

Cryocapacitation and Apoptosis Like Change in Haryana Bull Spermatozoa at Different Stages of Freezing and Thawing

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Abstract Proposed study was carried out to evaluate the acrosomal integrity, capacitation, acrosome reaction (AR) and mitochondrial transmembrane potential (MTP) during different stages of semen freezing and thawing in Haryana bull semen. Forty ejaculates were collected from four Haryana bulls during the period of study. Each ejaculate was analyzed after each step of initial and final dilution with standard Glycerolated Egg Yolk Tris (GEYT) extender, freezing

in liquid N₂ for 24 h and after thawing. Acrosome integrity was evaluated by Fluorescent Isothiocyanate-Pisum Sativum Agglutinin (FITCOPSA) staining, whereas, capacitation status was evaluated by Chlotetracycline (CTC) assay and MTP was evaluated by using commercial Miticapture JC I kit. Significant ($p < 0.05$) reduction in percent spermatozoa having intact acrosome was seen after thawing as compared to initial and final diluted semen samples, while, significant ($p < 0.05$) increase in percent of capacitated spermatozoa (B-pattern) along with acrosome reacted (AR-pattern) spermatozoa were observed after thawing. Spermatozoa after thawing revealed significant ($p < 0.05$) decrease in percent of spermatozoa having high MTP. Freezing-thawing as well as different stages of sperm processing found to induce capacitation like changes in bull spermatozoa along with increased probability of apoptosis as evident from low MTP. Suitable strategies are required to overcome the cryocapacitation and apoptosis like changes in sperm cells opted for freezing and thawing.

Keywords Spermatozoa, Haryana bull, Chlortetracycline, Cryopreservation, Mitochondrial transmembrane potential.

Introduction

Cryopreservation of semen is used for long term storage of spermatozoa and serves as an important tech-

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nique for dissemination of superior germplasm among the farm animals [1]. Ultra-low temperature storage of semen makes the spermatozoa quiescent, metabolically inert with simultaneous induction of various intracellular and membrane alterations. Most of the reported changes are irreversible and damaging to spermatozoa which reduce their fertilizing competence [2].

Successful mammalian fertilization requires an intact acrosome to exhibit exocytosis of its contents during acrosome reaction and sperm-oocyte interaction [3]. This interaction leads to release of enzymes which lyses the limiting layers, complexes and membrane of the oocyte. The process leads to the penetration of spermatozoa head and its fusion with oocyte to form zygote. Therefore, the intactness of acrosome is one of the key factors which decide the future and the fate of fertilization [4]. Cryopreservation induces irreversible alterations in sperm membrane with the efflux of membrane cholesterol, change in membrane fluidity, reduction in cholesterol and phospholipid ratio, and eventually leads to induction of intracellular signaling events causing cryocapacitation [5].

The process of freezing and thawing also induce generation of free radicals which are highly reactive and primarily target the membrane phospholipids. With their interaction, there is onset of lipid peroxidation events which cause membrane damage in sperm cells [6]. Free radical mediated oxidative stress targets the membrane lipids, acrosome, membrane proteins, and spermatozoa chromatin causing induction of apoptosis in spermatozoa. Premature capacitation like changes in the spermatozoa during freezing and thawing events shortens the life span of spermatozoa and also compromises the fertilizing ability of spermatozoa [7].

Studies have demonstrated stage and time dependent alterations in spermatozoa during freezing and thawing process in terms of cryocapacitation [5, 7]. Induction of apoptosis during freezing and thawing are also mediated by generated free radicals which cause peroxidative damage to the mitochondria membrane and thereby reduce transmembrane mitochondrial potential (MTP). With the reduction in MTP, there is release of mitochondrial enzymes which initiate the

process of apoptosis [8, 9].

Apoptosis like changes in the sperm cells include translocation of phosphatidyl serine to the outer leaflet of plasma membrane, activation of caspases, fragmentation of DNA and lower inner transmembrane potential. Mitochondria serve as the major regulator of sperm cell apoptosis by altering its transmembrane potential and leakage of mitochondrial enzymes. Studies have shown that lowering of transmembrane potential acts as the potential initiator of apoptosis [10, 11]. Looking to the scenario of climate change, indigenous animals have been given priority in terms of conservation and propagation of germplasm. Semen cryopreservation and artificial insemination are the best tools for the dissemination of germplasm. Haryana bulls are indigenous cattle breed and are best breeds for draught. No studies have been conducted till date up to our best of knowledge regarding cyocapacitation like changes during different stages of semen processing in these Haryana bulls along with the dynamics of mitochondrial transmembrane potential. Looking to this, the proposed study was designed with two major objectives, first, to study the acrosome intactness along with capacitation, and acrosome reaction and secondly, to study the inner mitochondrial transmembrane potential (MTP) of spermatozoa in Haryana bull spermatozoa during different stages of sperm processing for freezing.

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Materials and Methods

Ethical permission

No ethical permission was required to conduct the study as the method of semen collection was non-invasive. Due care has been taken not to provide any stress to the bulls during the period of semen collection by artificial vagina (AV).

Table 1. Percent of spermatozoa showing intact acrosome (%) in hariana bull spermatozoa at different stages of cryopreservation (mean \pm SE, n=40). (Means with different superscripts (lower case) differ significantly ($p < 0.05$) in between the rows).

Bulls	Fresh stage	After final dilution	Post thaw (After 24 h)
H-531	81.09 ^a \pm 0.98	70.07 ^b \pm 1.15	55.62 ^c \pm 1.09
H-533	79.75 ^a \pm 1.10	71.15 ^b \pm 0.92	52.00 ^c \pm 1.03
H-580	79.95 ^a \pm 1.40	70.09 ^b \pm 1.34	52.93 ^c \pm 1.60
H-623	79.19 ^a \pm 0.96	70.73 ^b \pm 1.53	51.95 ^c \pm 1.11
Overall (n=40)	79.49 ^a \pm 0.57	70.76 ^b \pm 0.62	52.86 ^c \pm 0.65

Experimental animals

Present study was conducted on four Hariana bulls of the aged between 5.5 - 6.5 years and weighing 450–500 kg. The bulls were reared at the University Instructional Livestock Farm Complex (ILFC), College of Veterinary Sciences, Up Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura which is situated in a semiarid zone of Northern part of India, in the state of Uttar Pradesh. Bulls were fed as per the standard guidelines of firm. Bull were apparently free of infection and were vaccinated as per the prescribed manual of certified semen laboratory.

Semen collection

Semen samples were collected twice a week during January to March. Semen was collected directly into a clean dry graduated centrifuge tube attached to the latex cone of the AV. Immediately after collection, tube containing semen were marked, transferred to the laboratory and placed in the water bath at 32–34°C for physio-morphological studies.

Evaluation of seminal attributes

The collected semen samples were evaluated (as per the standard guidelines framed in the semen biology laboratory, DUVASU, Mathura) and those which fulfill the criteria for cryopreservation were extended with Glycerolated Egg Yolk Tris (DEYT) extender. Three stages of spermatozoa evaluations were carried out

Table 2. Percent of spermatozoa showing different CTC patterns in hariana bull spermatozoa at different stages of cryopreservation (mean \pm SE, n=40). (F pattern- Percent uncapacitated spermatozoa; B pattern- Percent capacitated spermatozoa and AR pattern -percent acrosome reacted spermatozoa). (Means with different superscripts (lower case) differ significantly ($p < 0.05$) in between the rows; Means with different superscripts (upper case) differ significantly ($p < 0.05$) in between the columns).

Stages of Cryopreservation	CTC Pattern (Overall n=40)		
	F-Pattern	B-Pattern	AR-Pattern
Fresh stage	79.09 ^{aA} \pm 0.57	20.07 ^{bA} \pm 0.50	0.84 ^{cA} \pm 0.10
After final dilution	69.97 ^{aB} \pm 0.58	27.87 ^{bB} \pm 0.52	2.27 ^{cB} \pm 0.10
Post thaw (After 24 h)	52.09 ^{aC} \pm 0.71	44.2 ^{bC} \pm 0.71	3.94 ^{cC} \pm 0.16

viz., (i) Neat/fresh semen, (ii) after final dilution with extender and (iii) after 24 h of cryopreservation and thawing. Acrosome intactness was evaluated by FITC-PSA staining and capacitation patterns were evaluated by CTC assay. The sperm mitochondrial transmembrane potential (MTP) was evaluated by using Mitocapture JCI kit as per kit specifications.

Processing of semen for greezing

The extended semen samples were taken for cryopreservation using a biological freezer (liquid nitrogen vapour freezing). These samples were processed for cryopreservation as per the standard protocol (lowering of temperature from 4°C to -10°C @ 5°C/min., -10°C to -100°C @ 40°C/min., -100 to -140°C @ 20°C/min) developed in the semen biology laboratory, DUVASU, Mathura [12].

Post-thaw evaluation of spermatozoa

After 24 h of cryopreservation, the straws containing the semen were thawed in thawing unit (IMV, France) maintained at 37°C with holding time kept as 45 seconds. After thawing the straws were processed for retrieval of the spermatozoa which was followed by evaluation of acrosome intactness, different patterns of CTC showing capacitation and mitochondrial trans-

membrane potential (MTP).

Evaluation of acrosomal status (Fluorescent Isothiocyanate – pisum sativum agglutinin, FITC-PSA labeling)

Acrosomal integrity was evaluated by highly sensitive FITC-PSA labeling technique as described earlier for bucks with little modification for bulls [13]. Spermatozoa at a concentration of ($1 \times 10^6/\text{ml}$) were suspended in PBS (Ca^{2+} and Mg^{2+} free) and the spermatozoa samples were centrifuged at $300 \times g$ for 5 minutes. Spermatozoa samples were washed three times in PBS ($\text{pH}=7.4$). After three washings, final spermatozoa pellet was resuspended in PBS ($\text{pH}=7.4$). Ten μL of the sample was taken and a thin and uniform smear was made on a clean grease free glass slide. The slide was allowed for air drying. Air dried slides were fixed in cold methanol for 15 minutes and then allowed for air drying. Air dried slides were incubated with FITC ($100 \mu\text{g}/\text{ml}$ in PBS, $\text{pH}=7.4$) for 30 minutes at room temperature in dark. Slides were removed and air dried. After air drying, slides were observed using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under FITC/blue filter. Two hundred spermatozoa were differentiated according to the fluorescence pattern of their acrosome (bright fluorescence acrosome- intact, no fluorescence or only fluorescence of the equatorial segment means loss of Scrosome).

Evaluation of capacitation status (Chlortetracyclin, CTC Assay)

Capacitation status and acrosome reaction were assessed using Chlortetracycline (CTC) staining as described earlier with little modification [14]. Chlortetracycline (CTC) binds with membrane calcium, whose distribution appears to change through capacitation, and is readily visualized by fluorescence microscopy. Hence, Chlortetracycline (CTC) distribution pattern seems to be related to the capacitation stage as well as status. Briefly, spermatozoa at a concentration of ($1 \times 10^6/\text{ml}$) were suspended in TALP and were centrifuged at $200 \times g$ for minutes. The collected spermatozoa pellet was washed three times with TALP and final pellet of spermatozoa was resuspended in 0.5 ml of TALP to serve as the spermatozoa stock solution.

CTC stock solution was prepared by dissolving 1 mg of CTC in 1 ml of PBS ($\text{pH}=7.4$). 250 μL of spermatozoa suspension was mixed with 250 μL of CTC stock solution and was incubated in the incubator in dark at 37°C for 30 minutes. 5 μL of CTC labeled spermatozoa suspension was taken on a clean grease free slide and loaded with antifading medium followed by cover slip. Slides were observed using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm by using $40 \times$ objective). A total of 200 spermatozoa per slide were observed and different patterns of spermatozoa were evaluated as established in literature. Three different forms of CTC pattern were observed namely, a. F pattern: Even distribution of fluorescence over the entire head (uncapacitated spermatozoa; Pattern F), b. B pattern: Fluorescence-free band in the post acrosomal region (capacitated, acrosome intact spermatozoa; pattern B), fluorescence in anterior portion of the head, c. AR pattern: Fluorescent free head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells, pattern AR), In all cases, fluorescence in the middle piece of the flagellum was observed as well.

Evaluation of mitochondria transmembrane potential

Mitochondria transmembrane potential was evaluated by using Mito Capture Apoptosis detection kit (Merck-Millipore, Germany). The standard protocol specified the kit and as described earlier [15] with little modifications was followed for the evaluations of the sperm MTP. The kit employs highly sensitive fluochrome JC I for the detection of mitochondria transmembrane potential. With a higher inner transmembrane potential, JC I accumulates inside mitochondria and emits orange red fluorescence, whereas, a low transmembrane mitochondria potential causes JC I to get its monomer form and fluoresces green. Slides were observed using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm by using $40 \times$ objective). A total of 100 sperm per slide were observed and different patterns of sperm were evaluated as per the site of fluorescence on the sperm.

Table 3. Percent of spermatozoa showing high mitochondrial transmembrane potential (TMP) (%) in Hariana bull spermatozoa at different stages of cryopreservation (mean \pm SE, n=40). (Means with different superscripts (lower case) differ significantly ($p < 0.05$) in between the rows).

Bulls	Fresh stage	After final dilution	Post thaw (After 24 h)
H-531	87.29 ^a \pm 1.90	74.28 ^b \pm 1.65	57.68 ^c \pm 1.49
H-533	89.70 ^a \pm 1.40	76.25 ^b \pm 1.94	56.84 ^c \pm 1.23
H-580	88.90 ^a \pm 1.80	77.46 ^b \pm 1.44	58.90 ^c \pm 1.40
H-623	87.28 ^a \pm 1.96	76.68 ^b \pm 1.83	57.94 ^c \pm 1.60
Overall (n=40)	87.49 ^a \pm 1.60	74.86 ^b \pm 1.62	57.18 ^c \pm 1.68

Statistical analysis

Analysis of variances was carried out by comparing the means and significance was tested at 5% level ($p < 0.05$). The data obtained were presented as Mean \pm SE. Mean values were compared by using 2-way ANOVA with Post Hoc Tukey Test by using SPSS 16.1 version, Chicago, USA.

Results and Discussion

Percent of spermatozoa showing intact acrosome was evaluated at 3 different stages of semen processing and results obtained from the study have been presented in Table 1. Semen samples of all the four bulls exhibited significant ($p < 0.05$) difference of percentage spermatozoa having intact acrosome at fresh stage, after final dilution and post thaw after 24 h of freezing. When the bull ejaculates were compared for overall mean, similar trend was seen at all the three stages of semen processing. There was significant ($p < 0.05$) reduction in the percent of sperm having intact acrosome after the process of thawing.

Chlortetracycline fluorescent staining was carried out to evaluate the non capacitated (F), capacitated (B) and acrosome reacted (AR) spermatozoa and the results are given in Table 2. The fresh semen (initial dilution) exhibited significantly ($p < 0.05$) high percent of F-pattern of spermatozoa as compared to B and AR pattern. Similar trend of capacitation pattern was seen in spermatozoa samples (after final dilution) and post thaw. F-pattern of spermatozoa was signifi-

cantly ($p < 0.05$) higher in fresh spermatozoa as compared to after final dilution and post thaw. Both B-pattern and AR-pattern of spermatozoa was significantly ($p < 0.05$) higher in post thaw semen samples.

Percent spermatozoa having high transmembrane mitochondrial potential was significantly ($p < 0.05$) decreased in post thaw sperm as compared to spermatozoa of fresh semen with initial dilution and final dilution and final dilution (Table 3).

Present study evaluated acrosomal integrity, capacitation status and mitochondrial transmembrane potential (MTP) in Hariana bull semen opted for ultra low freezing. Significant loss of spermatozoa with intact acrosome was observed in post thaw semen as compared to neat semen with initial dilution and after final dilution. Percent of sperm showing B-pattern (capacitated) in CTC stain was significantly higher in post thaw sperm cells.

Acrosome serves as a limiting structure of fertilization and its intactness decides the fate of fertilization. In spite of advances in the techniques of sperm cryopreservation, there is 30 to 50% loss of acrosome damage occurs during freezing and thawing of sperm in different species of animals. Loss of acrosome intactness makes the sperm incapable of bringing fertilization and there by serves as one of the most common reasons of poor conception after artificial insemination with frozen semen [3]. Oxidative stress generally leads to loss of motility, swelling and blebbing of the acrosomal membrane [16]. The process of handling of semen, initial and final dilution, and stages of freezing are the major factors responsible for generation of free radicals which target the acrosome and sperm membrane to bring peroxidative damage. Due to very less antioxidative protection of the sperm cells, the sperm cells show free radial mediated acrosome damage.

From the study, it was evident that, cooling and thawing induced acrosomal damage as reported earlier in bulls and other species of animals [7, 17]. Results of the present study are similar to the earlier [18—20] in bulls. Cryocapacitation occurs during freezing and thawing process which is characterized by premature acrosome reaction and reduction in func-

tional viability of spermatozoa in female genital tract [5]. The capacitation-like changes in spermatozoa affect the fertility of cryopreserved semen, by rendering the cells less stable in the reproductive tract, after artificial insemination and therefore relatively short-lived. In the present study, different patterns of spermatozoa were seen after staining them by CTC. Significant increase in B and AR pattern indicated that freezing and thawing process induced capacitation and acrosome reaction.

Free radicals generated during the process of freezing, thawing along with changes in temperature, liquid gel transitions are responsible for induction of morphological changes in the spermatozoa. With the progression of freezing process, spermatozoa membrane exhibits change in membrane fluidity due to efflux of cholesterol; membrane becomes permeable to Ca^{++} occurs to the spermatozoa cells. Intracellular pH increases and flagellar reorganization of proteins and phosphorylation of tyrosine containing proteins lead to cryocapacitation. Similar changes have occurred during the process of freezing during our study and therefore B and AR pattern significantly increased after thawing as compared to sperm with initial and final dilution.

Kadirvel et al. [8] reported that cryopreservation and thawing of buffalo spermatozoa induced capacitation like changes and these changes were associated with reduction in cholesterol level, increase in intracellular Ca^{++} and increase in fluidity of sperm plasma membrane after cryopreservation and thawing.

During apoptosis, reduction in mitochondrial transmembrane potential (MTP) results due to the opening of membrane pores present in the mitochondria membrane. The open pores allow the transfer of various pro-apoptotic factors leading to activation of caspases [21]. Change in mitochondrial transmembrane potential also causes leakage Bax and Bel-2 proteins into cytoplasm of spermatozoa inducing apoptosis.

Freezing and thawing processes induce apoptosis like changes in the spermatozoa [10, 11]. In our study, we observed significant reduction in the percent of

spermatozoa having high MTP and we suggested that it was due to the different stages of freezing. Similar to our results. Mortin et al. [11] reported increased percent of spermatozoa were having lower mitochondrial transmembrane potential due to cryopreservation of bovine spermatozoa while, Khan et al. [22] reported spoptosis in fresh and cryopreserved buffalo spermatozoa. Gadea et al. [18, 19] reported significant reduction in MTP in cryopreserved bull spermatozoa. The process of cooling and equilibration did not increase spermatozoa with apoptosis like changes significantly, however, after freezing and thawing the number of spermatozoa showing apoptosis like changes increased to 40% as against 25% during equilibration.

Conclusion

Capacitation and acrosome reaction in post thaw spermatozoa were significantly higher as compared to neat and extended semen. Higher percentage of spermatozoa exhibited lower inner transmembrane potential indicating apoptosis like changes in sperm cells. Combination of acrosomal damage and compromised transmembrane mitochondrial potential reduce the spermatozoa quality during freezing and thawing. Suitable strategies are warranted to develop extenders which will provide cryoprotection as well as will enhance the quality of semen opted for artificial insemination.

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