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Early Detection of Buffalo Sperm Apoptosis at various stages of Cryopreservation

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Abstract: The cryopreservation technique adversely affects the metabolism, motility and membrane integrity of the spermatozoa, thereby decreasing the fertility potential of the processed semen sample. In current study, we investigated the effect of cryopreservation on buffalo sperm apoptosis at five different stages of cryopreservation. We employed a combination of fluorescence-based microscopic as well as flow cytometric assays and assessed the quality of spermatozoa undergoing apoptosis. Semen ejaculates from Murrah buffalo bulls were collected and cryopreserved using standard protocol. Annexin-V/Propidium Iodide (AN/PI) assay was used to detect the externalization of phosphatidylserine (PS) across the plasma membrane of buffalo spermatozoa, a marker to monitor the early apoptosis. Four different sperm subpopulations were identified: viable, early apoptotic, late apoptotic, and necrotic spermatozoa. Cooling at 4 °C and freeze-thawing significantly decreased the motility percent, plasma membrane integrity ($P<0.01$), the percentage of viable spermatozoa ($P < 0.001$), and dramatically increased the percentage of late apoptotic spermatozoa ($P < 0.01$), but not of early apoptotic spermatozoa and necrotic spermatozoa. Moreover, frozen thawed semen showed an increase in PS translocation index ($P < 0.05$) as compared to that of fresh semen. Several new classes of AN+ sperms were identified where Annexin-V stained the buffalo spermatozoa in head, midpiece and tail. Our results suggests that cooling and freeze-thawing steps during cryo-preservation result in apoptosis in buffalo spermatozoa, which might be responsible for the decreased fertilizing potential of cryopreserved spermatozoa. Efforts are therefore required to minimize cryodamage and maximize the fertility of cryopreserved buffalo spermatozoa by using various cryoprotectants.

Keywords: apoptosis; spermatozoa; cryopreservation; flow cytometry; phosphatidylserine externalization

I. INTRODUCTION

Cryopreservation of buffalo semen is widely used as a method of preserving male genome [1]. However, freezing and thawing invoke a plethora of changes in spermatozoa including capacitation-like effects [2], oxidative stress [3] reduction in integrity of the plasma and acrosome membranes [4], diminished motility, ability to penetrate cervical mucus *in vitro*[5], and apoptosis-like changes [6]. These changes impair the ability of spermatozoa to traverse the female tract and fertilize the oocyte, resulting in decreased fertility. Buffalo spermatozoa is rich in polyunsaturated fatty acids [7], therefore, it is more prone to oxidative damage as compared to cattle spermatozoa [8], resulting in relatively higher freezing and thawing associated damage and poor fertility [9]. Apoptosis contributes to the decrease of sperm vitality during cryopreservation as the proportion of apoptotic sperms in fresh and cryopreserved bull semen have been related to fertility [10]. Therefore, evaluation of apoptosis in buffalo spermatozoa is a key factor in achieving better cryopreservation results.

The sperm plasma membrane is one of the key structures affected by cryopreservation that displays apoptotic features [11-14]. During the early phases of the disturbed cell membrane, asymmetry of the membrane phospholipids occurs, before the integrity of the plasma membrane is progressively damaged [15], leading to the onset of apoptosis mechanism and eventually necrosis. Apoptosis and necrosis are two forms of cell death. Necrosis results from injury and affects large number of cells, causing cell swelling and rupture. On the other hand, apoptosis is programmed cell death that affects single cells [16]. Apoptosis comprises of three stages: initiation, execution and degradation. Externalization of phosphatidylserine (PS) from inner leaflet of plasma membrane to the outer leaflet of plasma membrane provides a chance to detect cells which are in the early stage of apoptosis [15, 17]. Recent studies in human [18], bull [10], boar [19], ram [20], equine [21], canine [22] and buffalo spermatozoa [23] have shown that cryopreservation is associated with induction of membrane PS translocation thus, indicating cryopreservation is a cause

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of apoptosis [6]. Annexin V is a Ca^{2+} dependent, phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Annexin V conjugated to fluorescein isothiocyanate (FITC) retains its high affinity for PS and, therefore, serves as a sensitive probe that can be used for flow cytometric detection of cell death. Use of Annexin V-FITC in combination with propidium iodide (PI) allows the simultaneous detection of apoptotic and necrotic sperm populations [10, 24, 25].

At present, the information regarding apoptosis in buffalo sperm at various stages of cryopreservation is scarce. Therefore, the present study was designed to detect and assess early apoptosis in buffalo spermatozoa at five different stages of cryopreservation by using fluorescence-based assays and to correlate the quality of spermatozoa undergoing apoptosis. This information could be used to improve *in vitro* semen-handling protocols, which would minimize cryo-damage and maximize the fertility of cryopreserved buffalo spermatozoa.

II. MATERIALS AND METHODS

A. *Semen collection and cryopreservation*

The Murrah buffalo bulls (3-5 years) were maintained at Central Artificial Breeding Station, Jammu, India, under uniform nutritional conditions. Semen was collected, twice a week, from three buffalo bulls using artificial vagina (IMV, France) maintained at 42 °C. Immediately after collection, mass motility of semen was assessed by light microscopy. Semen ejaculates having progressive motility more than 80% were used in this study. Each ejaculate was divided into two aliquots. One aliquot was used for fresh semen studies and the other aliquot was processed for cryopreservation. Semen was diluted in Tris-based egg yolk extender (Tris 24.2 g/L, Citric acid 13.8 g/L, Fructose 10 g/L, Egg yolk 20% (v/v), Glycerol 7%, Benzyl penicillin 10,00,000 IU/L, Streptomycin 1 g/L) to a final concentration of approximately 80 X 10^6sperms/mL. Diluted samples were aspirated into medium-size French straws (0.25 mL), sealed with polyvinyl alcoholpowder and equilibrated at 4 °C for 4 h. Immediately after equilibration, the rack containing filled semen straws was transferred to a bio-freezer (Digit cool-5300, IMV Technologies, France) where the temperature was brought down from 4 to -140 °C in 7 minutes. The freezing rate used was as follows: 6 °C/min from 4 °C to -2 °C, 4° C/min from -2 °C to -6 °C, 12 °C/min from -6 °C to -18 °C, 37 °C/min from -18 °C to -55 °C, 44 °C/min from -55 °C to -99 °C, 18 °C/min from -99 °C to -115 °C and 25 °C/min from -115 °C to -140 °C. The straws were then transferred to pre-cooled plastic goblets and plunged in to liquid nitrogen (-196 °C). After 4 weeks of storage, the straws were thawed at 37 °C for 30 secin a water bath and used immediately to analyse the semen quality parameters and externalization of PS.

B. *Evaluation of sperm quality parameters*

Motility, plasma membrane integrity and viability of buffalo semen samples was assessed at different stages of cryopreservation - fresh neat semen, cooled (4 °C) semen and frozen-thawed (FT) semen samples. For evaluation of motility, 10 µL semen was placed on a warm slide and cover slip was placed on it. The percentage of motile spermatozoa was determined by observing a minimum of 200 sperms, in at least six different fields using the bright-field microscope at X 400 [26]. The viability of spermatozoa was assessed by means of the eosin-nigrosin staining [27]. Sperm suspension (50 µL) was mixed with 5 µL of 5% eosin B and 5 µL of 10% nigrosin on a warm slide and the stain was spread with a second slide and allowed to dry. Viability was assessed by counting 200 spermatozoa under a bright-field microscope at X 400. Spermatozoa displaying partial or complete purple staining were considered nonviable and spermatozoa showing strict exclusion of stain were counted as viable. Buffalo sperm plasma membrane integrity (SPI) was assessed by using Hypo Osmotic Swelling Test (HOST) [28]. Solution for HOST consisted of 0.73 g sodium citrate and 1.35 g fructose in 100 mL distilled water (osmotic pressure = 190 mOsmol/Kg). To assess SPI, 50 µL of frozen thawed semen was mixed with 500 µL HOST solution and incubated at 37 °C for 40 min. A drop of semen sample was placed on glass slide and covered with cover slip. A minimum of 200 spermatozoa were counted per slide for different types of swelling pattern under phase contrast microscope at X 400. Sperms with swollen or coiled tails were considered intact.

C. *Evaluation of externalization of PS with* **Annexin** *V/PI assay using fluorescence microscopy*

An Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, USA) was used to assess externalization of PS according to the manufacturer's instructions with slight modifications. The Murrah bull semen samples were analysed at five different stages of cryopreservation i.e. fresh neat semen sample (A), after dilution with glycerol (B), after cooling to 4 °C for 4 h (C), after exposure to liquid nitrogen (LN_2) vapour (D) and after freeze-thawing (E). The sperm suspension collected from each stage of cryopreservation was washed three times with PBS [Phosphate Buffer Saline; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.4)] by centrifugation at 400 x g for 5 min. The final sperm pellet was resuspended in 1X Annexin V binding buffer [10 mM HEPES/NaOH (pH 7.5), 140 mM NaCl, and 2.5 mM $CaCl_2$] at room temperature to a concentration of 1 x 10^6 sperms/mL. Aliquots [100 µL each (1 x 10^5 cells)] of the sperm suspension were transferred to polypropylene tubes and 5 µL of Annexin V-FITC (AN) and 3 µL of PI were added to each sample. The tubes were mixed gently and incubated at room temperature for 10 min in the dark. After incubation, an additional 400 µL of 1X Annexin V binding buffer was added to each tube. Flow cytometric analysis

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was carried out within 5 min. The different labelling patterns in the AN/PI analysis were classified as follows: viable (AN-/PI-); viable but PS translocated (AN+/PI-); nonviable and PS translocated (AN+/PI+); and nonviable and necrotic sperm (AN-/PI+). We defined the ratio between the AN+/PI- sperm and the total living (PI-) sperm as the PS translocation index. The various patterns of sperm samples staining were confirmed by using a fluorescent microscope (Olympus BX51, Singapore).

D. ***Flow cytometric analysis***

All fluorescence signals of labelled spermatozoa were analysed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Argon laser excitation at 488 nm was coupled with emission measurements using >630-nm long pass filter (far red; propidium iodide), followed by 500- and 530-nm band pass (green) filters. Approximately 10, 000 sperm cell events were examined with a flow rate of <200 events/second and analysed using Cell Quest Pro software. Acquisition gate was applied in the forward scatter (FSC) and side-scatter (SSC) two-dimensional histogram to restrict the analysis to spermatozoa, and to eliminate small debris and other particles from further analysis. For the gated cells, the percentages of Annexin V negative or positive (AN- or AN+), and PI negative or positive (PI- or PI+), as well as double positive cells were evaluated, based on quadrants determined from single-stained and unstained control samples.

E. ***Statistical analysis***

Least-squares model was used to estimate the possible effects of cryopreservation of buffalo semen on sperm motility, plasma membrane integrity, viability, apoptosis and necrosis. Data were analysed by analysis of variance and least squares mean differences were determined by Duncan's multiple range test (DMRT). A difference with value $P < 0.05$ was considered statistically significant. Coefficient of correlation, combined at all stages for each variable was determined between different semen assays using Pearson correlation. All analysis was done using SYSTAT-12 software package [29].

III. RESULTS

A. ***Effect of cryopreservation on the semen quality parameters: motility, viability and plasma membrane integrity***

The results of sperm quality parameters in fresh, cooled and frozen-thawed semen are shown in Figure 1. The cooling phase of the cryopreservation protocol induced several significant changes in most of the semen quality parameters of buffalo spermatozoa tested. Cooling to 4 °C resulted in the decline ($P < 0.01$) in the percent motility in the cooled semen (72.50 ± 0.66) as compared to the fresh semen (86.50 ± 0.66). Motility percentage was further declined ($P < 0.01$) after freezing and thawing (45.12 ± 0.66). The viability percentage of the cooled and cryopreserved spermatozoa (74.33 ± 1.23 and 52.13 ± 1.23) declined ($P < 0.01$) due to freeze thaw procedures as compared to the fresh semen (87.66 ± 1.23). The percentage of spermatozoa with coiled tails (membrane intact) was reduced ($P < 0.01$) in cooled and cryopreserved semen (75.01 ± 1.74 and 50.21 ± 1.74) as compared to the fresh semen (85.55 ± 1.74).

B. ***Externalization of PS***

In the present study, the AN/PI analysis was used to identify the different sperm populations at five different stages of buffalo semen cryopreservation using flow cytometer (Figure 2). Annexin V/PI analysis identified four distinctive spermatozoa populations: early apoptotic spermatozoa, labelled with Annexin V-FITC, but not with PI (AN+/PI-); late apoptotic spermatozoa, labelled with both Annexin V-FITC and PI (AN+/PI+); necrotic spermatozoa, labelled with PI but not with Annexin V-FITC (AN-/PI+); and viable spermatozoa, neither labelled with Annexin V- FITC nor PI (AN-/PI-).

From the data presented in Table 1, the mean percentage of viable spermatozoa (AN-/PI-) did not differ between fresh and diluted semen samples and were 88.46 ± 0.34 and 86.66 ± 0.77, respectively. However, a significant decline ($P < 0.001$) was observed when the semen samples were cooled (4 °C), exposed to LN_2 vapour (LNV) and finally plunged in liquid nitrogen and cryopreserved for future use (81.60 ± 0.77, 74.85 ± 0.77 and 56.02 ± 0.77, respectively). Although the mean percentage of early apoptotic spermatozoa (AN+/PI-) did not differ during the entire process of cryopreservation, the percentage of late apoptotic spermatozoa (AN+/PI+) increased ($P < 0.01$) when the semen samples were cooled at 4 °C (9.35 ± 1.48), exposed to LNV (14.74 ± 1.48) and frozen-thawed (31.78 ± 1.48). Moreover, after freezing and thawing, the PS translocation index increased ($P < 0.05$) from 2.52 ± 0.41% to 6.98 ± 0.51% as compared to the fresh semen. This is in agreement with previous finding in canine [22]. On the contrary, the mean percentage of necrotic spermatozoa increased non significantly from 5.28 ± 0.40 to 8.06 ± 0.89 during the entire process of cryopreservation.

When visualized under a fluorescence microscope, Annexin V stained the buffalo spermatozoa in the head and the midpiece portion in more than 90% of the early apoptotic sperms (Figure 3). We observed several new classes of Annexin V-positive spermatozoa where Annexin V stained the sperm at various portions such as midpiece only, midpiece with tail combined, and apical area of head plasma membrane with midpiece.

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C. **Correlation between different semen assays**

Correlations between different semen assays used in this study are summarized in Table 2. Percent motility was positively correlated to proportion of viable (AN-/PI-) spermatozoa ($r = 0.98$, $P < 0.01$) and plasma membrane integrity ($r = 0.96$, $P < 0.01$) during five different stages of cryopreservation. Moreover, percentage of late apoptotic spermatozoa were negatively correlated to percent motility ($r = -0.97$, $P < 0.01$), and plasma membrane integrity ($r = -0.95$, $P < 0.01$). Percent motility and plasma membrane integrity were found negatively correlated ($P < 0.05$) to the percentage of necrotic spermatozoa ($r = -0.48$ and $r = -0.47$, respectively).

IV. DISCUSSION

Sperm cryodamage is caused by each step or a combination of the steps of cryopreservation. In the current study, we evaluated the effect of five different stages of cryopreservation on the quality of buffalo spermatozoa undergoing apoptosis. We observed that cooling and freeze-thawing significantly decreased the post thaw motility, viability and membrane integrity of buffalo spermatozoa and dramatically increased the apoptosis in the buffalo spermatozoa. We assume that this might be the reason for poor fertilization potential of frozen-thawed semen when introduced into the reproductive tract at the time of artificial insemination. Similar results were obtained in earlier studies [4, 23, 32-34]. We also identified several new classes of buffalo spermatozoa where externalization of PS was observed at different domains of the cell membrane like head, midpiece, tail and apical area of head plasma membrane. To the best of our knowledge, this is the first report, which assess the early apoptosis in buffalo sperm at five different stages of cryopreservation.

The percentage of late apoptotic spermatozoa started to increase during cooling stage and showed significant rise during exposure to liquid nitrogen vapour phase and after freezing and thawing (Table 1). Cold shock impairs the function of membrane proteins that are necessary for structural integrity or ion metabolism. Watson *et al.*, 2000 [3] reported that major changes in the bovine spermatozoa occur near 15 to 5 °C, and do not happen below 0 °C. This suggests that chilling at 4 °C greatly affected the late apoptotic sperms such that their population almost doubled each time when the cooled (4 °C) spermatozoa were exposed to the vapours of liquid nitrogen and finally, frozen-thawed. Though the percentage of late apoptotic cells increased significantly and the proportion of viable cells decreased, the percentage of early apoptotic cells remained constant during the process of cryopreservation. This suggests that the viable, Annexin V-positive spermatozoa died during freezing/thawing to become dual stained cells. At the same time, a fraction of live, Annexin V-negative cells become Annexin V-positive after cryopreservation [37]. Hence, this preliminary observation might prove the hypothesis that early apoptotic cells represent a transitory step between live and necrotic cells [38, 39]. Furthermore, the proportion of these transitory cells is dependent on conditions to which the spermatozoa are exposed during the cryopreservation process and might represent the rate at which sperm cells undergo apoptosis *in vitro*. Alterations of cell-membrane components [40] and cold shock result in reduced motility and lead to necrosis [41]. In our study, the percentage of necrotic spermatozoa showed a non-significant increase throughout the process of cryopreservation. Our results corroborate well with previous findings [18, 19, 21, 22, 42]. However, our results differ from Khan *et al.*, 2009 [23] who reported significant decline in the percentage of necrotic cells after freezing and thawing. Moreover, Khan *et al.*, 2009 [23] did not report any significant difference in motility, integrity, percentage of viable and apoptotic spermatozoa in fresh and cooled (4 °C) buffalo semen samples. We also identified PS externalization in buffalo spermatozoa around head, midpiece and tail, our results are in agreement with previous studies [18, 43, 44].

Correlation of different assays indicated that percentage of viable spermatozoa correlated positively with the percent motility and membrane integrity whereas sperm apoptosis correlated negatively with sperm motility and plasma membrane integrity. Our results are consistent with previous studies [45, 46]. Efforts are therefore required to minimize cryodamage and maximize the fertility of cryopreserved buffalo spermatozoa by using various cryoprotectants.

V. CONCLUSION

In the current study, we observed that cooling and freeze-thawing significantly decreased the quality of buffalo spermatozoa and dramatically increased the apoptosis. We also identified several new classes of buffalo spermatozoa where externalization of PS was observed at different regions of the cell membrane like head, midpiece, tail and apical area of head plasma membrane.

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Figures and Legends

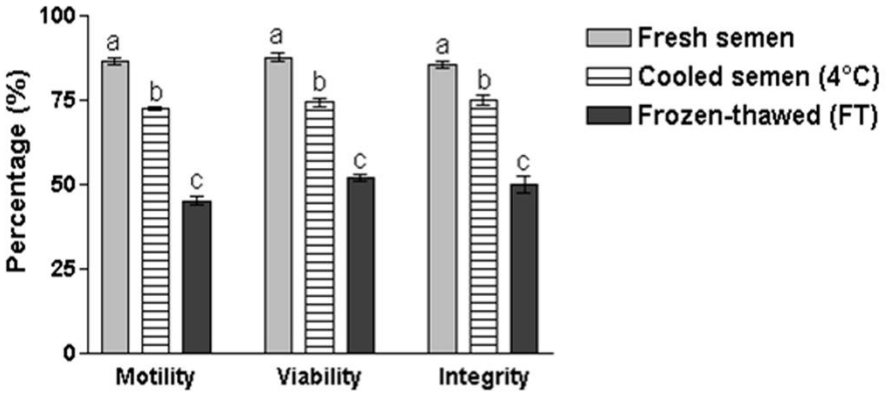

Figure 1: Sperm motility, viability and membrane integrity in fresh, cooled and frozen-thawed (FT) semen. Data are presented as LSM ± SE. [a,b,c]Dissimilar superscripts indicate significant differences between fresh, cooled and frozen-thawed semen within each parameter ($P < 0.01$).

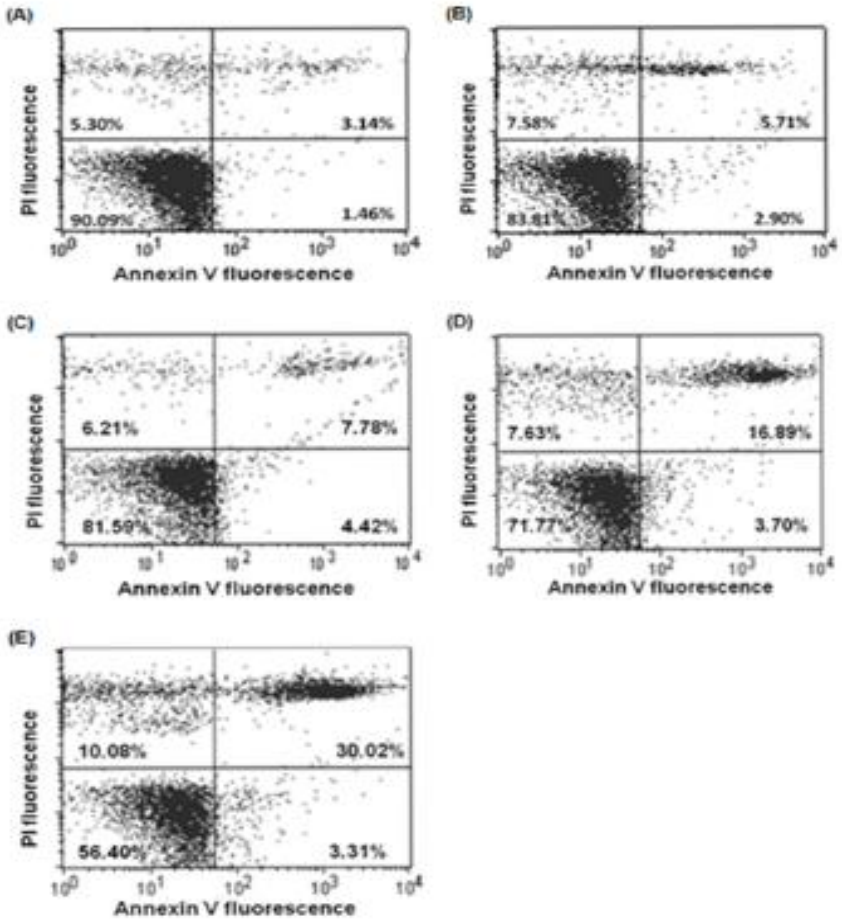

Figure 2: A representative result of one of the six independent experiments showing Annexin V (AN) / Propidium Iodide (PI) bivariate analysis used to detect apoptosis in buffalo spermatozoa during five different stages of cryopreservation. The lower left quadrant of each graph contains AN-/PI-, viable, non-apoptotic sperm. The lower right quadrant shows AN+/PI- early apoptotic sperm. The upper right quadrant represents AN+/PI+ late apoptotic sperm. The upper left quadrant contains AN-/PI+ necrotic spermatozoa. Data from flow cytometry: Fresh neat semen sample

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(A), semen diluted with glycerol (B), semen cooled to 4 °C (C), semen sample exposed to LN_2 vapour (D) and frozen-thawed semen sample (E).

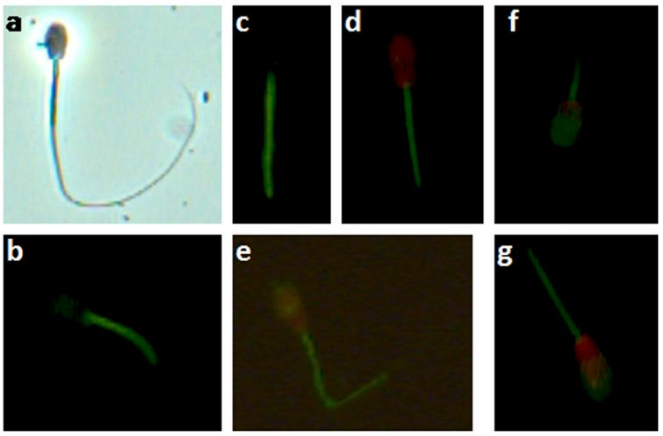

Figure 3: Localization of PS on buffalo spermatozoa : (a) A spermatozoa as seen under phase contrast microscope. (b - g) Representative patterns of Annexin V-FITC binding sites: (b) head plus midpiece, (c) midpiece, (d) midpiece along with PI in head, (e) midpiece plus tail, (f, g) apical area of head and midpiece. Magnification X 400.

Table 1: Staining of sperms with Annexin V-FITC/ Propidium Iodide (PI) during different stages of the cryopreservation process (LSM ± SE)

	AN-/PI-, % [1]	AN+/PI-, % [2]	AN+/PI+, % [3]	AN-/PI+, % [4]
Fresh semen	88.46 ± 0.34[a]	2.29 ± 0.33	3.98 ± 0.66[a]	5.28 ± 0.40
Diluted semen	86.66 ± 0.77[a]	2.86 ± 0.73	5.26 ± 1.48[a]	5.22 ± 0.89
Cooled (4 °C)	81.60 ± 0.77[b]	3.28 ± 0.73	9.35 ± 1.48[ab]	5.80 ± 0.89
LNV	74.85 ± 0.77[c]	3.55 ± 0.73	14.74 ± 1.48[c]	6.39 ± 0.89
FT	56.02 ± 0.77[d]	4.14 ± 0.73	31.78 ± 1.48[d]	8.06 ± 0.89

[1]Nonstained cells (AN-/PI-) correspond to viable spermatozoa; [2]AN+/PI- spermatozoa are early apoptotic;
[3]AN+/PI+ spermatozoa are late apoptotic; [4]AN-/PI+ spermatozoa are necrotic cells
[a,b,c,d]Different superscripts within a column indicate significant differences ($P < 0.01$)
where LNV refers to Liquid nitrogen vapour and FT refers to Frozen –thawed

Table 2: Correlation between sperm quality parameters and the Annexin V / PI binding assay

	Motility	Viability	Integrity	AN-/PI-	AN+/PI-	AN+/PI+
Viability	0.962**	-				
Integrity	0.966**	0.956**	-			
AN-/PI-	0.985**	0.941**	0.954**	-		
AN+/PI-	-0.385	-0.280	-0.270	-0.374	-	
AN+/PI+	-0.964**	-0.943**	-0.948**	-0.974**	0.234	-
AN-/PI+	-0.483*	-0.420	-0.475*	-0.520*	0.164	0.365

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level