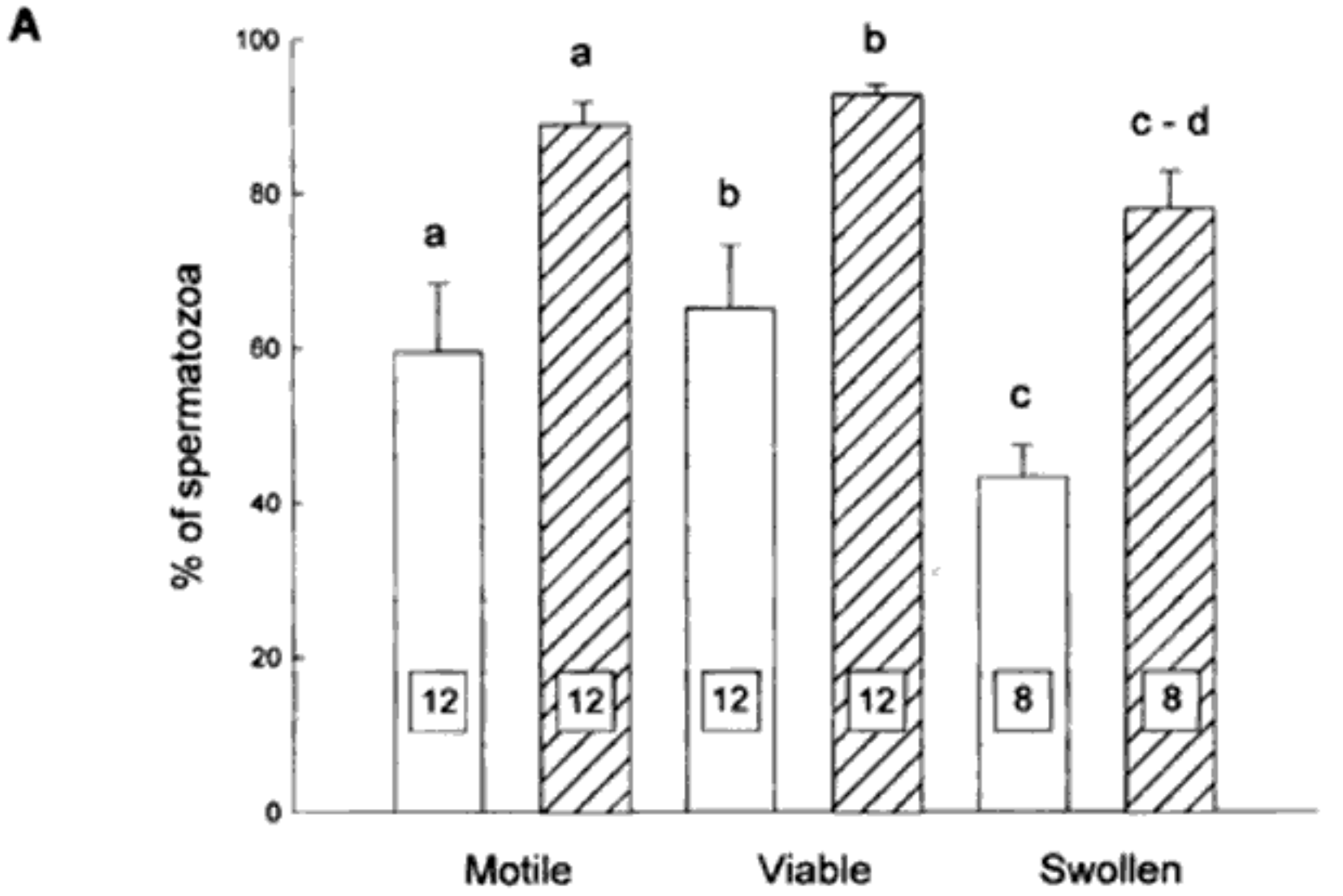


FIG. 1. Functional parameters of *C. lanigera* spermatozoa stored with TES–Tris egg yolk–glycerol (□) or TES–Tris egg yolk–ethylene glycol ▨ for 24 h at 4°C and incubated for 0 (A) or 4 h (B) at 37°C. Results are expressed as means ± SEM. Numbers of animals are indicated at the bottom of each column. Same letters indicate significant differences: a, b, d, and e, $P < 0.01$; c, $P < 0.05$.



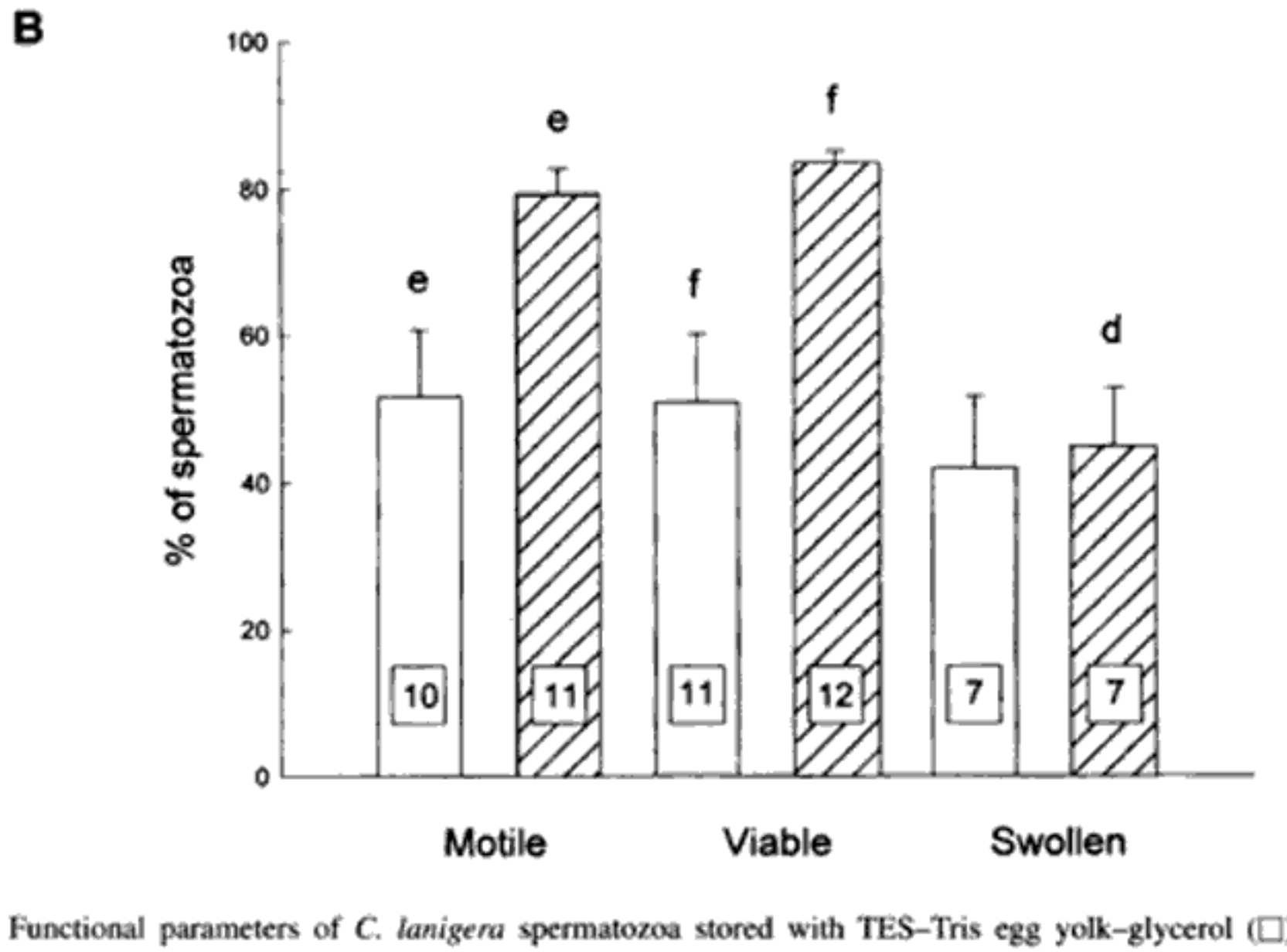


FIG. 2. Functional parameters of *C. lanigera* spermatozoa stored with TES–Tris egg yolk–glycerol (□) or TES–Tris egg yolk–ethylene glycol ▨ for 72 h at 4°C and incubated for 0 (A) or 4 h (B) at 37°C. Results are expressed as means ± SEM. Numbers of animals are indicated at the bottom of each column. Same letters indicate significant differences: a–f, $P < 0.01$.

TABLE 2
Acrosomal Status of *Chinchilla lanigera* Spermatozoa after 24 or 72 h Storage at 4°C with Glycerol or Ethylene Glycol and Incubated at 37°C for 0 or 4 h

	4°C storage time	37°C incubation time	Mean ± SEM (%)			
			Viable acrosome intact	Dead acrosome intact	Viable acrosome reacted	Dead acrosome reacted
Glycerol	24 h	0 h (n = 11)	51.8 ± 8.6[a]	3.3 ± 1.7	5.9 ± 2.2	38.9 ± 9.5[b]
		4 h (n = 7)	45.7 ± 11.7	1.7 ± 0.8	6.5 ± 2.5	45.5 ± 13.3
	72 h	0 h (n = 10)	52.1 ± 10.0[c]	2.1 ± 0.6	3.9 ± 1.1	42.0 ± 10.5[d]
		4 h (n = 11)	38.2 ± 9.5[e]	3.1 ± 1.0	4.2 ± 1.8	54.6 ± 10.0[f]
Ethylene glycol	24 h	0 h (n = 11)	82.9 ± 6.9[a]	1.8 ± 1.1	4.8 ± 1.6	10.5 ± 5.5[b]
		4 h (n = 6)	71.3 ± 5.4	1.4 ± 0.8	10.0 ± 3.5	17.4 ± 4.2
	72 h	0 h (n = 12)	81.9 ± 4.1[c]	0.9 ± 0.3	5.3 ± 1.2	11.8 ± 3.1[d]
		4 h (n = 10)	69.7 ± 7.1[e]	2.0 ± 0.8	9.8 ± 5.8	17.9 ± 3.8[f]

Note. C. lanigera spermatozoa were stored at 4°C with TES–Tris egg yolk–glycerol or TES–Tris egg yolk–ethylene glycol for 24 or 72 h and incubated at 37°C for 0 or 4 h. Results are expressed as means ± SEM. Numbers of animals are indicated in parentheses.

Same letters indicate significant differences, $P < 0.01$.

for sperm storage than glycerol, because it retains sperm functional activity without important changes over several days of storage. Furthermore, storing chinchilla spermatozoa at 4°C for short periods of time presents an interesting possibility that should be tested on spermatozoa from other species, because it is a useful, practical, and inexpensive method of short-term spermatozoa storage.

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