Analysis of chromatin integrity and DNA damage of buffalo spermatozoa

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Summary

This study was conducted to determine chromatin integrity and DNA damage by DNA electrophoresis and comet assays of buffalo fresh and frozen semen. Semen samples were collected from four buffalo bulls and evaluated after freezing for semen motility, viability, sperm abnormalities, chromatin integrity and DNA damage. A significant variation was found in semen parameters after thawing. Highly significant differences ($P<0.001$) in chromatin integrity were observed between fresh and frozen semen. For the fresh semen, there was no significant difference between the bulls for chromatin integrity; however, a significant variation ($P<0.05$) was detected in their frozen semen. No DNA fragmentation was observed by agarose gel electrophoresis. The percentage of sperm with damaged DNA detected by comet assay differed significantly between fresh and frozen semen. A significant negative correlation was recorded between motility and DNA damage ($r=-0.68$, $P<0.05$). Sperm abnormalities and DNA fragmentation were significantly positively correlated ($r=0.59$, $P<0.05$). In conclusion, DNA damage evaluation can provide reassurance about genomic normalcy and guide the development of improved methods of selecting spermatozoa with intact DNA to be used in artificial insemination.

Key words: Buffalo bull, Chromatin integrity, DNA damage, Semen quality

Introduction

Artificial insemination is one of the major reproductive biotechnologies extensively used for rapid genetic improvement of livestock in developed countries. To raise the genetic potential of livestock, AI with frozen-thawed spermatozoa was introduced in most developing countries more than three decades ago, yet it has not been applied in buffaloes on a large scale (Andrabi, 2009). Semen evaluation is extremely important for the success of AI with cryopreserved semen, by which hundreds of straws are produced from an ejaculate (Barros *et al*., 2007).

Sperm genome anomaly is one of the important factors involved in conception failure (Filatov *et al*., 1999). Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins, which are condensed and insoluble in nature. These features protect genetic integrity and facilitate the transport of the paternal genome through male and female reproductive tracts (Manicardi *et al*., 1998).

Spano *et al*. (2000) suggested that markers of sperm DNA integrity may be better measures of male fertility potential than conventional measures. The impact of DNA fragmentation on fertilization and pregnancy rates has been reported in the past (Virro *et al*., 2004). Sperm DNA fragmentation tests are liable indices that may help identify animals at risk of failing to initiate a healthy pregnancy (Agarwal and Said, 2003). In Egypt, a number of studies were conducted to evaluate sperm DNA damage in buffalo using the acridine orange test (El-Sheshtawy *et al*., 2010) and the comet assay (Abd El-Fatah *et al*., 2008). Few reports exist on DNA damages in animal frozen semen as related to semen parameters. Therefore, this study was conducted to determine chromatin integrity and DNA damage by acridine orange test, DNA electrophoresis and comet assays in buffalo frozen semen and its relationship with conventional semen parameters.

Materials and Methods

Semen collection

Using the artificial vagina method, semen samples were collected from four healthy buffalo bulls, at Abassia Frozen Semen Centre, General Organization for Veterinary Services, Egypt. The Egyptian buffalo bulls were 4-8 years old. Two ejaculates were collected 10 min apart twice weekly, all over the year, assessed for subjective motility analysis. Semen was checked for volume, sperm motility, sperm abnormalities and sperm cell concentration. All seminal attributes were within the acceptable limit.

Semen cryopreservation

Each ejaculate was diluted with prewarmed (37°C)

Bioxcell extender (IMV Technologies-France) to provide a concentration of 60 million sperm/ml. The extended semen was examined for individual motility at 37°C. Semen with more than 65% motile sperm was used for further processing. The diluted semen was cooled from 37°C to 5°C in a cold cabinet for 2 h, and packed into 0.25 ml polyvinyl French straws (0.25 ml; IMV, L'Aigle, France). The straws were placed on trays for at least 4 h at (5°C) for further equilibration, and were then kept at -95°C in a temperature controlled chamber for at least 8 min. The straws were then plunged into liquid nitrogen at -196°C (Mohammed *et al*., 1998).

Post-thaw semen evaluation

Semen was thawed in a water bath at 37°C for 45 s. The percentage of motile sperm was estimated at 37°C using a heated stage, by viewing 5-6 fields per slide with the aid of a television monitor attached to a phase contrast microscope (×400). Percentages of live and abnormal spermatozoa in stained smears were recorded using eosin-negrosine stain (Campbell *et al*., 1956).

Chromatin integrity by acridin orange test (AOT)

Frozen semen was washed in 5 ml of phosphate buffered saline. After centrifugation, the sperm pellet was re-suspended in 0.5 ml of phosphate buffered saline. A small aliquot (50 µL) of the sperm suspension was then glass smeared. Three smears from each sample were prepared on glass slides, air dried and then fixed overnight in Carnoy's solution (methanol/acetic acid, 3:1) according to Liu and Baker (1994). Once rinsed and air dried, the slides were stained for 5 min with freshly prepared acridine orange (AO) stain as follows: 10 ml 1% AO in distilled water was added to a mixture of 40 ml 0.1 M citric acid and 2.5 ml 0.3 M $Na_2HPO_47H_2O$. The AO solution was stored in a dark place at 4°C for four weeks. After washing and drying, the slides were examined using a fluorescent microscope (Leitz, Germany; excitation of 450-490 nm). Sperm with intact chromatin or normal DNA content had green fluorescence, whereas sperm with an abnormal DNA content emitted fluorescence in a spectrum varying from yellow-green to red.

DNA electrophoresis

DNA was extracted from frozen semen of four buffalo bulls according to Giusti *et al*. (1986) with some modifications. After centrifugation of sperm cells at 5000 rpm for 5 min, 500 µL of a lysis buffer (50 M Tris-HCl, pH 8 + 10 mM EDTA, pH 8 + 100 mM NaCl + 1% SDS) and 15 µL of a proteinase K solution (20 mg/ml) were added. Cells were incubated overnight at 55°C. Nucleic acids were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), followed by extraction with chloroform-isoamyl alcohol (24:1). After centrifugation, the top layer was carefully transferred to another tube for the next extraction. Then, 1/10 of a volume of 3 M sodium acetate (pH = 5.2) and two volumes of 96% cold ethanol (stored at 20°C) were added to precipitate DNA. The supernatant was removed and the pellet (DNA) was washed with 1 ml of 70% ethanol. The DNA was dissolved in an appropriate volume of 1 X Tris EDTA (TE) buffer. After RNAse (40 µg/ml) incubation for 2 h at 37°C, the samples were applied and analyzed on agarose gel (1.5%) with ethidium bromide (0.5 µg/ml).

Comet assay (single cell electrophoresis assay)

Sperm DNA damage was investigated using the single cell gel electro-phoresis (COMET) assay, generally performed at neutral conditions. The neutral comet assay allows the detection of double strands, breaks by subjecting lysed cell nuclei to an electrphoretic field at neutral pH. The protocol was similar to Bucak *et al*. (2010) with few modifications. The straws were thawed by gently shaking in a 37°C water bath for 10 s, and centrifuged at 600 g for 10 min. The remaining sperm cells were washed with PBS (Ca_2 + and Mg_2 + free). Each pre-cleaned slide was pre-coated with a layer of 1% normal melting point agarose in PBS and dried at room temperature. Approximately, 100,000 sperm cells (18 µl) were mixed with 0.8% low melting point agarose (50 µl) at 37°C, and this suspension was dropped onto the first agarose layer. Slides were allowed to solidify for 20 min at 4°C. The cover slips were removed and the slides were immersed in freshly prepared cold lysis buffer. The slides were then incubated at 37°C in the lysis buffer with 20 mg/ml proteinase for 2 h. The lysis buffer contained NaCl, EDTA, TRIS, DTT, Triton X 100 and 20 mg/ml protienase K. The slides were incubated for 2 h at 4°C followed by 24 h at 37°C. They were then removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with a fresh neutral electrophoresis buffer for 20 min incubation to allow the DNA to unwind. Electrophoresis was performed at room temperature, at 25V for 20 min. Following electro-phoresis, the slides were neutralized with a TRIS-HCl buffer (pH = 7.5) for 5 min and subsequently stained with 50 µl of 10 µl/ml probdium iodide and covered with a cover slip. The images of 200 randomly chosen nuclei were analyzed visually. Observations were made at a magnification of 400 using a green light fluorescent microscope (Olympus, Japan).

Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a "comet" pattern, whereas whole sperm heads without any comet were not considered as damaged. The high molecular weight, unbroken DNA were found to remain in the sperm head, while smaller broken pieces of DNA migrated out to take on the form of a comet, thus the name.

Statistical analysis

The results were tabulated in to indicate the mean values of the various studied parameters and their standard errors. Data were subjected to an ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by Duncan's Multiple Range Test. Correlation coefficients

among different semen parameters were calculated. Differences were considered to be significant at P<0.05.

Results

Post thaw semen characteristics are shown in (Table 1). Post thaw sperm motility ranged between 39.33 and 45.87%. Live sperm percentage ranged between 58.73 and 65.67%. For the third bull, the percentage of spermatozoa with abnormalities achieved the highest percentage (17.07%). A significant variation could be found among bulls in semen parameters after thawing (Table 1). Live sperm percentage and sperm abnormalities were significantly different (P<0.05) among the bulls. A highly significant difference (P<0.01) for motility was also found.

Table 1: Post-thaw semen characteristics in buffalo bulls (means ± SE)

Bull number	Motility (%)	Live sperm (%)	Sperm abnormalities (%)
1	45.87±1.12^{a}	65.67±0.58^{a}	12.87±0.80^{a}
2	43.84±0.90^{ab}	63.33±2.84^{ab}	14.67±1.24^{ab}
3	39.33±0.88^{c}	59.30±2.6^{ab}	17.07±0.77^{b}
4	41.00±1.15^{bc}	58.73±0.93^{b}	16.17±1.11^{ab}
Total means	42.51±0.88	61.76±1.22	15.19±0.64

Values with different superscript within the same column differ significantly (P<0.05-P<0.01)

The chromatin integrity of sperm was analyzed using acridine orange staining (Table 2). The overall means of spermatozoa with intact chromatin were 99.25% and 97.21% for fresh and frozen semen, respectively. Highly significant differences in chromatin integrity were observed between fresh and frozen semen (P<0.001). There were no differences between the bulls for chromatin integrity in fresh semen but a significant variation among bulls was detected in frozen semen (P<0.05). Sperm with normal intact chromatin or normal DNA had a green flourscence, whereas sperm with damaged chromatin or abnormal DNA content had a red flourscence. Green sperm indicated that the stain intercalates into native DNA and flourscence green, but the sperm with single stranded DNA or damaged chromatin turned flourscence red (Fig. 1).

Table 2: Percentage of buffalo sperm cells with intact chromatin detected by acridine orange test in semen from four buffalo bulls (mean ± SE)

Bull number	Fresh	Frozen
1	99.67 ± 0.33	98.33 ± 0.88^{a}
2	99.50 ± 0.28	97.50 ± 0.29^{ab}
3	99.00 ± 0.58	97.00 ± 0.58^{ab}
4	98.83 ± 0.44	96.00 ± 0.58^{b}
Total means	99.25 ± 0.21**	97.21 ± 0.37

Values with different superscripts within the same column differ significantly (P<0.05). ** P<0.001 (t-test) between fresh and frozen semen

Sperm DNA was extracted from the frozen semen of the four buffalo bulls and analyzed on agarose gel. DNA fragmentation could not be observed when measured by agarose gel electrophoresis in the frozen semen of buffalo bulls as DNA appeared normal (Fig. 2).

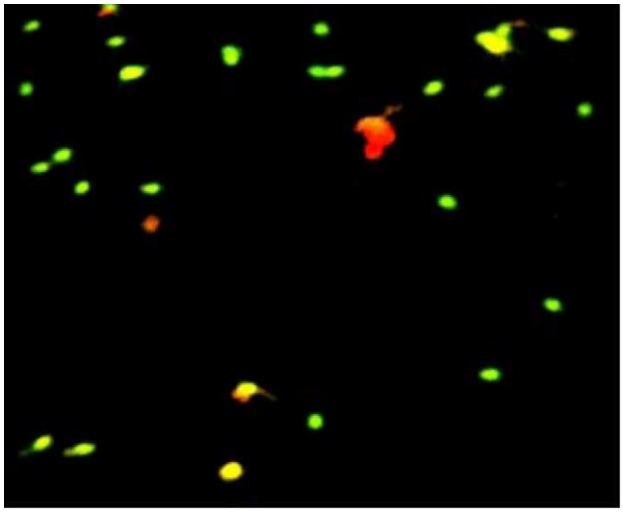

Fig. 1: Fluorescence microscopy displaying buffalo spermatozoa with damaged chromatin as shown by yellow or red colours of the acridine orange stain, and normal chromatin with green colour (×400)

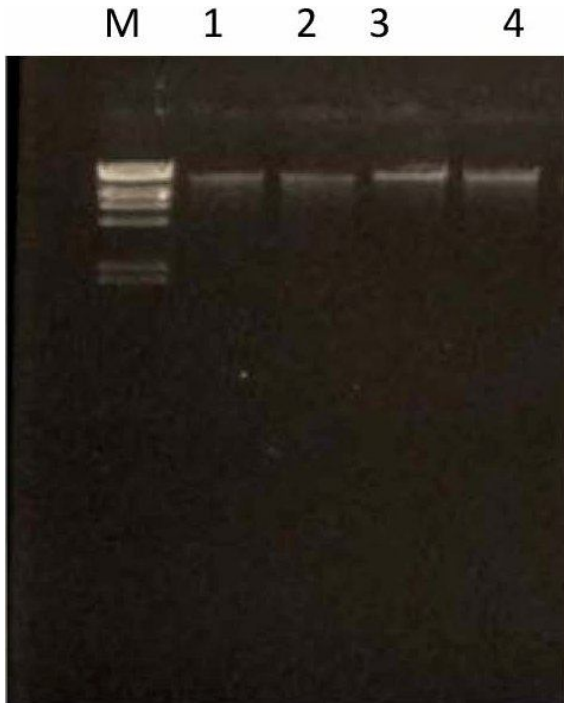

Fig. 2: DNA profile of frozen semen after electrophoresis. M is λ DNA hind III molecular weight marker (23130, 9416, 6557, 4361, 2322, 2027 and 564 bp). 1, 2, 3 and 4 represent frozen semen samples of four buffalo bulls

Low levels of DNA damage assessed by comet in fresh and frozen spermatozoa are presented in Table 3. The percentage of spermatozoa with damaged DNA ranged from 1.73 to 2.37 for frozen semen and 0.63 to 1.00 for fresh semen. The highest comet percentage in frozen semen was for bull three and the lowest for bull one. A significant difference was observed in DNA

damage between fresh and frozen semen by the comet assay (P<0.01). The intact DNA sperm and DNA damage with DNA migration from nuclei is shown in Figs. 3A and B.

Table 3: Analysis of DNA fragmentation percentage by comet assay of four buffalo bulls (means ± SE)

Bull number	Fresh	Frozen
1	0.67 ± 0.20	1.73 ± 0.35
2	0.63 ± 0.27	2.07 ± 0.18
3	0.63 ± 0.18	2.37 ± 0.20
4	1.00 ± 0.12	2.07 ± 0.37
Total means	0.68 ± 0.10	2.06 ± 0.14**

** P<0.001 (t-test) between fresh and frozen semen

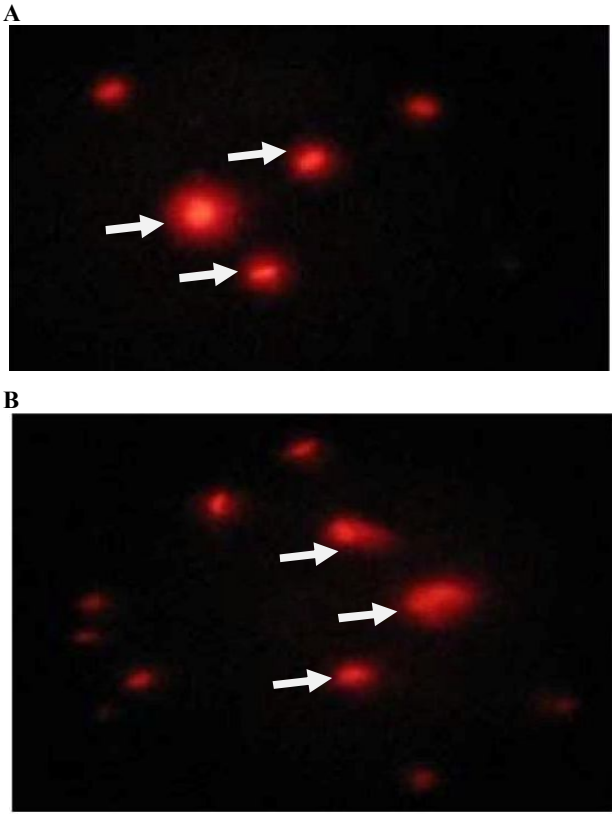

Fig. 3: Fluorescence photomicrograph of buffalo spermatozoa (×400) following neutral microgel electrophoresis showing different forms of fragmentation (A and B). Spermatozoa with non-fragmented DNA do not have a comet (without arrows) and spermatozoa with fragmented DNA exhibit the characteristic formation of comet (arrows)

Correlation coefficients between semen parameters are presented in Table 4. Significant negative correlations were found between motility and sperm abnormalities (r=-0.64, P<0.05) and motility and DNA damage (r=-0.68, P<0.05), whereas a significant positive correlation (r=0.59, P<0.05) was reported between sperm abnormalities and DNA fragmentation. On the other hand, no significant correlation was found between chromatin integrity evaluated by acridine orange stain and other sperm parameters including DNA fragmentation detected by comet assay.

Discussion

In the present study, motility and live sperm percentage significantly reduced by freezing as much as 42.5% and 61.76%, respectively. Similar findings for a decline in motility due to freezing and thawing have been reported for buffalo (El-Sisy *et al*., 2007). Watson (1995) also reported that more than 50% of spermatozoa are usually injured by the cryopreservation process. These injuries are most likely due to the formation of ice crystals in the extra and intracellular environment, increasing solute concentration (Mazur, 1984). Ice crystal formation in mitochondria and axonemes during cryopreservation impairs sperm motility (Courtens *et al*., 1989). The post-thawing semen characteristics including motility, live sperm percentage and sperm abnormalities in the current study were within the range reported in Egyptian buffalo bulls (Scholkamy *et al*., 2009; El-Sheshtawy *et al*., 2010). A significant variation was also found in semen parameters among bulls after thawing, which is in accordance with the report by Scholkamy *et al*. (2009) on buffaloes.

In this study, chromatin integrity detected by the acridine orange stain was affected by freezing. Similarly, Mukhopadhyay *et al*. (2011) reported significant differences in the percentage of sperm nuclear DNA fragmentation in fresh and cryopreserved semen samples (P<0.01). Conversely, Koonjaenak *et al*. (2007) maintained that previously frozen thawed buffalo sperm chromatin integrity is not seriously damaged by cryopreservation. Other researchers found that the cryopreservation of bull semen had no effect on sperm chromatin stability or DNA integrity (Martin *et al*., 2004). The study of Duran *et al*. (1998) indicated that when acridine orange score was normal (<11%), the likelihood of fertilization was found to be superior to that of all other parameters. Barth and Oko (1989) stated that the presence of sperm nuclear chromatin damage in freshly ejaculated semen cannot be accepted at levels greater than 15-20% of spermatozoa.

This study revealed no damage to any of the bulls sperm DNA detected by agar gel electrophoresis. Martin

Table 4: Correlation coefficients between post-thaw sperm parameters and DNA damage

Parameters	Motility	Live sperm (%)	Sperm abnormalities	Chromatin intact	DNA damage
Motility		0.482 (0.112)	-0.642* (0.024)	0.510 (0.090)	-0.681* (0.015)
Live sperm (%)			-0.308 (0.330)	0.564 (0.056)	-0.094 (0.772)
Sperm abnormalities				-0.414 (0.181)	0.585* (0.045)
Chromatin integrity					-0.301 (0.342)
DNA damage					

* Correlation is significant at the 0.05 level (2-tailed)

et al. (2004) reported that cryopreservation had no effect on DNA fragmentation. Mammalian sperm nuclei are very stable and highly condensed with a unique DNA organization (Yanagimachi, 1994), with a 6-fold more compact and a 40-fold lower volume than somatic cells (Ward, 1994). This unique DNA packing is essential to protect the cell and minimize damages caused by exogenous agents before fertilization. Perreault *et al*., (1988) suggested that the bovine sperm has a more stable nuclear packing than other species.

In the present investigation, the percentage of spermatozoa with damaged DNA detected by comet averaged from 0.63 to 1.00 and 1.73 to 2.37 for fresh and frozen semen, respectively. A similar low percentage of DNA damage was previously recorded in Egyptian buffalo semen (Badr *et al*., 2010). The mean percentage of comet-detected spermatozoa with damaged DNA was significantly higher for frozen than fresh semen. This level of DNA damage shows that the neutral comet assay may be used as an additional parameter for assessing the quality of frozen semen. Compared to gel electrophoresis and acridine orange test, comet assay appears to be the most sensitive of all the procedures for detecting DNA fragmentation.

Mukhopadhyay *et al*. (2011) added that a sperm nuclear DNA protamination below 10% in cattle and buffalo bulls was not an important cause of semen poor quality. It has been shown that spermatozoa which carry an altered genome are able to achieve fertilization, thereby posing concerns about the transmission of abnormal genetic material to the offspring (Barroso *et al*., 2009).

The obtained results showed that motility may be a marker for semen quality as significant correlations were found between motility and many sperm parameters such as sperm abnormalities and DNA damage. In this respect, Ramos and Wetzels (2001) maintained that motility may be a relevant physiological marker for DNA intact sperm. The present study revealed a significant negative correlation between DNA damage and post-thawing motility. This result was in accordance with (Badr *et al*., 2010) in Egyptian buffalo semen. Sperm motility is often used as a predictive measure in semen analyses; high motility being a prerequisite of normal sperm parameters. Irvine *et al*. (2000) reported sperm samples with low motility to carry higher loads of DNA damage. In contrast, Morris *et al*. (2002) revealed that the higher the semen sperm motility, the higher the DNA damage load carried by the sperm populations.

In conclusion, DNA damage evaluation may provide reassurance about genomic normalcy and improve methods of selecting spermatozoa with intact DNA to be used in artificial insemination.

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