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Sperm Characteristics in Cryopreserved Buffalo Bull Semen and Field Fertility

Research Article

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ABSTRACT

Semen quality and its relationship to fertility are major concern in animal production. The aim of this study was to assess the relationship between frozen thawed sperm characteristics and fertility following *in vivo* fertility through Artificial Insemination (AI). Semen samples were collected from four buffalo bulls. Semen volume and sperm concentration appeared to be significantly different ($P<0.05$) among bulls, while sperm motility and live sperm percentage did not vary in fresh semen. Frozen thawed semen was evaluated for motility, viability, sperm abnormalities, membrane integrity and *in vivo* fertility. A significant variation was found in semen parameters among bulls after thawing. Highly significant ($P<0.001$) differences in membrane integrity between fresh vs. frozen semen samples were noticed. Pregnancy rate was significantly ($P<0.05$) different among bulls. Significant correlations were found between motility and sperm abnormalities ($r=-0.64$; $P<0.05$) and membrane integrity ($r=0.64$; $P<0.05$). A significant negative correlation ($r=-0.73$; $P<0.01$) has been reported between sperm abnormalities and membrane integrity. In addition, a correlation between pregnancy rate and live sperm percentages ($r=0.65$; $P<0.05$), has also been reported. In conclusion, in this study motility was correlated with sperm abnormalities and membrane integrity. Live sperm percentage was the only parameter correlated with fertility. Motility and live sperm percentage can be used as a predicative measure in semen evaluation.

KEY WORDS buffalo bull, *in vivo* fertility, membrane integrity, semen quality.

INTRODUCTION

Semen cryopreservation and artificial insemination (AI) offer many advantages to the livestock industry, particularly in conjunction with genetic evaluation and selection programs (Maxwell, 1984). Failure of fertilization and embryonic mortality, particularly after AI, has long been recognized as potential sources of loss in breeding cows (Gordon, 1996). However, some studies have reported that impaired sperm quality leads to a lower percentage of embryos that develop to blastocysts (Shoukir *et al.* 1998), poor blastocyst quality (Janny and Ménézo, 1994) and relatively high rates of pregnancy failure (Sanchez *et al.* 1996). Saacke *et al.* (2000) suggested that failure in fertilization and subsequent bovine embryonic development are of seminal origin. In line with this study, Zhang *et al.* (1998) reported that the factors with the highest predictive index for success within *in vitro* fertilization procedures were sperm motility, morphology, and the percentage of sperm cells with intact acrosomes.

Many *in vitro* methods of semen quality evaluation have been developed for predicting fertility of bull semen in routine AI practice. Conventional semen evaluation has some limitations due to the difficulty to detect some functional sperm cell impairments, which are responsible for decreased fertility (Aitken, 2006). Bavister (1990) and Rowe

et al. (1993) reported that examination of semen characteristics, such as morphology, concentration, and progressive motility are routinely performed for evaluation of semen quality in assisted reproductive technologies. Morphology evaluation is widely used for predicting fertility potential in farm animals (Januskauskas *et al*. 1996; Correa *et al*. 1997; Johnson *et al*. 1998; Chacon, 2001) and humans (Kruger *et al*. 1986; Vawda *et al*. 1996).

The hypoosmotic swelling (HOS) test developed by Jeyendran, 1984 was designed to evaluate the function of the sperm membrane. Its principle is based on the observation of the morphological alterations (size increase) in spermatozoa exposed to hypoosmotic conditions (Drevius and Eriksson, 1966). During the HOS test, spermatozoa with intact and functionally active plasma membranes will undergo swelling (due to the influx of water) and subsequently increase in volume to establish equilibrium between the extra- and intracellular compartments. The use of this inexpensive and simple assay has been recommended as an additional fertility parameter (Jeyendran *et al*. 1984; Correa and Zavos 1994; Johnston *et al*. 1995).

The birth of live offspring is the ultimate test to evaluate *in vivo* or *in vitro* fertility of a given sire, but this entails too many practical and economical drawbacks. Therefore, *in vivo* bull fertility is mostly expressed as non-return rates at 56 days after AI. Non-return rates are affected by sperm quality parameters (Soderquist *et al*. 1991) and can be improved by intervening on other factors related to cow and environment such as farm management, AI technician and season (Den Daas, 1992). Moreover, fertility following AI with frozen-thawed semen is poorer when compared to the use of fresh semen (Januskauskas and zilinskas, 2002) and when is correlated with conventional semen parameter (García Álvarez *et al*. 2010). Therefore, the aim of this study was to investigate the relationship between frozen thawed semen characteristics and *in vivo* fertility of buffalo bulls.

MATERIALS AND METHODS

Buffalo bulls

Four mature buffalo bulls housed at the Abassia Frozen Semen Centre, General Organization for Veterinary Services, Egypt, were used in this study. The bulls were 4-8 years old and 400-600 kg body weight. They were kept under uniform nutritional conditions and regular semen collection schedule for use commercially in AI in Egypt.

Semen collection and evaluation

Two semen ejaculates were collected 10 min apart twice weekly before feeding at 8.00 a.m. A bull was used as teaser animal for sexual preparation. Two successive ejaculates from each bull were collected every third day to avoid deterioration of spermatozoa (Ollero *et al*. 1996) by an artificial vagina at 42 ˚C. Each ejaculate was transferred to the processing laboratory rapidly and kept in a water bath at 37 ˚C for performing evaluation tests. Immediately after semen collection, each ejaculate was evaluated according to (Blom, 1983) for ejaculate volume, sperm cell concentration, sperm motility and live / dead sperms.

Semen cryopreservation

Each ejaculate was further diluted with pre-warmed Bioxcell extender (IMV technologies-France) to the desired semen concentration per milliliter. The extender is composed of carbohydrates, mineral salts, buffer, antioxidants, glycerine, antibiotics (gentamycin, tylosin, lincomycin and spectinomycin), phospholipids and ultra pure water. All extended semen was examined for individual motility at 37 ˚C with the aid of a television monitor connected to a microscope. Semen with greater 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 hours, semen was then packed into 0.25 mL polyvinyl French straw (0.25 mL; IMV, L'Aigle, France) by filling and sealing machine (IMV, Cedex, France). Straws were placed on trays for at least 4 hours at (5 ˚C) for further equilibration (Mohammed *et al*. 1998). Then they were moved in a temperature controlled chamber for at least 8 minutes at -95 ˚C. The straws were then plunged into liquid nitrogen (-196 ˚C) and packaged in plastic goblets for storage in the liquid nitrogen container.

Post-thawing semen evaluation

Thawing of semen was carried out in a water bath at 37 ˚C for 45 seconds. 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 (400X). Percentages of live and abnormal spermatozoa in smears using eosin nigrosin stain were recorded according to Campbell *et al*. (1956).

Plasma membrane integrity test (PMI).

Plasma membrane integrity of spermatozoa was assessed using a hypo-osmotic solution (HOS) assay (Jeyendran *et al*. 1984). Sodium citrate (0.735 g; Merck) and fructose (1.351 g; Merck) were dissolved in 100 mL distilled H_2O to prepare an HOS solution (osmotic pressure ~150 mOsmol/kg), following the method of Rasul *et al*. (2000), and maintained at 37 ˚C for 5 minutes before use. Fifty µL of each semen sample was mixed with 500 µL of HOS solution and incubated at 37 ˚C for 30 minutes. After incubation, 5 µL of formalin solution (10%) was added in order to stop reaction and fix the sperm cells (Barros *et al*. 2007).

The semen samples were evaluated using a phase contrast microscope (400X). Two hundred spermatozoa were assessed for their swelling ability in HOS, and at least five different fields were examined in each preparation. The swollen spermatozoa characterized by coiling of the tail were considered to have an intact plasma membrane.

***In vivo* fertility**

Cyclic buffalo cows were observed for estrus twice daily, and AI was performed on a conventional a.m. / p.m. rule. Buffalo cows would be inseminated by the farmer once standing heat is observed, although the best time for insemination is traditionally considered to be 12 hours after detection of heat. A total number of 3173 buffalo cows were artificially inseminated with frozen semen from the four buffalo bulls. The number of inseminated buffaloes used for the evaluation of fertility rating per bull ranged from 711 to 865. Pregnancy was diagnosed by rectal palpation at 60 days after insemination. The data of pregnancy rates were collected over an interval of one year from the two provinces of Behira and Domiate.

Statistical analysis

Results are expressed as mean values and standard errors for the various parameters taken into consideration. Data were subjected to ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by Duncan's Multiple Range Test. Correlation coefficient among different semen parameters was calculated. Differences were considered to be significant at $P<0.05$ and $P<0.01$.

RESULTS AND DISCUSSION

In Table 1 biophysical characteristics of fresh semen from four buffalo bulls are listed. Semen volume ranged from 2.7 to 3.5 mL. Mean sperm concentration was 1079.2×10^6 cells/mL. Mean percentage of fast forward progressive sperm motility was 65.8 ± 0.9% (range: 65.0-68.3%). Percentage of live spermatozoa was 70.9% (range: 69.0-73.3%). Semen volume and sperm concentration appeared to be significantly different ($P<0.05$) among bulls, while sperm motility and live sperm percentage did not vary in fresh semen.

The post thawing semen characteristics and every significant variation in all semen parameters among bulls are shown in Table 2. Post thaw sperm motility ranged between 39.3 and 45.9%. Live sperm percentage ranged between 58.7 and 65.7%. The percentage of spermatozoa with abnormalities in the third bull achieved the highest percentage (17.1%). Significant differences could be found in semen parameters among bulls after thawing (Table 2). In fact, live sperm percentage and sperm abnormalities were significantly different ($P<0.05$) among bulls. A highly significant difference ($P<0.01$) for motility was also reported. The percentage of curled spermatozoa with intact membrane in fresh and frozen semen was assessed by hypo-osmotic swelling test (Table 3). The sperm cell component most affected by freeze-drying was the plasma membrane, as evidenced by highly significant ($P<0.001$) differences between fresh vs. frozen semen. Significant differences ($P<0.05$) were also observed for intact membrane among bulls in both fresh and frozen semen. Pregnancy rates from different bulls are reported in Table 4. The overall pregnancy rate was 44.5%, ranging from 41.19% to 47.40%. Pregnancy rates were significantly ($P<0.05$) different among the four bulls. Correlation coefficients between semen parameters are shown in Table 5. Significant correlations were found between motility and sperm abnormalities ($r=-0.64$; $P<0.05$) as well as motility and membrane integrity ($r=0.64$; $P<0.05$). Moreover, a significant negative correlation ($r=-0.73$; $P<0.01$) was reported between sperm abnormalities and membrane integrity. Correlation between pregnancy rate and live sperm % ($r=0.65$; $P<0.05$) has also been reported.

Semen evaluation is extremely important to try to predict fertility from a given sperm sample that will generate hundreds of straws, especially when AI with cryopreserved semen is employed (Barros *et al.* 2007). In the present study, motility and live sperm percentage was significantly reduced by the process of freezing, down to 42.5% and 61.8%, respectively. Similar findings for a decline in motility due to freezing and thawing have been reported in the buffalo species (Rasul *et al.* 2001; El-Sisy *et al.* 2007). Watson (1995) reported also, 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, and increasing solution concentration (Mazur, 1984). Ice crystal formation in mitochondria and axonemes during cryopreservation impairs sperm motility (Courtens *et al.* 1989).

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 (El-Sheshtawy *et al.* 2009; Scholkamy *et al.* 2009; El-Sheshtawy *et al.* 2010). Also, a significant difference was reported in semen parameters among bulls after thawing. This result is in accordance with that previously reported by Scholkamy *et al.* (2009) on buffalo bulls.

In the present study the plasma membrane of frozen semen was affected by the freezing process as detected by HOST. Similar observation has been reported in frozen thawed buffalo sperm cells (Rasul *et al.* 2001).

Table 1 Biophysical characteristics of fresh buffalo semen (Mean±SEM; n=4)

Bull	Semen volume (mL)	Sperm concentration (10^6/cc)	Sperm motility %	Live sperm %
1	3.5±0.3^{a}	1100.0±36.5^{ab}	65.0±1.8^{a}	70.3±1.7^{a}
2	2.7±0.4^{b}	1016.7±45.9^{b}	65.0±1.8^{a}	71.0±1.1^{a}
3	2.7±0.1^{b}	1166.7±10.5^{a}	68.3±2.1^{a}	73.3±1.5^{a}
4	2.8±0.1^{ab}	1033.3±42.2^{b}	65.0±1.8^{a}	69.0±1.3^{a}
Total means	2.9±0.1	1079.2±21.0	65.8±0.9	70.9±0.7

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 2 Post-thawing buffalo semen characteristics (Mean±SEM)

Bull	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

The means within the same column with at least one common letter, do not have significant difference (P>0.01 and P>0.05).

Table 3 Mean percentages ±SEM of curled spermatozoa with intact membrane in fresh and frozen buffalo semen

Bull	Fresh	Frozen
1	89.26±1.90^{a}	56.48±0.95^{a}
2	84.25±1.06^{ab}	52.93±1.09^{ab}
3	83.06±1.99^{b}	50.78±1.27^{b}
4	81.62±1.13^{b}	50.58±1.19^{b}
Total means	84.54±1.09**	52.70±0.86

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
** P<0.001; total differences between columns (*t*-test).

Table 4 Pregnancy rate after artificial insemination using post-thawing buffalo semen (Mean±SEM; n=4)

Bull	Buffalo Cows inseminated	Pregnancy rate (Means±SE)	
		NO.	%
1	696	330	47.40±1.51^{a}
2	819	367	44.04±1.62^{ab}
3	779	342	45. 19±1.48^{ab}
4	880	364	41.19±0.76^{b}
Total means	3174	1403	44.45±0.90

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 5 Correlation coefficients between post-thawing sperm parameters and pregnancy rate

Parameters	Motility	Live sperm %	Sperm abnormalities	Membrane integrity	Pregnancy rate
Motility	-	0.482 (0.112)	**-0. 642*** (0.024)	**0.640*** (0.025)	0.291 (0.360)
Live sperm%	-	-	-0.308 (0.330)	0.480 (0.114)	**0.648*** (0.023)
Sperm abnormalities	-	-	-	**-0.725**** (0.008)	-0.021 (0.948)
Membrane integrity	-	-	-	-	0.337 (0.285)
Pregnancy rate	-	-	-	-	-

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

It has been reported that some characteristic features of the biological membrane are represented by the asymmetrical arrangement of lipids within the bilayer components. The lipid composition of the plasma membrane of mammalian sperm cells is markedly different from those of mammalian somatic cells. The fact that sperm cells contain a high content of polyunsaturated fatty acids makes the membranes more susceptible to peroxidative damage. It is suggested that the membrane is thought to be a primary target of freezing or cold shock damage in cells (Aitken *et al*. 1989; Alvarez and Storey, 1995). The hypoosmotic swelling test (HOST) is a rapid test available to evaluate sperm membrane integrity for selecting spermatozoa with a high degree of membrane resistance. Plasma membrane functional activity is one of the most important aspects of sperm biology. It is involved in metabolic exchanges with the surrounding medium, and also plays an important role in several events taking place during fertilization (e.g. capacitation, acrosome reaction, sperm fusion with the oocyte). Investigation of this aspect seems to offer more information about sperm tail membrane functionality (Stanger *et al*. 2010) and potential semen fertility (Jeyendran *et al*. 1984).

Pregnancy rate from different bulls ranged from 41.2% to 47.4%. Similarly, Barile *et al*. (1999) reported 45.2% pregnancy rate in Italian buffalo after artificial insemination with frozen semen. In another study, the fertility rate in buffaloes inseminated with frozen semen was 33% (Chohan *et al*. 1992). Conception rate in buffaloes inseminated with frozen–thawed semen under field condition is approximately 30% (Anzar *et al*. 2003). Published reliable studies on the fertility of liquid stored buffalo semen seem not to be available (Sansone *et al*. 2000). However, few reports indicate a pregnancy rate of approximately 60% with liquid semen after AI in buffaloes (Tomar and Singh, 1970; Akhter *et al*. 2007). Therefore, a pregnancy rate higher than 50% is regarded as a good result after AI with frozen–thawed spermatozoa in buffalo (Vale, 1997). In the present study, all bulls have been considered fertile and have been used for artificial insemination in many provinces in Egypt. In this respect, Al Naib *et al*. (2011), classified bulls with pregnancy rate of about 50% to be considered of high fertility, and the sperm of high fertility bulls tends to be more effective in penetrating artificial mucus and to have an increased ability to fertilize oocyte *in vitro*.

Our results demonstrated that pregnancy rates were different among bulls. Similarly, Haugan *et al*. (2006) found that the success of fertilization with frozen–thawed spermatozoa varies considerably between species and among individuals of the same species. These findings are in agreement with *in vitro* fertilization procedures where semen from different bulls has been used with varying capacity to fertilize oocytes (Mahmoud *et al*. 2004; Abdel Dayem *et al*. 2009). It is clear, taking into account the available evidence, that male variability in IVF is likely to be a general feature of farm animals. Male variation in fertilization rates may be possibly attributed to differences in breed (Sumantri *et al*. 1996), season, age of animal, ejaculate sperm quality and other factors. Also, such differences may be linked to metabolic characteristics of sperm cells (Brackett and Oliphant, 1975), seminal plasma content, and the ratios of seminal plasma volume to sperm number (Fukui *et al*. 1988) and even, within batches of semen from the same bull (Otoi *et al*. 1993).

Results obtained in this study show that motility may be a candidate marker for semen quality, considering that significant correlations were found between motility and both sperm abnormalities and membrane integrity. Ramos and Wetzel's (2001) reported that motility may be a relevant physiological marker for DNA integrity within sperm cells. The present study revealed no significant correlation between pregnancy rate and other sperm parameters except live sperm percentage. The current data concur with El-Sisy *et al*. (2010) who found no significant correlations between semen parameters and conception rate in buffalo. Similarly, no correlations were found between pregnancy rates and vitality (Barros *et al*. 2007). The result of the present study confirmed that some routine semen evaluation protocols do not correlate with fertility. This may be due to the physiological heterogeneity of sperm cells, as well as to the inability of routine semen evaluation techniques to access sperm functional competence (Lewis, 2007; Petrunkina *et al*. 2007).

CONCLUSION

Sperm motility is correlated with sperm abnormalities and membrane integrity. Live sperm percentage is the only parameter correlated with fertility. Motility and live sperm percentage could be used as a predicative measure for fertility in semen analysis of buffalo.

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