Original Research

Cryopreservation Induced Sperm Cryoinjuries in Hariana Bull Semen

Chetna Gangwar[1*], Atul Saxena[2], Sarvajeet Yadav[3], S. P. Singh[4], Vijay Singh[5] and Akhil Patel[5]

[1]AP & R Division, ICAR- Central Institute for Research on Goats, Makhdoom, Mathura-281122, Uttar Pradesh, INDIA
[2]Department of Animal Reproduction & Gynaecology, College of Veterinary Sciences, DUVASU Mathura, Uttar Pradesh, INDIA
[3]Department of Physiology, College of Veterinary Sciences, DUVASU, Mathura, Uttar Pradesh, INDIA
[4]Department of Anatomy, College of Veterinary Sciences, DUVASU, Mathura, Uttar Pradesh, INDIA
[5]Deptt of ARGO, CVSc & Animal Husbandry, SVPUA&T Meerut, Uttar Pradesh, INDIA

***Corresponding author:** chetnaom82@gmail.com

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Abstract

Cryopreservation causes breach in the plasma membrane which culminates into loss of membrane permeability, change in membrane fluidity, exudation of acrosomal enzymes, and modification in mitochondrial membrane potential. Keeping these views in mind the current study was conducted to evaluate the apoptosis like changes or cryoinjuries in spermatozoa during cryopreservation. In the present study semen from four Hariana bulls after dilution was evaluated for various parameters, thereafter semen was cryopreserved. Various sperm characteristics were further evaluated 24 hrs after cryopreservation. The findings of the current study revealed that there is significant reduction in progressive motility, viability, acrosomal integrity, plasma membrane integrity and mitochondrial membrane potential of spermatozoa after semen cryopreservation. Thus, we concluded that cryopreservation induced cryoinjuries in spermatozoa and investigation regarding mechanism involved in these changes is key factor to improve the success of semen cryopreservation.

Key words: Cryoinjuries, Cryopreservation, Hariana Bull, Semen

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Introduction

Artificial insemination is arguably the most important tool contributing to the advancement of modern animal production (Ombelet and Robays, 2010). Although, research on semen preservation has a long history over centuries, the problem regarding restriction of post-thawed survival to about 50% or even less of sperm population still persists even with best cryopreservation techniques available to date. Moreover, most of the viable spermatozoa have alerted characteristics, which differentiate them from spermatozoa before cryopreservation (Watson, 2000). At present it is generally accepted that the sequelae of the sperm injury caused by cryopreservation procedures are impaired sperm transport, reduced longevity and poor survival in the female reproductive tract compared to the fresh spermatozoa (Salamon and Maxwell, 1995). To optimize AI and to maximize conception rate following AI, not only good quality of semen but also a very good post thaw quality of semen is required in achieving the target of AI.

Inspite of its great practical benefits for reproduction, the process of cryopreservation and thawing inflicts certain damage to spermatozoa and its organelle. This vandalism includes swelling and breach in plasma membrane, loss of membrane selective permeability (Francisco *et al.*, 2016), change in membrane fluidity, rearrangement of phospholipids and protein, diminution in motility and enzyme activity and viability which results in adverse changes in membrane lipid conformation, acrosome status and vitality. It also causes an increase in sperm DNA damage, reduction or removal of certain transcripts and changes related to apoptosis. More recently an apoptosis-like phenomenon has been identified (Agrawal and Said, 2005; Kadirvel *et al*., 2012). This describes not only cellular death but also the divergent degree of subtle cellular damages that most of the surviving population of spermatozoa encounters after thawing. The mechanism behind cryodamage or apoptosis may be due to osmotic stress, cold shock, intracellular ice crystal formation, excessive production of reactive oxygen species, modification in anti-oxidant defense systems and amalgamation of these conditions (Saeid *et al.*, 2016). These changes during cryopreservation prejudicially affect the viability and the fertilizing capability of bull spermatozoa (Gangwar *et al.*, 2018). Therefore, there is a need to debate in depth the major component influencing the successful cryopreservation of cattle bull semen. Further exploration regarding mechanism involved thereof and the inhibition of early apoptosis will hopefully lead to the enhancement of the fertility of cryopreserved semen.

Material and Methods

Experimental Animals

Present study was conducted on four Hariana bulls of the age group of 5.5–6.5 years and weighing 450-500 kg, reared at the Semen Biology Lab, University Instructional Livestock Farm Complex (ILFC), College of Veterinary Sciences, U. P. Pandit Deen Dayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura which is located in a semi-arid zone of

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Northern part of India, in the state of Uttar Pradesh. The experiments were carried out in accordance with the guidelines set out by the Institute Ethics Committee.

Semen Collection

Semen was collected biweekly interval from individual bull with the help of separate artificial vagina (AV) on dummy. Each bull was given one false mount before actual collection. Semen was collected directly into a clean dry graduated centrifuge tube attached to the latex cone of the AV. Immediately after collection, tubes containing semen were marked and placed in the water bath at 35°C for further evaluation.

Evaluation of Semen

Semen was evaluated for following seminal attributes.

Live and Dead Spermatozoa Count

The method described by Bloom (1950) and Hancock (1951) was followed. One small drop of semen sample (kept at 35°C) was mixed with 2 to 3 drops of Eosin-Nigrosin stain on a clean glass slide kept on a thermostatically warm stage (34-35ºC). This mixture was kept for 2 min. Then a smear was made from the mixture on a clean and grease free glass slide. It was dried in air and examined under the bright field 100× oil immersion objective of phase contrast microscope. Around 200 sperms were assessed. The sperms that were colorless (unstained) were classified as live and those that showed any grade of pink colour were classified as dead.

Progressive Motility

The progressive motility of the spermatozoa was observed under high power phase objective lens (40×) on a thermostatically controlled stage maintained at 34-35^{0}C**.** A small drop of diluted (1:100) semen was put on a clean grease free slide and was covered with a cover slip. The slide was examined to observe vigorously motile spermatozoa exhibiting progressive path.

Plasma Membrane Integrity

Plasma membrane integrity is judged by the hypo osmotic swelling test (HOS). The HOS test is based on swelling ability of functioning sperms after being exposed to hypo-osmotic solution. Spermatozoa with functionally defective membrane do not swell and their tail does not invaginate by Jayendran *et al.* (1984). One ml of hypo-osmotic solution, having an osmotic strength of 100 mOsm/L was mixed with 0.1 ml of semen and incubated in a water bath at 37°C for one hour. Following incubation, a drop of well mixed solution was taken on a clean dry glass slide and covered with a cover-slip. Sperm tail curling is recorded as an effect of swelling due to influx of water. A total of 200 spermatozoa were counted in different fields at 400× magnification under phase contrast microscope.

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Acrosomal Integrity (FITC-PSA Assay)

The acrosomal integrity of the spermatozoa was detected using Fluorescence isothiocyanate – Pisum sativum agglutinin (FITC-PSA) staining procedure as standardized by Mendoza *et al.* (1992). Sperm suspension used for preparation of smear for FITC-PSA were washed 2-3 times with DPBS (protein, calcium and magnesium free DPBS) solution. The smear prepared was air dried and dipped in absolute methanol for 15 minutes and then allowed to dry rapidly. Methanol treated smears were then incubated for 30 minutes at room temperature in dark moisture chamber with FITC labeled PSA (50 µg/ml in DPBS). The slides were then washed in distilled water (rinsed with distilled water and further dipped in distilled water for 15 minutes) to remove unbound probe. The smear was air dried and examined immediately under a fluorescence microscope. At least 200 spermatozoa were counted in the prepared smear and differentiated according to the fluorescence pattern of their acrosome.

Evaluation of Capacitation Status by Chlortetracycline Assay (CTC Assay)

Capacitation status and acrosome reaction were assessed using Chlortetracycline (CTC) staining. The method as described earlier by Fraser *et al.* (1995) for goats, was used with slight modification. Chlortetracycline (CTC) binds with membrane calcium, whose distribution appears to change during capacitation, and is readily visualized by fluorescence microscopy. Slides were observed using Nikon Eclipse TE 2000-S microscope under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm by using 40× objective). A total of 200 sperm per slide were observed and different patterns of sperm were evaluated as established in literature.

Evaluation of Mitochondrial Transmembrane Potential

Mitochondrial transmembrane potential was evaluated by using Mito Capture TM Apoptosis detection kit (Sigma, France). For mitochondrial transmembrane potential JC-1, assay was used. The method used was little modification over the method as earlier described by Garner *et al.* (1994). 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. Working solution of JC-1 was prepared by dissolving JC-1 in DMSO (1mg/ml). After 3 times washing of sperm suspension in DPBS, 0.3 µL of JC-1was added in 500 µL of sperm suspension and incubated for 30 min in dark. 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× objective). A total of 200 sperm per slide were observed and different patterns of sperm were evaluated as per the site of fluorescence on the sperm.

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Evaluation of Sperm Viability through SYBR-14/ PI Assay

For cell viability and plasma membrane integrity, SYBR-14 and PI assay was used. The method used was little modified from earlier described by Garner *et al.* (1994). SYBR-14 penetrates the sperm independently of the membrane integrity status, while PI only stains cells with compromised membrane integrity. Working solution of SYBR green was prepared by dissolving SYBR green in HEPES buffer (1mg/ml). After 3 times washing of sperm suspension in DPBS, 5µL of SYBR green was added in 500 µL of sperm suspension and incubated for 15 min in dark. Then 2 µL of PI was added in the suspension and incubated for 10 min. Slides were observed using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under 40× objective. A total of 200 sperm per slide were observed. The percent cell viability was determined by counting the number of green cells out of a 100 cells in a field.

Results and Discussion

Sperm Viability

The overall mean of live spermatozoa in the fresh ejaculate of Hariana bull was 87.09±0.67 percent. The respective value in equilibrated and post thaw semen were 83.38±0.66 and 65.59±0.99 percent. A significant (P<0.01) decrease was observed at equilibrated and post thaw stage (Table 1).

Table 1: Percent progressive viability, motility and HOS response of spermatozoa in fresh, equilibrated and post thaw semen of Hariana bull (Mean ± SEM = 32)

Parameters	Fresh	Post Equilibration	Post Thaw
Live spermatozoa (%)	$87.09^{c}\pm 0.67$	$83.38^{b}\pm 0.66$	$65.59^{a}\pm 0.99$
Progressive Motility (%)	$83.28^{c}\pm 0.91$	$75.46^{b}\pm 0.88$	$57.03^{a}\pm 0.77$
HOS reactive spermatozoa (%)	$79.00^{c}\pm 0.61$	$72.85^{b}\pm 0.57$	$61.56^{a}\pm 0.91$

Means with small alphabet differ significantly (P < 0.05) within row.

Progressive Motility

The overall mean progressive motility of spermatozoa in the fresh ejaculate of Hariana bull was 83.28±0.91 percent. The respective value in equilibrated and post thaw semen were 75.46±0.88 and 57.03±0.77 percent. A significant (P<0.01) decrease was observed at equilibrated and post thaw stage (Table 1).

Acrosomal Integrity (FITC-PSA)

The overall mean of spermatozoa with intact acrosome was 83.75±0.54 and 76.21±0.59 percent at fresh and post thaw stage respectively. A significant (P<0.01) decrease was observed at post thaw stage (Table 2, Fig. 1).

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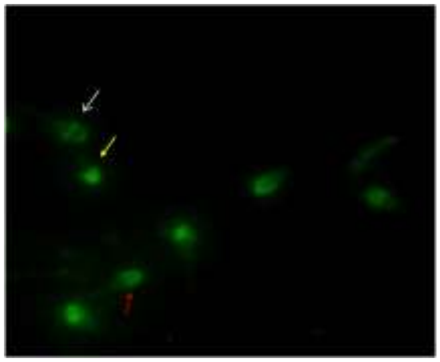

Fig. 1: Photomicrograph showing acrosomal integrity of spermatozoa in Hariana bull semen (FITC-PSA Stain, Magnification 40X); Intact acrosome (red arrow); Damaged acrosome (yellow arrow)

Hypo Osmotic Swelling Test (HOST)

The overall mean of hypo osmotic swollen spermatozoa in the fresh, equilibrated and post thaw semen of Hariana bull were 79.00±0.61, 72.85±0.57 and 61.56±0.91 percent respectively. A significant ($P<0.01$) decrease was observed at equilibrated and post thaw stage (Table 1).

Mitochondrial Membrane Potential

The overall mean of spermatozoa with active mitochondria in fresh semen of Hariana bull was 81.66±0.71 and overall mean of spermatozoa with inactive mitochondria was 18.13±0.64 percent. The respective values in post thaw semen were 59.90±0.75 and 40.09±0.77 percent. A significant ($P<0.01$) decrease was observed in active mitochondria at post thaw stage (Table 2; Fig. 2).

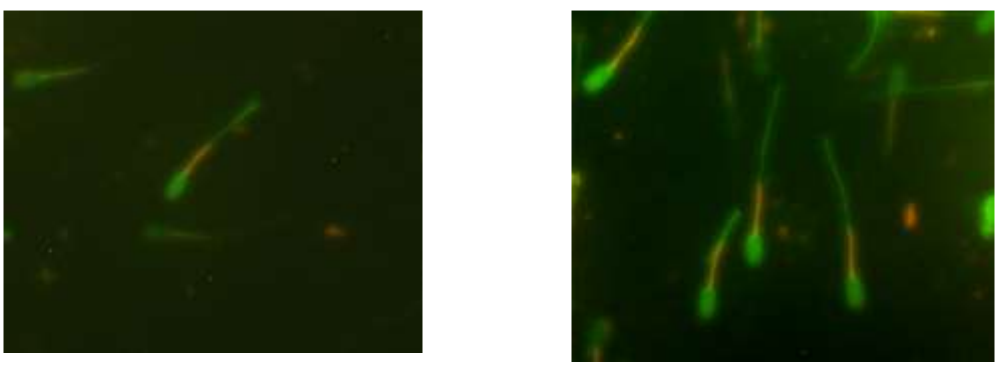

Fig. 2: Photomicrograph showing mitochondrial transmembrane potential in Hariana bull semen (JC-1 stain, Magnification 40 X); High transmembrane potential (orange mid piece); Low transmembrane potential (green mid piece)

Capacitation like Changes (CTC Method)

The overall mean of spermatozoa showing F pattern (non-capacitated) was 79.59±1.05, spermatozoa showing B pattern (capacitated) was 19.50±0.59 and spermatozoa showing AR pattern (acrosome reacted) was 0.16±0.07 percent in fresh semen. The respective values in post thaw semen were 65.06±1.35,

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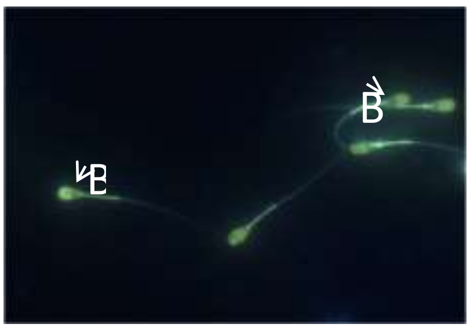
33.56±1.37 and 0.53±0.13 percent. A significant (P<0.01) difference was observed between fresh and post thaw stage (Table 2; Fig. 3).

Table 2: Various parameters in fresh and post thaw semen of Hariana bull (Mean ± SEM =32)

Parameters	Technique	Fresh	Post Thaw
Intact Acrosome (%)	FITC-PSA	$83.75^{b} \pm 0.54$	$76.21^{a} \pm 0.59$
Capacitated sperm (%)	CTC-ASSAY	$19.50^{a} \pm 0.59$	$33.56^{b} \pm 1.37$
Non capacitated sperm (%)		$79.59^{b} \pm 1.05$	$65.06^{a} \pm 1.35$
Acrosome reacted sperm (%)		$0.16^{a} \pm 0.07$	$0.53^{b} \pm 0.13$
Active mitochondria (%)	JC-1 ASSAY	$81.66^{b} \pm 0.71$	$59.90^{a} \pm 0.75$
Inactive mitochondria (%)		$18.13^{a} \pm 0.64$	$40.09^{b} \pm 0.77$
Live sperm (%)	SYBR GREEN-PI ASSAY	$79.03^{b} \pm 0.96$	$50.75^{a} \pm 1.15$
Dead sperm (%)		$11.180^{a} \pm 0.66$	$30.71^{b} \pm 0.96$
Moribund sperm (%)		$10.28^{a} \pm 0.67$	$18.56^{b} \pm 0.89$

Means with small alphabet differ significantly (P < 0.05) within row.



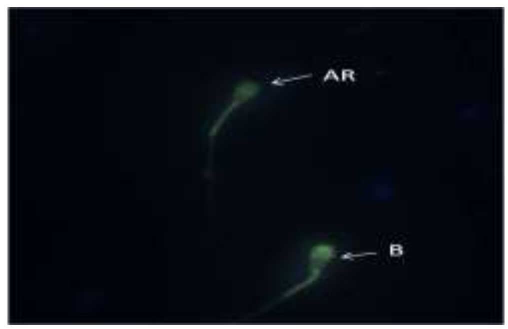


Fig. 3: Photomicrograph showing various pattern of chlortetracycline (CTC) in Hariana bull semen, Magnification 40X); 'F' – Non capacitated; 'B' – Capacitated; 'AR' – Acrosome reacted

Changes in Viability (SYBR Green-PI Method)

The overall mean of live spermatozoa in fresh semen was 79.03±0.96, moribund spermatozoa were 10.28±0.67 and dead spermatozoa were 11.18±0.66 percent. The respective values in post thaw semen were 50.75±1.15, 18.56±0.89 and 30.71±0.96 percent. A significant (P<0.01) difference was observed between fresh and post thaw stage (Table 2; Fig.4).

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Fig. 4: Photomicrograph showing apoptotic changes in Hariana bull semen (SYBR Green –PI assay, Magnification 40 X); Live sperm (Bright green fluorescence); Dead sperm (Red fluorescence); Moribund sperm (Orange green fluorescence)

Cryopreservation reduces fertility of frozen-thawed semen by leading to various structural and functional sperm modifications. Some of these alterations are non-lethal but can lead to early capacitation of sperms. Therefore when thawed, many of the sperms have a reduced time period for functional ability to attach and fertilize oocytes (Watson, 2000). Fertilization success cannot be accredited solely to the total number of live, motile and morphologically normal spermatozoa. The procedure underlying the culmination in fertility of cryopreserved sperm is not fully understood, but recently there has been increasing attentiveness towards apoptosis like changes or cryoinjuries that occur during cryopreservation. The mean percentage of live spermatozoa in the present study reduced drastically from fresh semen to post thaw semen and the values were 87.09±0.67 and 65.59±0.99 percent respectively in Hariana bulls. In agreement with our findings Rajoriya *et al.* (2016) documented that 88.23±1.82 percent viability in fresh semen while 80.70±2.86 and 70.03±1.88 percent viability at prefreeze and post thaw stage respectively in Tharparkar bulls. Similarly, Malik *et al.* (2015) reported mean percentage of live spermatozoa in fresh semen of HF bulls as 88.79±3.50. However, they reported higher percentage of live spermatozoa in post thaw semen.

Progressive motility percentage in the present study declined gradually from fresh to post equilibration and post thaw stage and respective value were 83.28±0.91, 75.46±0.88 and 57.03±0.77. In agreement with our findings Rajoriya *et al.* (2016) documented 84.00±3.03 percent forward progressive motility in fresh semen while 75.67±1.04 and 51.33±1.04 percent forward progressive motility at pre freeze and post thaw stage respectively, in Tharparkar bulls. However, Malik *et al.* (2015) reported lower initial and post thaw progressive motility (76.23±4.66and 48.33±0.31 percent) in semen of HF bulls. Likewise, Zodinsanga *et al.* (2015) also reported the lower (41.5±1.1 percent) value of sperm motility at post-freeze semen. But all the researchers found that there is highly significant drop in sperm motility after cryopreservation. A significant correlation was found between volumetric parameters and sperm-oviduct binding capacity Khalil *et al.* (2006). In the present study, the mean percentage of HOS response positive spermatozoa was 79.00±0.61 in the fresh semen. Nadeem *et al.* (2017) reported a higher mean value (84.95±0.76) in fresh semen of Hariana bulls. We found 61.56 ± 0.91 percent host reactive spermatozoa in post thaw semen,

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however, Patel *et al.* (2015) reported the respective parameter as 57.34±0.74 percent at the same stage in Hariana bulls. In agreement with our findings Rajoriya *et al.* (2016) documented 81.43±1.49 percent HOS reactive spermatozoa in fresh semen while 69.57±1.94 and 51.13±1.22 percent HOS reactive spermatozoa at pre freeze and post thaw stage respectively in Tharparkar bulls. Similar to other workers Karunakaran *et al.* (2017) also reported reduced number of HOS reactive spermatozoa (55.61±2.70) percent at post thaw stage.

Acrosome plays a vital role in ovum fertilization by dispersing the enzymes into the interstitium of the zona cells for successful fertilization. In the present study, the mean percent spermatozoa showing intact acrosome were 83.75±0.54 and 76.21±0.59 in fresh and post thaw stages, respectively. Similarly, Srivastava *et al* (2013) evaluated the acrosomal integrity by FITC-PSA assay and reported higher number of spermatozoa with intact acrosome (71.00±1.48) in fresh semen while acrosomal integrity decreased (61.58±1.70) after thawing of bull semen. Similar trend of acrosomal damage was also reported by Nadeem *et al.* (2017) during cryopreservation in Hariana bulls. Üstüner *et al.* (2015) also reported that defective acrosome percent (54.0 ± 1.9) increased during cryopreservation. Several mechanisms have been attributed to the reduced fertility of cryopreserved semen, however, cryopreservation-induced capacitation-like changes in frozen-thawed spermatozoa gained momentum recently (Thomas *et al.*, 2006). Frozen-thawed spermatozoa have modified membrane state, which is practically similar to capacitated and/or acrosome reacted sperm Maxwell and Watson (1996). In the present study, we found 79.59±1.05 percent non capacitated, 19.50±0.59 percent capacitated and 0.16±0.07 percent acrosome reacted spermatozoa in fresh semen while the respective parameter in post thaw semen was 65.06±1.35, 33.36±1.37 and 0.53±0.13 percent. Srivastava *et al.* (2013) reported more number of capacitated and acrosome reacted spermatozoa in fresh as well as in frozen thawed semen but, they also demonstrated that there is significant increase in capacitated (53.58±1.27) and acrosome reacted (30.58±1.87) spermatozoa after cryopreservation.

Mitochondria play a crucial role in sperm apoptosis. In the present study we found the mean value of mitochondria with higher transmembrane potential as 81.66±0.71 percent while the respective value in post thaw semen was 59.90±0.75 percent. Higher values were reported by Baishya *et al.*, 2014 in fresh semen (88.40±0.95) and post thaw semen (81.67±1.20) of boar. Similarly, Fraser *et al.* (2014) documented that cryopreservation affects the sperm motility, mitochondrial function and plasma membrane integrity. Thus, we propose that the impaired mitochondrial function caused by freezing is one of the mechanisms that modulate bovine sperm resistance to the whole cryopreservation procedure.

The evaluation of sperm membranes is an appropriate indicator of the success of cryopreservation since sperm membranes are extremely susceptible to cryoinjury. In the present study, mean value of percent live spermatozoa detected by SYBR green PI was 79.03±0.96 percent in fresh semen and 50.75±1.15 percent in post thaw semen of Hariana bull. Similarly, Fraser *et al.* (2014) also reported that apoptotic spermatozoa

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percent increased significantly during cryopreservation. Garner and Johnson (1995) using SYBR14-/PI stain reported 49.2% of sperm stained with SYBR-14, whilst 45.2% were labeled with PI. In another study, Makarevich *et al*. (2010), the percentage of SYBR-14 stained sperm cells was higher (63–66%) than in the above mentioned study. This difference may be explained by different incubation conditions (type of semen extender and temperature) and analysis (fluorescent microscopy versus flow cytometry) used in our study and the study of the mentioned authors. Some variations could be due to sperm origin. Thus, we attribute our results to the association of the freezing thawing induced overall increase of ROS. Thus, cold shock causes plasma membrane damage because the lipid bilayer becomes unstable at cool temperatures (Holt, 2011).

Conclusion

The results of this study demonstrated that cryopreservation compromised post-thaw sperm motility, mitochondrial function and plasma membrane integrity in the bulls. Successful cryopreservation with retention of fertilizing ability requires that these changes are minimized. Therefore, evaluation of cryodamages and apoptosis like changes are key factor in achieving better cryopreservation results.

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