MOTILITY AND ACROSOMAL INTEGRITY OF FROZEN RABBIT SPERMATOZOA AS AFFECTED BY DIFFERENT EXTENDERS, CRYOPROTECTANTS AND PACKAGING METHODS

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ABSTRACT

In a first trial semen from twenty NZW rabbit bucks was used to study the effects of different extenders (Tris–yolk fructose, lactose–yolk citrate and sucrose–yolk citrate), type and level of cryoprotectant (glycerol or dimethylsulfoxide) on motility and acrosome integrity percentages of frozen rabbit spermatozoa. In a second trial the effect of packaging method (straws or pellets) of frozen rabbit semen on sperm motility and acrosome integrity was also studied. The results showed that sucrose–yolk citrate and tris–yolk fructose extenders were significantly ($P<0.01$) better than lactose–yolk citrate extender in maintaining high motility and acrosomal integrity. The 2% glycerol or 4% dimethylsulfoxide levels gave significantly ($P<0.01$) higher motility and acrosomal integrity for frozen rabbit bucks spermatozoa. Post–thaw motility and acrosomal integrity were significantly better when semen of rabbit bucks was packaged in straws rather than in pellets.

Key words: Spermatozoa, Freezing, Cryoprotectants, Packaging.

INTRODUCTION

Artificial insemination offers one of the most effective means for large distribution of good hereditary males over a large number of females. Applying this technique in rabbit production field is now widely used in developed countries. In less developed countries, applying this technique is still practiced on a very limited scale of rabbit farms. One of the main constraints is the processing and conservation of rabbit semen for prolonged periods, while still preserving acceptable fertilizing capacity. Different aspects of rabbit buck spermatozoa cryopreservation have been studied (Miller *et al.*, 1969; Hanada and Nagas, 1980; Fisher and Fairfull, 1984; Chen *et al.*, 1989a; El-Gaafary, 1990; Awad *et al.*, 2000). However, it seemed that previous researches, especially in less developed countries where a simplified and low cost technique is needed, did not fully cover all aspects of rabbit artificial insemination. The present work aimed to study the effects of different extenders (tris yolk fructose, lactose yolk citrate and sucrose yolk citrate), cryoprotectants (glycerol and DMSO) and packaging methods (straws and pellets) on post freezing motility and acrosomal integrity of rabbit buck spermatozoa.

MATERIALS AND METHODS

Trial 1

Semen was collected twice a week from each of twenty NZW rabbit bucks for six weeks by means of an artificial vagina. Collected semen was immediately evaluated and only semen samples with no less than seventy percentage progressive motility were used. Semen was then pooled and was divided into three equal portions. Each portion was initially extended with one of three extenders; tris yolk fructose (TYF), lactose yolk citrate (LYC) or sucrose yolk citrate (SYC). TYF is composed of citric acid anhydrous 1.675, fructose 1.250 and tris aminomethane 3.028 g per 100 ml distilled water; LYC is

composed of sodium citrate dihydrate 2.90, citric acid anhydrous 0.04, lactose 1.25 g per 100 ml distilled water while SYC is composed of sodium citrate dehydrate 2.90, citric acid anhydrous 0.04 and sucrose 1.25 g per 100 ml distilled water. Egg yolk was added as 20% to all extenders. Level of glycerol or DMSO varied in different trials. 500 IU/ml penicillin + 500 microgram /ml streptomycin were added to each extender.

Extension of semen was carried out in two steps; in the first step each portion of semen was extended with one from the three extenders free of glycerol or DMSO to half the final extension rate (one semen to four extender). Each extended portion was subdivided into six portions three of them were extended finally with the same volume from the respective extender (B) containing twice the required level from glycerol or DMSO each with three final levels; 2, 4 or 6%.

Extended semen was packaged in 0.25 ml straws and then left at 5°C for two hours as an equilibration period. Straws were then exposed to liquid nitrogen vapor at 4 cm distance above liquid nitrogen level for ten minutes at about -90 to -100°C. Straws were then dipped in liquid nitrogen (-196°C). After 24 hours straws were removed from liquid nitrogen container and were thawed by dipping them in water bath at 37°C for 30 seconds. Thawed samples were then examined for progressive motility (%) PTM) and acrosome integrity expressed as percent of spermatozoa with abnormal acrosome as described by Watson and Martin (1975).

Trial 2

Semen was extended in SYC with 4% DMSO which gave the best results in trial one using the same procedures except that extended semen was packaged in straws and pellets form. Pelleting was carried out using 0.25 ml graves in paraffin wax blocks. The paraffin wax blocks containing semen samples were maintained at liquid nitrogen vapor for ten minutes and then were dipped in liquid nitrogen container for 24 hours. Semen samples packaged in straws were frozen and thawed as the same method described in trial one. Frozen semen in pellets was thawed by dipping pellets in test tubes containing suitable volumes of sodium citrate (2.9%) as a thawing solution to obtain similar extension rate as straws. The samples were kept in water bath at 37°C for 30 seconds. The thawed semen samples were soon examined for progressive motility and acrosomal integrity percentages (zero time of incubation) and after one and two hours of incubation.

Statistical analysis of data was carried out using Least Squares Analysis of variance according to Snedecor and Cochran (1982). The significant differences between means were evaluated by Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

The results of trial one (Table 1) showed that type of extender and cryoprotectant agent type and level and their interactions had highly significant effect on PTM of buck spermatozoa. The highest ($P<0.01$) values of PTM were obtained when glycerol and DMSO were used at 2 and 4% levels, respectively. The other levels of glycerol or DMSO gave unsatisfactory results. It is worth noticing that this trend was found irrespective of the extender used. Statistical analysis of data confirmed this since extender, cryoprotectant type and level interactions did not reach a significant level. The significant interaction was shown between both protectant type and level.

Acrosomal integrity (%) as expressed by percentage of spermatozoa with abnormal acrosome was studied also under the influence of type of extender and cryoprotectant type and level (Table 1). The results showed that significantly ($P<0.01$) the least acrosomal abnormality (%) values were noticed when SYC and TYC were used in comparison with LYC which showed the highest ($P<0.01$) acrosomal abnormality values. In a trend similar to that was shown in PTM results, the incidence of spermatozoa with normal acrosome increase significantly ($P<0.01$) when 2% glycerol and 4% DMSO were implemented to the extenders. Significant ($P<0.01$) extender, cryoprotectant type and levels

interaction effects on acrosome integrity was noticed. Results of present study demonstrated that SYC and TYC were better (P<0.01) in maintaining PTM and acrosome integrity of frozen rabbit spermatozoa than LYC. Several authors supported these findings in that TYC and SYC are most compatible for rabbit spermatozoa if freezing is applied (Eschlorn, 1985; Strauss *et al*., 1986; Dee Leeaw *et al*., 1993; El-Gaafary *et al*., 1993; Viudes-de Castro and Vicente, 1996; Awad *et al*., 2000). It has been mentioned in some reports (Strauss *et al*., 1986) that sucrose is the most protective disaccharide to prevent freeze – thaw bilayer destabilization. Similarly sucrose has the capacity to act as non penetrating cryoprotective agent by direct interaction with cell membrane.

Table 1: Post thaw motility (%) and abnormal acrosomes (%) of frozen buck spermatozoa as affected with different extenders and cryoprotectants

Extenders	Cryprotectants					
	Glycerol (%)			DMSO (%)		
	2	4	6	2	4	6
	Post thaw motility (%)					
Tris yolk fructose	33.40±^{a}1.67	26.60±^{b}1.20	26.80±^{b}1.46	35.20±^{a}2.84	40.20±^{b}2.40	28.40±^{c}2.17
Lactose yolk fructose	30.20±^{a}1.75	25.10±^{b}2.13	21.80±^{c}2.10	30.20±^{a}1.75	36.10±^{b}1.38	25.20±^{c}1.75
Sucrose yolk citrate	38.50±^{a}1.64	30.10±^{b}2.11	25.20±^{c}1.82	38.70±^{a}1.80	43.80±^{b}1.35	30.80±^{c}3.80
	Abnormal acrosome (%)					
Tris yolk fructose	20.10±^{a}0.84	24.20±^{b}1.26	26.20±^{c}0.88	19.80±^{a}1.23	17.40±^{b}1.21	21.20±^{a}1.64
Lactose yolk citrate	20.70±^{a}1.12	24.60±^{b}0.93	27.30±^{c}1.30	19.80±^{a}1.27	18.27±^{a}0.82	22.70±^{b}0.99
Sucrose yolk citrate	14.70±^{a}1.23	21.70±^{b}0.82	23.60±^{c}1.40	19.82±^{b}1.920	16.20±^{b}1.15	20.50±^{a}1.40

^{a,b,c}Means within the same raw in the same classification bearing different superscripts differ significantly (P<0.01)

Glycerol and DMSO results as cryoprotectants for rabbit spermatozoa are confirmed also by previous reports (Fisher and Fairfull, 1984; El-Gaafary *et al*., 1993; Viudes-de-Castro and Vicente, 1996; Amer, 1999). Unlike other mammals, high levels of glycerol during freezing of rabbit bucks spermatozoa were found to have unfavorable effects on PTM or acrosome integrity. These findings may be due the toxic effect of high level of glycerol that reduce cryosurvival rabbit spermatozoa during freezing process (Unal *et al*., 1978). It is of interest to notice also from these results that using DMSO during freezing rabbit spermatozoa generally gave better results than glycerol, a fact which was previously proved (Chen *et al*., 1989; Castellini *et al*., 1992; El-Gaafary, 1993).

The results of trial 2 (Table 2) showed that PTM and acrosomal integrity of rabbit spermatozoa are significantly (P<0.01) affected by the two packaging methods (straws or pellets). Higher PTM and lower abnormal acrosomes values were recorded when rabbit buck semen was packaged and frozen in straws than in pellets. These results agreed with El-Gaafary (1993) and Awad (2000) and might be attributed to that straws act as a coat around the extended semen causing gradual cooling and freezing while pellets have direct contact to the surrounding cold media.

Although this is the condition for packaging rabbit buck spermatozoa in pellet form, the obtained PTM and acrosome integrity results are still in the acceptable range and acceptable fertilizing ability could be obtained from rabbit spermatozoa if frozen and packaged in pellets.

Semen frozen in both straws or pellets if incubated at 37°C a showed a significant increase in PTM after one hour incubation and then a decrease after two hours at a significant level. This phenomenon led us to think that optimum fertilizing ability for spermatozoa might be within one hour at natural mating.

Concerning changes in percent of abnormal acrosomes during two hours incubation at 37°C, it increased from zero to two hours but the increase was sharp from one to two hours. Therefore, for none understandable reasons both PTM and abnormal acrosomes percentages differ from each other in respect with changes pattern during two hours incubations of semen after freezing and thawing.

Table 2: Post thaw motility (%) and abnormal acrosomes (%) of rabbit buck spermatozoa as affected by packaging methods during incubation at 37°C for 2 hours

Incubation time (hrs)	Packaging methods	
	Straws	Pellets
	Post thaw motility (%)	
0	37.90 ± 2.90^{a}	32.42 ± 2.68^{a}
1	40.10 ± 5.72^{b}	35.22 ± 5.41^{b}
2	28.42 ± 4.39^{c}	24.37 ± 4.19^{a}
Mean	33.5^{A}	30.69^{B}
	Abnormal acrosome (%)	
0	19.03 ± 0.53^{a}	21.42 ± 0.89^{a}
1	21.45 ± 1.33^{b}	24.43 ± 1.57^{b}
2	28.20 ± 1.83^{c}	29.55 ± 1.99^{a}
Mean	22.89^{A}	25.13^{B}

a, b, c: Means within the same column (a, b, c) or raw (A, B, C) within the same classification bearing different superscripts differ significantly (P<0.01)

CONCLUSIONS

In conclusion rabbit buck semen could be successfully extended in sucrose yolk citrate or tris yolk fructose with 2% glycerol or 4% DMSO as cryoprotectants. Packaging frozen rabbit buck spermatozoa in straws, although was better than pellets, the later form for packaging gave acceptable PTM results and may be considered more cheap and simplified technique for developing countries.

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