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Research Article

ASSOCIATIONS BETWEEN PHYSICO -MORPHOLOGICAL CHARACTERISTICS AND ANTIOXIDANTS IN MURRAH BUFFALO SEMEN

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ABSTRACT

This paper describes the associations between physico-morphological characteristics and antioxidants in Murrah buffalo semen. Fresh diluted semen samples (n=52) from 44 Murrah bulls were subjected to various tests. Out of these (n=52), thirty four semen samples of good phenotypic quality were included in Group I (Good Phenotypic Quality Samples). The same good phenotypic quality semen samples were processed and maintained at -196 ºC. Those frozen samples in straws after thawing, were also subjected to various tests and included in Group II (Good Phenotypic Quality Frozen Thawed Samples). The remaining 18 semen samples subjected to various tests and not processed for freezing and preparation of straws were included in Group III (Poor Phenotypic Quality samples). There was significant difference in the motility percentage, live sperm percentage, acrosomal integrity, HOS in groups I & II and also between group I & III. Resazurine dye reduction assay showed significant difference in time required for reduction of the dye between group I and group III of buffalo bulls. The catalase activity in group I and group III was found to be 1.05 ± 0.02 and 0.92 ± 0.05. However, catalase activity was not detected in group II. There was significant (P≤0.01) reduction in SOD activity in group II (12.52 ± 0.22 U/109sperm) compared to group I (25.42 ± 0.79 U/109sperm). Similarly there was significant decrease in intracellular GPx in group II (5.58 ± 0.104 U/109sperm) compared to group I (9.79 ± 0.06 U/109sperm). The antioxidant enzymes had positive correlation with sperm motility, sperm livability, membrane integrity and acrosome integrity.

Keywords: Antioxidants, Buffalo bull, Cryopreservation, Semen

Among the various causes, oxidative stress is major contributing factor for poor fertility status of spermatozoa, which leads to buffalo infertility. Under aerobic conditions, spermatozoa also produce reactive oxygen species (ROS) viz., hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and superoxide anion (O_2-) during its normal metabolic activity (Kadirve *et al.*, 2014), which are continuously generated and effectively eliminated. However, there may be imbalance between its production and safe disposal leading to oxidative stress on spermatozoa. H_2O_2 is known to arrest motility and block oxidative metabolism in sperm, decrease oocyte penetration ability of sperm and block sperm–egg fusion. In buffalo species, spermatozoa are more prone to oxidative damage because of high amount of poly unsaturated fatty acids (PUFA) compared to cattle leading to relatively higher freeze thaw associated damage, lower post thaw motility and conception rate (Nair *et al.*, 2006). Seminal plasma is a powerful source of antioxidants such as super oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) that counteract the toxic effect of O_2- and H_2O_2. The SOD converts the O_2- to H_2O_2 then the CAT converts H_2O_2 into H_2O and O_2, eliminating the potential ROS toxicity (El-Sisy *et al.*, 2008). The concentrations of these antioxidative enzymes are differed amongst species both in seminal plasma and spermatozoa (Kadirve *et al.*, 2014). Furthermore, concentration of these antioxidants is reduced during dilution and storage that affect the semen quality (Bansal and Cheema, 2016). Considering the importance of antioxidants to reduce the oxidative stress, its association with physico-morphological characters viz., motility percentage, viability, acrosome damage, plasma membrane integrity and DNA integrity was studied before and after preservation of good and poor quality Murrah buffalo semen.

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MATERIALS AND METHODS

The current research work was carried out over a period of six months from March 2017 to August 2017 at Frozen Semen Laboratories, Pune and Aurangabad (Grade 'A') approved by Government of India. Freshly diluted first ejaculates (n=52) collected from 44 Murrah buffalo bulls (Age: 2 to 7 years; Body wt: 500 to 900 kg) were subjected to various macroscopic (volume, colour, consistency and density), microscopic (mass motility, total sperm count, live sperm percentage and abnormal sperm percentage), sperm function [Resazurine Reduction test (RRT), Hypo Osmotic Swelling Test, Percentage Intact Acrosome (PIA by Geimsa staining), DNA Integrity Test (Acridine Orange staining)] and antioxidative enzyme tests (Catalase, Superoxide dismutase and Glutathione peroxidase). On the basis of macroscopic and initial motility, 52 semen ejaculates were divided into three groups. Thirty four semen samples of good phenotypic quality were included in Group I (Good Phenotypic Quality Semen Samples, n=34). The same semen samples

of good phenotypic quality were further processed and maintained at ultra low temperature (-196 ºC). Those frozen semen samples in straws after thawing, were also subjected to microscopic, sperm function and antioxi dative enzyme tests and were included in Group II (Good Phenotypic Quality Frozen Thawed Semen Samples, n=34). The remaining 18 semen samples out of fifty two ejaculates, which were already subjected to all the above mentioned various tests, were included in Group III (Poor Phenotypic Quality Semen samples, n=18). These 18 semen samples were not processed further for freezing and preparation of straws. Each parameter was analyzed using two replicates of each buffalo bull. The statistical analysis was done by using WASP 2 basic statistical analysis software maintained by ICAR GOA for analysis of descriptive statistics. Two sample t-test for analysis of Group I and III and paired t-test analysis of Group I and II, correlation analysis and linear regression analysis and Analysis of variance (ANOVA) was performed to examine differences between and within bulls. Student 't' test was used to evaluate significant difference between fresh and frozen-thawed spermatozoa for various parameters.

RESULTS AND DISCUSSION

There was significant ($p \leq 0.01$) reduction in sperm motility, live sperm percentage and intact acrosome after cryo-preservation and thawing in the present study (Table 1). This might be due to increase in the production of ROS during cryopreservation which deteriorated the quality of semen (Fig. 1 and 2). Sperm motility percentage and intact acrosome percentage recorded in this study was slightly higher than the observation reported by Kadirve *et al.*, (2014) in fresh and frozen thawed semen. Mean spermatozoa percentage responding to HOST in Fresh semen (76.52 ± 0.47) was significantly more than frozen thawed semen (61.52 ± 0.86) which indicated that quality is reduced after cryopreservation (Fig. 3 and 4).

Table 1

Seminal & Biochemical Parameters in Fresh and Frozen thawed Buffalo Semen

Sr.No.	Parameters	Group I Fresh semen (n=34)	Group II Frozen thawed semen (n=34)
1	Progressive Motility %	83.17 ± 0.98**	56.17 ± 0.89
2	Live Sperm %	86.87 ± 0.73**	58.82 ± 0.88
3	Acrosomal Integrity % (PIA)	95.92 ± 0.65**	83.23 ± 0.78
4	HOST %	76.52 ± 0.47**	61.52 ± 0.86
5	DNA Integrity % (n=6)	98.25 ± 0.30**	91.75 ± 0.53
6	SOD (U/109 sperm)	25.42 ± 0.79**	12.52 ± 0.22
7	GPx (U/109sperm)	9.79 ± 0.06**	5.58 ± 0.104

Significance *($p \leq 0.05$); ** ($p \leq 0.01$) within rows

Mean DNA integrity percentage in Fresh semen was significantly more than frozen thawed semen (Table 1). This showed that semen quality was better before cryopreservation. There was significant ($P \leq 0.01$) reduction in SOD activity in frozen thawed semen (12.52 ± 0.22) compared to Fresh semen (25.42 ± 0.79). Similarly, there was significant decrease in intracellular GPx after freezing and thawing (5.58 ± 0.104) as compared to fresh semen (9.79 ± 0.06). These findings were in close agreement with those reported by Bilodeau *et al.* (2000) and Slaweta *et al.* (1988) in bulls. The reduction in the levels of SOD and GPx after cryopreservation may be due to altered membrane integrity and increased permeability as a result of membrane damage due to freezing thawing stress.

The mean average volume of fresh semen did not differ significantly between Group I (4.46 ± 0.25 ml) and Group III (4.44 ± 0.59 ml) of Murrah buffalo bulls (Table 2). Most of the fresh semen buffalo ejaculates were milky white in colour, thick in consistency with better density. Mean mass motility of semen recorded in Group I was higher than Group III (Table 2) which differed significantly at 1% level of significance ($p \leq 0.01$) between the two groups of Murrah buffalo bulls. Mean sperm concentration of fresh semen samples in Group I was numerically higher than Group III (Table 2) but

Table 2

Seminal & Biochemical Parameters in Good Quality (Group-I) and Poor Quality fresh semen (Group III) (mean ± SE)

Sr.No.	Parameters	Group I Good quality Fresh semen (n=34)	Group III Poor quality fresh semen (n=18)
1	Volume (ml)	4.46 ± 0.25	4.44 ± 0.59
2	Mass activity (0-5 scale)	3.58 ± 0.08**	2.05 ± 0.09
3	Sperm Concentration (106/ml)	1351.02 ± 99.86	1183.11±162.91
4	Initial Progressive Motility %	83.17 ± 0.98**	60.94 ± 1.11
5	Live Sperm %	86.87 ± 0.73**	58.52 ± 1.12
6	Abnormal Sperm %	11.79 ± 0.28	13.28 ± 0.32
7	HOST %	76.52 ± 0.47**	64.77 ± 1.07
8	RRT- Time in (min)	15.97 ± 0.38**	19.38 ± 0.24
9	Acrosomal Integrity	95.92 ± 0.65**	89.44 ± 0.93
10	DNA Integrity (n=6)	98.25 ± 0.30**	92.58 ± 0.83
11	CAT (U/109 sperm)	1.05 ± 0.02**	0.92 ± 0.05
12	SOD (U/109 sperm)	25.42 ± 0.79**	20.53 ± 0.70
13	GPx (U/109 sperm)	9.79 ± 0.06**	8.91 ± 0.05

Significance *($p \leq 0.05$); **($p \leq 0.01$) within rows

Table 3

Correlation coefficient (r) among different Seminal Parameters and Antioxidants enzymes in Buffalo Spermatozoa

Sr. No	Parameters	Group I (n=34)	Group II (n=34)	Group III (n=18)
1	Motility and CAT concentration	0.1729	-	0.4881
2	Motility and SOD concentration	0.4817	0.4389	0.7136
3	Motility and (GPx concentration	0.5144	0.917**	0.9643**
4	Live sperm percentage and CAT conc.	0.3146	-	0.5023
5	Live sperm percentage SOD concentration	0.5614	0.6325*	0.729
6	Live sperm percentage and GPx conc.	0.579	0.9**	0.9665**
7	Percent Intact Acrosome and CAT conc.	0.2719	-	0.4622
8	Percent Intact Acrosome and SOD conc.	0.5503	0.595	0.6544
9	Percent Intact Acrosome and GPx conc.	0.5387	0.8781	0.9616**
10	HOS positive sperm % and SOD conc.	0.3723	0.9001**	0.7088
11	HOS positive sperm % and GPx conc.	0.2653	-	0.9664**

*($p \leq 0.05$), **($p \leq 0.01$) differ significantly within columns; (Correlation coefficient r: $0.7 < |r| \leq 1$ strong correlation, $0.4 < |r| < 0.7$ moderate correlation, $0.2 < |r| < 0.4$ weak correlation and $0 \leq |r| < 0.2$ no correlation)

statistically there was no significant difference between two groups with respect to mean sperm concentration. Abnormal sperm count percentage of Group I and Group III differed significantly at 1% level of significance ($P \leq 0.01$). However, the abnormal sperm percentage recorded in Group I and III was under permissible limit of

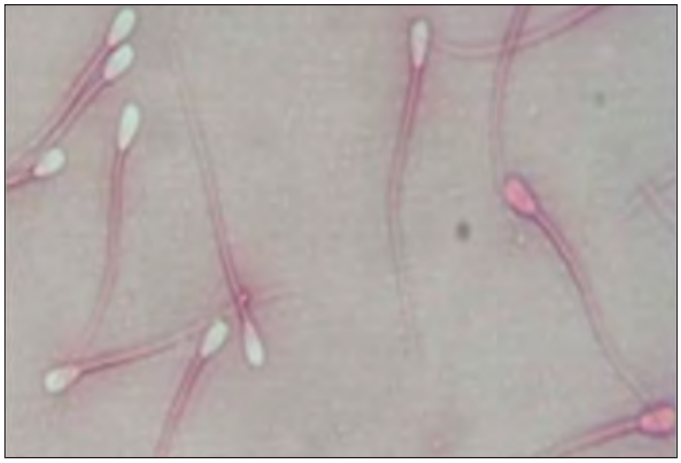


Fig. 1. Live & Dead Spermatozoa

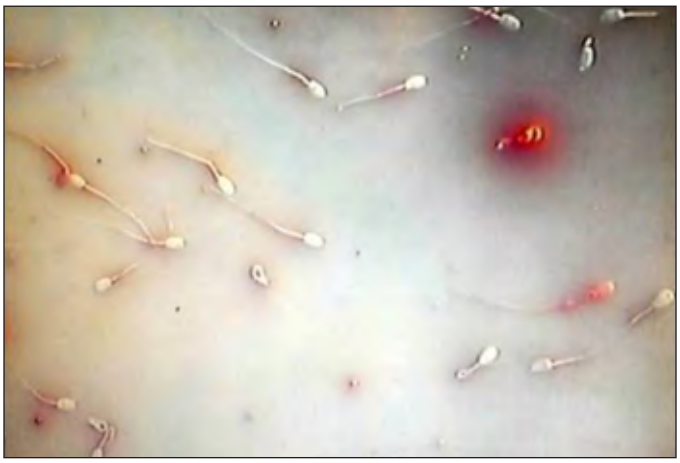
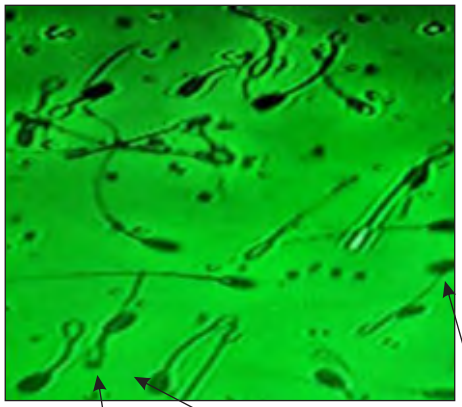
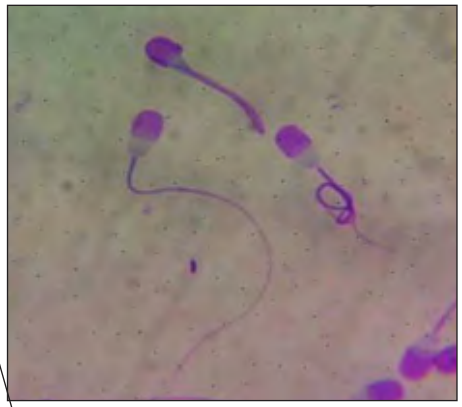


Fig. 2. Abnormal Spermatozoa

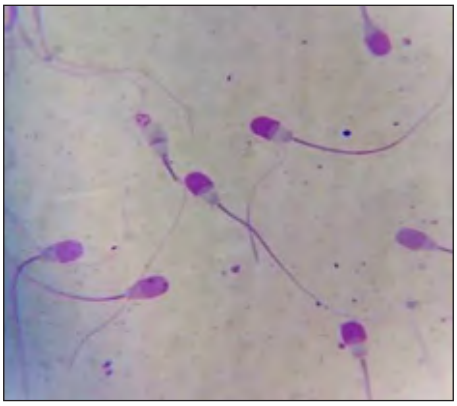


Swollen Plasma Membranes and Curled Tips of the Distal end of Tails of Sperms

Fig. 4. Hypo Osmotic Swelling Test (HOST)



Loose Acrosomal Cap



Complete Loss of Acrosomal Cap

Fig. 5. Acrosome Integrity of Spermatozoa

abnormal sperm concentration in semen sample. There were less than 70 per cent of total motile sperms, less viable sperm per cent and less number of sperm with intact acrosome and plasma membrane (HOS positive) present in the semen samples which were grouped in poor quality prefreezed extended semen. Hence, the group III semen samples were rejected for further processing. Higher percentage of HOS positive sperm is indicative of better sperm plasma membrane integrity.

Resazurine dye reduction assay showed significant difference in time required for reduction of the dye between good quality and poor quality semen groups of buffalo bulls. The mean total time taken for change in colour of semen samples from blue to white indicated good quality semen in group I. Intact acrosome percentage was significantly higher in the Group I than Group II and Group III (Table 2). Higher percentage of sperm with intact acrosome in Group I is indicative of good quality semen samples. The overall percentage of spermatozoa with intact DNA in fresh semen samples of Murrah buffalo bulls in Group I reduced significantly after freezing. Also, there was significant difference ($p \leq 0.01$) between two groups of fresh semen samples i.e. Group I and Group III (Table 2). The catalase activity in Group I and Group III was found to be 1.05± 0.02 and 0.92±0.05. However, catalase activity was not detected in frozen thawed semen of Group II.

Antioxidant enzymes like GPx, SOD and Catalase of buffalo spermatozoa had significant positive correlation with sperm motility, sperm livability, membrane integrity and acrosome integrity in the present study. Similar findings were also reported by Nair *et al.*, (2006). Antioxidants like SOD, CAT and GPx are responsible for suppression of ROS. The loss in activity of spermatozoa is due to acute damage caused by freeze-thaw process, resulting in loss of intracellular components.

From the observations of the present study, it may be concluded that, reduction in the quality of semen after cryopreservation might be due to excessive stress on spermatozoa and reduction in the level of intracellular antioxidants. It is also necessary to establish reference values for antioxidants so that these antioxidants can be supplemented in extender to improve semen quality in male infertility.

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