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SPERM DNA DAMAGE AND EMBRYONIC DEVELOPMENT AS RELATED TO FERTILITY POTENTIAL OF BUFFALO BULLS

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ABSTRACT

Conventional semen analysis are not sufficient to evaluate bull's fertility, however it might be used as a preliminary evaluation for predicting fertility potential. Sperm DNA integrity may give a better evaluation of bull fertility potential. In the current study, six mature buffalo bulls were divided into two groups (based on a threshold of 70% motility; as high or low potential fertility). The first objective of the current study was to evaluate DNA fragmentation (measured as Comet value) in the two groups of bulls (three bulls/group) in relation to its potential fertility. The results showed that low potential fertility bulls had significantly ($P<0.05$) more sperm DNA damage, higher ($P<0.05$) sperm abnormal morphology and lower ($P<0.05$) sperm viability than that of sperm from the high potential fertility bulls.

Our second objective was to assess whether a relationship exists between bull's sperm DNA damage and bull *in vitro* fertility. A significant variations ($P<0.05$) in the developmental competence of the embryos to the morula and blastocyst stages were observed. The results also showed a clear negative correlation between bull's potential fertility and DNA integrity at and the ability of the fertilized ova to sustain its development to the morula ($r=-0.756$) and blastocyst stage ($r=-0.643$). These results indicate that measuring DNA integrity could be a powerful marker of bull's fertility. Therefore, screening bulls for DNA damage should be emphasized along with routine semen analysis in selecting bulls either for natural mating or artificial insemination.
Keywords: Buffaloes, sperm, DNA, Comet value, embryo development.

INTRODUCTION

Differences in bull fertility have been demonstrated earlier either *in vivo* or *in vitro* . These differences have been suggested to be due to semen quality that might be compensated by increasing sperm number deposited in the female reproductive tract (Saacke *et al*., 1988; DeJarnette *et al*., 1992) as defective sperm with poor functional traits might be selected against during its transport in the female reproductive tract and never reach the site of fertilization (Saacke *et al*., 1988). Bull fertility increases with increased sperm number until a plateau is reached. At this point, compensable factors are no longer have any effect on fertility as it is the case during natural mating and non-compensable factors comes into act. In this case, the sperm might reach the site of fertilization and initiate the egg activation process, but it might fail to sustain zygotic, embryonic or fetal development (Tesarik *et al*., 2004), pointing at some sperm-endogenous factors that determines its potential for sustaining further development of the activated egg and the presumptive zygote (Eid, 1995 ; Hourcade *et al*., 2010).

Routine semen analysis including concentration, motility and viability gives an approximate evaluation of the functional competence of spermatozoa but does not always reflect the quality of its genetic material (DNA). Mature mammalian spermatozoa transport their genetic package of DNA in a tightly packaged form. This package is largely condensed because of the substitution of greater part of histones by protamines. The integrity of sperm DNA is of prime importance for the paternal genetic contribution to normal offspring. Spermatozoa with defective DNA compromise sperm motility as stated by Huang *et al*. (2005), and Hourcade *et al*.,(2010), suggesting that defects in the DNA-chromatin packaging or fragmentation of DNA are likely to inhibit sperm motility, as a result of changes in the overall sperm morphology, and eventually impede fertilization or subsequent embryo development (Lewis and Aitken, 2005; Fatehi *et al*., 2006). Several techniques have been developed to detect DNA abnormalities for assessing sperm competence (Fraser and Strzezek, 2004). These assays include the single cell gel electrophoresis assay (COMET), as described by Haines *et al*. (1998) and Ivrine *et al*., (2000). It also has been suggested that sperm DNA integrity may be a more objective marker of sperm function as opposed to the standard semen analysis (Saleh *et al*., 2003; Avdatek *et al*., 2010), and it is more reliable to predict the potential fertility of semen using a combination of laboratory tests for prediction of the sperm different attributes.

Therefore, in the present study, our first objective was to use the alkaline Comet assay since it is highly sensitive and reproducible than other assays (Irvine *et al*., 2000; Simon *et al*., 2011) in measuring DNA fragmentation in two groups of bulls with different potential fertility based upon a threshold of 70% sperm motility. Our second objective was to determine the predictive power of sperm DNA damage in relation to sperm ability to sustain embryonic development.

MATERIALS AND METHODS

Animals:

Six sexually mature buffalo bulls with an average of 59.17 months of age and 692.5 kg body weight belonging to the International Livestock Management Training Center, Sakha, Animal Production Research Institute (APRI; Egypt) were used in this study. Bulls were housed individually under semi-open sheds and partially roofed with asbestos. All bulls were healthy and clinically free of external and internal parasites, free of physical defects and venereal diseases. Palpation of the external genital tract of each animal showed that they were typically normal. The testicular tone was glandular, all epididymal regions were present and both testes were almost normal in size and moved freely up and down within the scrotal pouches. Copulatory patterns for all tested bulls, at the beginning of the trials were judged to be normal.

Throughout an experimental period (16 weeks), semen was collected from all bulls twice a week using an artificial vagina. Percentage of motility,

livability, and abnormality of spermatozoa as well as sperm concentration were determined in the collected ejaculates according to Barth,(2001).

According to the results of the performed evaluation, bulls were divided into two groups, 3 bulls had semen of satisfactory quality (≥70% sperm motility) were served as high potential fertility group. However, three bulls had less than the above values (unsatisfactory quality; <70% sperm motility), were considered as low potential fertility group.

Chemicals and reagents:

All chemicals used in this study were purchased from Sigma (Madrid, Spain), unless otherwise indicated.

Single-Cell Gel Electrophoresis (SCGE) or Comet Assay:

The Comet assay procedure was performed according to the method described by Haines *et al*. (1998), with slight modifications.

Slide preparation and lysis:

Agarose solutions of normal and low-melting point gels (Bioshop Canadian, Burlington. No. L7L4Y8) were prepared at 0.5% and 0.7%, respectively. Agarose powder was mixed with DPBS (Dulbecco's phosphate buffered saline) and the tubes were boiled for 1 to 2 minutes. Both agarose mixtures were kept at 37°C to maintain a liquid state until the time of use. Slides were prepared by applying a thin layer of the 0.5% normal agarose gel to fully frosted slides (Fisher, Pittsburgh, Pa) to ensure adhesion of subsequent layers. A 50-µL drop of the agarose was spread across the length of the slides with a coverslip by scraping off the excess, and the slides were allowed to air dry. For the second agarose layer, 5 µL of the washed sperm (approximately 10^6–10^7 sperm/mL) in DPBS/PVA was mixed well with 95 µL of 0.7% low- melting point agarose gel. Seventy-five micro liters of this sperm-agarose mixture was pipetted onto the first layer of agarose on each slide and a 24 X 50 mm glass coverslip was placed over each sperm-agarose drop to facilitate spreading. The agarose was allowed to solidify at 4°C for 10–15 minutes. After careful removal of the coverslips, a third layer, 75 µL of 0.7% low-melting point agarose gel was pipetted onto the slides. As before, coverslips were placed and the gel solidified at 4°C for 10–15 minutes. The slides were removed from the refrigerator, loaded into a perspex rack and gently immersed in 500 mL of lysis buffer. The lysis buffer contained 2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base (Tris (hydroxymethyl) aminoethane, American Bioanolytical), (pH 10.3), 1% (w/v) Triton X-100, 40 mM DTT (dithiothreitol) and 500 µL of 10 µg/mL Proteinase K. The slides were incubated for 1 h at room temperature, followed by 2.5 h at 37°C. After that incubation, the slides were then washed three times at 20 min intervals with distilled water by transferring the rack from one container to another, to remove traces of salt and detergent. The container was covered with aluminum foil to minimize light.

Following cell lysis, all the slides were placed in a horizontal electrophoresis tank filled with electrophoresis buffer (10 mM Tris containing 0.08 M boric acid and 0.5 M EDTA; pH 8) and was kept for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at 25 V adjusted to 300 mA. When electrophoresis was completed, the slides were

drained and flooded with neutralization buffer (0.4 mol/l Tris; pH 7.5). After neutralization step, the slides were stained with ethidium bromide (20 µg/ml in distilled water) and mounted with a cover slip. Cells were visualized at 200× using a fluorescent microscope (CETi., Belgium).

Sperm cells were assessed by a single observer as either positive or negative for DNA damage. Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a ‘‘comet’’ pattern, whereas whole sperm heads, without a comet, were considered as not damaged. A total of 100 cells per slide were assessed for comets in these experiments.

***In vitro* oocyte maturation (IVM):**

Oocytes were collected and matured *in vitro* essentially as described by Sirard *et al*., (1988). Buffalo ovaries were collected at slaughter house and transported to the laboratory in saline solution (0.9% NaCl) supplemented with penicillin G (100 IU/ml) and streptomycin sulfate (100 µg/ml) at 30°C. Cumulus oocyte complexes (COCs) were aspirated from 2-8 mm follicles with an 18 Guage needle, then pooled and placed in Petri dishes. The COCs were recovered and selected using a stereomicroscope. Only COCs with a homogenous cytoplasm and ≥3 layers of cumulus cells were used. The selected COCs were washed three times in maturation medium (TCM-199 supplemented with 10% fetal calf serum (FCS; v/v), 20 IU/ ml of PMSG (Folligon, Intervit International BV. Boxmeer - Holland), 10 µg/ml of LH, 1 µg/ml of E_2 and 50 µg/ml of gentamycin), and transferred to a 200 ml drop of maturation medium under mineral oil and incubated for 24 h at 39 °C in 5% of CO_2 in air with 95% humidity.

***In vitro* fertilization (IVF) and embryo culture:**

Fresh semen collected by artificial vagina from the six buffalo bulls were swim-up separated as described by Parrish *et al*., (1986) and diluted to 25×10^6 sperm / ml. Swimup separated sperm diluted with IVF-TALP medium were coincubated with 10 µg/ml heparin (heparin sodium) for 30 min in CO_2 incubator at 38°C, 5% CO_2 in air and humidified atmosphere.

Matured oocytes were washed three times in TL- HEPES medium (Parrish *et al*., 1989) and twice in fertilization medium (IVF-TALP). Oocytes were inseminated with capacitated semen (1.5×10^6 sperm/ml). Oocytes and spermatozoa were cocultured in fertilization medium and incubated for 22 h at 38.5 °C, 5 % CO_2 in air with maximum humidity.

After co-incubation, presumptive zygotes were stripped of cumulus cells, washed three times in embryo culture medium, (TCM–199 medium supplemented with 20 mMol Na- pyruvate, 3 mg/ml BSA, and 50 µl/ml Gentamycin sulphate) and cultured in pre-equilibrated embryo culture medium in 4 well Petri dishes and overlaid with sterile mineral oil, then incubated at 38.5 °C, 5% CO_2 with 95% humidity (Eyestone and First, 1989). Half of the medium was replaced every 48 hour with fresh medium. Development of the fertilized oocytes and cleavage rates were recorded on d 7 after fertilization.

Statistical Analysis:

Data are reported as means ± SE. The comparisons between two groups were tested by T-test using SPSS (1999) computerized program

v.14.0 to calculate the analysis of variance (ANOVA) for the different parameters. Pearson correlation coefficient was calculated between sperm parameters; embryo development and Comet value.

RESULTS AND DISCUSSION

Semen quality:

In the current study, bull potential fertility was determined based on a threshold of 70% sperm motility (mass progression) into two groups. The first group of high potential fertility (≥ 70%), and the second group of a lower potential fertility (<70%). The mean of semen analysis parameters and Comet value for the two groups of bulls differ in their *in vivo* potential fertility (based on the motility of fresh semen) are summarized in Table (1). As shown in Table (1), there were no significant differences in the mean of ejaculate volume (ml), and sperm concentration (10^6/ml). However, the percentage of live spermatozoa was significantly (P<0.05) higher in the group of high fertility bulls than that of lower fertility bulls (72.50±2.12% vs. 59.44±2.97%). The mean percentage of sperm abnormalities in the group of high fertility bulls was significantly lower (P<0.05) than that of low fertility bulls (7.61±0.48% vs. 11.39±0.75%). The mean Comet value in the sperms of high fertility bulls was lower than that of low fertility bulls (8.94±0.51 vs. 18.72±0.53 (P<0.05; Table 1).

Table (1). Sperm Characteristics, DNA fragmentation (comet value) of two groups of bulls with different potential fertility.

Semen Characteristics	High potential fertility bull (Mean±SE)	Low potential fertility bull (Mean±SE)
Ejaculate volume (ml)	2.14±0.17[a]	2.44±0.26[a]
Sperm motility (%)	74.17±1.29[a]	55.28±0.95[b]
live sperm(%)	72.50±2.12[a]	59.44±2.97[b]
abnormal sperm(%)/ml	7.61±0.48[a]	11.39±0.75[b]
Sperm concentration sperm×106/ml	1.12±0.11[a]	1.42±0.16[a]
Comet value	8.94±0.51[a]	18.72±0.53[b]

Means within rows with different superscripts are significant at least (P<0.05).

The present results show that sperm DNA damage (indicated as Comet value) in the low fertility bulls was significantly (P<0.05) higher than in bulls with higher fertility, accompanied with higher sperm abnormal morphology and lower levels of sperm motility (Table1). These results provided evidence of an important relationship between some of semen characteristics and sperm DNA damage as detected by Pearson correlations. A high correlation was recorded between poor semen quality and higher levels of sperm DNA damage (Table 2). A very clear negative correlation was found between sperm motility and DNA damage for the two groups of bulls (r=--0.940). However, positive correlation was observed between sperm morphological abnormalities and DNA damage (r= 0.722). Moderate inverse

correlation was found between sperm livability and DNA damage in the current study (r= -0.299). Contradictory results were found between ejaculate volume, sperm concentration /ml and the amount of damaged DNA (Table 2). The current results are agreeable with the results demonstrated by Ivrine *et al*., (2000); Gandini *et al*., (2000); Giwercman *et al*., (2003); Sheikh *et al*., (2008) and El-Sisy *et al*., (2010). They showed that sperm motility was inversely correlated with the percentage of sperm with fragmented DNA. In these studies, no significant correlations were detected between DNA fragmentation, sperm morphology and concentration by Gandini *et al*., (2000) and Giwercman *et al*., (2003). It can be suggested that defective DNA packaging resulting from increased DNA fragmentation can lead to morphological abnormalities that may inhibit sperm motility, thus sperm with DNA damage may exhibit lower functional potential that explain the lower potential fertility of these bulls.

Table (2). Pearson correlations between semen characteristics and DNA integrity measured by Comet value in high and low potential fertility bulls.

Semen trait	High potential fertility	Low potential fertility	Overall
Ejaculate volume (ml)	0.044[a]	-0.064[b]	0.142
Sperm motility (%)	-0. 667[a]	-0.710[a]	-0.940
Sperm livability(%)	-0.161[a]	-0.292[a]	-0.299
Sperm abnormalities (%)	0.472[a]	0.646[b]	0.722
Sperm concentration (106/ml)	-0.059[a]	-0.591[a]	0.088

Sperm DNA damage and Embryonic development competence:

Evidence from the current results suggests that spermatozoa with higher DNA damage may compromise the ability of the developing embryos fertilized by sperm from the group of bulls with the low potential fertility (Table 3). The development of fertilized oocytes was followed up to 7 days and the average percentages of embryonic development to different stages at day 7 post fertilization are shown in Table (3). There were no differences between the two groups of bulls with different potential fertility in the percentage of embryos blocked their development at the two cell stage, 4-cell stage or even 8-16 cell stage (Table 3). However, the differences in the developmental competence began to show a significant ($p<0.05$) variation at the morula and blastocyst stages. Increased levels of sperm DNA fragmentation have been proposed as a possible cause of lower developmental competence (Tesarik *et al*., 2004). The current results supported the hypothesis that paternal effects on fertility may go beyond events of fertilization and completion of the first 2-3 cleavages, but involve the ability of preimplantation embryos to sustain its development to the later stages since high negative correlations were found between the level of DNA damage and ability of the fertilized oocytes to develop to morula and blastocyst stages (Table 4). This finding was also reported by different studies (Fatehi *et al*., 2006 ; Chaveiro *et al*., 2010). These results also indicated that oocytes may be capable of repairing

paternal damaged DNA to a certain degree at the earlier stages of embryonic cleavage (Fatehi *et al*., 2006; Huang *et al*., 2005). On the other hand, the first steps of development are subjected to maternal control and the expression of paternal genes begins at the 4-8 cell stage in most of the domestic species (Tahtamouni *et al*., 2009) and might go beyond that stage in the buffalo embryo, and thus impair embryonic development at these stages. In support of this (*in vitro*) only 20-40% of the oocytes fertilized ever develop to the morula stage (Eid, 1995), and this percentage decreased in the oocytes fertilized by spermatozoa from the bulls of low potential fertility. It might be possible to confirm that the embryos which fail had more damaged DNA, since they were fertilized by sperm from bulls with low fertility potential. The current results may also explain the early undetectable *in vivo* embryonic loss as a consequence of the presence of paternal damaged DNA and/or the inability of the activated oocyte to repair paternal DNA damage.

Table 3. Relationships between bull fertility and embryo developmental competence in two groups of bulls with different potential fertility.

Embryonic developmental stage	High potential fertility bulls (Mean±SE)	Low potyential fertility bulls (Mean±SE)
2-Cell	8.97±1.08[a]	9.43±0.89[a]
4-Cells	10.08 ± 0.89[a]	11.01 ± 1.11[a]
8-16 Cells	11.28 ± 1.04[a]	8.30 ± 1.15[a]
Morula	16.77 ± 0.97[a]	7.94 ± 0.87 [b]
Blastocyst	14.85 ± 1.65[a]	6.73 ± 0.95 [b]

Means within rows with different superscripts are significant at least (P<0.05).

Table 4. Pearson correlations between semen characteristics and DNA integrity measured by comet value and embryonic developmental competence.

Developmental stage	High potential fertility	Low potential fertility	Overall
2-cell embryo	0.008	0.185	0.087
4-Cell embryo	0.219	0.113	0.165
8-16 Cell embryo	-0. 141	-0.106	-0.333
Morula	-0.349	-0.357	-0.756
Blastocyst	-0.177	-0.512	-0.643

In summary, we have demonstrated that there were negative correlations between sperm motility, morphology and sperm DNA damage. The observation that oocytes fertilized with spermatozoa from bulls with higher damaged-DNA might pass the first 2-3 cleavages (until 8-16 cell stage) prior to activation of the embryonic genome and don't have the ability to go through the later stages of embryonic development pointing at the parental genome despite of the active mechanism of DNA repair in the oocyte. Therefore, the current study emphasizes the importance of including

bull screening for DNA damage in combination with routine semen analysis in selecting bulls for either natural mating or freezing semen for artificial insemination.

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تكسر المادة الوراثية فى الحيوان المنوى وعلاقتها بخصوبة الطلوقة ومقدرة الأجنة على التطور

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تعتبر الطرق التقليدية لتقييم السائل المنوى غير مرضية لتحديد خصوبة الطلائق، بينما يمكن استخدامها كمحدد مبدئي لتوقع خصوبة الطلائق. حيث يوجد الآن عدد من الإختبارات التى عن طريقها يمكن قياس أفضل لمعدل تكسر المادة الوراثية وبالتالى خصوبة الطلائق منها اختبار (المذنب) Comet. وفى هذه الدراسة استخدم ستة طلائق جاموسى سليمة وناضجة قسمت إلى مجموعتين (استنادا إلى النسبة المئوية للحركة الجماعية 70 ٪)، وبناء عليه قسمت إلى طلائق محتمل ان تكون مرتفعة الخصوبة والمجموعة الثانية أكثر احتمالية فى إنخفاض الخصوبة. وكان الهدف الأول من الدراسة الحالية هو تقييم تكسر الحمض النووي (تقاس قيمة المذنب) في المجموعتين من الطلائق (ثلاثة طلائق لكل مجموعة) وفيما يتعلق بخصوبتها المحتملة .وأظهرت نتائج هذه التجربة أن الطلائق المحتمل أن تكون منخفضة الخصوبة كانت الحيوانات المنوية المنتجة منها بها المزيد من الحمض النووي التالف وكذلك كانت النسبة المئوية للحيوانات المنوية غير الطبيعية أكبر وانخفاض حيوية الحيوانات المنوية عند مقارنتها بالحيوانات المنوية المنتجة من الطلائق الأكثراحتمالية فى الخصوبة .

وكان هدفنا الثاني تقييم ما إذا كانت هناك علاقة بين تلف الحمض النووى للحيوانات المنوية و الخصوبة المعملية للطلائق .وأظهرت النتائج أنه توجد اختلافات في الكفاءة التطورية للجنين إلى مراحل التوتية والكيسة الأريمية .كما أظهرت النتائج أيضا وجود علاقة سلبية واضحة بين الخصوبة المحتملة للطلوقة وسلامة الحمض النووي مشيرا إلى أن قياس سلامة الحمض النووي يمكن أن يكون عاملا قويا فى تقدير الخصوبة. ولذلك ينبغي التأكيد على فحص الحمض النووي للحيوانات المنوية مع تحليل السائل المنوي وذلك عند اختيار طلائق سواءعند إستعمالها فى التلقيح الطبيعي أو التلقيح الاصطناعي.

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