EFFECT OF SEASON AND FREEZING ON SPERM VELOCITY, PLASMA MEMBRANE INTEGRITY AND ACROSOMAL STATUS OF EGYPTIAN BUFFALOES SPERMATOZOA

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SUMMARY

This work aimed at studying the impact of season of the year and cryopreservation process on the spermatozoa characteristics of the Egyptian buffalo bulls. Year was divided into two periods; cold (September - March) and hot (April - August). Semen was collected from six intact buffalo bulls twice weekly. One hundred – 40 outstanding semen samples representing two periods of the year were used. Semen was diluted using Tris-based extender and stored in French straws (0.25 ml). Semen straws were frozen in liquid nitrogen. Characteristics of spermatozoa motion were determined before and after freezing using computer-assisted sperm analysis (CASA).Plasma membrane integrity and acrosomal status were also evaluated.

Progressive motility (%) of spermatozoa was higher (P< 0.05) in the semen collected during the cold period (87.1 ± 0.6) compared to that in the hot period (78.2 ± 0.5) by about 9%. Period of the year had no effect on sperm curved line velocity (VCL), average line velocity (VAP) and straight line velocity (VSL) (um/sec). Semen samples collected during the hot period indicated a significant (P<0.05) increase in the percentage of deformitive spermatozoal plasma membrane (10.9 ± 0.7) compared to the cold period (7.9 ± 0.6). Meanwhile, percentage of spermatozoa that had partial or complete acrosomal damage were higher (P<0,05) in the semen collected during the hot period (6.9 ± 0.4 and 2.5 ± 0.2, respectively) compared to the semen collected during the cold period (5.5 ±0.4 and 1.2 ±0.2, , respectively).

VCL, VAP and VSL were higher (P<0.05) in the pre-frozen semen compared to those after (159.7 ± 1.3 vs. 72.4 ± 0.8; 83.6 ± 0.6 vs. 45.9 ± 0.4 and 68.4 ± 0.3 vs. 35.7 ± 0.4, respectively). Percentage of spermatozoa with intact plasma membrane was higher (P <0.05) in pre-frozen compared to the post-frozen semen by 4 %. Significant increase (P <0.05) was obtained in partially and completely damaged spermatozoa in the post frozen samples compared to those before freezing.

Keywords: Buffaloes, semen, freezing, season, plasma membrane integrity, acrosomal damage

INTRODUCTION

Application of artificial insemination (AI) in buffaloes is not as extensive as in cattle (FAO, 2005). The conception rate in buffalo under AI system was reported to

range between 43.4 - 60.8 % (Sosa *et al*., 2003), due to the low percentage of viable spermatozoa after freezing (Barkawi *et al.,* 2006), season of the year (Bhavsar *et al*., 1989) and/or type of extenders (Sosa *et al*., 2003).

Post-thawing sperm motility was found to affect fertility rate (Zhang *et al*., 1998; Januskauskas *et al*., 2001, Rasul *et al.,* 2001, Januskauskas *et al*., 2003, and Rodriguez-Martinez, 2003). This is due to that semen freezing has an adverse effect on the integrity of plasma membrane and acrosome of spermatozoa (Barkawi *et al.,* 2006). Plasma membrane plays a significant role before and during fertilization processes (Azam *et al*., 1998). Thus, damage of plasma membrane and / or acrosome represent the main biological factors that control acrosome reaction during fertilization process (Graham and Moce, 2005).

Studies have shown that there are significant relationships between aspects of sperm movement, cervical mucus penetration (Mortimer *et al*., 1990), and fertilization in vitro (Holt *et al*., 1985; Chan *et al*., 1989; and Sukcharoen *et al*., 1995, 1996) and *in vivo* (Barratt *et al*., 1993; Irvine *et al*., 1994; and Macleod and Irvine, 1995) fertilization. Computer-assisted sperm analysis (CASA) was used widely during the last two decades to determine precisely the physical characteristics of semen (Koonjaenak *et al*., 2007 b) and to assess the kinematics of individual spermatozoa (Budworth *et al*., 1987; Januskauskas *et al*., 1999 Mandal *et al*., 2003 and Hallap *et al*., 2004). The CASA instruments have also been used for the kinematic analysis of capacitating sperm populations to identify the proportion exhibiting hyperactivated motility.

Based on the traditional evaluation practice, many studies were conducted to evaluate the physical characteristics of Egyptian buffalo bull semen with particular reference to the season of the year (Barkawi *et al.,* 2006 and Abdel-Khalek *et al*., 2008) However, no data are available describing the individual kinematics of buffalo spermatozoa.

The aim of this study was to assess the kinematics of individual buffalo bull spermatozoa and to evaluate the integrity of acrosome and plasma membrane in relation with cryopreservation process.

MATERIALS AND METHODS

Animals and management:

Six healthy and sexually matured Egyptian buffalo (*Bubalus bubalis)* bulls (3-5 years old) with intact testes and average body weight of 592 ± 55 kg were used. The calendar of the year was divided according to environmental temperature into two periods; hot (April - August) and cold (September - March). Experimental bulls were kept tied in a semi-shaded yard, and fed on concentrate feed mixture, rice straw and green fodder according to their live body weight according to NRC (1995).

Semen collection:

The semen was collected early in the morning at the rate of two ejaculates/bull/week using an artificial vagina covered by a rubber sheath. Ejaculates were transferred immediately to the laboratory and kept in a water bath at 37 ˚C until the respective examination. The outstanding ejaculates with more than 70% progressive motility (n=140) were used to evaluate motion characteristics using

Computer-Assisted Sperm Analysis (CASA) (SpermVision™ software minitube, Hauptstraße 41, 84184 Tiefenbach, Germany).

The recorded motion characteristics include: the distance curved line (DCL, microns); distance average path (DAP microns); distance straight line (DSL, microns) ; velocity curved line (VCL, Point to point velocity, microns/sec); velocity average line (VAP, point to point velocity on a path constructed using a roaming average, microns/sec) as well as, the velocity straight line spermatozoa (VSL, the average path and the point reached that is furthest from this origin during the measured time period, microns/sec).

Semen processing and freezing:

Selected semen ejaculates were diluted using Tris-based extender at a rate of 1:10 (v/v) (Osman, 1996). Diluted semen was packed into French plastic straws (0.25 ml) using an automatic machine for filling and sealing (LPE Branson generator IMV Technologies, France) and kept into the refrigerator (5˚C) for four hours as equilibrium period. Thereafter, straws were mounted horizontally on a metal rack placed in freezing floating boat to liquid nitrogen (LN_2) vapor. After 15 min, the loaded straws were plunged directly into the liquid nitrogen and stored until respective evaluation.

Evaluation of plasma membrane and acrosomal integrity:

Plasma membrane integrity was determined using Hypo-Osmotic Swelling Test (HOST) according to the method described by Jeyendran *et al.* (1984). A minimum of 200 sperm were checked under phase contrast microscopy (400x). Sperm storage displaying different types of swelling (coiling of tails) were considered as positive for the HOST. Percentage of hypo osmotic sperm swelling was calculated as the total number of spermatozoa with coiled tails / total number of sperm (coiled and normal tails) X 100.

Assessment of acrosome status of spermatozoa was executed according to the procedure outlined by Watson (1975). The percentage of intact acrosome was calculated for 200 spermatozoa selected randomly from each slide as no. of intact spermatozoa / total number of checked spermatozoa X 100. Integrity status of spermatozoa was classified into three categories: intact (when the stain was clearly distributed over the anterior part of the sperm to the equatorial segment), partially damaged (when the stain partially covered the acrosome region) and completely damaged (sperm with acrosome completely lost) when the sperm had no acrosome.

Statistical analysis:

The data of motion characteristics were analyzed using the General Linear Model procedure (SAS, 1998) to test the effects of the period of the year (cold and hot) and freezing on the measured traits applying the following model:

$$Y_{ijkl} = \mu + S_i + F_j + (S_i * F_j)_{ijk} + e_{ijkl}$$

Y_{ijkl} = The measured trait.

μ = Overall mean.

S_i = Effect of period of the year (Cold = 1 and Hot = 2).

F_j =Effect of freezing (pre freezing =1, post-freezing=2).
$(S_i * F_j)_{ijk}$ = the interaction between the period of the year and freezing
e_{ijkl} = Experimental error supposed to be randomly distributed

RESULTS AND DISCUSSION

Characteristics of spermatozoa kinetics:

Effect of period of the year:

Total and progressive motility of spermatozoa were significantly higher ($P< 0.05$) in the semen collected during the cold compared to the hot period of the year. The studied velocity traits showed non-significant difference between the two studied periods of the year (Table 1).

Table 1. Traits of sperm motility (Mean ± SEM) in fresh semen of buffalo bulls as affected by the period of the year

Traits	Period of the year	
	Hot	Cold
Motility (%)		
Total motility	84.6^{b} ± 0.4	91.3^{a} ± 0.5
Progressive motility	78.2^{b} ± 0.5	87.1^{a} ± 0.6
Velocity types		
DAP (um)	36.7^{a} ± 0.3	37.1^{a} ± 0.4
DCL (um)	71.5^{a} ± 0.6	70.5^{a} ± 0.7
DSL (um)	29.9^{a} ± 0.3	30.2^{a} ± 0.4
VAP (um/s)	83.0^{a} ± 0.7	84.8^{a} ± 0.8
VCL (um/s)	161.4^{a} ± 1.3	160.9^{a} ± 1.6
VSL (um/s)	67.7^{a} ± 0.7	69.2^{a} ± 0.8

Means having different superscripts within the same row differ significantly at 5% level
DCL: distance curved line (microns); DAP: distance average path (microns); DSL: distance straight line (microns); VCL: velocity curved line (microns/sec); VAP: velocity average line (microns/sec) ;VSL : velocity straight line (microns/sec).

Plasma membrane and acrosomal integrity:

Effect of period of the year:

Period of the year had a significant ($P<0.05$) effect on the percentage of spermatozoa with intact plasma membrane. The semen collected during the hot period of the year showed lower percentage of intact plasma membrane relative to that collected during the cold period by about 3% (Table 3). This trend comes in agreement with the results obtained by Barkawi *et al.* (2006) who reported higher percentage of intact plasma membrane in buffalo bull spermatozoa collected during the cold *vs* the hot period.

The effect of the period of the year on the total and progressive motility comes in accordance with the findings of Barkawi *et al*. (2006) and Koonjaenak *et al*. (2007a) who reported that the hot period of the year had adverse effect of the quality of produced semen either from Egyptian or Swamp buffalo. This feature may be attributed to the low released concentrations of GnRH and testosterone (Younis, 1998) and / or LH basic concentration (Mandal *et al*., 2000) released during the hot period of the year.

Table 2. Post-freezing sperm motility traits (Mean ±SEM) of buffalo bull semen analyzed by computer-assisted sperm analysis (CASA) as affected by the period of the year

Traits	Pre-freezing	Post-freezing
Motility (%)		
Total motility	87.5[a] ± 0.4	63.4[b] ± 0.8
Progressive motility	81.9[a] ± 0.5	47.4[b] ± 0.8
Velocity types		
DAP (um)	36.8[a] ± 0.3	19.4[b] ± 0.2
DCL (um)	70.4[a] ± 0.6	30.8[b] ± 0.4
DSL (um)	30.1[a] ± 0.3	15.1[b] ± 0.2
VAP (um/s)	83.6[a] ± 0.6	45.9[b] ± 0.4
VCL (um/s)	159.7[a] ± 1.3	72.4[b] ± 0.8
VSL (um/s)	68.4[a] ± 0.3	35.7[b] ± 0.4

Means having different superscripts within the same row differ significantly at 5% level
DCL: distance curved line (microns); DAP: distance average path (microns); DSL: distance straight line (microns); VCL: velocity curved line (microns/sec); VAP: velocity average line (microns/sec) ;VSL : velocity straight line (microns/sec).

Effect of freezing:

Cryopreservation of semen was found to be negatively affect ($P<0.05$) all the studied traits of spermatozoal motility compared to the cryopreservation estimates pre-freezing. The percentage of motile spermatozoa and progressive motility decreased post-freezing by 24 and 35% in comparison to the values recorded pre-freezing, respectively (Table 2). This result cames in agreement with that reported by Budworth *et al*. (1988), Mandal *et al*. (2000) and Barkawi *et al*. (2006).

The pattern of sperm motion reflects the biochemical environment and physical conditions imposed on spermatozoa. The reduction in spermatozoal motility and velocity (VCL, VSL and VAP) were most probably attributed to the percentage of injured spermatozoa during the cryopreservation process (Watson, 1995). Sperm velocity was found to reflect its mitochondrial function (Graham *et al.*, 1984) and subsequent fertility of bulls (Budworth *et al.,* 1988; and Kjaestad *et al*., 1993). The damage was mainly attributed to the formation of ice crystals (Mazur, 1984) in the mitochondria and axonemes resulting in impaired sperm motility (Courtens *et al*., 1989). Such changes increase the levels of intracellular calcium resulting in increased circular sperm motility (Suarez *et al*., 1993)

Percentage of spermatozoa that had intact acrosomes was higher ($P<0.05$) by about 2.7% in the semen samples collected during the cold period of the year compared to those collected during the hot period. On the other hand, the percentage of spermatozoa with partially damaged or completely lost acrosomes were higher in the hot period compared to the cold period by 1.4 and 1.3%, respectively (Table 3 and Plates 1 and 2).

Table 3. Effect of period of the year (mean ±SEM) on plasma membrane and acrosomal integrity of buffalo bull spermatozoa

Traits	Period of the year	
	Cold	Hot
Plasma membrane integrity (%)		
Intact	92.1 [a] ± 0.6	89.1 [b] ± 0.7
In-intact	7.9 [a] ± 0.6	10.9 [b] ± 0.7
Acrosomal integrity (%)		
Intact	93.3 [a] ±0.4	90.6 [b] ±0.5
Partially damaged	5.5 [a] ±0.4	6.9 [b] ±0.4
Completely lost	1.2 [a] ±0.2	2.5 [b] ±0.2

Means having different superscripts within each factor within the same column differ significantly at 5% level.

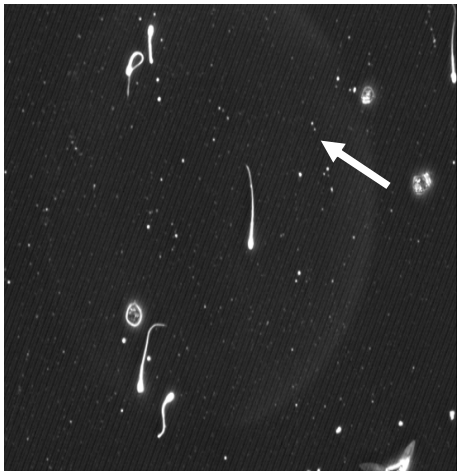

Plate 1: Buffalo spermatozoa with in-intact plasma membrane as pointed with arrow (400x).

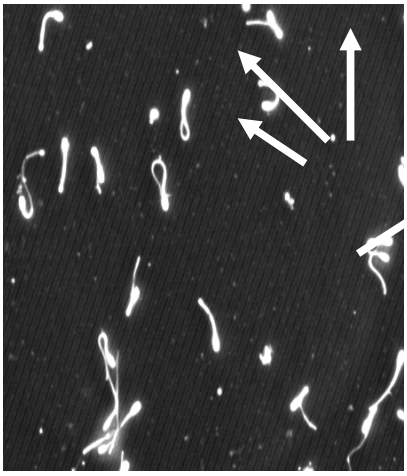

Plate 2: Buffalo spermatozoa with intact plasma membrane as pointed with arrow (400x).

The significant reduction in the percentage of the acrosomal integrity of buffalo spermatozoa during the hot period were in consensus with the findings of Mandal *et al*. (2000), who reported a similar trend.

Effect of freezing:

Percentage of spermatozoa with intact plasma membrane insignificantly ($P< 0.05$) decreased in the frozen semen by about 4 % compared to that recorded before freezing (Table 4). This reduction coincided with the increased incidence of spermatozoa with partial or complete damage of acrosomes ($P<0.05$) post-freezing compared to pre-freezing by 1 and 0.8%, respectively, (Table 4 and Plates 3 and 4).

Table 4. Effect of freezing (mean±SEM) on plasma membrane and acrosomal integrity of Egyptian buffaloes spermatozoa

Traits	Pre-freezing	Post-freezing
Plasma membrane integrity (%)		
Intact	92.6 [a] ± 0.5	88.5 [b] ± 0.7
In-intact	7.4 [a] ± 0.5	11.5 [b] ± 0.7
Acrosomal integrity (%)		
Intact	92.1 [a] ±0.6	91.8 [a] ±0.3
Partially damaged	5.6 [a] ±0.5	6.7 [b] ±0.3
Completely lost	2.2 [a] ±0.3	1.4 [b] ±0.1

Means having different superscripts within each factor within the same column differ significantly at 5% level.

The obtained percentage of spermatozoa with intact plasma membrane was higher than those obtained by Azam *et al*. (1998), Koonjaenak *et al*. (2007a) and Selvaraju *et al*. (2008) in Surti buffalo bulls.

The decrease in the incidence of post-frozen intact spermatozoa was most probably due to the destabilization of plasma membrane during cryopreservation process when exposed to low temperature and high salt concentration (Holt and North, 1994 and Shannon and Vishwanath, 1995). This phenomenon was reported to be associated with the loss of plasma-lemma over the entire acrosome, a marked projection in the anterior part of outer acrosomal membranes, and extensive vesiculation and disruption of plasma-lemma and outer acrosomal membranes (Krogenaes *et al*., 1994).

The recorded post-freezing percentage of spermatozoa with intact acrosomes (91.0 %) was higher than the values reported previously by Singh *et al*. (1989); Aguiar *et al*. (1994); Kumar *et al*. (1993) and Barkawi *et al. (* 2006) (81.9 - 90%). However, there is a common agreement among them that cryopreservation of semen has an adverse effect on the integrity of spermatozoa.

The recorded percentage of the spermatozoa with intact acrosome before freezing is lower than the value reported by Krishna and Rao (1987) in Murrah buffalo bulls (93.5%). The obtained percentage of post-frozen intact spermatozoa (91.8 %) in this study was higher than the values found by Ismail (1993); Osman (1996); Barkawi *et al*. (2006) and Abdel-Khalek *et al*. (2008) in Egyptian buffalo bulls and Azam *et al*. (1998); Pratap *et al*. (2000); Panghal *et al*. (2002) and Maurya *et al* .(2003) in Murrah buffalo bulls and Selvaraju *et al*. (2008) in Surti buffalo bulls. They recorded percentage from 11.5 to 73.5%. Meanwhile, it was lower than the percentages reported by Sosa *et al*. (2003) for Egyptian buffalo bulls (93.9 %) and Koonjaenak *et al*. (2007a) for Swamp buffalo bulls (98.9%).

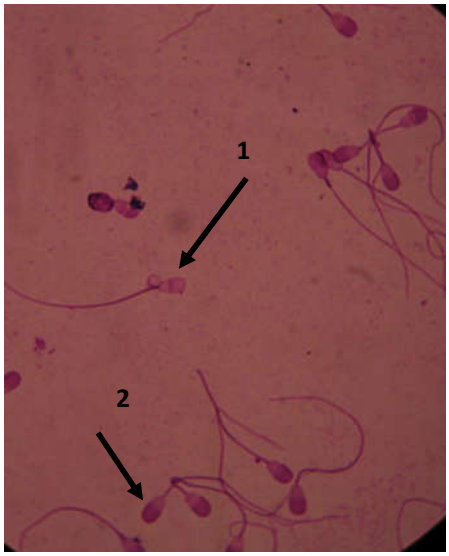

Plate 3: Buffalo spermatozoa with completely lost acrosomes (1) as compared to intact one (2) (1000x).

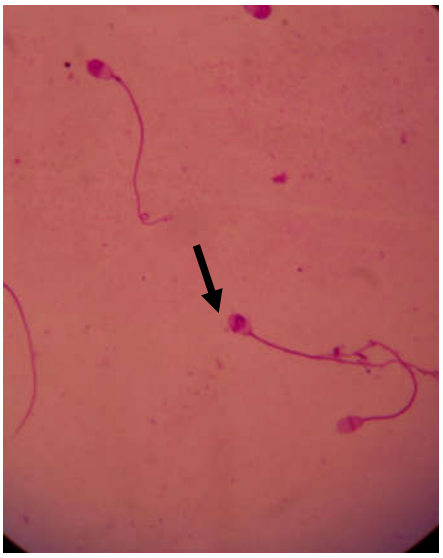

Plate 4: Buffalo spermatozoa with partially damaged acrosomes as pointed with arrow (1000x).

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تأثير الموسم والتجميد على حركة وسلامة الغشاء البلازمى وحالة الأكروسوم فى الحيوانات المنوية للجاموس المصرى

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استهدفت هذه الدراسة دراسة تاثير موسم السنة والحفظ بالتجميد على الحيوانات المنوية للجاموس المصرى. قسم العام إلى فترتين, البارد (سبتمبر- مارس) والحار (أبريل – أغسطس). تم جمع السائل المنوى من عدد ٦ طلائق جاموسى مرتين أسبوعياً أنتخب (عدد = ١٤٠ عينة) على مدار العام. تم تخفيف السائل المنوى بإستخدام مخفف التريس والتخزين فى قصيبات (٠,٢٥ مل). ثم التجميد فى النيتروجين السائل. تم تقدير حركة الحيوانات المنوية قبل وبعد التجميد بإستخدام (CASA). كما تم تقييم سلامة الغشاء البلازمى والأكروسوم للحيوانات المنوية.

حدث زيادة معنوية للحركة الفردية للحيوانات المنوية خلال الموسم البارد (٨٧,١ ± ٠,٦) مقارنة بالموسم الحار (٧٨,٢ ± ٠,٥) أى بما يعادل ٩%. لم يكن لموسم السنة تأثير معنوى على (VCL) , (VAP) , (VSL) (ميكرون / الثانية). أظهرت العينات التى جمعت خلال الموسم الحار زيادة معنوية فى نسبة الحيوانات المنوية ذات الخلل فى الغشاء البلازمى (١٠,٩ ± ٠,٧) مقارنة بالموسم البارد (٧,٩ ± ٠,٦). أظهرت نسبة الحيوانات المنوية ذات التلف الجزئى والكلى للأكروسوم زيادة معنوية خلال الموسم الحار (٦,٩ ± ٠,٤ و ٢,٥ ± ٠,٢ على التوالى) مقارنة بالموسم البارد (٥,٥ ± ٠,٤ و ١,٢ ± ٠,٢ على التوالى).

زادت معدلات الحركة VCL , VAP, VSL معنوياً خلال مرحلة ما قبل التجميد مقارنة بما بعد التجميد (١٥٩,٧ ± ١,٣ مقابل ٧٢,٤ ± ٠,٨ ; ٨٣,٦ ± ٠,٦ مقابل ٤٥,٩ ± ٠,٤ و ٦٨,٤ ±٠,٣ مقابل ٣٥,٧ ± ٠,٤ على التوالى). زادت معنوياً نسبة الحيوانات المنوية ذات الغشاء البلازمى السليم قبل التجميد بمعدل ٤% مقارنة بمرحلة ما بعد التجميد. كما حدث زيادة معنوية فى نسبة الحيوانات المنوية ذات التلف الجزئى والكامل للأكروسوم بعد التجميد.