The Indian Journal of Animal Reproduction; 29(2):169-176; December 208

Research Article

Effect of Follicular Proteins on Buffalo Sperm Functions during Cryopreservation

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ABSTRACT

A study was undertaken to assess the effect of follicular proteins on freezability of buffalo spermatozoa. Follicular fluid was aspirated from the follicles (>5mm) of buffalo ovaries obtained from slaughterhouse. The follicular fluid was centrifuged (3000 rpm; 30 minutes), filtered (0.2μ) and frozen at -20°C. The proteins in pooled follicular fluids were precipitated over night using ammonium sulphate, centrifuged (10000 rpm; 30 minutes) and dialyzed (> 10kDa). After protein estimation, aliquots of samples containing 10 mg proteins were lyophilized in cryovials and stored frozen at -20°C. Six pooled good quality ejaculates from two Murrah buffalo bulls were used as split ejaculates. After routine semen analysis, each pooled ejaculate was splited into five parts and extended in Tris-Egg yolk-Citrate extender (20% egg yolk: 7% glycerol), so that final dilution yielded approximately 60 million sperm per ml and cryopreserved in 0.5 ml French straws (30 million sperm cells/straw) in LN_2 (-196°C). Prior to freezing, one part of each ejaculate was added with follicular proteins at the rate of 1mg/ml of extended semen while another part was frozen without any added proteins (control). Semen samples (pre-freeze and frozen-thawed) were evaluated for motility, viability and intact acrosome percentages, sperm penetration distance in cervical mucus (BCMPT) and swollen sperm percentage in response to hypo-osmotic solution (HOSST). Besides this, frozen thawed sperms were washed and incubated in sperm-TALP at 37°C for 6 hrs. The sperm motility, viability and intact acrosome percentages were evaluated at hourly interval. Addition of follicular proteins maintained significantly (P<0.05) higher post-thaw motility, viability and acrosomal integrity percentages than the control. Similarly the BCMPT and HOSST values were also higher in follicular protein added group than the control group. Sperm motility and viability percentages were significantly (p<0.05) high throughout the incubation period in the follicular protein treated group than the control group. But, the trend was not observed in case of sperm acrosomal integrity percentage. It was inferred that inclusion of follicular proteins before freezing had beneficial effects on post thaw characteristics of buffalo spermatozoa.

Key words: Buffalo, Follicular proteins, Cryopreservation, Spermatozoa.

INTRODUCTION

Follicular fluid has been reported to exert significant beneficial effects on motility and other functions of mammalian sperm (Kumar, 1995; Lapointe *et al.*, 1996 and Kumaresan *et al.*, 2003). In the last 2 -3 decades, many investigators have been studying the motility stimulating factor(s) present in the follicular fluid. Although several suggestions have been made regarding the factor in follicular fluid responsible for modulation of sperm functions it has been identified as protein in nature (Lee *et al.*, 1992; Ram Soondar *et al.*, 1995).

Lapointe *et al.* (1996) fractioned bovine follicular fluid by dialysis and reported that semi purified sub fraction of follicular fluid protects the spermatozoa during freezing and thawing and maintained good motility. Although, several literatures are available on *in vitro* capacitation, hyperactivation and acrosomal reaction activity of follicular fluid, only little information is available on the effect of addition of follicular fluid on freezability and *in vitro* fertility of spermatozoa. Recently, it has been reported that buffalo follicular fluid improved the preservability of spermatozoa at 5°C (Agarwal, 1997). However, information regarding the use of follicular proteins to modulate the buffalo sperm function during cryopreservation could not be traced out during perusal of literature. Hence, the present

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investigation was carried out to assess the effect of inclusion of follicular proteins in extender before freezing on post-thaw sperm characteristics in buffaloes.

MATERIALS AND METHODS

The present study was carried out at Artificial Insemination Laboratory and Germ Plasm Center of Indian Veterinary Research Institute.

Preparation of follicular proteins

Buffalo ovaries, obtained from slaughterhouse immediately after slaughter, were brought to the Laboratory on ice. Ovaries were washed with normal saline and wiped with filter paper. Follicular fluid was aspirated from follicles of > 5mm diameter, pooled, centrifuged (3000 rpm; 20 min.) and the supernatant was stored at –20^{0}C until used.

Proteins in the concentrated oviductal fluids were separated by following the procedure given by Boquest *et al.* (1999). Briefly, the protein was precipitated by adding solid ammonium sulphate to the fluid on a magnetic stirrer over a 30 min period until 80% saturation (52.3 g/100 ml) was reached. All the following procedures were performed at 4^{0}C unless otherwise specified. The protein was allowed to precipitate overnight. The precipitated proteins were pelleted by centrifugation at 10000 rpm for 30 min. Pellets were resuspended in a two pellet volumes of PBS (pH 7.4) and dialyzed overnight against 4 L of PBS in four changes. (Dialysis tubing, Spectrapor, Los Angles). The retentates (>10 kDa) were filter sterilized through sterile mini start (Sartorius) 0.2-µ non-pyrogenic filter units. The protein content was estimated as per the procedure given by Lowry *et al.* (1951). Aliquots of samples containing 10 mg of protein were dispensed in 1 ml cryovials and lyophilized to dryness and stored frozen at –20^{0}C. Control tubes were prepared by lyophilizing cryovials containing PBS only.

Extension and Freezing of Semen

Six good quality pooled ejaculates from two buffalo bulls were utilized in the study (Six replications). Semen was collected by Artificial Vagina method and extended in Tris-egg yolk-citrate extender (Davis *et al.*, 1963). The rate of extension was kept in such a way that each straw contained approximately 30 million spermatozoa. One part of the split ejaculate was added with follicular proteins at the concentration of 1 mg per ml of extended semen (concentration selected based on our previous study; Kumaresan, 2002) while the second part remained as control (with no added proteins). Extended semen was frozen in LN_2 (-196^{0}C) as per the standard method in 0.5 ml French straws with an equilibration time of 4 h at 5^{0}C.

Semen evaluation

Randomly selected semen straws, at least two from each group and each ejaculate, were assessed for sperm motility, viability and acrosomal integrity percentages before freezing as well as after thawing. Sperm motility was evaluated at X400 magnification based on the visual estimation of the percentage of sperm possessing progressive motility and the percentage was rounded to the nearest 5%. Percent live sperm cells were evaluated using Eosin-Nigrosin staining method (Campbell *et al.*, 1953). Partly stained cells were also considered as dead. Percent intact Acrosome was evaluated under X1000 magnification by Giemsa staining method as per the procedure given by Watson (1975). For assessing live sperm and acrosomal integrity percentages, at least 100 sperm cells/smear were counted using a tally counter. At all times, at least two smears per group were assessed. All the semen evaluation was done by a single person to avoid individual variations.

Bovine cervical mucus penetration test (BCMPT) and hypo-osmotic sperm swelling test (HOSST) were performed as per the procedure given by Matousek *et al.*(1989) and Jeyendran *et al.*(1984), respectively. Each sample was assessed in duplicate.

For BCMPT, the capillary tubes (8 cm in length) were loaded with mucus collected from estrus buffaloes. One end of the capillary tube was sealed with haemoseal (Shandilya chemical Pvt. Ltd. Bombay) while the other end was left free. 0.5 ml of semen was taken in a small test tube into which the loaded capillary tubes in duplicate were placed so that the free end remained at the bottom of the tubes and incubated for 60 min. at 37^{0}C. After incubation, the capillary tubes were removed from the test tubes, wiped clean and placed on a graduated glass slide and the distance travelled in millimeter by the vanguard spermatozoa was measured under high power magnification (40x).

For HOSST, 2 ml of hypo-osmotic solution (Sodium citrate dehydrate – 735 mg; Fructose – 1351 mg in 100 ml distilled water) and 0.1 ml of semen was mixed in a small test tube. The suspension was incubated at 37°C for 60 minutes. After incubation, a small drop from the suspension was placed on a clean, dry and grease free glass slide and covered with a cover slip. The slide was examined under the high power magnification (40 x) of a phase contrast microscope. A minimum of 100 spermatozoa were counted per slide for different types of swelling pattern.

Randomly selected straws from treatment and control groups were thawed at 37°C for 30 seconds and the semen was pooled and washed twice by centrifugation in protein free sperm-TALP (pH 7.3). Then the sperm pellet was dissolved in incubation media i.e. sperm TALP containing 6 mg/ml of bovine serum albumin (BSA) and incubated for 6 h at 37°C. The motility, viability and acrosomal integrity were assessed at hourly interval to find out the effect of follicular proteins on spermatozoan characteristics during incubation.

Statistical analysis:

Two semen straws from each treatment and each of six separate (pooled) ejaculates were examined for sperm characteristics. The data thus obtained were subjected to Analyses of variance. Means from motility, live % and acrosomal integrity analysis that were different at $p<0.05$ were tested using Fisher's least significant difference test (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

Effect of inclusion of follicular proteins in extender at the concentration of 1mg per ml before freezing on spermatozoan freezability is shown in Table 1.

The post-thaw motility, viability and intact acrosome percentages were significantly ($P<0.05$) higher in follicular proteins included semen samples than the control samples. The beneficial effect of follicular proteins on the maintenance of sperm acrosomal integrity was observed at pre-freeze evaluation itself as the intact acrosome percentage was significantly ($P<0.05$) higher in follicular protein added samples than the control. The sperm penetration distance in follicular proteins added group was higher, but not significantly, than the control group both at pre-freeze and post-thaw evaluation. The distance traveled by vanguard spermatozoa in cervical mucus in follicular protein treated group was 22.50 ± 0.57 and 17.25 ± 2.06 mm at pre-freeze and post-thaw evaluation, respectively. The swollen sperm percentage was significantly ($P<0.05$) higher in follicular proteins included semen samples (30.75 ± 0.95) than the control samples (22.00 ± 1.63) in post-thaw evaluation. There was no significant difference at pre-freeze evaluation (Fig 1).

At 0h of incubation, the motility rating of follicular proteins treated group was similar with control group. But at 1h and onwards during incubation the follicular proteins included group maintained significantly ($P<0.05$) higher motility than the control group. At 6h incubation the per cent motility in the follicular proteins treated group was 11.25±2.50 where as in the control group the corresponding percentage was only 3.75±2.50 (Fig 2). Sperm viability percentage in follicular proteins treated group was always significantly ($P<0.05$) higher than the control group during incubation up to 6h. At 0h the viability percentage was 70.75±1.71 in follicular protein treated group and in control group it was 58.25±3.31. The corresponding values at 6 h were 22.50±1.29 and 11.25±1.71, respectively (Fig 3). No significant effect of follicular proteins on percent intact acrosome was observed during incubation (Fig 4). The initial acrosomal integrity percentage was 69.75±10.65 in follicular proteins treated group and 66.25±5.44 in control group. The corresponding values at 6 h incubation were 39.00±1.40 and 36.25±4.50, respectively.

At the time of ovulation, follicular fluid passes into oviduct and modulates sperm functions (McNutt and Killian, 1991). Inclusion of follicular fluid has been shown to maintain sperm motility, viability and acrosomal integrity in refrigerated storage of buffalo (Agarwal, 1997) and cattle (McNutt and Killian, 1991) spermatozoa. Although several suggestions have been made regarding the factor responsible for modulation of sperm functions, it has been identified as a protein (Lee *et al.*, 1992; Ram Soondar *et al.*, 1995). Though few studies have used buffalo follicular fluid as an additive to improve

Fig 2: Ef

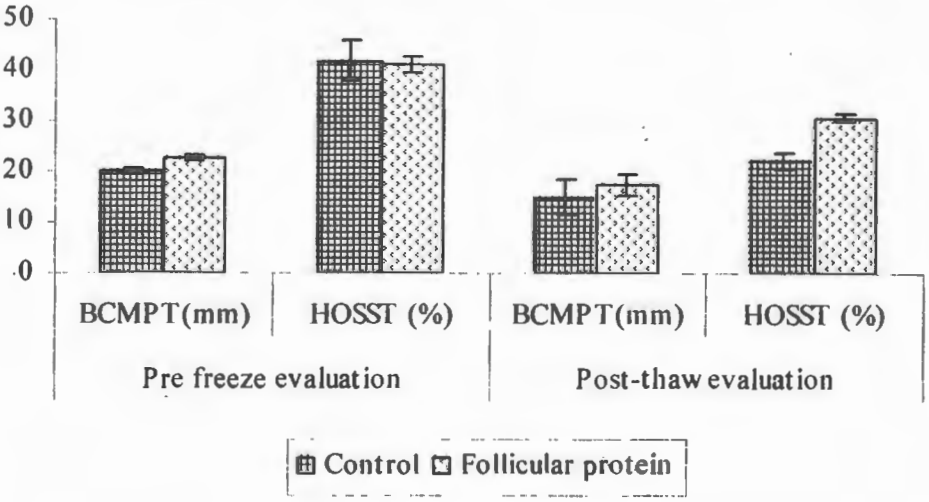

Figure 1: Effect of follicular proteins on BCMPT and HOSST values

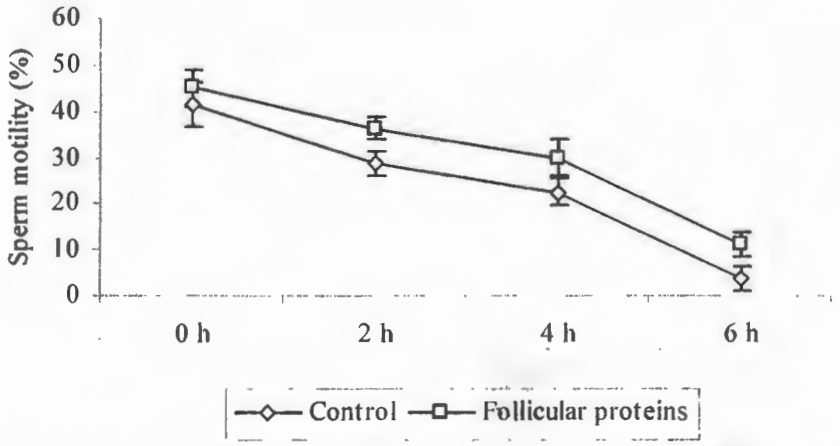

Fig 2: Effect of follicular proteins on sperm motility during incubation. Data shown all means ± SE.

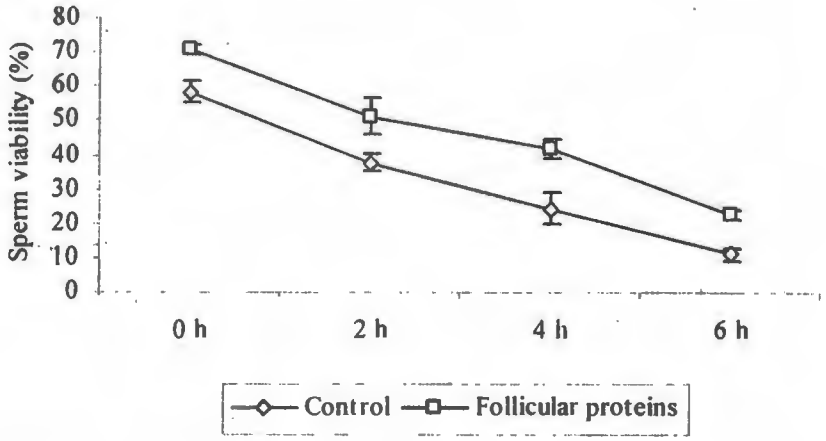

Fig 3: Effect of follicular proteins on sperm viability during incubation. Data shown all mean ± SE.

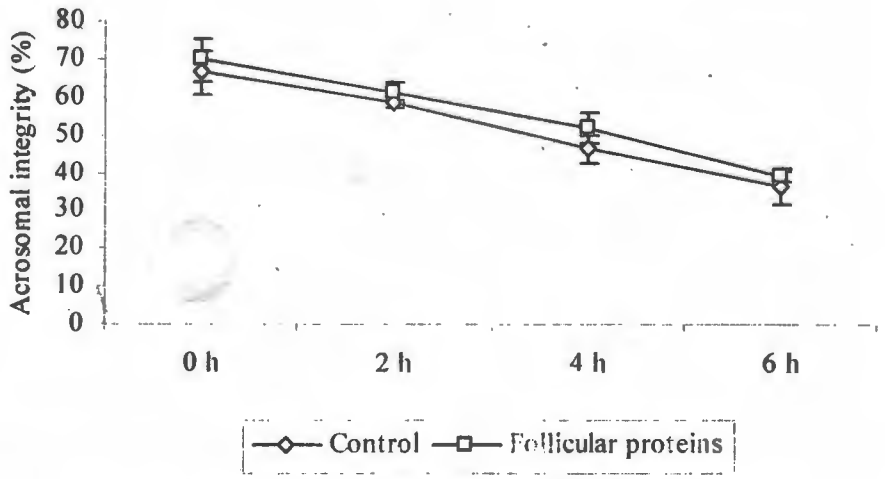

Fig 4: Effect of follicular proteins on sperm acrosomal integrity during incubation. Data shown all mean ± SE.

Table 1. Effect of follicular proteins on pre-freeze and post thaw spermatozoan motility, viability and acrosomal integrity percentages. Data shown all mean ± SE

Sperm Characteristics	Pre freeze evaluation		Post-thaw evaluation	
	Control	Follicular proteins	Control	Follicular proteins
Motility (%)	52.50 ± 2.89[a]	60.00 ±4.08[a]	40.00 ±4.08[a]	50.00 ±5.77[b]
Viability (%)	73.75 ±6.55[a]	75.00 ± 2.58[a]	53.25± 6.54[a]	68.75± 3.30[b]
Acrosomal integrity (%)	73.25 ±3.20[a]	79.00 ±0.81[b]	63.75 ±6.07[a]	71.50 ±1.29[b]

FP- Follicular proteins included semen

Mean with different superscript in a row within pre-freeze and post-thaw evaluation differ significantly (P<0.05).

semen quality (Kumar, 1995; Agarwal, 1997), the effect of follicular proteins on freezability and post thaw characteristics of spermatozoa in buffaloes need to be studied in detail.

This study reports the beneficial effects of follicular proteins when added at the concentration of 1mg/ml of extended semen on cryopreservation of buffalo spermatozoa. The follicular protein concentration was selected based on another experiment (Data not shown). Follicular proteins included semen samples had significantly (P< 0.05) higher post-thaw motility, viability and acrosomal integrity percentage compared to control. It suggests that follicular proteins have a protective effect on spermatozoa during freezing. These findings are in agreement with Kumar (1995) who reported the beneficial effects of follicular fluid on cryopreservation of spermatozoa and with our previous study in buffaloes (Kumaresan *et al.*, 2003). The beneficial effect of follicular proteins on maintaining the sperm motility and viability was visible during incubation also (Fig 2 &3). Significantly (p<0.05) higher motile and viable sperms were observed in follicular protein treated group than in the control group even up to 6 hr incubation, suggesting that the follicular proteins exert protective effect on sperm after thawing also. Studies on human sperm have shown that follicular fluid proteins enhanced the sperm motility for a prolonged period (Falcone *et al.*, 1991). Follicular fluid is known to stimulate the motility and respiration of spermatozoa to a considerable extent. However, there was no significant difference between the intact acrosome percentages of control and follicular proteins treated groups during incubation. This might be due to the capacitating action of follicular proteins on spermatozoa. Mc Nutt and Killian (1991) reported that cow follicular fluid might contain both capacitating and acrosome reaction inducing factors and required less time to capacitate the spermatozoa. Follicular fluid has been known for many years to be a rich source of glycosaminoglycans, which induces capacitation of spermatozoa. Moreover, the level of albumin in buffalo follicular fluid was significantly (P<0.05) higher when compared to oviductal fluid (Kumaresan *et al.*, 2003). It is possible that the higher levels of albumin might have induced capacitation since albumin acts as a sink for the removal of cholesterol from the sperm plasma membrane (Cross, 1998) and this may be the

reason for higher percentage of capacitated spermatozoa in follicular fluid proteins treated group. Kumaresan *et al.* (2005) also reported that follicular proteins treated buffalo spermatozoa had significantly higher acrosome reacted spermatozoa than the control group at 4 h of incubation at 37°C.

Penetration of spermatozoa into the mucus of the cow has been used for human studies in which a failure of spermatozoa to swim could be correlated with fertility problems. Though non-significant, higher sperm penetration distance in the cervical mucus was observed in follicular proteins included group than the control group. These observations are in consonance with those of Lapointe *et al.* (1996). They reported that follicular proteins had a beneficial effect on post-thaw motility and viability of spermatozoa. Further, they observed that addition of follicular proteins to the extender enabled more spermatozoa to penetrate the cervical mucus *in vitro*. This might be due to the fact that follicular proteins hyper activate sperm and once hyper activated, the sperm develop greater hydrodynamic thrust and pushed harder against their surroundings than those with symmetrical beats (Katz and Drobnis, 1990), helping them to penetrate more distance. During the HOSST, the spermatozoa having intact and biochemically functional membrane undergo swelling to establish equilibrium between the fluid compartment within the spermatozoa and extracellular environment (Jeyendran *et al.*, 1984). These osmotic changes induce typical morphological alterations characterized by different patterns of swelling in tail region. These changes have been considered as an indicator of the membrane integrity and normal functional activity of spermatozoa. The swollen sperm percentage was also higher in follicular proteins included semen samples indicating the membrane integrity. The higher percentages of swollen sperm and intact acrosome in post-thaw semen analysis suggest that inclusion of follicular proteins in the extender protects the sperm membrane during cyropreservation.

ACKNOWLEDGEMENTS

The authors thank the Director, Joint Director, IVRI and Head, Animal Reproduction Division for the necessary facilities provided during this work.

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