Early apoptotic changes and acrosomal integrity of cryopreserved *Hariana* bull spermatozoa on modified cryopreservation methods

Alok Kumar[1*] Atul Saxena[2] , Mukul Anand[3], Gyandev Singh[4] and Sumit Singhal[5]
[1]Department of Veterinary Gynaecology and Obstetrics, Bihar Veterinary College, BASU, Patna. Bihar INDIA
[2] Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, DUVASU, Mathura, INDIA
[3]Department of Veterinary Physiology, College of Veterinary Science, DUVASU Mathura, UP, INDIA
[4]Department of Veterinary Clinical Complex, Bihar Veterinary College, BASU, Patna. Bihar INDIA
[5] Centre of excellence on indigenous breed, Piprakothi, Bihar, INDIA
***Corresponding author: Gyandev Singh; Email: drgyandev26079@gmail.com**

ABSTRACT

Semen cryopreservation involves two major steps i.e. cooling and freezing. These steps play a significant role in post-thaw semen quality. The objective of the experiment was to compare different cooling and cooling-freezing combinations on survival, membrane integrity, and apoptotic changes in *Hariana* bull sperm. Forty ejaculates were collected from four bulls (ten collections/bull) using an artificial vagina at the biweekly interval. Extended semen was then split into three parts and subjected to different cooling regimens i.e. from 35°C to 4°C temperature drop at 2.21°C/min (rapid), 0.48°C/min (moderate), and 0.25°C/min (slow cooling). Each cooled part was again split into three parts and subsequently subjected to different freezing rates for each group i.e. rapid (from 4°C to -10°C @ 20°C/ min), moderate (from 4°C to -10°C @ 10°C/ min) and slow (from 4°C to -10°C @ 5°C/ min) using a programmable biological freezer. Samples were evaluated at pre-freeze and post-thaw stages for % viability, motility, membrane integrity, and apoptotic changes. The result showed that slow cooling and slow cooling-freezing combination were more effective as slow cooling resulted in 73.58±2.00% viability, 70.17±1.75% acrosomal integrity, 68.83 ± 1.79% plasma membrane integrity, and 61.96±2.17% non-apoptotic sperm. Similarly, the slow-cooling slow-freezing combination showed 62.50±1.61% viability, 61.01±1.71% acrosomal integrity, 59.25±1.68% plasma membrane integrity, and 51.46±1.51% non-apoptotic sperm. In conclusion, the slower rate of cooling followed by slower freezing rates were more efficient than rapid and moderate rates for cryopreservation of *Hariana* bull semen.

Keywords: Annexin-V; Apoptosis; Cooling; Freezing; *Hariana* bull spermatozoa

DOI Number: 10.14704/nq.2022.20.8.NQ22984 **NeuroQuantology 2022; 20(8): 9662-9677**

1. Introduction

The genetic improvement, achieved through artificial insemination using cryopreserved semen of an elite bull with high production traits, could be the paramount strategy to improve the indigenous dairy cattle milk production. The cryopreservation procedure causes physical and functional damage to a large fraction of the sperm population [47]. The major detrimental effects of cryopreservation procedures include an increased number in spermatozoa with subtle changes in plasma and acrosome membranes [27], altered membrane fluidity [22], impaired calcium influx, and marked changes in the biochemical activities [22]. The decrease in temperature from 25°C to 4°C reduces the metabolic activity of the cell thus preserving the cell. The damaging effects of freezing attain critical values as the temperature reaches about –10°C [16] [17]. The increased risk of intracellular water freezing can confer ice crystals-induced cellular disruptions from mechanical injuries [16]. However, it has been demonstrated that spermatozoa can sustain a critical temperature range of -5ºC to -50°C [28] [29]. The cells will remain either in equilibrium with the extracellular plasma or will be gradually cooled which results in an increased risk of intracellular ice crystal formation and hence challenging the cell viability [44]. Physiological processes in fertilization (acrosome reaction, capacitation, fusion of sperm with ovum) needed an active membrane which explains the functional and structural integrity of sperm membranes are crucial for sperm fertilizing potential [28]. Cryopreserved semen must have sufficient spermatozoa with a full range of functions (the ability to survive, reach the oviduct, interact with the oviductal epithelium, attach to and penetrate the zona pellucida, and interact with the oocyte) to achieve satisfactory fertility. In the recent past, the apoptotic changes, the earliest stage of cell damage, in spermatozoa during the cryopreservation have been demonstrated in various animal species [33] [34]. The apoptotic changes begin at the plasma membrane level, resulting in the externalization of phosphatidylserine (PS) which is generally referred to as early apoptotic changes. The physio-biochemical activities for instance exposure to cooling and freezing during the cryopreservation, heat (thawing), radiation, acid, alkali, hydrogen peroxide, and endocrine dysfunction can induce apoptosis in the spermatozoa [1] [2] [4]. A marked negative correlation between sperm apoptosis and fertilization has been recently reported in clinical and experimental studies [32]. Moreover, sperm apoptosis could retard sperm oocyte penetration, hence, affects negatively to the conception rate [42].

Early apoptotic changes are commonly observed in the cells with intact plasma membrane thus plasma membrane integrity remains unaffected. The externalization of PS is a common phenomenon in moribund cells which creates confusion about the clear-cut differentiation of early apoptotic cells from the rest of the cell population. Therefore, the incorporation of other specific markers for the identification of spermatozoa with lost plasma membrane integrity is essential. A supravital fluorescent dye, propidium iodide (PI), serves the purpose and thus combination of Annexin-V with Propidium iodide (PI) could successfully indicate the compromised spermatozoa membrane i.e. early apoptotic with non-viable cells [25]. The apoptotic changes in the spermatozoa population due to alteration in semen pH, and thermal fluctuation during the standard cryopreservation protocols have been investigated earlier. However, to the best of our knowledge, early apoptotic changes leading to subtle changes in the bull sperm plasma

membrane by altering the rate of cooling and freezing (modification in cryopreservation protocols) have not yet been explored. Therefore, the present study aimed to determine the effect of modified cryopreservation methods on the bull spermatozoa plasma membrane integrity through detection of early apoptotic changes and the acrosomal integrity along with viability and motility of the spermatozoa in comparison with traditional cryopreservation protocol i.e. Cooling of extended semen from 35ºC to 4ºC in 65 minutes followed by equilibration in cold handling cabinet at 4°C for 4 hours then freezing of sample using biological cell freezer in following sequence i.e. from 4°C to -10°C @ 10°C /min, -10°C to -100°C @ 40°C/min, -100°C to -140°C @ 20°C/min

2. **Materials and methods**

Unless otherwise described all chemicals were purchased from Sigma Aldrich, St Louis, MO, USA. Ethical permission from the Institutional Animal Ethics Committee for the collection of semen from bulls using an artificial vagina was obtained before the start of the present study.

2.1 Animal selection

Four *Hariana* bulls of 6.5 to 7.5 years of age and 450-500 kg body weight, were enrolled in the present study for semen collection. The participated bulls were maintained at the Semen Biology Lab, Instructional Livestock Farm Complex (ILFC) of the College of Veterinary Sciences, Mathura, India. The bulls were being fed the balance ration available at the farm. The participated bulls were healthy, vaccinated against the common livestock diseases, and routinely dewormed.

2.2. Semen collection and handling

Ten ejaculates from each bull totaling forty collections were included in the present study. Semen was collected biweekly i.e. two times a week keeping 3 days rest between two collections, from each of the bulls through an artificial vagina (AV). Immediately after collection, the semen samples were transferred to the Semen Biology Lab and the ejaculates containing higher than 80% motile spermatozoa were selected for further processing.

2.3. Semen processing (initial evaluation and cooling protocol)

Several trials were carried out to standardize the protocol, for this purpose temperature drop in samples kept in a cold handling cabinet was recorded manually. The semen extended in Tris egg-yolk citrate extender with the addition of 7% glycerol as a cryoprotectant, having 80 million sperm/ml was divided into three parts and kept in measuring cylinders (25ml) which are marked as rapid, moderate, and slow. All the three portions were subjected to three cooling regimens. Sample rapid was cooled from 35°C to 4°C for 14 min with a temperature drop rate of 2.21ºC/min (extended semen at 35°C (in measuring cylinder) was kept in a 2000 ml capacity glass beaker filled with 800 ml of water at 4°C which was subsequently placed in cold handling cabinet at 4°C). Sample moderate was cooled down from 35°C to 4°C in 65 minutes, and the temperature drop rate of 0.48°C/min (extended semen at 35°C (in measuring cylinder) was kept inside a cold handling cabinet without any water jacket). Sample slow was cooled from 35°C to 4°C in 122 min, with temperature drop rate of 0.25°C/min (extended semen at 35°C (in measuring cylinder) was kept in a 2000 ml capacity glass beaker filled with 800 ml of water at 35°C which was subsequently placed in cold handling cabinet at 4°C).French mini straws (0.25 ml capacity, 135 mm length, and 2 mm diameter, (IVM, France) were used for the storage of semen samples using an automatic filling and sealing machine (IVM, France).

2.4. *Freezing of semen straws*

Each cooling protocol was subjected to equilibration at 4ºC for 4 hours followed by three different freezing under liquid nitrogen vapor in a programmable biological cell freezer (IVM Technology, France). In the first freezing protocol, the equilibrated samples were cryopreserved from 4°C to -10ºC @ 20°C/ min

(rapid freezing), in the second freezing protocol the samples were cryopreserved from 4ºC to -10°C @ 10°C/ min (moderate freezing) and in the third freezing protocol the samples were cryopreserved from 4ºC to -10°C @ 5ºC/ min (slow freezing). The rest of the freezing rates for all the samples were the same. For convenience the three freezing rates can be expressed as;

2.4.1. *Rapid freezing*
From 4ºC to -10°C @ 20°C /min
From -10ºC to -100ºC @ 40°C/min
From -100ºC to -140°C @ 20ºC/min

2.4.2. *Moderate freezing*
From 4°C to -10ºC @ 10°C /min
From -10ºC to -100°C @ 40ºC/min
From -100°C to -140ºC @ 20°C/min

2.4.3. *Slow freezing*
From 4ºC to -10°C @ 5ºC /min
From -10°C to -100ºC @ 40°C/min
From -100ºC to -140°C @ 20ºC/min

The cryopreserved straws were transferred into goblets with the help of cryo gloved hand and the goblets were stored in separate canisters in the liquid nitrogen (LN_2) in cryovessel, for a minimum 72 hours duration, the latter was always kept to the desired level of LN_2 by replenishing it time to time. The protocol involving moderate cooling followed by moderate freezing is the conventional method of bovine semen cryopreservation in our lab.

2.5. *Evaluation of semen*

The frozen semen at a concentration of 80 million/ml (20 million/straw) was thawed in a thawing unit (IVM, France) maintained at 37°C withholding time kept as 45 seconds. Semen samples were evaluated at post-equilibration (pre-freeze) and post-thaw stages for various seminal attributes viz. progressive motility, viability, sperm plasma membrane integrity, spermatozoa with intact acrosome, and apoptotic changes in the plasma membrane.

2.5.1. Progressive motility

The progressive motility of the spermatozoa was observed under a high power phase objective lens (40 X) on a thermostatically controlled stage maintained at 34-35°C**.** A small drop of diluted semen was put on a clean grease-free slide and a glass coverslip was mounted over it. The slide was examined to observe the vigorously motile spermatozoa exhibiting a progressive path. The progressive motility of spermatozoa was then calculated using mathematical formula i.e. number of progressively motile spermatozoa divided by the total no of sperm observed per field.

2.5.2. Sperm Plasma Membrane Integrity

The HOST is based on the swelling ability to function spermatozoa after being exposed to a hypo-osmotic solution. Fluid percolates into the cell through the plasma membrane of spermatozoa, trying to achieve a balance between intracellular and extracellular spaces; functionally intact membrane begins to swell starting at the tail of spermatozoa, referred to as swelled or HOS reactive/positive indicating functionally intact membranes. Spermatozoa with functionally compromised membranes do not swell and their tail does not evaginate [32]. One ml of hypo-osmotic solution, osmotic strength of 150mOsm/L was mixed with 0.1 ml of thawed semen and incubated in a water bath at 37°C for one hour. Following incubation, 20µl of the well-mixed solution was taken on a clean dry glass slide and covered with a coverslip. Sperm tail curling is recorded as an effect of swelling due to an influx of water**.** A total of 200 spermatozoa were counted in different fields at 400X magnification under a phase-contrast microscope. The spermatozoa were classified according to the presence of swelling patterns [69]. Briefly, the spermatozoa without swelling were categorized as HOS negative, whereas the spermatozoa with swelling at the midpiece region or complete tail curling were categorized as HOS positive

2.5.3. Viability of spermatozoa

One small drop of semen sample (kept at 35°C) was mixed thoroughly with 2 to 3 drops of Eosin-Nigrosin stain on a clean glass slide kept on a thermostatically warm stage (34-35ºC) and allowed to stand for 3 minutes. Further, a

smear was made from the mixture on a clean grease-free glass slide. It was dried in air and examined under the bright field 100X oil immersion objective of a phase-contrast microscope. Around 200 sperms were assessed. Sperms that were colorless (unstained) were classified as viable and those that showed pink coloration were classified as non-viable. The proportion of viable spermatozoa was calculated using mathematical formulae i.e. the number of unstained spermatozoa divided by the total no of sperm (unstained as well as pink stained) observed per field.

2.5.4. Apoptosis

Annexin V-FITC (AN-FITC) apoptosis detection kit (Catalog no- APOAF, Sigma Aldrich, USA) was used to detect the apoptotic changes in the spermatozoa membrane as per the manufacturer's specification with slight modification.

Spermatozoa were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS-calcium and magnesium-free, centrifuged at 500×g 10 minutes at 25^{0}C). Sperm pellet re-suspended in 100 µl AN binding buffer. (10 mM Hepes/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM $CaCl_2$) and concentration adjusted to approx 1×10^6 spermatozoa/ml, further transferred into 2.5 ml Eppendorf tubes then supplemented with 5µl of AN-FITC conjugate and 10 µl of the PI solution to each tube, incubated at the room temperature for 15 min in dark. Spermatozoa suspension in 10 µl volume was placed over the clean grease-free slide, covered with a glass coverslip, and examined under a phase-contrast microscope (40 X) Nikon 140 Eclipse TE 2000-S (Nikon, Japan) with epifluorescence using a dual filter, at least 200 spermatozoa per slide with the different fluorescent pattern were observed i.e. early apoptotic spermatozoa showed green fluorescence over entire sperm plasma membrane, apoptotic spermatozoa that have lost membrane integrity were showing red fluorescence (PI) throughout the head and green fluorescence (FITC) on the midpiece and tail region (plasma membrane) moreover, necrotic spermatozoa were showing red fluorescence only, were counted. (Figure 1)

2.5.5. Acrosomal Integrity

Acrosomal integrity of the bull spermatozoa was evaluated by using FITC labeled with *Pisum sativum* agglutinin (PSA) under phase contrast microscope Nikon 140 Eclipse TE 2000-S (Nikon, Japan) with epifluorescence.

Sperm suspensions were washed (centrifugation with 1000 rpm for one minute) 2-3 times with DPBS (calcium and magnesium-free DPBS) solution as traces of protein on smear react with FITC labeled PSA and produces a strong fluorescent background which makes the evaluation nearly impossible. Spermatozoa suspension in 10 µl volume was poured over a clean non-greasy slide and a thin and uniform smear was prepared. The smear prepared was air-dried and dipped in absolute methanol for 15 min and allowed to dry. The smears were further incubated for 30 min at room temperature in the dark moist chamber with FITC labeled PSA (50 µg/ml in DPBS). The slide smears were rinsed with distilled water and dipped into distilled water for 15 min to remove the excessive and unbound probe. The smears were air-dried and examined immediately, without mounting, under an epifluorescence microscope using a blue filter. A total of 200 spermatozoa per slide were counted in the prepared smear and differentiated according to the Fluorescence pattern. Spermatozoa showing bright apple green fluorescence at acrosome indicated intact acrosome (fig 2A), whereas, the absence of fluorescence or fluorescence restricted at equatorial segment indicated loss of acrosome(Figure 2B).

2.5.6. Statistical analysis

The raw data were analyzed using the statistical package SPSS V 22 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) and expressed in the form of the mean and standard error of the mean (Mean ± SEM). Comparisons were made by employing ANOVA.

*e*ISSN1303-5150 www.neuroquantology.com

3. Results

3.1. *Pre freeze evaluation*

The proportion of progressive motility, viability, HOS positive spermatozoa as well as spermatozoa with intact acrosome (FITC-PSA) differed significantly (p<0.01) amongst the different cooling rates (rapid, moderate, and slow) with maximum value observed for slow cooling (Table 1A). Four categories of sperm population were classified using AN/PI assay. The proportion of viable spermatozoa with the intact plasma membrane (AN-/PI-) and necrotic spermatozoa with subtle changes in the plasma membrane (AN+/PI+) differed significantly (p<0.01) amongst the different cooling rates. Overall mean indicated maximum proportion of AN-/PI- and minimum proportion of AN+/PI+ sperm population for slow cooling regimen. The proportion of spermatozoa with early apoptotic changes (AN+/PI-) did not differ significantly amongst different cooling rates. However, the proportion of only non-viable spermatozoa (AN-/PI+) differed significantly (p<0.05) amongst different cooling rates and was lowest in the slow cooling process (Table 1B). Among the studied protocols, the slow cooling group had the least subtle changes.

3.2. *Post-thaw evaluation.*

3.2.1. *Different cooling combinations with Rapid freezing*

Progressive motility, viability, HOS positive, and spermatozoa with intact acrosome (FITC-PSA), differed significantly (p<0.01) when compared among the different cooling protocols subjected to rapid freezing (rapid cooling-rapid freezing, moderate cooling-rapid freezing, and slow cooling-rapid freezing). Maximum values for aforesaid parameters were observed in a slow cooling-rapid freezing combination (Table 2A).

Assay (AN/PI) demonstrated the viable spermatozoa with the intact plasma membrane (AN-/PI-) and necrotic spermatozoa with the subtle changes in the plasma membrane (AN+/PI+) differed significantly (p<0.01) for different cooling combinations with rapid freezing and maximum and minimum values of above-mentioned categories observed for slow cooling-rapid freezing combination. Values indicating early apoptotic spermatozoa (AN+/PI-) did not differ significantly within the group. However, the proportion of only non-viable spermatozoa (AN-/PI+) differed significantly (p<0.05) for different cooling combinations with rapid freezing. The least damage was observed with the slow cooling-rapid freezing combination (Table 2B). Overall values indicate that the plasma membrane incurred the least early apoptotic changes in the slow cooling-rapid freezing group, however, variations in early apoptotic changes were not recorded among the studied cooling protocols.

3.2.2. Different cooling combinations with Moderate freezing

Progressive motility, viability, HOS positive spermatozoa, and the spermatozoa with intact acrosome (FITC-PSA) differed significantly (p<0.01) amongst the different cooling protocols with moderate freezing. Maximum values for aforesaid parameters were observed for slow cooling-moderate freezing combination (Table 3A).

Assay (AN/PI) demonstrated the viable spermatozoa with the intact plasma membrane (AN-/PI-) and necrotic spermatozoa with subtle changes in the plasma membrane (AN+/PI+) differed significantly (p<0.01) for different cooling with moderate freezing combinations. The maximum and minimum values of the above-mentioned categories were observed for the slow cooling-moderate freezing combination. Values indicating early apoptotic spermatozoa (AN+/PI) did not differ significantly for different cooling with moderate freezing combinations. However, values of only non-viable spermatozoa (AN-/PI+) differed significantly (p<0.05) for different cooling combinations with moderate freezing. The least damage was observed with the slow cooling-moderate freezing combination (Table 3B). Overall values indicate that plasma membrane damage was least in the slow cooling-moderate freezing group, moreover, early apoptotic

changes do not show much variation amongst all combinations.

3.3.3. Different cooling combinations with slow freezing

Progressive motility, viability, HOS positive, as well as spermatozoa with intact acrosome (FITC - PSA), differed significantly ($p<0.01$) amongst the different cooling with slow freezing protocols. The maximum values for the above-mentioned parameter were observed for the slow cooling-slow freezing combination (Table 4A), indicating that the present group exerts the least damage on basic sperm physiology.

Assay (AN/PI) resulted in viable spermatozoa with the intact plasma membrane (AN-/PI) and non-viable sperm with subtle changes in the plasma membrane (AN+/PI+) differ significantly ($p<0.01$) for different cooling combinations with slow freezing. Maximum and minimum values of the above-mentioned categories were observed for the slow cooling-slow freezing combination. The proportion of spermatozoa with the only subtle changes in the membrane (AN+/PI-) did not differ significantly for different cooling combinations with slow freezing. However, values of only non-viable spermatozoa (AN-/PI+) differed significantly ($p<0.05$) for different cooling combinations with slow freezing. The least damage is observed for the slow cooling-slow freezing combination (Table 4B). Overall values indicate that the plasma membrane was least affected in the slow cooling-moderate freezing group, moreover, early apoptotic changes do not show much variation amongst all combinations.

Post-freeze-thaw evaluation section all together consists of nine different protocols. Out of all, the rapid cooling-rapid freezing group seems to be more damaging whereas the slow cooling-slow freezing group succumbs to limited cell damage.

4. Discussion

Cryopreservation involves temperature reduction for the preservation of spermatozoa which exerts physical and biochemical modification causing loss of seminal attributes [13, 47]. Similarly ROS production during semen processing causes nonlethal damage which affects fertilizing potency of spermatozoa [64].

Our study demonstrated a significant difference in the proportion of progressively motile spermatozoa for the three cooling rates at the pre-freeze stage. Drop-in progressively motile spermatozoa were observed between rapid to moderate and moderate to slow cooling. Slow cooling yielded maximum progressively motile spermatozoa. Rapid (2.22°C/min) and moderate (0.48°C/min) cooling of extended *Hariana* bull semen between 35°C to 4°C should not be a choice for cryopreservation as it could result in maximum cold shock damages. The decrease in motility following cooling indirectly reflects mitochondrial damage of spermatozoa, ATP needed for oxidative phosphorylation could not be available [25] and thus attributed to poor spermatozoa motility [10] [13] [13]. It was further addressed that rapid cooling from 30°C to 15°C has no major impact on spermatozoa however when the condition continues for 10°C or below the effect are harmful [26] [30]. Decrease in temperature up to 5°C leads to change in the fluidity of plasma membrane due to phase transition of lipid within plasma membrane from liquid to semisolid or gel-like state. The re-organization of membrane lipids elicits significant sperm damage, resulting in aberrant or complete loss of motility, impaired energy generation, reduced metabolic activity, and acrosomal as well as plasma membrane damage. All these changes are referred to as cold shock [47]. The cooling rate significantly influences the degree of cold shock. The rapid rate of cooling causes maximum cold shock damage [12] whereas slow cooling from37°C to 5°C has been associated with better seminal attributes [10] [16]. Our study evaluated the maximum proportion of viable spermatozoa with slow cooling. The trend was similar to

progressive motility. Our findings are in agreement with Medeiros et al [38], who demonstrated that slow cooling results in less damage to the viable spermatozoa. However, the test for the viability of spermatozoa does not provide the functional integrity of the membrane. HOS test is a further indicator of potential sperm plasma membrane function and is considered to be related to the fertilizing ability of the spermatozoa [7] [8]. In the present study, the significant difference in HOS reactive spermatozoa was observed amongst different cooling groups. Thus, slow cooling could be a better option as it preserved more number of viable spermatozoa with a functionally active membrane. Our findings are in agreement with the report of Keel and Webster [36] where a slow rate of cooling resulted in better viability and plasma membrane integrity.

A true acrosome reaction, which precedes fertilization, occurs only in acrosome intact spermatozoa. Several techniques have been proposed to differentiate acrosome intact from acrosome-reacted spermatozoa. In the present study, we employed FITC–PSA to differentiate the intact acrosome from reacted one [22] [23] and hence is a more accurate index for the intact acrosome. The acrosome membrane suffers mostly from cold shock and is accountable for fertility failure [8]. Our result for the effect of cooling revealed that the slow cooling group contains more acrosome intact spermatozoa compared to rapid and moderate cooling at the pre-freeze as well as post-thaw stage and such finding has been reported by other workers too [5].

Cold shock results in the change of lipid orientation of the plasma membrane which is also an early indication of cell death (apoptosis). The early sign of apoptosis is characterized by the translocation of phosphatidylserine (PS), from its normal location on the inner leaflet of the plasma membrane to the outer side of the membrane bilayer [6] [24]. Apoptotic and necrotic changes can be identified through observing externalization using AN/PI assay by flow cytometry or fluorescence microscopy. Observing our data for the pre-freeze stage evaluation using AN/PI assay provides results towards the lower side, thus providing a clear picture of reorganization of lipids within sperm plasma membrane which in turn indicates the functional status of the membrane. The cooling process has a detrimental effect on the spermatozoa plasma membrane and the adoption of rapid, moderate, or slow cooling results in a total loss (dead, acrosome reacted, and subtle changes) of spermatozoa. Thus the subtle change in the plasma membrane is evident as proposed by other workers [18] [47]. Generally, changes in plasma membrane integrity and motility are both indicators of sperm viability and metabolic activity [40] [41]. When different cooling rates were subjected to rapid freezing, the post-freeze thaw results for all the parameters under study were found to differ significantly, however, a minimum freezing loss for all the parameters was observed with the slow cooling-rapid freezing combination. Moreover, the losses to spermatozoa were maximum between 35°C to 4°C temperature, and between these stages, the cooling rate has a pronounced effect. Thus our study suggests that slow freezing with slow cooling will be the better protocol for cryopreserving the *Hariana* bull semen. Cooling is a major stressor, as a result of which membrane-bound phospholipids reorient themselves into a different configuration that disrupts membrane function and permeability [9] [3] [31]. Drop-in temperature occurs during the cooling process and sperm react to this temperature change by manifesting a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoa motility, and an increase in the number of dead spermatozoa. The severity of the cold shock depends upon the final temperature and the rate of temperature drop. The cellular damage resulting from cooling or freezing affects both the structure and function of the cells [39] [43]

[45]. Our results are in agreement with Kumar et al [37] who reported that losses to spermatozoa during cooling from 35°C to 4°C were maximum for extended *Hariana* bull semen. Kamber et al [27] demonstrated that if cooling time is increased for bringing extended semen from 30°C to 5°C then the post-thaw motility was maximum for delayed cooling rather than rapid cooling [35].

E.A.R. Dias et al 2018 [12], observed that Using a programmable freezer high freezing rate (50°C/min) between -15 to -60°C and a lower rate (30-40°C/min) till the end, using a programmable bio cell freezer provided good results. In the present study, a slower rate of freezing i.e. temperature drop of 5°C/min between 4°C to -10°C resulted in better cryo-survival. Our results are in agreement with Kumar et al [29] who employed three freezing rates (-1°C/min, -30°C/min, and -50°C/min) for freezing the extended semen from -5°C to -50°C. He observed that -30°C/min was best in terms of preserving intact acrosome character. Similarly Dies et al [12] observed better post-thaw seminal attributes on slow freezing rate i.e. -15°C/minutes in comparison with -19°C/minutes [46]. Contrary to that Forero-Gonzalez et al [15] observed non-significant differences between rapid -19°C/min and slow -15°C/min freezing rates on sperm cryo-survival, moreover provision of equilibration for one hour and the presence of different levels of cryo-protectant should also be considered before interpretation of results [15]. Thus our study demonstrates that for cryopreserving *Hariana* bull semen, slow cooling from 35°C to 5°C in 122 minutes, followed by equilibration of 4 hours duration [11], and subsequent freezing of semen from 4°C to -10°C @ of 5°C/min, from -10°C to -100°C @ of 40°C/min and from -100°C to -140°C @ of 20°C/min is a better choice compared to the other cooling and freezing rates.

5. Conclusion.

Cooling and freezing rates have profound effects on various seminal attributes i.e. viability, progressive motility, acrosomal integrity as well as subtle changes of the plasma membrane and its integrity. In the present study, we observed slow rate of cooling (35°C to 4°C in 122 minutes @ 0.25ºC/min) followed by slow rates of freezing (From 4°C to -10ºC @ 5°C /min,from -10°C to -100°C @ of 40°C/min and from -100°C to -140°C @ of 20°C/min, finally plunging in LN_2) confer less subtle changes thus more functional spermatozoon, as compared to other protocols.

Acknowledgment

The author expresses his sincere regards to, the Hon'ble Vice-Chancellor, DUVASU Mathura INDIA for providing the necessary facilities to carry out the research work.

References

[1] R.J. Aitken, M.A. Baker, Causes and consequences of apoptosis in spermatozoa, contributions to infertility and impacts on development, Int. J. Dev. Biol. 57 (2013) 265-272.

[2] R.J. Aitken, A.J. Koppers, Apoptosis and DNA damage in human spermatozoa, Asian J.Androl. 13 (2011) 36–42.

[3] L. Anel, P. de. Paz, M. Alvarez, C.A. Chamorro, J.C. Boixo, A. Manso, M. Gonzalez, M. Kaabi, E. Anel, Field and inVitro assay of three methods for freezing ram semen,Theriogenology 60 (2003) 1293-1308.

[4] M. Anzar, L. He, M.M. Buhr, T.G. Kroetsch, K.P. Pauls, Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol. Reprod. 66 (2002) 354-360.

[5] G.P. Byrne, P. Lonergan, M.J. Wade, P. Duffy, A. Donovan, J.P. Hanrahan, M.P. Boland, Effect of freezing rate of ram spermatozoa on subsequent fertility in vivo and in vitro, Anim. Reprod. Sci. 62 (2000) 265-275.

[6] S. Chatterjee, E. de Lamirande, C. Gagnon, Cryopreservation alters membrane sulfa hydroxyl status of bull spermatozoa:

protection by oxidized glutathione, Mol. Reprod. Dev. 60 (2001) 498–506.
[7] Padrik, P., Hallap, T., Kaart, T., Bulitko, T., & Jaakma, Ü. Relationships between the results of hypo-osmotic swelling tests, sperm motility, and fertility in Estonian Holstein dairy bulls. Czech J. Anim. Sci, (2012). 57(10), 490-497.
[8] N. Deneke, A. Lemma, T. Yilma, Study on the efficiency of conventional semen evaluation procedure employed at Kaliti National artificial insemination center and fertility of frozen-thawed bull semen. As part of M.Sc. thesis, Faculty of Veterinary Medicine, Addis Ababa University, Ethiopia 2010.
[9] S. Desagher, J.C. Martinau, Mitochondria as the central control point of apoptosis, Trends in cell biology 10 (2000) 369-377.
[10] A.J. Dhami, K.L. Sahni, A comperative evaluation of certain processing procedures (cooling, equilibration, and thawing in deep freezing) of cattle and buffalo semen under tropical climate, Ind. J. Anim. Sci. 64 (1994) 933-938.
[11] Dhami AJ, Sahni KL. Evaluation of different cooling rates, equilibration periods and diluents for effects on deep-freezing, enzyme leakage and fertility of bull spermatozoa, Theriogenology 40(1993) 1269-1280.
[12] E.A.R. Dias, S.P. Campanholi, G.F. Rossi, C.P. F. DellAqua, J.A. DellAqua junior, F.O. Papa, M.F. Zorzetto, C.C.P. dePaz, L.Z. Olivera, M.E.Z. Mercadante, F.M. Monteiro, Evaluation of cooling and freezing system of bovine semen, Anim. Rep. Sci. 195 (2018) 102-111
[13] N.K. Duru, M. Morshedi, A. Schuffner, S. Oehninger, Cryopreservation-thawing of fractionated human spermatozoa and plasma membrane translocation of phosphatidylserine,Fertil. Steril. 75 (2001) 263–268.
[14] M.R. Fernandez-Santos, M.C. Esteso, A.J. Soler, V. Montoro, J.J. Garde, Effects of Egg Yolk and Cooling Rate on the Survival of Refrigerated Red Deer (Cervuselaphushispanicus) Epididymal Spermatozoa,Reprod. Dom. Anim. (41) 2006 114–118.
[15] R.A. Farero-Gonzalez, E.C.C. Celeghini, C.F. Raphael, A.F.C. Andrade, F.F. Bressan, R.P. Arruda. Effect of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia 44 (2012) 154-159.
[16] D. Gao, P. Mazur, J.K. Critser, Fundamental cryobiology of mammalian spermatozoa. In: Karow AM, Critser JK (eds.), Reproductive Tissue Banking. San Diego: Academic Press. (1997) 263-327.
[17] R.H. Hammerstedt, J.C. Andrews, Metabolic support of normothermia. In: Karow AM, Critser JK (eds.), Reproductive Tissue Banking. San Diego: Academic Press. (1997) 139-166.
[18] R.H. Hammestedt, J.K. Graham, J.P. Nolan, Cryopreservation of mammalian sperm: what we ask them to survive, J.Androl. 11(1990) 73–88.
[19] J.L. Hancock, A staining techniques for the study of temperature shock in semen, Nature (London) 169(1951) 323-326.
[20] R.A.P. Harrison, B.M. Gadella, Membrane changes during capacitation with special reference to lipid architecture. In: Fenichel P, Parinaud J, editors. Human sperm acrosome reaction. Paris. (1995) 45–65.
[21] W.V. Holt, Basics aspects of frozen storage semen, Anim. Reprod. Sci. 62(2000) 3-22.
[22] J. Jankovičová, M. Simon, J. Antalíková, L. Horovská, Acrosomal and viability status of bovine spermatozoa evaluated by two staining methods, Acta Veterinaria Hungarica 56 (2008) 133–37.
[23] J. Jankovičová, M. Simon, J. Antalíková, Methods for evaluation of an acrosome reaction of bovine spermatozoa, Acta fytotechnicaet zootechnica. memorial cisco (2006) 118 -119

[24] A. Januskauskas, A. Johannisson, F. Rodriguez, H. Martinez, Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 60 (2003) 743- 758.

[25] A. Januskauskas, H. Zilinskas, Bull semen evaluation post-thaw and relation of semen characteristics to bull's fertility. Vet. Zootec. 17(2002) 29 – 36.

[26] R.C. Jone, D.L. Stewar, The effects of cooling to 50^{0}C and freezing and thawing on the ultrastructure of bull spermatozoa, J. Reprod. Fertil. 56 (1979) 233-238.

[27] D. Kamber, G.B. Öztürk, U. Cirit, H.H. Bozkurt, A. Aktaş, S. Birler, A.K. Kemal, S. Pabuccuoğlu, Effects of Cooling Rate on Membrane Integrity and Motility Parameters of Cryopreserved Ram Spermatozoa. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 21(2015) 61-67.

[28] V.R. Upadhyay, V. Ramesh, R.K. Dewry, G. Kumar, K. Raval, P. Patoliya, Implication of Cryopreservation on Structural and Functional Attributes of Bovine Spermatozoa, Andrologia 53(2021) 01-16

[29] S. Kumar, J.D. Millar, P.F. Watson. The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines. Cryobiology 46(2003) 246–253.

[30] B. Leboeuf, B. Restall, S. Salamon, Production and storage of goat semen for artificial insemination, Anim. Reprod. Sci. 62(2000) 113–141.

[31] C.S. Lessard, P.L. Parent, J.L. Bailey, R. Sullivan, Cryopreservation alters the levels of the bull sperm surface protein P25b, J. Androl. 21(2000) 700-707.

[32] C. Marchetti, M.A. Gallego, A. Defossez, P. Formstecher, P. Marchetti,stainingof human sperm with fluorochrome-labeled inhibitor of caspases to detect activated caspases: correlation with apoptosis and sperm parameters, Hum.Reprod. 19(2004) 1127–1134.

[33] G. Martin, N. Cagnon, O. Sabido, B. Sion, G. Grizard, P. Durand, R. Levy, Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa, Hum.Reprod. 22 (2007) 380–388.

[34] G. Martin, O. Sabido, P. Durand, R. Levy, Cryopreservation induces an apoptosis like mechanism in bull sperm, Biol. Reprod. 71(2004) 28-37.

[35] I.C.A. Martin, Effect of rate of cooling to 5°C, storage at 5°C, glycerol concentration, sodium chloride, fructose and glycine on the revival of deep-frozen bull spermatozoa, J. Agric. Sci. 64(1965) 425 – 432.

[36] A.C. Mathur, G.S. Bishta, G. Mohan, K.L. Sahni, Development of some rapid test freezing methods for predicting the freezability of bovine semen, Ind. J. Anim. Reprod. 12(1991) 6-9.

[37] A. Kumar, A. Saxena, M. Anand, Effect of Cooling Rates on Cryopreserved Hariana Bull Spermatozoa, J. Anim. Res 8(2018) 149-154.

[38] C.M. Medeiros, F. Forell, A.T. Oliveira, J.L. Rodrigues, Current status of sperm cryopreservation: why isn't better, Theriogenology 57(2002) 327-344.

[39] M. Muratori, M. Luconi, S. Marchiani, G. Forti, E. Baldi, Molecular markers of human sperm functions, Int. J. Androl. (32) 2009 25-45.

[40] J.E. Parks, J.K. Graham, Effects of cryopreservation procedures on sperm membranes, Theriogenology 38 (1992) 209–222.

[41] J.E. Parks, D.V. Lynch, Lipid composition and thermo tropic phase behavior of boar, bull, stallion, and rooster sperm membranes, Cryobiology 29 (1992) 255-266.

[42] T. Said, A. Agarwal, S. Grunewald, M. Rasch, T. Baumann, C. Kriegel, L. Li, H.J.

Glander, A.J. Thomas Jr, U. Paasch, Selection of non-apoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: an in vitro model. Biol. Reprod. 74(2006) 530–537.

[43] M.K. Samplaski, A. Agarwal, R. Sharma, E. Sabanegh, New generation of diagnostic tests for infertility: review of specialized semen tests, Int. J. Urol.17 (2010) 839-847.

[44] J. Saragusty, H. Gacitua, M.T. Pettit, A. Arav, Directional freezing of equine semen in large volumes, Reprod. Dom. Anim. 42(2007) 610–615.

[45] P.F. Silva, B.M. Gadella, Detection of damage in mammalian sperm cells, Theriogenology 65(2006) 958-978.

[46] R. Talevi, S. Pelosi, G. Sansone, F. Graso, D. Matasino, Effect of different pre-freezing rates on buffalo sperm motility and ultrastructure preservation. In: Vale, WG, Barnabe VH, Mattos JCA, Proceedings of 4th World Buffalo Cong. Sao Paulo, Brazil. International Buffalo Federation, Rome Italy (1994) 537–539.

[47] P.F. Watson, The causes of reduce fertility with cryopreserved semen, Anim. Reprod. Sci. 60(2000) 481-492.

Table 1(A). Seminal characteristic with respect to different cooling protocol. (Mean ± SEM)

Seminal characteristic	Rapid cooling	Moderate cooling	Slow cooling
Progressively motile spermatozoa (%)	46.67^{c} ±1.72	56.46^{b} ±1.92	65.42^{a} ±2.08
Viable spermatozoa (%)	53.17^{c} ±1.78	64.08^{b} ±1.83	73.58^{a} ±2.00
Spermatozoa with intact acrosome (%)	52.75^{c} ±1.82	64.67^{b} ±1.88	70.17^{a} ±1.75
HOS Positive spermatozoa (%)	49.58^{c} ±1.53	61.21^{b} ±1.69	68.83^{a} ±1.79

Mean bearing different superscripts (a,b,c) differs significantly in a row

Table 1(B). Annexin V/PI assay showing effect of the different cooling protocol. (Mean ± SEM)

Annexin V/PI assay	Rapid cooling	Moderate cooling	Slow cooling
Double negative (AN-/PI-) spermatozoa (%)	43.38^{c}±1.55	54.79^{b} ±2.23	61.96^{a} ±2.17
Annexin V positive AN+/PI-) spermatozoa (%)	12.25 ±0.91	11.75 ±0.77	12.54 ±1.06
PI positive (AN-/PI+) spermatozoa (%)	14.04^{a} ±0.91	11.92^{ab} ±0.99	9.50^{b} ±0.96
Double positive (AN+/PI+) spermatozoa (%)	30.25^{a} ±1.64	21.54^{b} ±1.46	16.21^{c} ±1.12

●Means bearing different superscripts (a,b,c) differed significantly within the row

Table 2(A). Effect of different cooling rates (Rapid, Moderate and Slow) with rapid freezing on seminal characteristic following thawing of *Hariana* bulls semen. (Mean ± SEM)

Seminal characteristic	Rapid cooling-rapid freezing	Moderate cooling-rapid freezing	Slow cooling-rapid freezing
Progressively motile spermatozoa (%)	31.88^{c}±1.53	41.25^{b} ±1.54	47.92^{a} ±1.44
Viable spermatozoa (%)	37.67^{c} ±1.50	48.25^{b} ±1.85	54.54^{a} ±1.49
Spermatozoa with intact acrosome (%)	37.54^{b} ±1.25	50 .00^{a} ± 2.10	53.29^{a} ±1.61
HOS Positive spermatozoa (%)	34.38^{c} ±1.62	45.46^{b} ±1.86	51.38^{a} ±1.55

Mean bearing different superscripts (a,b,c) differs significantly in a row

Table 2(B). Annexin V/PI assay showing the effect of different cooling rates (Rapid, Moderate and Slow) with rapid freezing, following thawing of *Hariana* bulls semen. (Mean±SEM)

Annexin V/PI assay	Rapid cooling-rapid freezing	Moderate cooling-rapid freezing	Slow cooling-rapid freezing
Double negative (AN-/PI-) spermatozoa (%)	29.25^{c} ±1.39	39.83^{b} ±1.45	44.25^{a} ±1.60
Annexin V positive AN+/PI-) spermatozoa (%)	10.54 ±0.93	11.50 ±0.65	11.79 ±0.94
PI positive (AN-/PI+) spermatozoa (%)	18.79^{a} ±1.13	16.67^{ab} ±0.99	14.67^{b} ±1.01
Double positive (AN+/PI+) spermatozoa (%)	41.42^{a} ±1.22	33.00^{b} ±1.17	29.29^{c} ±1.33

● Means bearing different superscripts (a,b,c) differs significantly within row

Table 3(A). Effect of different cooling rates (Rapid, Moderate and Slow) with moderate freezing on seminal characteristic following thawing of *Hariana* bulls semen. (Mean±SEM)

Seminal characteristic	Rapid cooling-Moderate freezing	Moderate cooling-moderate freezing	Slow cooling-moderate freezing
Progressively motile spermatozoa (%)	32.29^{c} ±1.59	41.04^{b} ±1.53	48.54^{a} ±1.66
Viable spermatozoa (%)	38.79^{c} ±1.52	48.92^{b} ±1.64	56.04^{a} ±1.84
Spermatozoa with intact acrosome (%)	38.92^{c} ±1.48	49.42^{b} ±1.60	54.75^{a} ±1.93

HOS Positive spermatozoa (%)	35.04[c] ±1.51	44.88[b] ±1.42	53.67[a] ±1.74

- Mean bearing different superscripts (a,b,c) differs significantly in a row

Table 3(B). Annexin V/PI assay showing the effect of different cooling rates (Rapid, Moderate and Slow)with moderate freezing, following thawing of *Hariana* bulls semen. (Mean±SEM)

Annexin V/PI assay	Rapid cooling-moderate freezing	Moderate cooling-moderate freezing	Slow cooling-moderate freezing
Double negative (AN-/PI-) spermatozoa (%)	29.33[c] ±1.16	40.71[b] ±1.40	46.50[a] ±1.93
Annexin V positive AN+/PI-) spermatozoa (%)	11.83 ±0.86	10.75 ±0.56	11.71 ±0.86
PI positive (AN-/PI+) spermatozoa (%)	17.50[a] ±0.89	15.25[ab] ±0.94	14.17[c] ±1.01
Double positive (AN+/PI+) spermatozoa (%)	41.33[a] ±1.17	33.29[b] ±1.10	27.63[c] ±1.44

- Means bearing different superscripts (a,b,c) differs significantly within a row

Table 4(A). Effect of different cooling rates (Rapid, Moderate and Slow) with slow freezing on seminal characteristic, following thawing of *Hariana* bulls semen. (Mean±SEM)

Seminal characteristic	Rapid cooling-slow freezing	Moderate cooling-Slow freezing	Slow cooling-slow freezing
Progressively motile spermatozoa (%)	36.04[c] ±1.47	44.58[b] ±1.59	58.38[a] ±1.79
Viable spermatozoa (%)	43.50[c] ±1.49	54.13[b] ±1.58	62.50[a] ±1.61
Spermatozoa with intact acrosome (%)	43.00[c] ±1.43	55.00[b] ±1.73	61.00[a] ±1.71
HOS Positive spermatozoa (%)	39.63[c] ±1.48	50.38[b] ±1.76	59.25[a] ±1.68

- Mean bearing different superscripts (a,b,c) differs significantly in a row

Table 4(B). Annexin V/PI assay showing the effect of cooling rates (Rapid, Moderate, and Slow) with slow freezing, following thawing of *Hariana* bulls semen. (Mean ± SEM)

Annexin V/PI assay	Slow freezing-rapid cooling	Slow freezing-moderate cooling	Slow freezing – slow cooling
Double negative (AN-/PI-) spermatozoa (%)	34.54[a] ± 1.27	44.79[b] ± 1.35	51.46[a] ± 1.51
Annexin V positive AN+/PI-) spermatozoa (%)	11.04 ± 0.82	11.25 ± 0.76	12.38 ± 0.70
PI positive (AN/PI+) spermatozoa (%)	17.00[a] ± 1.00	15.88[a] ± 0.82	12.75[b] ± 1.01

Double positive (AN+/PI+) spermatozoa (%)	37.33^{a} ± 1.34	28.08^{b} ± 1.16	23.46^{c} ± 1.19

●Means bearing different superscripts (a,b,c) differed significantly within row

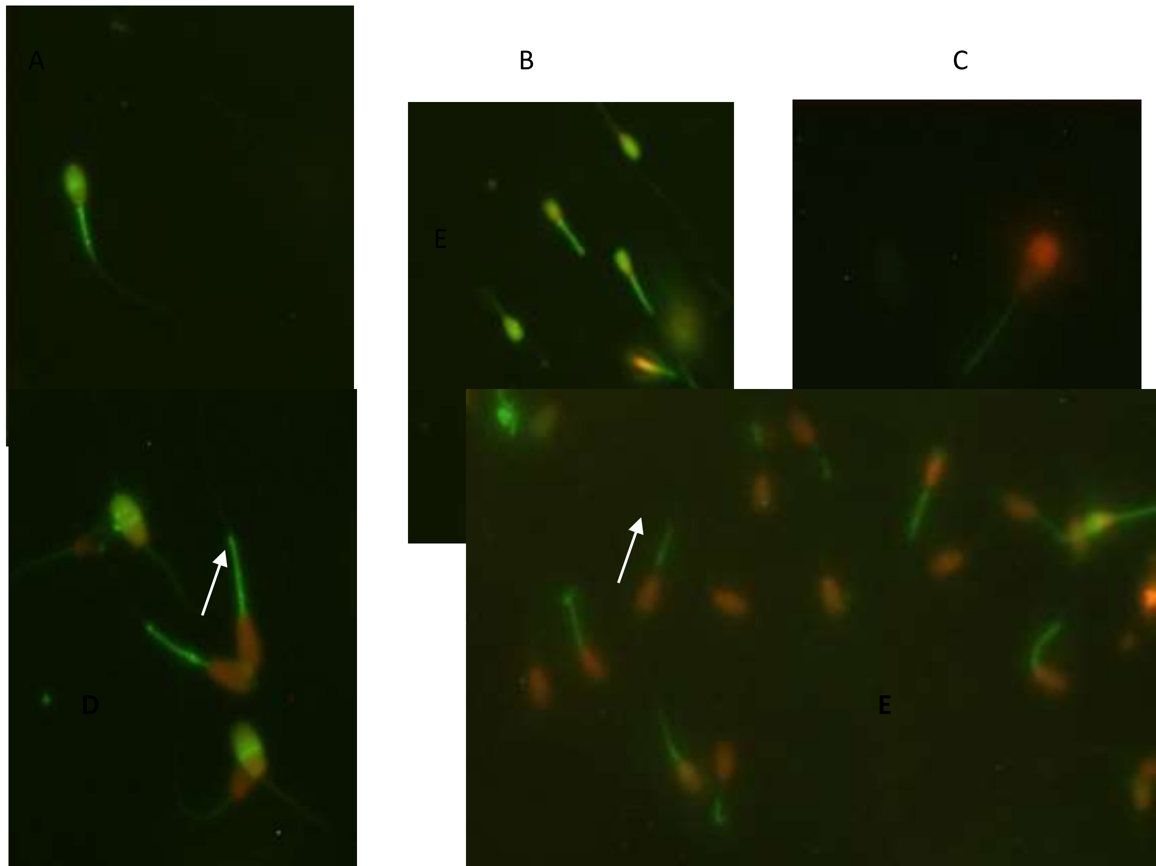

Figure 1. Photomicrograph showing Annexin-V/PI assay for plasma membrane integrity (A) & (B) showing green fluorescence over entire spermatozoa (AN+/PI-)**;** (C) & (D); Red and green fluorescence (AN+/PI+); (E) white arrow showing red fluorescence over head region only (AN-/PI+)

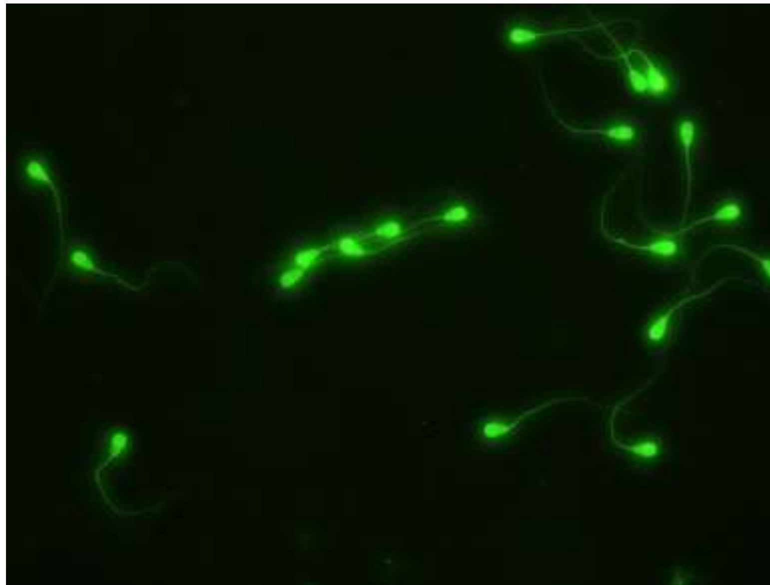

Fig 2A. Photomicrograph showing bright green fluorescense indicates intact acrosome.

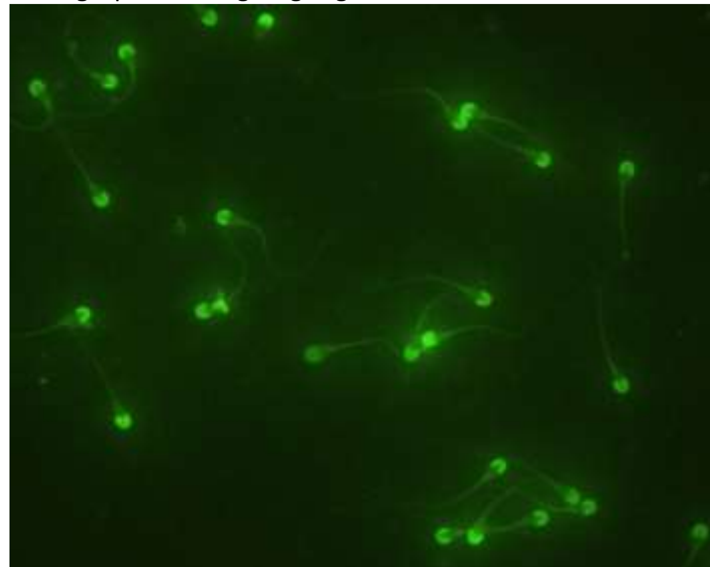

Figure 2(B). Photomicrograph showing absence of fluorescence or fluorescence restricted at equatorial segment indicated loss of acrosome