



Article

Cryopreservation-induced changes in buffalo spermatozoa with special reference to their DNA, membrane, acrosomal and mitochondrial integrities

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Abstract

The present study aimed to study the effect of cryopreservation on the membrane, acrosome, DNA, and mitochondrial integrities of buffalo bull spermatozoa. Two adult buffalo bulls were enrolled in this study. Semen samples were collected once weekly from each bull over a period of six successive weeks by means of artificial vagina. Samples were processed for cryopreservation. Individual motility, mitochondrial activity as well as membrane, acrosomal and DNA integrities of fresh and frozen-thawed spermatozoa were evaluated. Results showed that the procedures applied for freeze-thawing resulted in significant ($P \leq 0.001$) reduction in sperm motility, sperm membrane, acrosome, mitochondrial and DNA integrities by 26.67, 29.17, 17.66, 40 and 4.11%, respectively. In conclusion, the process of freezing and thawing significantly reduced semen quality of buffalo bull in terms of various functional parameters including motility, membrane, acrosome, DNA and mitochondrial integrities.

Keywords

Cryopreservation, Buffalo, Spermatozoa, DNA, Mitochondria

1. Introduction

Artificial insemination (AI) is the most important reproductive biotechnology that can be applied in a large scale for dissemination of superior genetic material of males. (Singh & Balhara, 2016) The quality of frozen semen is a major determinant to the successful of AI program. Accurate evaluation of fertility of bulls used for AI is of utmost importance since a single ejaculate provides several insemination doses (up to 500 doses. (Pesch & Hoffmann., 2007), and influences the reproductive potential of a herd. (Rodriguez-Martinez & Larsson., 1998)

Cryopreservation is a non-physiological and complex method that involves a high level of adaptation of biological cells to the thermal and osmotic shocks that occur both during the dilution, cooling-freezing and during the thawing procedures. (Holt, 2000) During cryopreservation, freeze-thaw damages are unavoidable that result in reduced semen quality. (Yoshida, 2000) The process of semen cryopreservation is stressful and around 40%- 50% of sperm lose their motility

and viability due to loss of structural and functional capabilities. (Mittal et al., 2019) As a result of cryopreservation, the characteristics of mammalian spermatozoa decreased because of the subject of spermatozoa to different stresses that lead to damage of the mitochondria, plasma membrane, and acrosome membrane of sperm cells. (Perteghella et al., 2017; Zhang et al., 2019)

An adequate evaluation of semen for breeding purposes has always been of great significance. Numerous methods have been developed over the years for the laboratory evaluation of semen quality and fertility. Some of these measure general characteristics of sperm (viability, motility patterns, morphology, sperm metabolism, membrane, and acrosomal integrities. (El-Badry et al., 2008). Besides acrosome intactness, membrane integrity is important not only for sperm metabolism but also plays a tremendous role in fertilization because a correct change in the properties of the membrane is required for sperm capacitation, acrosome reaction, and binding of the spermatozoa to the egg surface for which biochemically active membrane is required. sperm DNA contributes 50% of the zygote's genome, DNA integrity of fertilizing spermatozoa is vital for a successful embryonic development. (Agarwal & Allamaneni., 2004) sperm DNA contributes 50% of the zygote's genome, DNA integrity of fertilizing spermatozoa is vital for a successful embryonic development. (Agarwal & Allamaneni., 2004). Mitochondrial function has been focused to assess semen quality. (Marchetti et al., 2002) The mitochondrial sheath surrounding the midpiece of the sperm generates the energy that aids transit in the female reproductive tract, penetration, and fertilization of the egg. (Windsor, 1997; Kasai et al., 2002) Hence, assessment of the mitochondrial function than the standard viability tests is considered more useful to assess semen quality. (Turner, 2006; El-Badry et al., 2008).

The aim of this study was to evaluate the effect of cryopreservation on the membrane, acrosome, DNA, and mitochondrial integrities of buffalo bull spermatozoa.

2. Materials and Methods

Experimental animals

Two adult healthy buffalo-bulls were maintained on the experimental farm of Animal Reproduction Research Institute (ARRI). Their age ranged between 2.5 and 4 years and of body-weight 450-700 Kg. Throughout the experimental period, the animals were kept under similar conditions of nutrition and management, each animal received a daily ration composed of about 15 kg green berseem, 4-5 kg concentrates mixture in pellet form, and 4-5 kg rice hay. Water was allowed ad libitum.

Semen collection

Early in the morning, using the pre-warmed artificial vagina (AV, 40-42 oC), semen samples were collected once weekly from each bull over a period of six successive weeks (in February – March 2021). Two successive ejaculates with about 10 min intervals were collected from each animal on one occasion.

Fresh semen evaluation

Immediately after collection, semen samples were evaluated for ejaculate volume, individual motility (%) using a pre-warmed stage of phase-contrast system microscope. Sperm viability, abnormalities, and acrosomal status were evaluated by a dual staining procedure. (Didion et al., 1989) Briefly, spermatozoa were incubated with an equal volume of 0.2% trypan blue for 10 min and washed twice (centrifugation at 700g for 6 min) with PBS (phosphate buffer saline). Smears were made on glass slides and dried quickly on a warm stage. Slides were stained with 10% Giemsa stain for 40 min. They were rinsed under a stream of distilled water, air-dried, and covered with coverslips. At least 200 sperm cells were counted. Spermatozoa were classified as live [unstained post acrosomal region], dead [stained blue in the post acrosomal region], acrosome intact [light purple - dark pink acrosome] and damaged/lost acrosome [unstained or blue acrosome]. The abnormal spermatozoa were classified as head, mid-piece, and tail abnormalities. (Cevik et al., 2007) Sperm concentration was measured microscopically using a Neubauer counting chamber (hemocytometer).

Plasma membrane integrity

The procedure described by Jeyendran et al. (1984) was used to determine the percentage of HOS-positive cells in each sample. The hypo-osmotic solution is prepared as follows (0.735 g of sodium citrate dihydrate and 1.351 g of fructose in 100 mL of sterile, de-ionized water). A 100 µl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed hypo-osmotic solution. The mixture was incubated at 37°C for 30 minutes in a 1.5 ml micro-centrifuge tube. Following incubation, a small drop of the sample was placed on a microscope slide and cover-slipped for examination by using phase-contrast microscopy (400X) to evaluate 100 spermatozoa for evidence of swelling and curling changes of the sperm tail.

Sperm mitochondrial activity

MTT test, which determines the spermatozoal mitochondrial activity. For each fresh semen sample, six wells of the 96-well microplate were used. The 100 µl of semen sample plus 10 µl of MTT stock solution (5 mg MTT/ml of PBS) was placed in each well. The rates of MTT reduction were determined using an ELISA reader (Thermo Reader) at a wavelength of 550 nm. The optical density of semen samples was measured two times (immediately and after one hour of incubation at 37 °C). MTT reduction rates (optical density) for each semen sample were calculated by concurring the difference between the first and second reading (MTT1) of the ELISA reader.

Sperm cell DNA integrity

The alkaline comet assay (single-cell electrophoresis) for spermatozoa was carried out according to Hughes et al. (1996). Full frosted glass slides were covered with 100 µl of 0.5% normal melting point agarose (Sigma), a coverslip was added, and the agarose was allowed to solidify. The coverslips were removed and 1×10^5 sperm cells in 50 µl PBS (7.2 pH) were mixed with 50 µl of 1.2% low melting point agarose and used to form the second layer. The slides with coverslips removed were then placed in lysis buffer for 1 h (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% Triton X at a pH of 10). The slides were then incubated at 37°C in 100 µl/ml of proteinase K in lysis buffer overnight. After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA for 20 min to allow the DNA to denature. Electrophoresis was performed at room temperature, at 25 V (0.714 V/cm) and 300 mA, obtained by adjusting the buffer level, for 10 min. The slides were then washed with a neutralizing solution of 0.4 M Tris at pH 7 to remove alkali and detergents. After neutralization, the slides were each stained for 5 min with 50 µl of 20 µg/ml ethidium bromide and mounted with a coverslip. A total of 200 sperm cells were examined under a fluorescent microscope (Leica, Germany, 400X, at 254 nm wavelength). The percentage of non-fragmented (compact sperm head) and fragmented (elongated tail of a comet) sperm nuclear DNA was calculated. The intensity of the fluorescent stain in the comet tail region is presumed to be related to the DNA content, and DNA damage is estimated from measurements of the percent DNA in tail, tail length, and tail moment, using an image analysis system (Comet-Score program).

Semen processing

After the microscopic evaluation, the two ejaculates from each bull were pooled together. Each pooled semen sample was diluted with a Tris-based egg yolk extender (Tris-based buffer was prepared according to Reddy et al. (2010), which comprised Tris 33.2 g/l, citric acid 18.3 g/l, dextrose 7.8 g/l, 7% (V/V) glycerol, 20% (V/V) egg yolk and supplemented with 100 mMol of trehalose. (El-Sayed et al., 2010) and antibiotics (gentamycin sulphate 500µg/mL, tylosin tartrate 100µg/mL, lincomycin HCl 300µg/mL, and spectinomycin HCl 600µg/mL, Akhter et al., 2011) at 37 °C in an incubator in an appropriate dilution rate to obtain a concentration of 40×10^6 sperm/ml. Samples were cooled slowly to 5°C over a period of 1.5 hours. Semen was loaded in 0.25 ml straws (IMV, France) and placed 4 cm above liquid nitrogen in the vapor phase in a foam box for 15 minutes before being plunged into the liquid phase. (Khalifa, 2001) Straws were

stored in liquid nitrogen until thawing (one week after freezing) at 37°C in a water bath for 30 seconds.

Evaluation of frozen-thawed semen

Motility estimations were done at hourly intervals for a period of 3 hours. The viability index was calculated according to Milovanov (1962) to be equal to half of the post-thaw motility in addition to the summation of recorded motility at 1st, 2nd, and 3rd hours post-thawing.

Morphological abnormalities, mitochondrial activity as well as membrane, acrosomal, and DNA integrities of frozen-thawed spermatozoa were evaluated as mentioned above for fresh semen.

Statistical analysis

Data were presented as mean \pm SEM (standard error of the mean). Independent-samples T-test was done for the obtained data using SPSS program version 16.0 and $P \leq 0.05$ was considered as statistically significant.

3. Results

As presented in table 1, the mean volume of fresh semen and concentration of sperm cells were 4.13 ± 0.14 ml and 11342.00 ± 39.65 10⁶ sperm/ml, respectively. The individual motility, percentage of alive spermatozoa, sperm membrane, and acrosome integrities were 82.50 ± 1.11 , 86.67 ± 0.88 , 86.00 ± 0.58 , and 79.33%, respectively. The MTT-reduction rate was 1.15 ± 0.08 . Regarding the frozen-thawed spermatozoa, the post-thaw motility and viability index were $55.83 \pm 1.54\%$ and 153.50 ± 2.44 , respectively. The integrities of sperm membrane and acrosome were 56.83 ± 1.40 and $61.67 \pm 1.28\%$, respectively. The MTT-reduction rate of frozen semen was 0.69 ± 0.02 .

Processing of buffalo semen for cryopreservation resulted in 26.67%, 29.17, 17.66%, and 40.00% loss of sperm individual motility, membrane, and acrosomal integrities as well as mitochondrial activity, respectively.

Table 1. Fresh and frozen-thawed buffalo semen characteristics

Semen trait	Fresh semen	Frozen semen	p-value	Differences between fresh & frozen semen
Volume (ml)	4.13 ± 0.14	-	-	-
Concentration (10 ⁶ /ml)	11342.00 ± 39.65	-	-	-
Individual motility (%)	82.50 ± 1.11	55.83 ± 1.54	0.001	26.67
Alive spermatozoa (%)	86.67 ± 0.88	-	-	-
Viability index	-	153.50 ± 2.44	-	-
Membrane integrity (%)	86.00 ± 0.58	56.83 ± 1.4	0.001	29.17
Acrosome Integrity (%)	79.33 ± 1.05	61.67 ± 1.28	0.001	17.66
MTT reduction rate	1.15 ± 0.08	0.69 ± 0.02	0.001	40.00

Data regarding DNA integrity of fresh and frozen-thawed buffalo spermatozoa were presented in table 2. Cryopreservation of buffalo spermatozoa resulted in significant ($P < 0.001$) decrease in the percentage of sperm with intact DNA (97.00 ± 0.58 vs. $92.89 \pm 0.64\%$, respectively) and the percent of DNA in head of comet (97.77 ± 0.06 vs. $95.82 \pm 0.07\%$, respectively) and increase in percent of DNA in tail of comet (2.23 ± 0.06 vs. $4.18 \pm 0.07\%$, respectively), Comet tail length (4.26 ± 0.09 vs. 5.77 ± 0.26 pixels, respectively) and Olive tail moment (0.38 ± 0.01 vs. 0.47 ± 0.03 respectively).

Table 2. DNA integrity of fresh and frozen-thawed buffalo semen

Semen trait	Fresh semen	Frozen semen	p-value	Differences between fresh and frozen semen
Sperm with intact DNA (%)	97.00 ± 0.58	92.89 ± 0.64	0.001	4.11%

DNA in head of Comet (%)	97.77 ± 0.06	95.82 ± 0.07	0.001	1.95%
DNA in tail of Comet (%)	2.23 ± 0.06	4.18 ± 0.07	0.001	1.95%
Comet tail length (pixels)	4.26 ± 0.09	5.77 ± 0.26	0.001	1.51 pixels
Olive tail moment	0.38 ± 0.01	0.47 ± 0.03	0.001	0.09

4. Discussion

In the current study, Hypo-osmotic swelling test (HOS), Dual staining, MTT- and Comet assays were used to determine the integrity of plasma membrane, acrosome, mitochondria, and DNA of buffalo spermatozoa after freeze-thaw procedures.

The ejaculates of swamp buffalo which were collected in this experiment had normal characteristics (Individual motility: $82.50 \pm 1.11\%$) and were appropriate for cryopreservation. Similarly, Koonjaenak et al. (2007), Kaka et al. (2016) consider that ejaculates with over 70% of sperm cells with forwarding movement are the minimum standard for a good semen sample suitable for freezing. As routine, post-thaw motility is the most common parameter to assess the effects of freezing on spermatozoa and as an indicator governing the use or discard of semen for artificial insemination. The post-thaw motility of buffalo spermatozoa recorded herein ($55.83 \pm 1.54\%$) meets the recent recommendations for insemination of cattle which is at least 50% progressive forward motility following freezing and thawing. (ADR-Handbuch., 2008) The inevitable loss of motility associated with freezing and thawing in the current work was 26.67% which is the same as that recorded by El-Regalaty (2017) in buffaloes and lower than the reduction by 49% after thawing in buffalo sperm. (Rasul et al., 2001) Post-thaw motility is known to be influenced by a number of factors such as diluents, cryoprotectants, freezing procedures, age of the bull, and season of semen collection. Semen analysis is a valuable diagnostic tool to assess the fertility status of the male. However, the prediction of the potential fertility of a male on the basis of a single assay is not reliable. Conventional parameters used for the evaluation of semen have limited application because they only help to assess the structural integrity of the cell. (Neild et al., 1999) Each sperm cell consists of multiple subcellular compartments with different functions, all of which must be intact for successful fertilization. (Amann & Graham., 1993) In general, cryopreservation generates sublethal sperm injury due to chemical, osmotic, thermal, and mechanical stresses, which may result in loss of viability, motility, damage of DNA, destruction of acrosomal and plasma membrane. (NumanBucak et al., 2007; Rasul et al., 2001) that reduces cell survival, thereby reducing cell longevity and fertility compared with fresh semen. (Mittal et al., 2019) In buffaloes, it is well documented that motility, plasma membrane, acrosome, and DNA integrity of spermatozoa were significantly reduced after the process of cryopreservation. (Rasul et al., 2001; Kumar et al., 2011) Membrane integrity is important not only for sperm metabolism but also plays a tremendous role in fertilization because a correct change in the properties of the membrane is required for sperm capacitation, acrosome reaction and binding of the spermatozoa to the egg surface for which biochemically active membrane is required. The hypo-osmotic swelling test (HOST) also known as the osmotic resistance test was early developed by Jeyendran et al. (1984) to evaluate the sperm membrane function of human spermatozoa. Since its development, the HOS test has been used for evaluation of sperm membrane integrity in cattle. (Rota et al., 2000), buffalo (El-Badry et al., 2008), equine (El-Badry et al., 2017), canine (Rodriguez-Gil et al., 1994) and porcine (Perez-Llano et al., 2001). The test is based on the principle that when the sperms are subjected to a hypo-osmotic solution, the cells with intact membranes take up water apparently without a significant enlargement of their area, thus forcing the flexible apparatus of the tail to bend and coil. Viable sperm in a hypotonic solution has been shown to develop bent and coiled tails whereas dead sperm had straight tails probably associated with cell lysis. Therefore, it was hypothesized that the ability of the sperms to swell in hypotonic solution indicates its membrane integrity and normal function activity. (Jeyendran et al., 1984) Sperm plasmalemma contains polyunsaturated fatty acids (PUFA) and ROS attacks them leading to a cascade of chemical reactions known as lipid peroxidation. (Shah et al., 2016) Cryodamage during the

freeze-thawing process to buffalo semen is higher than cattle spermatozoa due to higher PUFA levels in the plasma membrane. (Nair et al., 2006; Andrabi, 2009) In the present study, there was a 17.66% reduction of acrosome integrity. The post-thaw proportion of sperms with intact acrosomes varied from 51.30 ± 0.56 to 64.10 ± 0.52 in one study. (El-kon et al., 2011) whereas in another study the proportion of spermatozoa with intact acrosomes varied from 76.9 to 84.0% immediately after thawing. (Andrabi et al., 2008) The first changes due to freezing processing are relevant mainly to the acrosomal region of the sperm that shows distension and loosening of the peri-acrosomal plasmalemma followed at late steps by ruffling and swelling. (Vale et al., 2014) The detachment of the acrosome results in a decrease in ATP and loss of intracellular proteins responsible for fertilization. (Pant et al., 2002) No significant difference in the acrosome structure of cattle and buffalo spermatozoa exists; however, certain enzymes responsible for fertilization like acrosin and hyaluronidase are known to be low in buffalo semen. (Agarwal & Tomer, 1998)

It is concluded that the process of freezing and thawing significantly reduced semen quality of buffalo bull in terms of various functional parameters including motility, membrane, acrosome, DNA, and mitochondrial integrities.

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الملخص العربي

التغيرات المستحثة بواسطة الحفظ بالتجميد في الحيوانات المنوية للجاموس مع الإشارة بوجه خاص إلى سلامته الوظيفية وسلامة الحمض النووي، الأكروسوم، والميتوكوندريا

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هذه الدراسة تهدف إلى تأثير الحفظ بالتجميد علي السلامة الوظيفية والحمض النووي والخلايا الحية للحيوانات المنوية للجاموس، تمت هذه الدراسة علي عدد ٢ جاموس تم جمع عينات الحيامن من الجاموس مرة كل أسبوع لمدة ٦ أسابيع متتالية وذلك بواسطة المهبل الصناعي كما تم تجهيز هذه العينات للتجميد وقمنا بفحص هذه العينات من حيث الحركة والسلامة الوظيفية والخلايا الحية والحمض النووي للحيوانات المنوية التي تم الحصول عليها بصورة مباشرة وكذلك بعد التجميد تم ملاحظة نقص في حركة الحيوانات المنوية في العينات التي تم تجميدها، وكذلك أيضا كان هناك اختزال بسيط في الحمض النووي والخلايا الحية والسلامة الوظيفية للحيوانات المنوية التي تم تجميدها مقارنة بالحيوانات المنوية الطازجة وبالتالي نستطيع أن نقول بأن الحفظ بالتجميد يؤثر علي جودة الحيوانات المنوية وذلك من خلال تأثير علي حركة الحيوانات المنوية وسلامته الوظيفية.