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Effects of Cryopreservation of Buffalo and Bovine Spermatozoa on Sperm DNA Damage and Early Embryonic Development

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ABSTRACT

Semen was collected using artificial vagina as per the standard practice from 3 healthy Egyptian buffalo and 3 Holstein Friesian bulls with good body condition, and their age ranged between 4 to 6 yr. Routine semen evaluation was performed. For a better prediction of buffalo and bovine sperm fertility, the first objective was to measure DNA fragmentation in buffalo and bovine bull semen using the alkaline Halo sperm assay. The second objective was to study the influence of cryopreservation process on sperm sustainability of the zygotic and embryonic development. The results revealed that the ejaculate volume and sperm concentration /ml differed ($p<0.05$) between the bovine and the buffalo bulls. Sperm physical characteristics including sperm motility, livability, percentage of dead sperm, abnormalities, acrosomal damage and values of sperm with halo were found to be different ($p<0.05$) depending on the species. Semen characteristics of bovine bull are almost better than there of buffalo.Sperm with halo (competent DNA) percentage was. There was a general decrease in post-thawing semen quality parameters including percentages of the live sperm, motility, dead, abnormality, acrosomal damage, and halo sperm value for buffalo and bovine. Moreover, evidence from the current results suggests that the percentage of embryos produced from insemination by fresh sperm had greater ($p<0.05$) values for the bovine in relation to the buffalo. The rate of development to morula or blastocyst, irrespective of the species or the status of the inseminated sperm (fresh or frozen/thawed) was comparable. It was concluded that (irrespective of the species) sperm freezing/thawing process significantly affected each of sperm physical characteristics and rate of DNA damage. It was also proposed that paternal effects on fertility may go beyond events of fertilization and completion of early stages of embryonic development, and might involve the ability of those embryos to sustain its development to the later stages.

Keywords: Buffalo, Bovine, bull semen, cryopreservation, embryonic development.

INTRODUCTION

The ultimate goal the animal's breeding is to transform superior germ-plasm creating a new individual that is superior to their parents. The use of successful biotechnologies enhances genetic progress, one of which is cryopreservation where spermatozoa from genetically superior bulls can be preserved. However, this mustn't be taken for granted in the livestock animals, ignoring the low ability of spermatozoa to withstand the freezing /thawing procedures, leading to damage of sperm membranes and probably its genetic material. Moreover, cryopreservation might be harmful to sperm capability and fertility (Lemma, 2011). A decrease in sperm viability by 50%, in addition to a sevenfold reduction in fertilizing capacity occurreddue to cryopreservation (Lessard *et al,* 2000; Muiño *et al.,* 2008; Chowdhury *el al.,* 2013). The viability and fertility of the sperm cells are influenced by premature acrosomal reaction, mitochondrial function, sperm motility and chromatin condensation (Chaveiro *et al.* 2006; Cooter *et al,* 2005; Watson, 2000; Wongtawan *et al,* 2006). It has been shown that various sperm organelles have been known to be affected by cooling stress occur during cryopreservation. Cold shock known asthe stress reaction occur due to the drop in temperature. Generally, a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoon motility and an increase in the number of dead spermatozoa are the different phenomena occur during cold shock. It's clear that disruption in sperm membrane configuration results from rearrangement of phospholipids bound to the sperm membrane into a different configuration resulting in disturbance of membrane function and permeability (Desai *et al.,* 2010). The disturbance of to the cellular membranes has a carry-over effect on other sperm cellular components and their functions.

The cryopreservation process has been also associated with increased production of reactive oxygen species (ROS), which detrimentally affects semen quality compared to fresh semen (Shiva Shankar Reddy *et al*., 2010), reslting in reduction in fertility of frozen– thawed buffalo spermatozoa (Lyashenko, 2015). High ROS levels induced lipid peroxidation, ATP depletion, DNA fragmentation, motility and viability reduction, which all finally affected fertility of spermatozoa (Kim *et al*., 2010). Also buffalo spermatozoa are susceptible to ROS induced damage due to absence of sufficient amounts of cytoplasmic antioxidants along with the presence of higher amounts of polyunsaturated fatty acid composition in the membrane structure (Nair *et al.,* 2006).

Successful fertilization of ova depends upon the morphologically normal spermatozoa. Since, various physical changes of spermatozoa like breaking of tail, cell surface destruction, loss of motility can lead to failure of the fertilization process. Also, DNA integrity of the spermplays an important role in the success of fertilization since it might interfere with the capability of the presumptive zygote to continue its cleavage and development normally (Eid and Parrish, 1995; Eid *et al*., 2011).

Buffalo sperm seems to be more sensitive to freezing damage due to lipid oxidation as compared to bull sperm because of its high content of polyunsaturated fatty acids [Garg *et al.,* 2009; Nair *et al.,* 2006], leading to relatively detrimental cryopreservation influence, reduced post thaw motility and conception rates (Kadirvel *et al.,* 2009).

Therefore, the current study, the first purpose was to measure DNA fragmentation in buffalo and cattle bull semen using the alkaline Halo sperm assay since it is highly sensitive and reproducible than other assays (Irvine *et al.,* 2000; Simon and Lewis, 2011). The second objective was to study the effects of cryopreservation

process on the ability of spermzoain sustaining embryonic development up to the blastocyst stage.

MATERIALS AND METHODS

The current study was carried out at the International Livestock Management Center (ILMTC), belonging to the Animal production Research Institute (APRI), Agriculture Research Center, Ministry of Agriculture and Land Reclamation.

Sperm DNA Fragmentation Assessment

Semen collected from 3 Egyptian buffalo and 3 Holstein Friesian bulls. DNA fragmentation of the sperm were assessed in fresh and frozen semen using the Halosperm test (INDAS laboratories, Spain), as described by Fernandez *et al.,* (2005).

In Vitro of the MaturationOocyte (IVM):

Buffalo and bovine ovaries were collected at slaughter house and transported to the laboratory in saline (0.9% NaCl; 30°C) supplemented with penicillin G (100 IU/ml) and streptomycin sulfate (100 µg/ml). Cumulus oocyte complexes (COCs) were then aspirated from 2-8 mm follicles with an 18 Guage needle, pooled and placed in a Petri dish. The COCs were recovered and selected using a stereomicroscope. Oocytes were then transferred to a 200 µl drop of maturation medium under mineral oil into sterile Petri dish as described by Sirard *et al.,* (1988).

In vitro fertilization (IVF) and embryo culture:

Fresh semen collected by artificial vagina from the 3 buffalo and 3 bovine bulls were separated by swim-up Parrish *et al.,* (1986) and diluted to 25×106 sperm / ml. Then, sperm were diluted with IVF-TALP medium, and incubated with 20 µg/ml heparin (heparin sodium) for 30 min at38°C, 5% CO2 in air and humidified atmosphere.

Matured oocytes were first washed three times in TL- HEPES medium (Parrish *et al*., 1989) and twice in fertilization medium (IVF-TALP). Oocytes were inseminated (~300 oocyte / treatment) with capacitated semen (1.5×106 sperm/ml). Oocytes and spermatozoa were co-cultured in fertilization medium and incubated for 22 h at 38.5 °C, 5 % CO2 in air with maximum humidity.

Then 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, they incubated in culture media for 48 hours according to (Eyestone and First, 1989). Development of the fertilized oocytes and cleavage rates were recorded on day 7 after fertilization (Table 3).

Statistical Analysis:

Statistical analysis of data was carried out using SAS (2008), according to the following model:

$$Yijkl = \mu + Ai + Cj + Bk + Eijkl$$

Where:

Yijkl : The observation lth of the ith type of animal in the jth case of semen in the kth bull number.

µ : Overall mean.

Ai:Fixed effect due to the type of animal (i: 1 = Egyptian buffalo, 2 = Holstein Friesian).

Cj : Fixed effect due to the case of semen (j: 1 = fresh semen, 2 = frozen semen).

Bk : Fixed effect due to the bull number (k: 1, 2, 3).

Eijklm : random error assumed N.I.D. (0, $\sigma^2 e$).

Differences among means were checked according to Duncan (1955).

RESULTS AND DISCUSSION

1. Comparison of different parameters of raw semen in the buffalo and bovine bulls:

In the current study, the mean ejaculate volume (ml), concentration of the sperm /ml of the ejaculate determined directly post collection for the buffalo bulls were given in Table 1. The results indicated that the ejaculate volume, Sperm concentration /ml are different between the bovine and the buffalo bulls. These results were found to be comparable to the results observed by Kadoom *et al.* (2016) in the bovine and buffalo bulls in Egypt. These findings are also allied to previous work of Chowdhury *et al.* (2013), where they compared the indigenous Gir with the Murrah buffalo.

Sperm physical characteristics including sperm total motility percentage, livability, percentage of dead sperm, abnormalities, acrosomal damage and values of sperm with halo were found to be different depending on the species (Table 1) the sperm characteristic of bovine semen was almost higher than buffalo semen.

Table 1. Values of different parameters (Mean±SE) of raw and frozen-thawed semen of the buffalo and bovine bulls.

Semen Trait	State	Buffalo	Bovine
Volume (ml)	Fresh	2.6 ± 0.2 ^{B}	4.0 ± 0.3 ^{A}
	Frozen	--	--
Sperm Concentration (106/ml)	Fresh	1.2 ± 0.1 ^{B}	1.5 ± 0.1 ^{A}
	Frozen	---	--
Sperm Motility (%)	Fresh	74.0± 0.5 ^{aA}	74.3± 1.5 ^{aA}
	Frozen	47.3± 1.5 ^{bA}	48.3± 1.5^{bA}
Live Sperm (%)	Fresh	73.3± 2.4a ^{B}	80.9± 1.5^{aA}
	Frozen	61.1 ± 3.2 ^{bA}	63.8 ± 0.8 ^{bA}
Sperm Abnormalities (%)	Fresh	7.3± 0.5 ^{bB}	12.0± 1.2 ^{bA}
	Frozen	26.7±1.4^{aA}	23.2± 0.8 ^{aB}
Sperm Acrosomal Damage (%)	Fresh	7.4± 0.7 ^{bB}	13.7± 0.9 ^{bA}
	Frozen	15.5± 1.4 ^{aB}	17.9± 0.9^{aA}
Halo sperm Value	Fresh	91.5± 2.5 ^{bB}	88.8± 1.2 ^{bA}
	Frozen	82.0±1.0 ^{aB}	80.5± 1.3 ^{aA}

Means within rows with different (small) superscripts are significantly different (p<0.05).

Means within column swith different (capital) superscripts are significantly different (p<0.05).

DNA integrity examinationin dicated as big halo using Halosperm test (Halotech DNA, Madrid, Spain) , a modified Sperm Chromatin Dispersion (SCD) (Fernández *et al.*, 2005) . The current study is focusing on spermatozoa DNA fragmentation expressed as big halo assuming that such spermatozoa has undamaged or slightly damaged DNA. The halos normally correspond to the relaxed DNA loops attached to the residual

nuclear structure. Moreover, the qualitative pattern observed in buffalo semen was almost double that observed in the bovine as shown in Table 1. Also, the percentage of sperm with halo (non fragmented DNA) was not different between buffalo and bovine bull semen as shown in Table 1.

Sperm progressive motility was found to be above 70%, envisaged that semen for both species were in good condition for further processing and preservation. The current results are higher than the results reported by Kadoom *et al.* (2016), in the bovine and buffalo bulls for the motility and livability, and a little lower for the abnormalities and damage of the acrosome in the two above mentioned species.

2- Effect of Freezing on Buffalo and Bovine Semen Parameters:

The quality of buffalo and bovine spermatozoa after the thawing process is shown in Table 1. There was a general decrease in post-thawing semen quality parameters such as percentages of the live sperm, motility, dead, abnormality, acrosomal damage, and halo sperm value for buffalo and bovine. No major differences were detected in the same trait following thawing process. Significant differences in the percentage of sperm abnormalities, dead sperm, and sperm with damaged acrosome, and values of sperm with halo were detected after thawing (Figure 1).The present results show that bovine spermatozoa had a higher rate of sensitivity to cryopreservation compared to the buffalo spermatozoa as shown in Table 2.

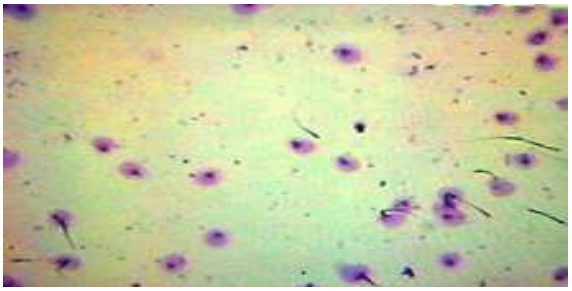

Figure1. Spermatozoa without DNA fragmentation (big halo spermatozoa and medium-sized halo spermatozoa), assessed by Halosperm test. Spermatozoa with DNA fragmentation included spermatozoa without halo, small halo, in addition to the degraded spermatozoa (image taken from the microscope).

Table 2. Percentage of change in sperm functional characteristics due to freezing/thawing in the buffalo and bovine

Semen Trait(%)	Buffalo	Bovine
Sperm Motility	36.1	35
Live Sperm	16.6	21.1
Sperm Abnormalities	265.7	93.3
Sperm Acrosomal damage	109.4	30.6
Halo sperm	7.3	6

The relative changes in sperm cells characteristics owing to cryopreservation, calculated as the proportions of the differences on the initial value of the trait.

As shown by Perteghella *et al.*, (2017), spermatozoa lost approximately 44% of their total motility (TM) after cryopreservation, whereas the diluted-encapsulated sperm lost approximately 38% of their TM.

Confounding with the current results, about 18% reduction in the motility was also obtained in a previous report of subjective assessment (Kumar and Atreja, 2012). The total motility was reduced by 49% after thawing in buffalo sperm (Rasul *et al.*, 2001). In cattle, the reduction in total motility was about 9% after cryopreservation (Thomas *et al.*, 1998). The decrease in sperm motility may be due to cholesterol loss that might reduce the survival rate after cryopreservation (Singh, 2016).These findings support earlier reports working with the same two species Kadoom *et al.* (2016). However, other studies using Gir bulls, and Murrah buffalo bulls (Chowdhury *et al.*, 2013), indicated that the percentage of motile sperm was significantly decreased up to 42% after thawing. Also, in Boer, sperm motility declined due to the cryopreservation process (Tuli and Holtz 1994) and Florida goats (Dorado *et al.*, 2010). The reduction in sperm motility could be due to biophysical injuries as a result of formation of ice crystals in the sperm extra- and intracellular environment and increasing solute concentration (Mazur 1984). Also, biochemical oxidative stress could result in irreversible damage to sperm structure, changes in membrane fluidity and enzymatic activity (Aitken *et al.*, 1989).

Sperm DNA isa highly compact cellular DNA insuring sperm chromatin protection since it undergoes chromatin remodeling during spermatogenesis (Ward and Coffey, 1991). Moreover,since sperm DNA contributes 50% of the zygote's genome, DNA integrity of fertilizing spermatozoa is vital for a successful embryonic development (Agarwal and Allamaneni, 2004).

Studies on buffalo and bovine spermatozoa were carried out to find out the causes behind the catastrophic effects of freezing/thawing (Castro *et. al.,* 2016 and Ahmad *et al.*, 2014). So far, the published data regarding the impact of freezing/thawing on sperm have shown that buffalo spermatozoa faces more damage due to higher content of polyunsaturated fatty acids (PUFA) in membranes which is highly vulnerable to lipid peroxidation and cholesterol efflux (Andrabi, 2009).

Sperm plasma membrane in the ram and the bull is known to contain high PUFA. Therefore, it is more susceptible to ROS causing reduction in sperm mobility, viability and sperm-oocyte fusion due to a reduction in axonemal protein phosphorylation (Jahanian *et al.*, 2014).

Jain and An (1976) observed that buffalo spermatozoa contained nearly equal distribution of saturated (47.8%) and unsaturated (49.8%) fatty acids, and high levels of PUFA. It was also reported that more than 70% of the total unsaturated fatty acids were in the form of PUFA. Moreover, the phospholipids' content of water buffalo spermatozoa and seminal plasma is much higher in the buffalo spermatozoa compared to that of the bovine bull. It has been reported that the effect of PUFA on sperm resistance to cooling and freezing procedures may be related to the type of PUFA content as well as compositions added to semen extender. The presence of

more fatty acids makes mammalian sperm highly sensitive to lipid peroxidation (Kothari*et al.*, 2010). Since, PUFA and cholesterol are the main targets for free radical (ROS) damage, resulting in an inverse relationship between lipid peroxides and sperm motility. Additionally, sperm membrane lipids particularly the cholesterol:phospholipid (C/P) ratio determines the sensitivity of sperm to cold shock (Garton *et al.*, 1961) are related to sperm membrane stability. Sperm having lower C/P ratio (such as buffalo sperm) are more susceptible to cryodamage than the sperm having high C/P ratio. Interestingly, studies carried out in bovine have also shown that sperm membranes of different species also vary in their cholesterol and phospholipid content (Garton *et al.*, 1961). Therefore, the differences in sperm membrane composition might account for the observed differential response of buffalo and bovine sperm to the freezing/thawing as shown in Table 2.

3-Effect of semen Freezing/Thawing on Buffalo and Bovine Early Embryonic Development:

The effect of buffalo and bovine bulls' semen cryopreservation was examined on success of embryonic development up to the blastocyst stage (Table 3). Evidence from the current results suggests that the percentage of embryos produced through insemination by fresh sperm was better ($p<0.05$) in the bovine compared to the buffalo as shown in Table 3. Early embryonic development up to blastocyst stages, irrespective of the species or the status of the inseminated sperm were comparable as shown in the current study (Table 3).

Table 3. Influence of freezing/thawing of the buffalo and bovine bulls' semen on early embryonic developmental competence in vitro

Item	Sperm	buffalo	Bovine
Number of inseminated	fresh	298	319
Oocytes	frozen	314	305
Cleavage rate	fresh	53.2 ± 2.7^{aB}	65.5 ± 3.6^{aA}
	frozen	41.6 ± 3.6^{bB}	56.0 ± 2.4^{bA}
Morulla stage	fresh	13.9 ± 0.8^{aB}	19.5 ± 1.9^{aA}
	frozen	12.1 ± 1.3^{aA}	13.9 ± 1.2^{bA}
Blastocyst rate	fresh	14.2 ± 1.5^{aA}	17.3 ± 2.0^{aA}
	frozen	11.3 ± 1.4^{aA}	12.9 ± 1.0^{aA}

Means within rows with different (small) superscripts are significant ($p<0.05$)

Means within columnswith different (capital) superscripts are significant ($p<0.05$)

The current results were found to be a little lower than the rate of early embryonic development for embryos inseminated in vitro with fresh semen in the Egyptian Buffalo (Eid *et al.*, 2011). The results also imply that frozen/thawed semen with higher DNA damage may compromise the ability of the developing embryos fertilized by frozen/thawed sperm to sustain its development (Table1 and 3). It can be suggested that defective DNA resulting from cryo-damage can lead to lower developmental potential of oocytes inseminated by frozen/thawed sperm in the bovine as well as the buffalo. Sperm freezing/thawing significantly affected only cleavage rate for the buffalo embryos. However, in the case of the bovine embryos, sperm freezing/thawing significantly affected cleavage rate and developing of the embryos to morula (Table 3). These interesting results might be related to the results presented in Table 2, where sperm freezing/thawing increased the rate of acrosomal damage and sperm abnormalities (109% vs. 30.6%) and (265% vs. 93%) in the buffalo and bovine bulls' sperm respectively. These results might also imply higher ability of the bovine oocytes to repair sperm DNA compared to the buffalo oocytes to a certain degree at the earlier stages of embryonic cleavage (Chaveiro *et al.*, 2010). The paternal influence of damaged DNA is more prominent after zygotic transcriptional activation. This paternal effect on the developing embryo may be due to the active repair mechanism present in oocyte's cytoplasm t to overcome the damaged paternal chromatin. The probability of eliminating an embryo fertilized by a sperm with damaged DNA is higher at the blastocyst stage than the cleavage stage (Simon *et al.*, 2014).

In conclusion, the current results generally (irrespective of the species) support the hypothesis that Sperm freezing/thawing significantly affected sperm physical characteristics, rate of DNA damage and that paternal effects on fertility may go beyond events of fertilization and completion of early embryonic development, and might involve the ability of those embryos to sustain its development to the later stages since a comparable rates of development were found between the DNA damage and ability of the fertilized oocytes to develop to morula and blastocyst stages (Table 3).

On the other hand, the first steps of development are subjected to maternal control and the expression of paternal genes begins around 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. 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.

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تأثير عملية حفظ السائل المنوى بالتجميد على سلامة DNA الحيوان المنوى والتطور المبكر للأجنة فى الأبقار والجاموس

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تم جمع السائل المنوي من ٦ طلائق سليمة من الناحية الصحية والفسيولوجية (٣ فحل جاموس ، ٣ ثور بقر فريزيان) تتراوح أعمارها بين ٤- ٦ سنوات ، وذلك باستخدام المهبل الصناعي . وكان النمط المستخدم في التنبؤ أثناء تقييم السائل المنوي في الجاموس والأبقار عن طريق خصوبة الإسبرم .إستهدف البحث تقديرمعدلات تكسرDNA الحيوان المنوى للجاموس والأبقار كنتيجة لعملية التجميد، وذلك من خلال تقدير Alkaline Halo Sperm . كذا فقد استهدف البحث دراسة تأثير عملية الاسالة على قابلية الحيوان المنوي للاستمرار إلى مرحلة الزيجوت والتطور الجنيني .أوضحت النتائج إختلاف حجم القذفة وتركيز الاسبرمات /مل بمعنوية عند مستوى معنوية($p<0.05$) ما بين ذكور الجاموس والأبقار .إختلفت الخصائص الطبيعية للحيوان المنوى متضمنة:- الحركة - ونسبة الاسبرمات الحية - ونسبة الاسبرمات الشاذة - الأكروسوم التالف - وقيم الـAlkaline Halo Sperm إعتماداً على نوع الحيوان ، بينما لم تختلف معدلات تجزؤ الDNA بين كل من الجاموس والأبقار .يوجد بصفة عامة نقص في قياسات نوعية السائل المنوي بعد عملية الإسالة والتي اشتملت على نسب الاسبرمات الحية وحركة الاسبرمات و نسبة الاسبرمات الميتة ونسبة الاسبرمات غيرالطبيعية ونسبة الضرر الذي يحدث في الأكروسوم وقيم Halo Sperm في الجاموس والأبقار .وجدت اختلافات معنوية في نسبة الاسبرمات غير الطبيعية والميتة والاسبرمات المحتوية على تلف في الأكروسوم ، وكانت قيم Halo Sperm مسجلة بعد التجميد والإسالة .أوضحت النتائج أيضاً ارتفاع معنوي في نسبة الأجنة المنتجة خلال التلقيح بسائل منوي طازج ($p<0.05$) في الأبقار مقارنةً بالجاموس .يتوقف معدل تطور الأجنة إلى مرحلة الموريولا أو البلاستوسيت المفرخة على نوع الحيوان وحالة الاسبرم المستخدم في التلقيح (طازج أم بعد التجميد والإسالة) .الاستنتاج: تتأثر الصفات الطبيعية للحيوان المنوى وكذا معدلات سلامةDNA الحيوان المنوى بعملية التجميد والإسالة - بغض النظر عن نوع الحيوان (بقرى / جاموسى).لايجب إغفال التأثير الأبوى على نتائج الإخصاب المتحصل عليها، وقدرة الحيوان المنوى على إكمال المراحل المبكرة لتطور الأجنة والاستمرار في التطور إلى المراحل الأخيرة.