

## Effect of Freezing on Proteins and Protein Profiles of Sperm Membrane Extracts and Seminal Plasma of Buffalo Bulls

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**ABSTRACT :** The total proteins were estimated in both deoxycholate (DOC)-extract of sperm membrane and seminal plasma of chilled as well as frozen semen obtained from five Murrah buffalo bulls. Proteins were further characterized by polyacrylamide gel electrophoresis (PAGE) in three bulls. The protein content of sperm membrane extract (SME) and that of seminal plasma (SP) decreased gradually with increase in freezing period from 6 to 24 mo when compared with the values observed in freshly chilled semen in all bulls. The total decrease in protein content of SME and SP varied from 30-40% and 28-59% respectively during 6-24 mo of freezing. The number of glycoproteins/proteins (GP/P) in SME varied from 4-8 in freshly-chilled semen of all bulls and reduced to 2-4 after 24 mo of freezing. In SP, the number of proteins varied from 6-10 in freshly chilled semen of all bulls and reduced to 3-8 after 24 mo of freezing. Some of the proteins in SME and SP disappeared, others got altered and appeared with change in molecular weight after different freezing times. These studies reveal that alterations in the sperm membrane proteins may be responsible for damage to their membrane during freezing and thus lowering their fertilizability. (*Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 12 : 1678-1682*)

**Key Words :** Buffalo, Semen, Freezing, Spermatozoa, Proteins

### INTRODUCTION

Freezing phenomenon involves complicated and varying effects of cold shock on live cells and very complicated intracellular changes. Electron microscopic observations of rapidly frozen semen suggest that intracellular ice formation particularly in the neck and tail of sperm causes heavy damages (Sharma, 1987). Freezing significantly increases the sperm abnormalities and there is leakage of certain enzymes in the seminal plasma due to damage of sperm membrane (Krishna and Rao, 1987). But so far no information is available on any alterations in the membrane components of buffalo spermatozoa during freezing. Therefore, a study on qualitative and quantitative changes in the sperm membrane proteins during freezing was conducted in buffalo bull semen.

### MATERIALS AND METHODS

#### Procurement and processing of the semen samples

Chilled as well as frozen semen of Murrah buffalo bulls was procured from the Dairy Farm, PAU, Ludhiana, India. The semen samples were collected from five buffalo bulls nos. 1,241, 1,315, 1,434, 1,437 and 1,446. Five semen samples including fresh (chilled) and frozen and stored for 6, 12, 18 and 24 mo in each bull were used. After thawing

the semen at 37°C, sperm motility and concentration were evaluated under the microscope. The freshly chilled as well as frozen semen was centrifuged at 6,000 rpm for 10 min. The spermatozoa and seminal plasma were separated and analysed for quantitative and qualitative estimation of proteins.

#### Sperm concentration and motility

The sperm concentration was measured with the help of a haemocytometer using 1% formalin in normal saline and 0.2% eosin. Sperm motility was assessed microscopically as a percentage of active spermatozoa out of total number of spermatozoa counted in a drop of semen placed on a slide and covered with cover slip.

#### Detergent extraction

Two ml of deoxycholate (DOC) detergent in 0.02 M Tris-HCl buffer, pH-8.0, containing 10 µl phenyl methyl sulfonyl fluoride (PMSF) (100 mM as protease inhibitor) was added to  $2 \times 10^9$  spermatozoa (Kinger et al., 1989). These were incubated for 1 h at 37°C on metabolic shaker, centrifuged at 6,000 rpm for 30 min at room temperature. The supernatant represented the extracted membrane components.

#### Quantitative and qualitative estimation of proteins

Total proteins were estimated in DOC extract of sperm membrane and in seminal plasma by using the method of Lowry et al. (1951). The glycoproteins and proteins were characterized in DOC-extracts of the sperm membrane by polyacrylamide gel electrophoresis (PAGE) and detected

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using Schiff's reagent periodic acid and coomassie brilliant blue stains respectively (Davis, 1964; Clarke, 1964).

**RESULTS**

**Effect of freezing on sperm motility**

On comparing the post thawing motility of frozen semen with the respective initial motility, it was observed that there was a 5-10% decrease in motility of spermatozoa after 6, 12, 18 and 24 mo after freezing. The initial as well as post thawing motility of bull nos 1,434 and 1,446 was lower (5-20%) than that of other bulls (30-60%) which are considered to be good for artificial insemination.

**Effect of freezing on proteins of sperm membrane extracts (SME) and seminal plasma (SP)**

The protein content decreased gradually with increase of freezing time in SME of all bulls. The rate of extraction of proteins was highest in freshly chilled spermatozoa of bull nos 1,241 and 1,434 (5.26-5.89 mg/10<sup>9</sup> spermatozoa) followed by 1,315, 1,446, 1,437 (4.63-4.84 mg/10<sup>9</sup> spermatozoa). A similar trend was observed with increase in time of freezing (table 1). The total protein content decreased more in SME from bulls nos. 1,315 followed by 1,241. The total percentage decrease in protein content of SME varied from 30-40% after 24 mo of freezing (table 1). The protein content of SP also decreased with increase of freezing time and it ranged from 3.87-5.13 mg/ml and 0.31-4.18 mg/ml in freshly chilled and frozen samples respectively (table 1). The total percentage decrease in SP

proteins varied from 28-59% after 24 mo of freezing (table 1). ANOVA two way test (table 1) indicated that the decrease in protein content with increase in freezing time was statistically significant (p<0.05).

**Effect of freezing on glycoprotein/protein (GP/P) profile of sperm membrane extracts**

The GP/P profiles of SME of bull nos. 1,241, 1,315 and 1,434 were evaluated. The number of GP/P reduced to 2-4 from 4-8 after 24 mo of freezing in all bulls (table 2). In bull no. 1,241, eight GP/P were observed in SME of the freshly chilled semen (table 2). This GP/P profile showed considerable variation in terms of molecular weight as the low molecular weight proteins (62, 45 and 17 kDa) disappeared after 6 mo of freezing and proteins with high molecular weight of (280 and 245 kDa) appeared as altered proteins. With increasing period of freezing, a few low molecular weight proteins (62, 45 and 19.5 kDa) reappeared. In bull no. 1,315, five GP/P were observed in the SME of freshly chilled semen which showed comparatively lesser alterations except that a high molecular weight protein (290 kDa) disappeared after 12 mo of freezing and two altered proteins (62 and 43 kDa) appeared. Another altered protein (245 kDa) appeared 18 mo after freezing. In bull no. 1,434, only four proteins were observed in the SME of freshly chilled semen. After 6 mo of freezing two proteins (290 and 90 kDa) disappeared and three altered proteins (225, 210 and 45 kDa) appeared. Later on only two GP/P were observed which showed a variable molecular weights (table 2).

**Table 1.** Effect of freezing on protein content of sperm membrane extracts (SME mg/10<sup>9</sup> spermatozoa) and seminal plasma (SP, mg/ml) of buffalo bulls

Times freezing (months)	Bull number											
	1,241		1,315		1,434		1,437		1,446		Mean±S.E.	
	SME	SP	SME	SP	SME	SP	SME	SP	SME	SME	SME	SP
0	5.89	3.97	4.84	4.18	5.26	5.13	4.63	3.87	4.84	3.97	*5.09±0.27	*4.22±0.21
6	4.95 (9.4)	3.87 (2.7)	3.79 (13.3)	4.09 (1.6)	4.78 (10.3)	4.18 (21.5)	2.32 (20.0)	2.19 (17.0)	2.64 (18.8)	3.76 (4.0)	*3.69±0.48	*3.61±0.33
12	3.69 (22.0)	3.34 (17.2)	3.27 (19.9)	3.24 (17.2)	4.11 (24.6)	4.09 (23.6)	1.90 (23.6)	2.08 (18.1)	2.23 (22.3)	3.13 (16.1)	*3.04±0.38	*3.12±0.29
18	2.53 (33.7)	3.03 (25.7)	2.74 (26.6)	2.92 (23.2)	3.79 (31.5)	3.97 (26.3)	1.59 (26.3)	1.03 (28.7)	1.59 (27.7)	2.08 (36.3)	*2.44±0.37	*2.60±0.44
24	2.43 (34.8)	1.98 (54.3)	1.99 (40.0)	1.03 (57.9)	3.69 (33.6)	3.87 (28.6)	1.17 (29.9)	0.31 (36.0)	1.17 (31.2)	1.77 (43.6)	*2.0±0.42	*1.70±0.54

0, Freshly chilled semen; Figures in parenthesis represent percent decrease in the protein content. \*, F cal<Ftable 16 value-calculated by ANOVA.

**Table 2.** Effect of freezing on glycoprotein/protein profile (molecular weight kDa) of sperm membrane extracts in buffalo bulls as determined by PAGE

Times of freezing (months)	Bull number		
	1,241	1,315	1,434
0	150*, 140*, 110*, 74, 71.5, 62*, 45*, 17*	290, 150*, 71.5*, 50*, 17	290*, 160*, 90*, 26*
6	280*, 245*, 160*, 74*	290*, 150, 71.5*, 33*, 17	225*, 210*, 170*, 45, 21
12	280*, 180*, 150, 80*, 62*, 45*	150*, 62*, 43*, 33*, 22*	245*, 62*
18	280*, 210*, 110, 83, 74*	290, 245*, 71.5*, 22*	140*, 62*
24	245*, 140*, 74*, 19.5	245*, 22*	285*, 210*

0, Freshly chilled semen; \* glycoproteins as determined by PAS.

#### Effect of freezing on protein profile of seminal plasma

The protein profile of SP of bull number 1,241 revealed that the proteins with molecular weight of 285, 210, 71.5 and 31.5 kDa persisted almost in all the samples, whereas 155 and 45 kDa proteins disappeared after 6 mo of freezing and reappeared 12 mo after freezing. The 325 and 61.5 kDa proteins appeared as altered proteins with changed molecular weight after 6 and 18 mo of freezing respectively, whereas 325, 200 and 160 kDa proteins disappeared after 24 mo of freezing (table 3). The protein profile of SP of bull number 1,315 showed that the number of high molecular weight proteins decreased from 4-2 and instead of medium molecular weight proteins (72 and 71.5 kDa), very low molecular weight proteins (51, 38, 23, 19 kDa) appeared 6 mo after freezing. After 12 and 18 mo of freezing, the number of proteins remained the same with little alterations in their molecular weight and only three proteins were present after 24 mo of freezing (table 3). The protein pattern of SP of bull number 1,434 revealed that a high molecular weight protein (340 kDa) and medium molecular weight (83 kDa) disappeared after 6 mo of freezing and after 12 mo a protein with molecular weight of 235 kDa disappeared whereas other six proteins appeared with little alterations in molecular weights similar changes were observed after 18 and 24 mo after freezing (table 3). Thus the GP/P profile of

SME and SP showed a similar trend of variable proteins after freezing in all the three bulls (figure 1).

#### DISCUSSION

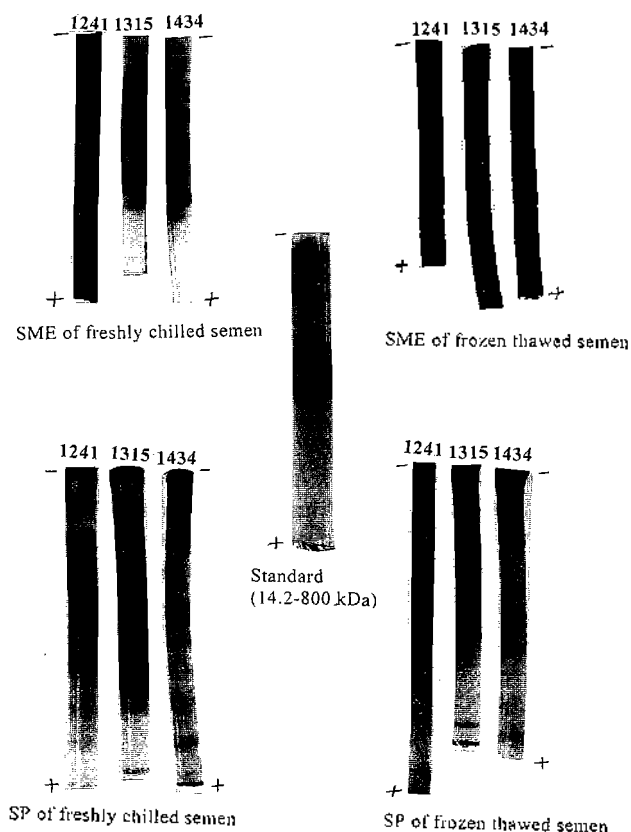
A gradual and consistent decrease in the motility of spermatozoa during the present studies reveals that in spite of protection of spermatozoa by cryoprotectants, a constant and gradual stress of freezing persists over a period of time, which might result in the reduction of energy giving substances of spermatozoa or a permanent damage to the tail region. Sahu and Pandit (1997) have also reported a significant decrease in the motility and number of live sperms of Murrah bulls after cryopreservation. The breakage of the plasma membrane and a reduction in electron density of the mitochondrial matrix in mid piece subsequent to freezing and thawing (Jones and Stewart, 1979) may be responsible for decrease in the motility of spermatozoa during freezing. However, the motility of spermatozoa remains within the permissible limits of fertility after freezing in present as well as the studies of Sahu and Pandit (1997).

The freezing and thawing processes have considerable deleterious effects on the membrane constituents. The damage to plasma membrane, acrosomal content and

**Table 3.** Effect of freezing on protein profile (molecular weight KDa) of seminal plasma in buffalo bulls as determined by PAGE

Times of freezing (months)	Bull number		
	1,241	1,315	1,434
0	285, 205, 155, 71.5, 45, 31.5	340, 245, 205, 140, 80, 72, 71.5, 60	340, 240, 210, 160, 83, 80, 72, 60, 59, 30
6	285, 215, 83, 71.5, 61.5, 31, 25.5	325, 210, 81, 51, 38, 23, 19	245, 235, 210, 100, 83, 80, 60, 45, 30
12	252, 245, 210, 155, 110, 83, 71.5, 45	290, 210, 84, 81, 43, 24	245, 210, 120, 100, 71.5, 62, 51
18	325, 285, 210, 200, 160, 80, 61, 38	340, 330, 225, 210, 80, 71.5	290, 250, 80, 50, 31.5
24	285, 210, 71.5, 51, 31.5	330, 51, 26	290, 240, 205, 165, 71.5, 50, 45, 24

0, Freshly chilled semen.



**Figure 1.** PAGE pattern of sperm membrane extracts (SME) and seminal plasma (SP) of freshly chilled and frozen semen

membrane of ram and boar spermatozoa by cold shock was reported by Ahmed et al. (1997) and the damage becomes decreased after the addition of egg yolk. The survival and the fertilizing ability of the bull spermatozoa is very low after freezing and it is definitely influenced by the presence of cryoprotectants in media during deep freezing (Kumar et al., 1998). It has been observed during the present studies that inspite of the addition of the cryoprotectants such as glycerol, freezing causes deleterious alterations in the plasma membrane constituents of buffalo bull spermatozoa. The protein content decreased gradually with the increase in freezing time in the frozen sperm membrane extract as well as seminal plasma. This shows that freezing exerts its deleterious effects on the protein molecules continuously throughout the duration of freezing. Our observation corroborates the ultra structural observations revealing loss and damage of sperm membrane during freezing of bull (Jones and Stewart, 1979), ram and boar spermatozoa (Ahmed et al., 1997).

It is widely accepted that the membrane viability, motility, survival and storage properties of spermatozoa are influenced by seminal plasma (SP) proteins (Dott, 1961; Moore and Hibbitt, 1976) and the SP of mammals play a

decisive role in the protection of sperm during cooling. The heteroplasmic and heterospermic studies (Ganguli, 1978) revealed that the SP plays a definite role in the sperm motility, survival and viability. In the present studies, the protein content of SP of the frozen semen also decreased, which may be responsible for decreased motility of the spermatozoa. Hibbitt and Benians (1971) reported that proteins may increase the permeability of biological membranes and it has been suggested that the basic proteins of seminal vesicles have a similar effect on the membrane of spermatozoa. It has also been reported that high concentration of SP proteins render the spermatozoa much more sensitive to the injuries during the cooling and deep freezing in boar (Lavon and Bourshell, 1971; Moore and Hibbitt, 1976). However Edwin et al. (1990) have shown a highly significant negative correlation between total protein content of SP and freezability of spermatozoa and suggested that a high protein content may cause an increase in the permeability of sperm membrane, thus promoting the shock and injury to spermatozoa during cooling and deep freezing. The decrease in the protein content of SP of frozen bull semen in the present studies may be having a stabilizing effect on sperm membrane during freezing, which may be responsible for maintaining the viability of the spermatozoa even after a long period of preservation, as evidenced by 20-34% of conception rate after AI with frozen semen of the bulls under investigation (observation of PAU, dairy farm).

The deleterious effects of the freezing and thawing injury on the membrane proteins and glycoproteins of the spermatozoa were also observed in terms of alterations in the quality of proteins, which showed a great variation in the number as well as molecular weight after all durations of freezing. This may perhaps be due to the injury caused by freezing and thawing processes resulting in breakage of certain high molecular weight proteins which disappear during freezing and some of them appear as low molecular weight proteins while others again as medium or high molecular weight proteins after union of the broken molecules. These molecular changes in the structural proteins may be responsible for the damage observed in the frozen spermatozoa by ultra structural studies reported by Jones and Stewart (1979) and Ahmed et al. (1997).

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