EFFECT OF TYPE OF FREEZING ON THE FUNCTIONAL CHARACTERISTICS OF FROZEN THAWED BULL SPERMATOZOA

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ABSTRACT

Programmable biological freezers can be used to control and optimize the freezing rate for successful cryopreservation of bull spermatozoa. The effect of freezing methods on the functional characteristics of frozen thawed bull spermatozoa was studied. Semen samples collected from Jersey cross (n=6) and Friesian cross (n=6) bulls were extended in Tris egg citric acid diluent. The samples were split and cryopreserved using static vapor freezing and programmed freezing. In both the methods of cryopreservation the post-thaw semen showed a highly significant (P<0.01) reduction in the functional characteristics such as progressive motility, acrosome status and membrane integrity. The post-thaw motility and percentage of intact acrosome were more in programmed freezing (P<0.01) than the static vapor freezing. A non-significant increase in hypo-osmotic swelling response and percentage of live spermatozoa with intact acrosome were obtained in the programmed freezing. The release of enzyme Aspartate transaminase into the seminal plasma was lower in the programmed freezing than in the static vapor freezing. The hypo-osmotic swelling test was relatively easy to perform and was simple, economical and efficacious in assessing the membrane integrity of bovine spermatozoa. The supravital triple staining technique was useful in simultaneous assessment of both membrane integrity and acrosome status of bull spermatozoa. It is evident from the present investigation that the programmed freezing results in enhanced post-thaw motility, acrosome retention and membrane integrity and might be adopted in frozen semen production.

Key word: Bull, Cryopreservation, Programmed freezing, Static vapour freezing.

INTRODUCTION

Post-thaw survival of cryopreserved bull spermatozoa is restricted to about 50 per cent of the sperm population. The reason assigned to the poor post-thaw survival is that there is no control over the freezing rate apart from setting the initial vapor temperature in the current cryopreservation procedures. With the advent of programmable biological freezers, it is possible to have control over freezing rates leading to higher post-thaw survivability of spermatozoa. Spermatozoal motility and acrosomal integrity are quality characteristics essential to normal fertility of cryopreserved semen after freezing and thawing. There is no satisfactory method to indicate the extent of damage that occurs to spermatozoa during freezing process. The biochemical tests of seminal plasma for leakage of enzymes after freezing and thawing could however be used as a marker for the sperm cell damage (Dhami *et al.* 1987). Hypo-osmotic swelling test has been evolved as a simple and economical procedure for assessment of cryosurvival (Jeyendran *et al.* 1992). Recent advances in staining technology have provided new means for assessing the functional capacity of sperms. Much emphasis has been directed towards using combination of stains to determine the sperm

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viability (Garner and Johnson, 1995). The present investigation was undertaken to compare the conventional method of cryopreservation using static vapor phase freezing and a programmed freezing using a biological freezer on crossbred bull spermatozoa and to assess the effect of cryopreservation on the acrosome as well as on the membrane integrity of bull spermatozoa

MATERIALS AND METHODS

Experimental animals and semen collection

Five semen samples (two ejaculates per collection) were collected from twelve sexually mature and healthy crossbred bulls [6 Jersey x Sindhi cross bulls and 6 Holstein-Friesian crosses (2 HF x Sahiwal, 2 HF x Sindhi, 1 HF x Gir and 1 HF x Tharparkar)] stationed at Nucleus Jersey and Stud Farm, Udhagamandalam, Tamil Nadu, India by artificial vagina (AV) using standard protocols. A total of 120 semen samples were analyzed.

Evaluation and extension of semen.

The ejaculates were examined for volume (ml), initial motility (% of motile spermatozoa) and concentration (millions per ml). The semen samples were extended using Tris-egg yolk-citric acid diluent (Mathew, 1984). The extended semen was further diluted with one step addition of 5 per cent glycerol at 5°C. The two ejaculates from each bull were pooled after the addition of glycerol. An equilibration period of 4 hours was allowed at the same temperature. The extended semen samples were filled into pre-labeled 0.25ml French mini straws and sealed using an automatic filling and sealing machine (Mini Tub, Germany). Dilution rate was fixed so that each dose had at least 25 million sperms. At the end of the equilibration period, the straws were cryopreserved using either of the freezing techniques.

Static vapour phase freezing

One set of straws from each bull were arranged in racks and placed inside a LR-440 container (MVE, Ohio, USA), by fixing the freezing rack at 4 cm from the level of the liquid nitrogen for 4 minutes. Immediately after freezing the semen straws were collected in goblets with liquid nitrogen and stored at -196°C.

Programmed freezing

The second set of the straws were frozen using a programmable bio-freezer DIGIT COOL 5300 (IMV, France). The program for the freezing rate was set as 4 hours of equilibration at +25°C to +4°C, followed by a freezing rate of 5°C / min to reach -12°C from +4°C and 6°C / min to reach -140°C. At the end of the freezing cycle the straws were collected into goblets and directly plunged into liquid nitrogen.

Pre and post-thaw evaluation of spermatozoa

The pre- and post-freeze evaluation of spermatozoa for motility, membrane integrity and acrosome status was performed during equilibration and after 20 days. The motility was assessed based on the percentage of progressively motile spermatozoa. Acrosome staining technique of Hancock (1952) using Giemsa stain was adopted to determine the percentage of intact acrosome before and after freezing. Supra vital triple staining was performed as described by Vazquez *et al.* (1992). The spermatozoa were classified as live spermatozoa with intact acrosome, live spermatozoa without acrosome, dead spermatozoa with intact acrosome and dead spermatozoa without acrosome based on the staining patterns. The plasma membrane integrity was assessed using Hypo-osmotic Swelling Test (Jeyendran *et al.* 1984) to determine the percentage of spermatozoa showing the hypo-osmotic swelling reaction before and after freezing. The concentration of enzyme Aspartate Transaminase (AST) released into the seminal plasma was estimated by colorimetric method as per Reitman and Frankel (1957) using Systronics® Colorimeter. The data collected were statistically analyzed using a computer programme Microstat®.

RESULTS AND DISCUSSION

Progressive Motility

The data on initial, pre-freeze and post-thaw motility (Mean ± SE) is given in Table 1. In the present study the mean initial motility (63.33 ± 0.53 per cent)

recorded in Jersey cross bulls was higher than the Friesian cross bulls (62.58 ± 0.53 per cent) and the difference was not significant. The results concurred with Tuli *et al.* (1988). However slightly higher values were obtained by Belorkar *et al.* (1993). Lower values were obtained by Sagdeo *et al.* (1990). The variation in initial motility could be due to the different agro climatic conditions under which the animals were kept and variation in seminal plasma composition (Ganguli, 1978).

A highly significant variation (P<0.01) between pre-freeze and post-thaw motility was recorded in both static vapour freezing and programmed freezing. The results concurred with Belorkar *et al.* (1993) who recorded 54.27 per cent post-thaw motility. However, Tuli *et al.* (1988) reported low post-thaw motility. This might be due to the lower initial motility recorded in their studies. In the present study the post-thaw motility was significantly higher in programmed freezing (60.58±0.43 per cent) than in the static vapour freezing (57.17±0.54). This is in agreement with the findings of Parkinson and Whitfield (1987), which compared the post-thaw motility between the two freezing methods in bull semen. Allen and Almquist (1981) recorded no difference in post-thaw motility between the two freezing methods, static vapor freezing and programmed freezing in a biological freezer. The difference in the post-thaw motility obtained in this study and others might be due to the difference in freezing rate adopted and due to the difference observed in the initial motility.

A better post-thaw motility in this study with the programmed freezing might have been due to reasons such as optimum cryopreservation cycle (Watson, 1975), greater control of freezing rate (Landa and Almquist, 1979) and the effect and level of cryoprotectant used (Kampschmidt *et al.* 1953). It can also be attributed to the rapid passage of the spermatozoa through the freezing point, which allows the production of large numbers of ice crystal seeds at a high temperature, thereby allowing minimum damage from large ice crystals to the cells but with control of the rate subsequent to freezing point. Therefore, the damage caused by the failure of dehydration seen in rapid, uncontrolled freezing is prevented and osmotic dehydration of the cells can take place at an optimal rate subsequent to the induction of ice crystallization (Parkinson and Whitfield, 1987).

Acrosome status

In this study, the Giemsa stain was used to differentiate sperms with intact acrosome, detached acrosome and lost acrosome. The pre-freeze and post-thaw acrosome status (Mean ± SE) during the equilibration period is presented in Table 2. The mean pre-freeze per cent of spermatozoa with intact acrosome obtained in this study was similar to the observation of Sharma *et al.* (1990) in cross bred bulls. The values were lower than those reported by Saacke *et al.* (1968). The process of cryopreservation had a highly significant (P<0.01) effect on the acrosome integrity of frozen thawed spermatozoa. The results were comparable with the findings of Watson (1975) who reported a highly significant reduction in per cent of intact acrosome (P<0.01). He reasoned that the freezing to -196°C was more destructive than freezing to -79°C (P<0.01. In the present study, the percentage of intact acrosome was significantly higher (P<0.01) in programmed freezing than the static vapour freezing. The results differed with observations of Gilbert and Almquist (1978) who did not find any significant difference in percentage of intact acrosome between the freezing rates. However, the results are in agreement with Landa and Almquist (1979) who reported an increase in acrosome retention when the freezing rate was optimum in programmable freezer. Almquist and Wiggin (1973) observed that wide variation in cooling occurred among successive freezes in the static vapour freezing.

Supra vital triple staining technique

The supra vital triple staining technique distingui shed four types of spermatozoa viz. Live spermatozoa with intact acrosome [pink acrosome with light brown post-acrosome region], live spermatozoa without acrosome [unstained acrosome with light brown post-acrosome region], dead spermatozoa with intact acrosome [pink acrosome with blue or dark brown post-acrosome region] and dead spermatozoa without

acrosome [unstained acrosome with light brown post-acrosome region].

The mean percentage (± SE) of live spermatozoa with intact acrosome in the equilibrated semen and post-thaw semen is presented in Table 1. There was highly significant difference in the percentage of live spermatozoa with intact acrosome between the pre-freeze and post-thaw semen (P<0.01). The percentage of live spermatozoa with intact acrosome was reduced in the post-thaw semen when compared to the pre-freeze semen and the reduction was highly significant (P<0.01). The difference between the breeds were also highly significant (P<0.01). The reduction in the percentage of live spermatozoa with intact acrosome is due to the cryopreservation process that causes damage to the plasma membrane as well as the acrosome (Landa and Almquist, 1979). The programmed freezing resulted in higher percentage of live spermatozoa with intact acrosome than in the static vapour freezing but the difference was not statistically significant (P>0.05). Vazquez *et al.* (1992) were of the opinion that the supra vital triple staining technique was an efficacious method for simultaneous assessment of sperm vitality and acrosome status. The present study also supports this conclusion.

Membrane integrity

The comparison between pre-freeze membrane integrity of equilibrated and post-thaw semen (Mean ± SE) using the hypo-osmotic swelling test is presented in Table 1. The mean pre-freeze hypo-osmotic swelling response obtained was similar to the observations of Sivaramalingam (1994). Higher values (89 per cent) were recorded by Fuse *et al.* (1993) in neat semen. This difference may be due to the effect of pre-freeze cooling to +5°C on the spermatozoa in the present study. Cell damage during freezing is usually ascribed to membrane rupture caused by the formation of intracellular ice crystals during rapid cooling or by osmotic effects or mechanical force due to intra cellular ice during slow cooling (Mazur, 1984). Saacke and Almquist (1961) suggested that during deep freezing the cytoplasmic membrane would be disrupted leading to loss of cellular solute and this fact might account for the inability of sperm to curl because damaged membrane would be unable to support osmotic swelling. It is plausible therefore; the hypo-osmotic swelling response could have predictive value for selecting cryopreserved semen samples for insemination.

Aspartate transaminase

Comparison between the effects of freezing on the loss of extra cellular enzyme Aspartate transaminase into the seminal plasma (Mean ± S.E) is presented in Table 1. The release of extracellular enzyme Aspartate transaminase into the seminal plasma was high in the post-thaw semen than in the pre-freeze semen (P<0.01). There was a highly significant (P<0.01) difference between the two freeing methods, where the release of enzyme Aspartate transaminase lower in programmed freezing using the biological freezer when compared to the static vapour freezing using liquid nitrogen.

The post-thaw motility was better in the programmed freezing method than in the static vapour freezing method. The programmed freezing in the biological freezer gives an increased percentage of spermatozoa with an intact acrosome while the membrane integrity was not compromised when compared to the static vapour freezing. The loss of Aspartate transaminase was also less in the programmed freezing when compared to the static vapuor freezing. Based on these observations, it is concluded that the programmed freezing in a biological freezer gives between sperm survivability than the static vapour freezing.

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Table 1: Comparison of effects of freezing (Mean ± SD) between static vapour freezing (SVF) and programmed freezing (PGF) on the functional characteristics of frozen thawed spermatozoa in Jersey cross and Friesian cross bulls. Means bearing same superscript do not differ significantly; * * Highly Significant (P<0.01), * Significant (P<0.05)

Parameter	Freezing Method	Bulls	Crossbred Bulls		F Statistics
			Jersey	Friesian	
Motility (%)	Prefreeze	62.96 ± 0.37	63.33 ± 0.53	65.58 ± 0.53	55.78**
	SVF	57.17 ± 0.54	56.83 ± 0.74	57.50 ± 0.79	
	PGF	60.58 ± 0.43	60.00 ± 0.59	61.17 ± 0.62	
Intact acrosome (%)	Prefreeze	71.78 ± 1.11	71.29 ± 1.68	72.30 ± 1.47	117.901**
	SVF	56.70 ± 0.79	54.87 ± 1.13	58.27 ± 0.99	
	PGF	59.36 ± 0.90	56.67 ± 1.34	62.06 ± 0.89	
Live spermatozoa with intact acrosome (%)	Prefreeze	56.81 ± 1.34	57.68 ± 1.93	55.94 ± 1.88	32.883**
	SVF	50.23 ± 1.22	50.86 ± 1.48	49.61 ± 1.71	
	PGF	51.11 ± 1.23	51.67 ± 1.54	50.54 ± 1.46	
Spermatozoa with intact plasma membrane (%)	Prefreeze	66.48 ± 0.66	66.45 ± 1.03	66.50 ± 0.86	39.090**
	SVF	58.01 ± 0.69	58.20 ± 1.07	57.81± 0.88	
	PGF	59.58 ± 0.66	60.02 ± 1.00	59.14 ± 0.87	
Aspartate transaminase (mmole/L)	Prefreeze	13.10 ± 4.70	12.92 ± 4.33	13.28 ± 5.12	145.520**
	SVF	39.32 ± 4.75	37.64 ± 7.69	41.00 ± 4.30	
	PGF	27.27 ± 7.73	23.92 ± 8.82	30.63 ± 4.54	

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