The Indian Journal of Animal Reproduction; 27 (2): 69-72; Dec. 2006

Research Article

Effect of stage of glycerolization on viability and acrosomal integrity of buffalo spermatozoa during cryopreservation

PAWAN SINGH[1], SAJJAN SINGH[2] AND INDERJEET SINGH[3]

Division of Physiology and Reproduction
Central Institute for Research on Buffaloes, Hisar - 125 001

Received : October 3, 2005
Accepted : November 4, 2006

ABSTRACT

This study was planned in order to evaluate the effects, if any, of the stage of glycerol addition on survival and acrosomal integrity of buffalo sperm cells undergoing cryopreservation. Two equal parts of tris-yolk extender were prepared, part 1 without and part 2 with 12.8% glycerol. Fifteen ejaculates, obtained from five breeding buffalo bulls using artificial vagina, were half diluted with part-1 extender immediately after collection. This half-diluted semen was then divided into two aliquots, to which part-2 extender (with glycerol) was added either at room temperature (Group 1) or after cooling to 5°C (Group 2) to give final glycerol concentration of 6.4%. The proportion of spermatozoa exhibiting forward and backward motility, live sperm count and percentages of spermatozoa with intact acrosome, were recorded. Significantly higher percentages of spermatozoa were recorded moving backwards, before freezing and post-thaw, when glycerol was added at 5°C, as compared to at room temperature, but no significant effect of stage of glycerolization was found on live sperm count and acrosomal integrity. It is concluded that addition of glycerol to buffalo semen either at room temperature or at 5°C during cryopreservation does not affect sperm survival rate and acrosomal integrity, in spite of altering the motility pattern of spermatozoa.

Key words: glycerolization, sperm, buffalo, cryopreservation

A high incidence of backward motility during cryopreservation of buffalo semen, particularly in summer months, prompted us to undertake this study. The problem has earlier been reported (Jindal *et al.*, 1995), but no attempt was made to identify the causes for the same, though high glycerol level was implicated in studies made in cow-bull (Philips and Kalay, 1984) and stallion (Watson and Morris, 1987). Our investigations revealed that the etiology of this problem can be ascribed to the stage when glycerol is added to extended semen (Singh *et al.*, 2006). The problem was checked when glycerol was added at initial stage (room temperature) of semen processing rather than after cooling extended semen to 5°C.

Progressive motility of sperm is essential for it to reach the site of fertilization and take active part in the process (Katz *et al.*, 1989). Sperm motility is also an indicator of viability. However, inspite of the spermatozoa being motile, a detrimental effect of cryopreservation on its functional parts, such as plasma membrane, acrosomal membrane and mitochondria, may not be evident. These structures have a great role in the process of fertilization. Therefore, a sperm cell's fertilizing competence requires that each of these membrane compartments remains intact.

The present study was, therefore, conducted to examine the effect of addition of glycerol, at room temperature or 5°C, on sperm plasma membrane through recording the proportion of live spermatozoa, with intact sperm plasma membrane. In addition, integrity of acrosomal membrane was also determined, besides the percent forward and backward motile spermatozoa, between the two groups.

MATERIALS AND METHODS

During peak summer months (May - June, 2004), 2 - 4 good quality ejaculates were obtained from each of the five Murrah breeding buffalo bulls (total ejaculates

[1]Senior Scientist and Incharge, Frozen Semen Laboratory,
[2]Technical Officer, [3] Principal Scientist and Head

= 15), using artificial vagina technique and evaluated for routine physical attributes. Each ejaculate was then divided into two aliquots before dilution. The extender (tris-yolk) was also divided into two equal parts. First part was non-glycerolated and added to all aliquots. The second part of extender, with 12.8 per cent glycerol, was added to these half-diluted aliquots of each ejaculate either at room temperature (RT group) or after cooling to 5°C (CT group), respectively. Thus the final glycerol concentration in the extended semen was 6.4 per cent. The semen extension rate was so adjusted as to contain 30 million spermatozoa with over 40 per cent motile spermatozoa post thaw in a insemination dose. Overall, standard cryopreservation protocol being followed in this laboratory (Jindal, 1994) was used, except for the glycerolisation step.

After dilution, glycerolisation and cooling of the semen to 5°C, followed by equilibration at 5°C for 3 h, pre-freezing motility was assessed in terms of per cent forward and backward motility. Similarly, post-thaw motility was also recorded after thawing frozen semen at 40°C for 30 sec. Integrity of the acrosomal membrane of spermatozoa (Watson, 1975) and per cent survival rate (Hancock, 1952) were determined for neat semen, before freezing and post-thaw. The values obtained for the two groups (RT and CT) were compared by analysis of variances using SPSS statistical package.

RESULTS AND DISCUSSION

In semen attributes' values in neat semen samples and after glycerolisation are given in Table. After glycerolisation, values for live count, intact acrosome and forward motile spermatozoa were similar in the two groups at pre-freezing stage. However, the percentage of spermatozoa with backward motility was significantly ($P<0.01$) higher in CT group as compared to RT group. Live spermatozoa and those with intact acrosome were similar between the two groups after freezing. However, the percentage of forward motile spermatozoa was higher in RT group ($P<0.05$), while the percentage of spermatozoa showing backward motility was significantly ($P<0.01$) higher in the CT group.

Perusal of data revealed that method of glycerolization did not influence survival rate and acrosomal integrity of the spermatozoa at pre and post freezing stages of cryopreservation, but the percentage of backward motile spermatozoa increased significantly when glycerolated at 5°C.

We have been practising buffalo semen freezing with glycerolisation at 5°C yielding acceptable post-thaw motility (Jindal, 1994). However, backward sperm motility was reported from this laboratory when 7% glycerol was added at 5°C and accordingly the glycerol concentration was kept at 6.4% thereafter (Jindal *et al.*, 1995). Inspite of the same, this phenomenon was again noticed during summer months and further investigations were made to minimize the ejaculate rejection rates on this account (Singh *et al.* 2006). Under low magnification, a significant proportion of spermatozoa appeared to be moving backwards, and this was found to be associated with the stage when glycerol was added (Singh *et al.* 2006). In cattle semen also similar phenomenon of spermatozoa appearing to be moving backwards was recorded with 7-14 per cent glycerol (Philips and Kalay, 1984), and also due to the cold shock in other species (Watson and Morris, 1987). However, the association of such backward motility with alterations in spermatozoal membranes integrity, has not been reported previously.

In the present study, proportion of backward motile spermatozoa was significantly ($P<0.01$) higher in CT than RT group - both at pre-freezing and post-thaw stages of cryopreservation, as reported previously (Singh *et al.*, 2006). Consequently, the percentage of forward motile spermatozoa at post-thaw stage was also significantly ($P<0.05$) lower in CT than RT group, possibly because a larger proportion of live spermatozoa were included in the backward motile category in CT group. Backward motility, therefore, appears to be a result of the cold shock to spermatozoa because the cryoprotectant was added only after cooling (CT). In comparison, when the cryoprotective effect of glycerol was available during cooling of extended semen (RT group), negligible incidence of backward motility was recorded.

Kataria and Tuli (1992) found little effect of glycerolization temperatures of 5, 22 or 37°C on

progressive motility as well as percentage of live and abnormal spermatozoa during and after freezing buffalo semen, though this report is silent on the phenomenon of backward motility. In cattle, Philips and Kalay (1984) studied this phenomenon in depth and ascribed it to tight bending of sperm tail exactly at 11 ìm distal to neck in a hairpin fashion, so that the flagellar movements propagated waves which made sperm head to appear moving backwards. Similar tight bending of sperm tail in a hairpin fashion at the neck region was observed in the present study (Figure). Flagella extended backwards over the head and it was propagating waves from its base to tip, thereby propelling the head backwards.

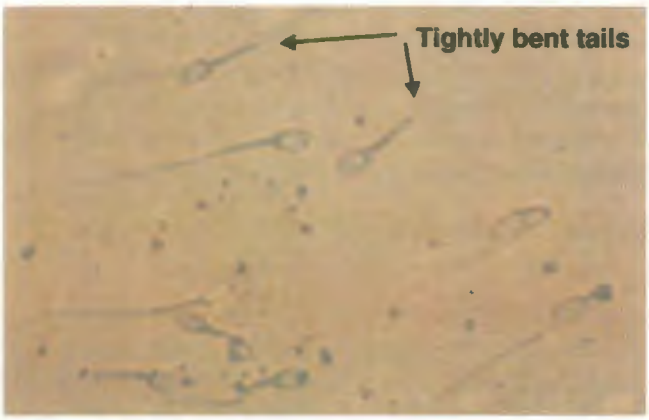

Fig 1: Microphotograph showing tightly bent tails of spermatozoa which exhibit backward motility

Table 1: Attributes of buffalo spermatozoa before and after freezing with two glycerolisation stages (RT and CT)

Attributes (%)	Stage of Glycerolisation	
	At room temp. (RT group)	At 5^{0}C (CT group)
Pre-freezing		
Live count	76.73±1.18	77.20±1.07
Intact acrosome	62.13±1.43	62.20±1.27
Forward motile	65.33±1.50	66.80±1.66
Backward motile	0.14±0.13**	3.26±0.83**
Post-freezing		
Live count	69.6±1.29	70.0±1.10
Intact acrosome	57.33±1.16	56.13±1.31
Forward motile	45.66±1.27*	39.20±2.48*
Backward motile	0.14±0.13**	3.27±1.10**

(*P<0.05; **P<0.01 in a row)

Glycerol acts as a cryoprotectant and early studies in cattle suggested that cellular entry of glycerol is rapid at higher temperatures, but this may also be detrimental to sperm survival (Sherman, 1963). Such doubts were negated by Clegg and co-workers (1965) who suggested similar motility and sperm survival rates when glycerolization was done at higher temperature (32^0 vs. 5^{0}C) but with longer equilibration time. Other studies (Dunn and Hafs, 1953) reported little differences in sperm survival when glycerol was added at room temperature or at 5^{0}C.

The incidence of backward motility lead to rejection of a high proportion of ejaculates thereby decreasing the frozen semen production (Singh *et al.*, 2006). The present study revealed that the sperm survival rate and acrosomal integrity did not differ between two stages of glycerolization inspite of significantly (P<0.05) higher backward motility in CT as compared to RT group.

Watson and Morris (1987) reported the incidence of spermatozoa moving backwards due to over bending of tail area, irreversible changes to the mid-piece and coiling of the tail due to cold shock. Similar findings were reported by Philips and Kalay (1984) that the spermatozoa which were observed moving backwards had their dense fibers and flagellar microtubules kinked or broken, presumably by the force that caused the mid-piece to bend at a point 11 ìm from the neck region. Although the sperm tails were kinked, the plasmalemma remained intact. They also suggested that only viable spermatozoa were caused to form hairpin shape and an intact membrane, therefore, appears to be needed to produce hairpin-shaped spermatozoa. Nevertheless, changes in the membrane could be involved in forcing the flagellum to bend and kink.

In the present study, however, there were no differences in proportion of sperm cells which were live and having intact acrosomal membranes between the two groups (RT and CT), suggesting no obvious effect of the two protocols on integrity of buffalo sperm membranes. However, ultrastructural changes in the membrane structure need to be investigated before it can be safely concluded that sperm membrane changes are not involved in the phenomenon of backward motility.

REFERENCES

Clegg, E.D., Pickett, B.W. and Gibson, E.W. (1965). Non-mechanical nitrogen vapour freezing of bull semen. *Connecticut Agriculture Experiment Station Research Report* 5 (Cited by Clegg *et al.*, 1965. Preliminary investigation on glycerol entry into bovine spermatozoa. J. Dairy Sci., **48**: 1709-1711).

Dunn, H.O. and Hafs, C. (1953). Extenders and techniques for freezing bovine spermatozoa. J. Dairy Sci., **36**: 577.

Hancook, J.L. (1952). The morphology of bull semen. J. Exp. Biol., **29**: 445-453.

Jindal, S.K. (1994). A simple and practical method for processing of buffalo semen. Indian Dairyman, **46**: 12.

Jindal, S.K., Jain, G.C. and Hooda, O.K. (1995). Effect of glycerol level on the freezability of buffalo semen. Indian J. Dairy Sci., **48**: 1.

Kataria, S.K. and Tuli, R.K. (1992). Freezability of buffalo spermatozoa as affected by method and temperature of glycerolization. Indian J. Dairy Sci., **45**: 488-490.

Katz, D.F., Drobnis, E.Z. and Overstreet, J.W. (1989). Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. Gamete Res., **22**: 443-469.

Phillips, D.M. and Kalay, D. (1984). Mechanisms of flagellar motility deduced from backward-swimming bull sperm. J. Exptl. Zool., **231**: 109-116.

Sherman, J.K. (1963). Questionable protection by intra-cellular glycerol during freezing and thawing. J. Cell. Comp. Physiol., **61**: 67-84.

Singh, P., Singh, I., Singh, S. and Sharma, R.K. (2006). Initial stage glycerolisation prevents the incidence of backward sperm motility during cryopreservation and increases buffalo semen freezability. Indian J. Anim. Sci., **76**: 777-779.

Watson, P.F. (1975). Use of Giemsa's stain to detect change in the acrosome of frozen ram spermatozoa. Vet. Rec., **97**: 12-15

Watson, P.F. and Morris, J.L. (1987). Cold shock injury in animal cells. In: Bowler, K.' Fuller, B.J. (Eds), Temperatures and Animal Cells. Symposium of the Society for Experimental Biology Vol. 41 The Company of Biologists Cambridge pp 311-340.