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Correlations Between Semen Parameters and Conception Rate in Buffaloes

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Abstract: The aim of the present study was to detect the sperm characteristics variations among different buffalo bulls in each stage of cryopreservation and study the correlation between those parameters and the conception rate. This study was carried out on four healthy buffalo bulls. Semen was collected weekly and tris-based extender was used for dilution of semen. Semen was subjected to freezing and evaluated for visual motility, plasma membrane integrity, live sperm and morphology, acrosomal integrity, assessment of capacitation and DNA fragmentation. Results revealed loss of a percentage of semen quality due to freezing-thawing process. Also, the present study revealed a significant variation in semen of bulls after freezing thawing process. In addition, non significant correlations between semen parameters and conception rate were recorded.

Key word: Buffalo · Sperm · Cryopreservation · Conception rate

INTRODUCTION

Semen cryopreservation is a widely used breeding technique in farm animals, especially bufaloes [1]. However, in this species the fertility of frozen semen remains poorer as compared to fresh semen and is recorded to be 33% in buffaloes [2].

It was confirmed that, sperm membrane functional status measurements as assessed by HOS test, seemed to be of clinical and practical importance in evaluating high and low fertility bulls and assessment of sperm membrane functional status should be considered as an additional parameter for the evaluation of viability and fertilizing capacity in frozen-thawed bovine spermatozoa used for all forms of Advanced Reproductive Technique [3].

Visual assessment of the proportion of motile spermatozoa is the most commonly used viability test to predict fertility. In Sweden, sperm motility is currently one of the quality criteria applied to detect viability of spermatozoa in frozen/thawed semen [4].

It was stated that the correlations obtained between results from semen assessment techniques and non-return rates are much lower within bulls than between bulls, so that prediction of bull fertility would be more reliable than prediction of the fertility of individual ejaculates. A bull with a high observed non-return rate would be expected to exhibit a correlation coefficient of 0.64 and 0.76 for thawed semen [5].

The routine evaluation of bull semen in the laboratory of an AI stud includes assessment of volume, concentration and motility and wave motion of the fresh semen and motility post-thaw are valuable in order to discard semen of poor quality, they appear of little value to predict the fertility of the individual males [6].

Freezing-thawing of buffalo spermatozoa causes considerable damage to DNA, motility apparatus, plasma membrane and acrosomal cap [7], leakage of intracellular enzymes [8] and thus, reduced fertility. The integrity of sperm DNA, whose stability largely depends on the integrity of the chromatin, was very important for the success of fertilization and the development of fetus and offspring [9]. It is recommended that chromatin integrity should be studied as an independent complementary parameter for the better assessment of sperm quality [10]. Sperm DNA integrity is important for the success of natural or assisted fertilization, including normal development of the embryo, fetus or offspring [9].

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It seems clear that others sperm features related to fertility could also be negatively affected during the freezing-thawing procedure in buffaloes [11]. Cryopreservation induced capacitation-like changes in frozen-thawed spermatozoa named as cryocapacitation [12-14]. Cryocapacitation is one of the major factors associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract [15,16], resulting in reduced fertility of frozen-thawed semen. The capacitation-like changes have been demonstrated by greater proportion of chlortetracycline fluorescent pattern "B" (capacitated) due to freezing-thawing in bull [17], boar [18], equine spermatozoa [14] and in buffalo bull semen [19].

Evaluation of DNA integrity must have a priority in the design of new freezing thawing procedures to increase fertilization rates and ensure good embryo development, health of the offspring and maintenance of the genotypes to be preserved. The objective of present study was to detect the sperm characteristics variations among different bulls in each stage of cryopreservation and studying of possible correlations between those parameters and the conception rate.

MATERIALS AND METHODS

Semen Collection and Initial Evaluation: Four healthy buffalo bulls maintained under common management conditions at Abassia Buffalo Semen Freezing Center (Animal Reproduction Division, General Organization for Veterinary Services, Ministry of Agriculture, Egypt) were used in the present study. Semen was collected using artificial vagina twice weekly for a period of 5 weeks and immediately after collection, semen samples were evaluated and those ejaculate having more than 70% progressive motility were used.

Sperm concentration was assessed by a hemocytometer in a 1:200 dilution.

Semen Processing

Preparation of Extender: The basic Tris extender consisted of 3.028 g Tris, 1.675 g citric acid monohydrate, 1.25 g fructose, 8 ml glycerol and 92 ml of glass redistilled water combined with 25 ml of egg yolk plus antibiotics (penicillin G sodium-1000 IU/ml; streptomycin sulphate 1000µg/ml).

Each ejaculate of individual bull was divided into two aliquots; one aliquot was used for fresh semen studies, while the other was processed for cooling and freezing at -196°C.

The semen was extended in Tris-egg yolk-citrate extender to yield approximately 120 million motile sperm cells/ml.

After dilution, the semen was cooled to 4°C in 2 h and equilibrated in an equilibration chamber for 4 hrs at 5°C (Minitub, Germany) before filling in 0.25 ml French straws. After filling and sealing, the semen straws were placed in a rack at 4 cm above liquid nitrogen in the vapour phase for 10 min and plunged into liquid nitrogen container (-196°C). Semen straws were stored in liquid nitrogen for 10 days. The frozen semen straws were thawed at 37°C for 30 s before evaluation.

Sperm Functional Assays: These assays were conducted in fresh, cooled and frozen-thawed buffalo spermatozoa

Visual Motility: A drop of thawed semen sample was placed on a pre-warmed glass slide and cover slipped. Sperm motility was evaluated at 400× magnification based on the visual estimation of the percentage of sperm possessing progressive motility and the percentage was rounded to nearest 5%.

Plasma Membrane Integrity: Plasma membrane integrity (PMI) of buffalo bull spermatozoa was assessed by hypo-osmotic swelling (HOS) assay as described earlier [20]. The solution of HOS contained sodium citrate 0.73 g and fructose 1.35 g, dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol kg^{-1}). The assay was performed by mixing 50 µl of frozen-thawed semen sample to 500 µl of HOS solution and incubated at 37°C for 40 min. After incubation, a drop of semen sample was examined under phase contrast microscope (X 400; Olympus BX40, Japan). Two hundred spermatozoa were counted for their swelling characterized by coiled tail indicating intact plasma membrane.

Live Sperm Percent and Morphology: Semen samples were evaluated using Eosin-Nigrosin staining method [21].

Acrosomal Integrity: Acrosomal integrity percentage was evaluated at 1000× magnification by Giemsa staining method [22].

Assessment of Capacitation Status: Chlortetracycline fluorescent staining was used to determine the capacitation status of buffalo sperm [23]. CTC solution

(750 mM CTC, 5 mM cysteine in 130 mM NaCl and 20 mM Tris acid, pH 7.4) was freshly prepared and pH adjusted to 7.8 and stored at 4°C under dark condition. Fifteen microliter of sperm suspension was mixed with equal volume of CTC solution on a glass slide at room temperature. After a few seconds, 1.5% of glutaraldehyde (12.5% (v/v) in 20 mM Tris-HCl, pH 7.4) was added. The slides were covered with coverslip and stored at 4°C overnight in the dark. Chlortetracycline fluorescence was observed under microscope equipped with phase contrast and epifluorescent optics (Olympus, Japan).

Each sample was assessed twice and at least 200 spermatozoa per slide were classified into one of three CTC staining patterns [23]: (1) uniform bright fluorescence over the whole head (characteristic of uncapacitated cells, pattern F); (2) fluorescence-free band in the postacrosomal region (capacitated cells, pattern B); and (3) dull fluorescence over the whole head except for a thin punctate band of fluorescence along the equatorial segment (acrosome reacted cells, pattern AR). For assessing live sperm % and acrosomal integrity, at least 100 sperm cells per smear were counted using a tally counter. At all times, at least two smears per group were assessed. All the semen evaluation was done by a single person to avoid individual variations.

DNA Fragmentation Using Acridine Orange Staining: Acridine orange staining was performed [24] A stock solution of 0.1% acridine orange (3, 6-bis [dimethylamino] acridine, hemi[zinc chloride]salt, Sigma Chemical Co., St. Louis, MO) was made and stored in the dark at 4°C until use. At the time of staining, a working solution was prepared by mixing 4 parts acridine orange stock solution with 16 parts 0.1 M citrate and 1 part 0.3 M Na_2HPO_4 $7H_2O$. The acridine orange final concentration of the resultant phosphate-citrate buffered solution was adjusted to 0.019% (pH 2.5).

First, spermatozoa were smeared on the glass slide. After being air dried, the samples were treated with acid alcohol (methyl alcohol-glacial acetic acid 3:1, vol/vol) for 2 hours. Immediate preparation is necessary to prevent natural oxidation of thiols in sperm nucleoprotein and the acid alcohol was made on the day of the experiment. Immediately after air drying, approximately 1 mL of working solution was mounted on each slide glass for 5 minutes at room temperature and the samples were then washed with distilled water. The samples were observed under an epifluorescent microscope (Olympus U-PMTVC5D 00637, Olympus, Tokyo, Japan) immediately after a coverslip was put in position. A total of 100 to 200 spermatozoa were observed and classified by type as green, red, or yellow, which is the intermediate type, based on differences in their fluorescent color.

Conception rate was calculated according to the following Table 1.

Statistical analysis data were analysed using the SPSS (2005) computerized program v. 14.0 to calculate the analysis of variance (ANOVA) for the different parameters between individual bulls. Number of straws examined represented the replicate for each bull (n=30). LSD was calculated for significant variance at $P<0.05$. Pearson correlation coefficient was calculated between parameters and the conception rates.

RESULTS

As shown in Tables 2- 4, freezing-thawing of buffalo spermatozoa causes considerable damages to DNA, motility and sperm viability decreases, increase in sperm abnormalities, damage of plasma membrane integrity and increase in the percentage of capacitated acrosome in all bulls.

No significant individual variation between bulls could be noticed in semen characteristics in fresh semen and in different stages of semen cryopreservation, but a significant variation ($P<0.05$) could be found in semen parameters among bulls after thawing.

Table 5, showed a correlation coefficient between different post-thawing sperm parameters and the conception rate. No significant correlation was observed between any of the different parameters and conception rate.

Table 1: The conception rate of inseminated buffalo cows

No. of bull	No. of inseminated animals	No. of conceived animals	Percentage
1	29	15	51.7
2	88	54	61.3
3	74	38	51
4	118	75	63.5

Table 2: Sperm characteristics, DNA fragmentation percentage and capacitation status of fresh semen from different buffalo bulls

	Bull number			
	1	2	3	4
Motility	83.0 ± 1.41[a]	80.0 ± 2.36[a]	84.0 ± 1.99[a]	82.0 ± 0.76[a]
Live%	91.0 ± 1.01[a]	91.0 ± 0.94[a]	89.0 ± 1.30[a]	91.4 ± 1.30[a]
Total abnormalities	4.78 ± 0.34[a]	5.10 ± 0.54[a]	6.04 ± 0.55[a]	5.48 ± 0.24[a]
Membrane integrity	88.1 ± 1.23[a]	88.8 ± 1.57[a]	88.1 ± 1.85[a]	88.4 ± 1.65[a]
Fragmented DNA%	2.1 ± 0.14[a]	2.7 ± 0.26[a]	2.5 ± 0.21[a]	2.3 ± 0.36[a]
Acrosome status				
Uncapacitated	79.7 ± 0.71[a]	87.9 ± 1.42[b]	85.4 ± 1.84[b]	84.4 ± 1.85[b]
Capacitated	8.0 ± 0.34[a]	7.5 ± 0.43[ab]	4.28 ±0.44[c]	6.6 ± 0.57[b]
Acrosome reacted	8.1 ± 0.22[a]	5.16 ± 0.62[b]	5.8 ± 0.31[b]	6.0 ± 0.71[b]

Means within rows within different letters are significance at least (P > 0.05)

Table 3: Sperm characteristics, DNA fragmentation percentage and capacitation status of cooled semen from different buffalo bulls

	Bull number			
	1	2	3	4
Motility	74.0 ± 2.17[a]	71.2 ± 1.18[a]	72.0 ± 2.55[a]	71.2 ± 1.48[a]
Live%	82.0 ± 1.41[a]	78.8 ± 1.37[a]	73.8 ± 2.54[a]	78.2 ± 3.21[a]
Total abnormalities	11.6 ± 1.29[a]	11.2 ± 1.27[a]	11.2 ± 1.14[a]	9.2 ± 1.18[a]
Membrane integrity	86.6 ± 1.42[a]	73.0 ± 2.97[b]	80.0 ± 2.36[ab]	78.8 ± 3.14[b]
Fragmented DNA%	3.0 ± 0.37[a]	3.0 ± 0.63[a]	2.8 ± 0.53[a]	4.6 ± 0.83[a]
Acrosome status				
Uncapacitated	71.6 ± 3.43[a]	75.2 ± 3.23[a]	77.0 ± 3.12[a]	74.0 ± 3.41[a]
Capacitated	10.8 ± 1.47[a]	9.8 ± 1.18[a]	9.0 ± 0.94[a]	10.6 ± 1.07[a]
Acrosome reacted	11.0 ± 0.76[a]	8.0 ± 0.94[b]	12.4 ± 1.24[a]	10.0 ± 0.94[ab]

Means within rows within different letters are significance at least (P > 0.05)

Table 4: Sperm characteristics, DNA fragmentation percentage and capacitation status of Frozen-thawed semen from different buffalo bulls

	Bull number			
	1	2	3	4
Motility	35.0 ± 1.23[a]	33.4 ± 0.91[a]	35.8 ± 1.37[a]	42.2 ± 1.14[b]
Live%	60.2 ± 0.86[ab]	57.2 ± 1.04[a]	57.4 ± 1.49[a]	61.0 ± 0.82[b]
Total abnormalities	17.2 ± 0.85[a]	17.4 ± 1.09[a]	19.2 ± 0.86[a]	16.4 ± 0.69[a]
Membrane integrity	53.8 ± 1.97[a]	55.2 ± 1.04[a]	57.4 ± 1.89[a]	61.2 ± 1.20[a]
Fragmented DNA%	10.2 ± 0.71[a]	10.4 ± 0.91[a]	9.8 ± 1.24[a]	10.4 ± 0.91[a]
Acrosome status				
Uncapacitated	43.4 ± 0.69[a]	44.2 ± 0.57[a]	42.4 ± 0.90[a]	48.6 ± 0.83[b]
Capacitated	23.4 ± 0.91[a]	24.2 ± 0.90[a]	28.4 ± 0.89[b]	25.6 ± 1.29[ab]
Acrosome reacted	17.4 ± 0.62[a]	18.2 ± 0.83[a]	18.2 ± 0.99[a]	20.2 ± 1.44[a]

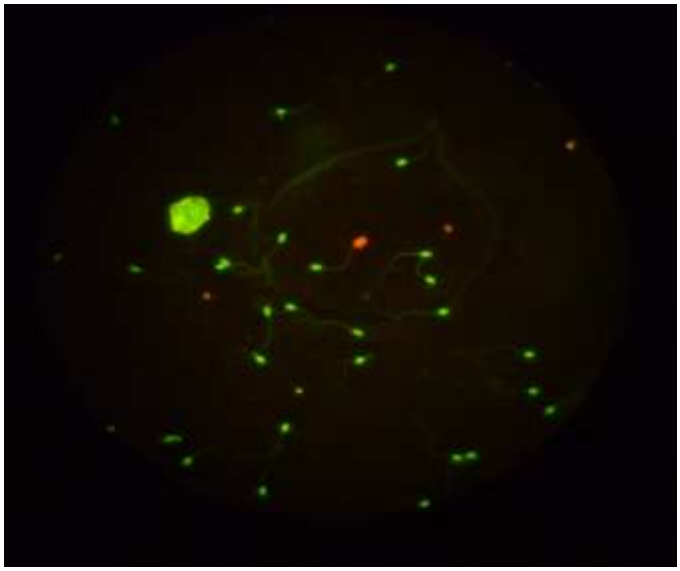
Means within rows within different letters are significance at least (P > 0.05)

Table 5: Correlation coefficient between different sperm parameters and the conception rate

		Motility	Live	Abnormality	HOS	DNA	Uncapacitated	Capacitated	Acrosome reaction	Conception rate
Motility	Pearson Correlation	1.000	.728**	.277	.918**	.383*	.548**	.529**	.649**	.422
	Sig. (2-tailed)		.000	.084	.000	.015	.000	.000	.000	.578
live	Pearson Correlation		1.000	.220	.681**	.309	.265	.234	.368*	.144
	Sig. (2-tailed)			.173	.000	.053	.099	.147	.019	.856
abn	Pearson Correlation			1.000	.340*	.694**	.224	.718**	.594**	-.755
	Sig. (2-tailed)				.032	.000	.164	.000	.000	.245
HOS	Pearson Correlation				1.000	.378*	.305	.593**	.599**	.580
	Sig. (2-tailed)					.016	.056	.000	.000	.420
DNA	Pearson Correlation					1.000	.470**	.742**	.887**	.607
	Sig. (2-tailed)						.002	.000	.000	.393
Uncap	Pearson Correlation						1.000	.257	.668**	.853
	Sig. (2-tailed)							.110	.000	.147
Capacit	Pearson Correlation							1.000	.808**	-.280
	Sig. (2-tailed)								.000	.720
Reacted	Pearson Correlation								1.000	.757
	Sig. (2-tailed)									.243

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

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



Red sperm head mean fragmented DNA. Green sperm head means normal DNA

DISCUSSION

The number of motile sperm inseminated, rather than the percentage of motile sperm is likely to be related to fertility (Motility of spermatozoa has been shown to be required for colonization of the isthmus of the oviduct) but the sperm number in the oviduct is related to the dose of motile cells rather than the proportion of these cells. Therefore, sperm motility has been classified as a compensative semen trait since a large insemination dose should compensate for a low percentage of motile cells. Accurate prediction of male fertility is necessary to increase breeding efficiency in animal production. However, prediction of male fertility is likely to depend on a number of sperm attributes rather than a simple semen evaluation as carried out in an AI stud. In particular, the un-compensative semen traits are factors which render a spermatozoon unable to sustain embryonic development after fertilization are likely to improve prediction of fertility when used in combination with other relevant techniques [6].

The current studies revealed that freezing-thawing process causes a considerable damage to DNA integrity, sperm motility, sperm viability, sperm abnormalities, plasma membrane integrity and percentage of capacitated acrosome in all bulls. This results was in close agreement with previous studies have shown that sperm acrosome capacitation and sperm DNA chromatin can undergo important changes after the freezing-thawing procedure in buffalo bulls [11], bull [25-26]. In addition, different changes in sperm chromatin structure could occur depending on the thawing procedure [27], the freezing package system or by an increase in the storage time [28]. It was previously accepted that cryopreservation induced capacitation-like changes in frozen-thawed spermatozoa named as cryocapacitation [12-14].

Concerning correlation coefficient among different sperm parameters and the conception rate, all laboratory tests showing a correlation with the conception rate, but did not reach to the level of the significance. Our results couldn't reach to the level of significance due to the low number of samples and the low number of inseminated females. When an additional test of sperm function was added to the routine spermiogram, the predictive value increased substantially. Evaluation of DNA integrity and assessment of membrane status increased the predictive value of bull fertility. A model comprised of a number of *In vitro* diagnostic tests of sperm function may prove the most accurate method of determining the fertilising ability of a bull *In vitro* [29]. Also, it was recorded that, the sperm function tests as acrosome reaction and hypo-osmotic swelling test extend the profiles of the sperm analysis and moreover, it was regarded that with its effective use in pre-determining the fertility potential, it provides great benefits for artificial insemination, *In vitro* fertilization, embryo transfer and infertility problems [30].

It was concluded that freezing-thawing process cause loss of a percentage of semen quality. Also, the present study revealed a significant variation in semen of different bulls after freezing thawing process. In addition, non significant correlations between semen parameters and the conception rate were recorded.

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