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Incubation of frozen-thawed ram spermatozoa*

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Ram spermatozoa are also susceptible to the stress of freezing and thawing. The viability after cryopreservation depends upon several interrelated factors (Fiser and Fairfull 1989, Pontbriand *et al.* 1989). However, their adverse effects are manifested on thawing (Holt *et al.* 1992). *In vitro* survival of frozen thawed spermatozoa at 37°C has been used as a critarion for evaluating the efficiency of cryopreservation. This study was undertaken to observe the viability of frozen thawed ram spermatozoa at 37°C.

Semen ejaculates (10) were obtained from 10 adult Rambouillet rams maintained under standard management conditions at Central Sheep and Wool Research Institute farm at Avikanagar (Rajasthan). After initial evaluation of semen quality these 10 samples of all rams were pooled and were extended in egg yolk tris glucose (EYTG) extender @ 1000×10^6 spermatozoa/ml. The extended semen was filled into 0.25 ml straws and were frozen in a programmable cell freezer @–25°C/min up to–125°C and then stored in liquid nitrogen for a fortnight. Thawing was done at 50°C for 10 sec. The thawed samples were subjected to incubation at 37°C for 6 h. Percentages of progressively motile and live spermatozoa were recorded at hourly intervals. Assessment of acrosomal integrity was done using Giemsa stain technique (Watson and Martin 1972). Absolute index of post-thaw sperm survival at 37°C was constructed using formula of Milovanof (1962); $\Sigma(T\times R)$, where Ia, absolute index of survival; T, time interval between motility evaluation, and R, average of every 2 consecutive motility evaluation. Data were analysed for analysis of variance (Snedecor and Cochran 1967). Evaluation of significant mean difference was done by the least significant difference method from the error term based $P<0.05$ or $P<0.01$ (Steel and Torrie 1960).

The mean per cent post-thaw motility before incubation (0h) declined significantly to 17% after 6h of incubation (Table 1). A progressive decline in the per cent motility and acrosome integrity at hourly intervals of incubation up to 4h at 37°C of frozen thawed ram semen was also reported by Joshi *et al.* (2002), where a significant ($P<0.05$) effect on motility was observed after 1 h of incubation. It was reported that higher survival of ram spermatozoa after 6 h of incubation was obtained after freezing in hypertonic diluents

Table 1. Effect of post-thaw incubation interval on progressive motility, live spermatozoa, abnormal spermatozoa and acrosomal integrity of ram semen (mean±SE, n=10)

Post–thaw incubation interval (h)	Semen characteristics			
	Progressive motility (%)	Live spermatozoa (%)	Abnormal spermatozoa (%)	Intact acrosomes (%)
0	$60.0^{a}\pm0.75$	$65.2^{a}\pm1.04$	4.7±1.08	$57.5^{a}\pm1.27$
1	$51.0^{b}\pm1.50$	$57.9^{ab}\pm1.46$	5.4±1.05	$55.3^{a}\pm2.10$
2	$44.0^{c}\pm1.94$	$51.0^{bc}\pm1.96$	6.1±0.31	$54.2^{ab}\pm1.11$
3	$38.5^{d}\pm1.98$	$44.3^{cd}\pm1.88$	6.8±1.22	$53.1^{b}\pm1.95$
4	$32.5^{e}\pm2.14$	$38.2^{de}\pm2.45$	6.5±1.68	$52.6^{b}\pm2.24$
5	$24.5^{f}\pm1.57$	$32.8^{ef}\pm2.28$	7.8±1.56	$50.7^{bc}\pm1.05$
6	$17.0^{g}\pm1.10$	$24.6^{f}\pm1.88$	8.4±2.17	$48.5^{c}\pm1.07$

Means within a column with different superscripts differ significantly ($P<0.01$) (Lsd, for; progressive motility, 3.77; live spermatozoa, 11.50; intact acrosomes, 3.50).

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containing sugar of high molecular weight (Salamon and Lightfoot 1969). Similarly ram semen frozen in BF_2F (Belts ville, Fs) and TEST extender having post-thaw motility of 49.0 and 45.0%, when subjected to incubation at 37°C, resulted into per cent motility of 46.0, 40.0 and 41.9, 34.4 after 3 and 6 h, respectively (Pontbriand *et al.* 1989). In the present investigation the absolute index of sperm survival was 229.5±5.21. In the only other study Ali *et al.* (1994) reported absolute index of liability in post-thaw incubated ram semen from 177.9 to 250.7 in semen samples frozen in tris citric acid fructose diluent containing varying levels of egg yolk and glycerol (5–10% and 5–7.5%, respectively). These results indicate that composition of extender and its osmolarity may have profound effect on post-thaw survival and absolute index of sperm survival, which may have a positive correlation with fertility.

Mean percentage of live spermatozoa declined from 65.2 at 0 h to 24.6% at 6 h of incubation, but the differences between any 2 successive hours were not significant. Interestingly, the percentage of abnormal spermatozoa was not affected by hours of incubation. Jones and Martin (1965) reported the drop in the percentage of live spermatozoa after incubation at 37°C in various extenders. These results again indicate that composition of extenders may have an important effect on post-thaw survival of ram spermatozoa. In this study, the acrosomal integrity declined significantly ($P<0.01$) with an increase in the post-thaw incubation interval from 0 to 6h. This is in agreement with earlier reports indicating substantial deterioration in acrosomal integrity of frozen-thawed bull and ram spermatozoa after prolonged incubation (Saacke and White 1972, Fiser *et al.* 1991). Our earlier study also revealed that incubation of frozen-thawed ram semen at 37°C for 4h resulted into a substantial decline in the proportion of spermatozoa with intact acrosome from 64.2 to 53.5% (Mathur and Joshi 1994).

It is established that thawing of frozen-ram semen results in a marked alteration in membrane structure (Quinn *et al.* 1969, Nath 1972) with major damage characterized by loosening of acrosomal cap (Pontbriand *et al.* 1989). It has been shown that after thawing membrane integrity is drastically reduced whereas effect on motility is not that evident and large population of membrane damaged spermatozoa which were motile were present in thawed semen (Valcarcel *et al.* 1994). Such spermatozoa are expected to lose their motility readily within few hours of incubation at 37°C. Hence, it was suggested that membrane damage, rather than incubation condition is the primary cause of low survival rate of frozen thawed spermatozoa at 37°C. Although membrane damage is especially manifested after thawing however, its severity and nature is governed by conditions of cooling and freezing (Holt and North 1994).

In view of these considerations it is reasonable to suggest that frozen ram semen should be used for AI immediately after thawing to avoid deterioration in semen quality with consequent loss of fertility. Since, membrane integrity plays a crucial role in fertility, cryopreservation protocols need to be developed towards improving membrane integrity at freeze-thawing and incubation, because duration of motility after thawing gives an indication of viability of frozen-thawed semen.

SUMMARY

Semen ejaculates were obtained from Rambouillet rams and after initial evaluation of semen quality these samples were cryopreserved. The thawed samples were subjected to incubation at 37° C for 6 h. Spermatozoal motility, per cent live spermatozoa and acrosomal damage were recorded at hourly interval. A decline in the per cent post-thaw motility with increase in the incubation time was observed and the differences among all the hours were highly significant. There was a progressive fall in the percentage of live spermatozoa at every hour of incubation, however decline in the percentage of live spermatozoa between successive hours was not significant. The mean values for abnormal spermatozoa were not significantly affected by incubation hours, but the percentage of intact acrosome declined with an increase in the post-thaw incubation interval and this decline was significant between 1h and 3, 4, 5 and 6 h of incubation. The overall deterioration in the semen quality was comparatively less marked up to 1 h of post-thaw incubation while beyond this time deterioration was very sharp.

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