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Effect of partial deoxygenation of semen extender on physico-morphological attributes and antioxidant profile of Murrah Buffalo (*Bubalus bubalis*) during cryopreservation

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Abstract
The present study semen extender was prepared freshly and divided into two sub extenders namely, Extender I: control (non deoxygenated) and Extender II: partially deoxygenated by using mechanical method). The estimation of dissolved oxygen (DO) level was done in both extenders. Semen sample was split into two groups viz., group I: diluted with extender I and group II: diluted with extender II up to 60×10^6 sperm/mL. The diluted semen samples were packed, kept for equilibration and then kept in automatic programmable freezer then plunging into LN_2. The evaluation of semen samples was carried out for various seminal attributes (sperm motility, live sperm count, acrosomal integrity (AI), and hypo-osmotic swelling (HOS response) and antioxidant profile (superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant capacity (TAC) at pre freeze and post thaw stage. Sperm motility, live sperm count, acrosomal integrity, HOS response were significantly (P<0.05) higher in group II as compared to group I. The average seminal SOD, GPx and TAC levels were significantly (P<0.05) higher in group II as compared to group I at pre freeze and post thaw stage. It is concluded that partial deoxygenation of the extender prior to its addition to semen enhances sperm quality in terms of sperm motility, live sperm count, AI and HOS response and also improves seminal antioxidant profile.

Keywords: Partial deoxygenation, semen extender, antioxidant profile, murrah buffalo

Introduction
Buffalo is the main milk producing animal in our country, as it contributes more than 55% of the total milk produced in India. Artificial insemination has been widely accepted reproductive technique for genetic improvement of animals only because of semen cryopreservation [1]. Comparatively, AI in buffalo with cryopreserved semen has been limited due to poor semen freezability [2, 3] and conception rate [4]. Cryopreservation is known to reduce sperm viability by 50% and fertilizing capacity by a factor of seven fold even with the most up to date techniques [5]. It causes several damages to sperm which is a combined effect of cold shock, osmotic stress and oxidative stress encountered during freezing-thawing process. One of the most important factors which deteriorate post-thaw semen quality has been reported to be oxidative stress [6]. Oxidative stress is associated with an increased rate of cellular damage induced by oxygen and oxygen derived oxidants commonly known as reactive oxygen species (ROS) [7]. buffalo bull semen owing to a higher oxidative stress though higher lipid oxidation rate, reduced activity of naturally present antioxidant enzymes. ROS produced during freezing-thawing process cause lipid peroxidation affecting membrane integrity of spermatozoa which leads to formation of a number of toxic by-products such as MDA, conjugated dienes and lipid hydroperoxides. Along with plasma membrane damage, mitochondrial membrane damage and nuclear DNA damage also occur due to ROS [8].

Oxygen is the most important component present in surrounding for a biological cell to survive but, excess present in the medium that leads to free radical formations which are highly reactive oxygen species that react with vital component of cellular structure i.e. carbohydrate, lipids and protein and damage the membrane system ultimately leading to cellular death and aging. The rate of lipid peroxidation in sperm and the rate of consequent motility loss are linear functions of the partial pressure of O_2 in the medium [9]. The storage of diluted semen under N_2 gas reduces the oxygen tension as well as substantially reduces the metabolic activity of spermatozoa without affecting the pH of the medium [19]. Oxygen tension present in semen extender can be reduced through partial deoxygenation process which was developed in this laboratory.

It is the process in which partial removal of oxygen is carried out through liquid nitrogen flushing [10].

The dissolved oxygen in the extender may act as a source for production of reactive oxygen species, and may alter seminal antioxidant profile which in turn may be responsible for impaired frozen thawed sperm quality and fertility. We reputed that flushing the extender with Liquid Nitrogen may reduce dissolved oxygen levels and enhance seminal antioxidant profile during cryopreservation. So the aim of the present study was carried out study the effect of partial deoxygenation of extender on seminal antioxidant profile of murrah buffalo during cryopreservation.

Materials and methods

Chemicals

The chemicals used in the study were procured from Sigma–Aldrich, Missouri (United States).

Partial deoxygenation of extender

Egg yolk-Tris-Glycerol (EYTG) extender (Tris 3.028 g, citric acid monohydrate 1.675 g, fructose 1.25 g, penicillin G sodium 1000 IU/mL, streptomycin sulphate 1000 µg/mL, double distilled water (DDW) up to 100 mL with 10% egg yolk and 7% glycerol added finally) was split into three sub extenders [Extender I (control; without deoxygenation), Extender II (partially deoxygenated mechanically by vacuum pump)]. The methods of partial deoxygenation are subsequently described

Partial deoxygenation by mechanical method (by applying negative pressure using vacuum pump)

In this process, negative pressure was applied at the rate of 550 mm Hg for 10 min to the flask containing Extender II using a modified vacuum pump (MV8000 Automotive Tune-up kit, Mityvac, Lincoln Helios, India). The process continued for 10 min and then the fl ask was sealed air-tight with Parafi lm. Measurement of extender DO occurred before and after treatment and also in semen from all groups at the post-thaw stage. The DO measurement was done by using a digital DO meter (Micro Teknik, Haryana, India).

Climatic conditions, experimental animals and semen collection

The present study was conducted at the Germ-Plasm Centre of Animal Reproduction Division, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (Uttar Pradesh). The location of the institute is at an altitude of 564 feet above the mean sea level at a latitude of 28° North and longitude of 79° east. Three healthy breeding Murrah buffalo bulls (between 4 and 6 years of age) that were maintained with a similar feeding and management regimen were used for the collection of the semen. During the morning hours, ejaculates were collected using an artificial vagina as per standard procedures. Ejaculates with a mass activity of ≥ 3+ (on a scale of 0 –5) and individual progressive sperm motility of 70% and greater were selected for further processing.

Semen processing and freezing

Each ejaculate was split into two group's viz., group I (control): diluted with Egg yolk-Tris-Glycerol (EYTG) Extender I, group II: diluted with Egg yolk-Tris-Glycerol (EYTG) Extender II (using a modified vacuum pump). Both groups were diluted up to 60 million sperm/mL. The extended semen was packaged in 0.25 mL French mini straws (Bal Krishan Plasikrafts, India) and subjected to a combined cooling with equilibration period of 3 h at 4 °C. For automated freezing, straws along with rack were transferred to Biological Cell Freezer (IMV, France). The freezing rate was -5 °C/min from +4 °C to -10 °C, -40 °C/min and from 10°C to -100 °C and 20 ◦C/min from 100 to −140°C. Until assessment for various parameters, straws were stored inside liquid nitrogen (−196◦C) until analyses for various parameters.

Evaluation of semen at pre freeze and post thaw stage

Semen samples from each group were evaluated at pre-freeze and post-thaw stage for various seminal attributes (sperm motility, sperm viability, acrosomal integrity and hypo-osmotic swelling (HOS) response) and antioxidants (superoxide dismutase, glutathione peroxidase and total antioxidant capacity).

Sperm motility and live sperm count

For assessing sperm motility, a uniform drop of semen (8 µL) was placed on a clean grease free slide maintained at 37 °C. Then a clean cover slip was put on the semen drop and observation was done at 400× magnification of phase contrast microscope (Nikon Eclipse E200, Japan). The number of sperm moving in forward direction were estimated and expressed in percentage. In three different fields semen samples were analyzed and a mean estimate of the three readings was taken as a final value of the sperm motility. Eosin-nigrosin stain was used to determine live sperm count. For preparation of stain 5 g of Eosin-Y (water soluble) and 10 g of Nigrosin were dissolved separately in 100 mL of 2.9% sodium citrate solution. The mixture was boiled for 15 min. The amount of volume which got evaporated during boiling was replaced with the buffer. Finally stain was prepared by mixing 10% nigrosin solution and 5% Eosin-Y solution in the ratio of 4:1 in a container. The mixture was thoroughly shaken and filtered through Whatman filter paper. One drop of semen sample (8 µL) was mixed with three drops (8 µL each drop) of stain and the semen-stain mixture was allowed to rest for about 1 min. After 1 min, a thin smear was prepared on a clean, grease free slide, air dried and then observed at 1000× magnification of phase contrast microscope. The sperm which appeared colourless or white were considered as live and those appeared partially or completely pink coloured were considered as dead. A total of 200 spermatozoa were counted in each slide and percentage of live sperm was determined.

Acrosomal integrity and hypo-osmotic swelling (HOS) response

The acrosomal integrity was evaluated by Giemsa staining as per the method described by Watson [24]. After staining, to determine percent intact acrosome (PIA), a total of 200 sperm were counted at 1000× magnification of phase contrast. Functional integrity of sperm plasma membrane was assessed by the hypo-osmotic swelling test (HOST) as per the procedure described by Jeyendran *et al*. [13] with some modifications. Briefly, to 100 µl of semen sample was added 1mL of the HOST medium (4.9 g of sodium citrate and 9.9 g of fructose were dissolved in 1 liter of distilled water) in an effendorf tube, followed by its incubation at 37 °C for 1 h. After incubation, a drop of semen sample (10 µL) was taken on a clean grease free slide and a cover slip was put and examined at 400× magnification. Sperm showing visible coiling of tail were considered as HOS responsive

spermatozoa. To determine percent HOS responsive spermatozoa, a total of 200 spermatozoa were counted.

Estimation of antioxidants

Preparation of Seminal Plasma

Semen was centrifuged at 5,000 rpm for 10 min, the supernatants were transferred into 1.5 mL tubes, re-centrifuged to eliminate the remaining cells and kept frozen (−20 ºC) until further analyses. The supernatant was used for the estimation of superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant capacity (TAC).

Estimation superoxide dismutase

Superoxide dismutase (SOD) in seminal plasma was estimated as per the method of Madesh and Balasubramanian [14] and modified by Lone *et al*. [17]. The reaction mixture consisted of 100 µL of seminal plasma, 60 µL of 1.25 mM MTT, 1280 µL of PBS (pH 7.4) and 15 µL of 1mM pyrogallol. Pyrogallol solution was prepared by dissolving 1.266 mg of pyrogallol in 10 mL phosphate buffer saline (PBS) containing 3.72 mg of EDTA. MTT was prepared by dissolving 20.64 mg MTT in 5mL of distilled water. Both Pyrogallol solution and MTT were prepared freshly. In blank, seminal plasma was replaced with same amount of PBS. The reaction of formation of formazan crystals by reduction of MTT was terminated by addition of 1.5 mL of DMSO and reading was taken spectrophotometrically at 570 nm using Double beam UV-VIS Spectrophotometer (DBS; Model-UV5704SS, ECIL, India). One unit of SOD was defined as the amount of protein required to inhibit MTT reduction by 50%. The total SOD activity was expressed in units per mg of protein present in seminal plasma.

Estimation of glutathione peroxidase (GPx) and total antioxidant capacity (TAC)

Glutathione peroxidase (GPx) and total antioxidant capacity (TAC) were estimated with glutathione peroxidase and antioxidant assay kits as per instructions of kit manufacturer (Cayman Chemical Company).

Statistical Analysis

The data obtained in this study were subjected to statistical analysis by one-way analysis of variance (ANOVA) using Stastical Analysis System (24) Software Programme, version 9.3 and results were expressed as mean ± SE. The mean values of dissolved oxygen (DO), sperm parameters, and antioxidants between control and treatment group were compared by independent t-test. P value ≤ 0.05 was considered statistically significant.

Results

The mean dissolved oxygen (DO) level in control (group I) and treated (group II) extenders were 7.54 ± 0.06 (Mean ± SEM, N = 30) and 4.47 ± 0.03 (Mean ± SEM, N = 30), respectively. The DO levels were significantly (P<0.05) higher in control (group I) than treatment group (group II). At pre-freeze and post thaw stage, sperm motility (%), was significantly (P<0.05) higher in group II as compared to control (Fig 1 and Fig 5). The percentage of viable sperm was significantly (P<0.05) higher in semen diluted with mechanical method extender (group II) than control (group I) at pre freeze and frozen thawed stage (Fig 2 and Fig 6). The sperm with intact membrane (HOS responsive) were significantly (P<0.05) higher in group II as compared to group I at pre freeze and post thaw stage (Fig 3 and Fig 7). The percent sperm with intact acrosome were significantly (P<0.05) higher in group II when compared to group I (Fig 4 and Fig 8).

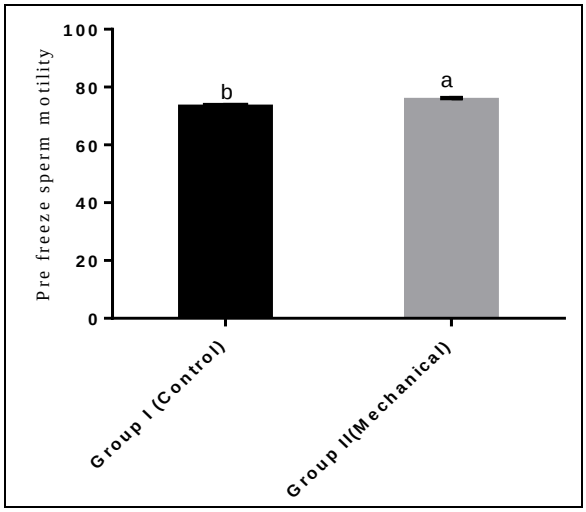

Fig 1: Pre freeze sperm motility in group I, group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

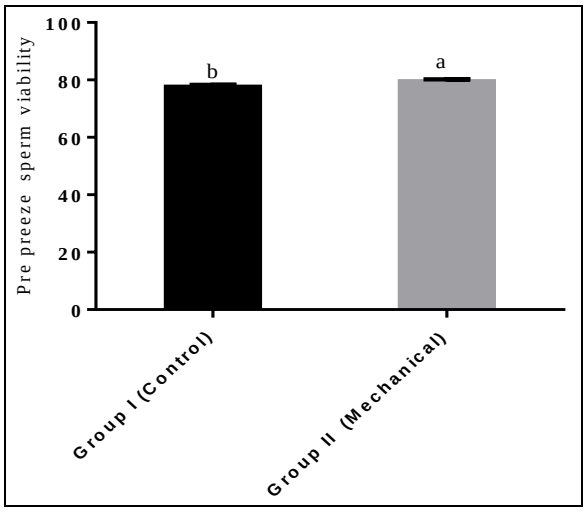

Fig 2: Pre freeze sperm viability in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

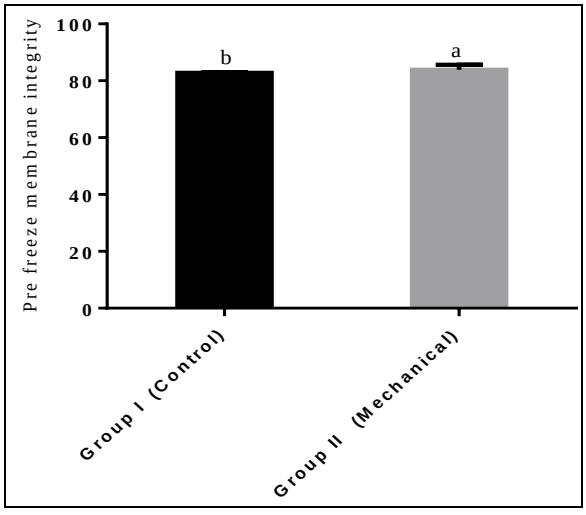

Fig 3: Pre freeze membrane integrity in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

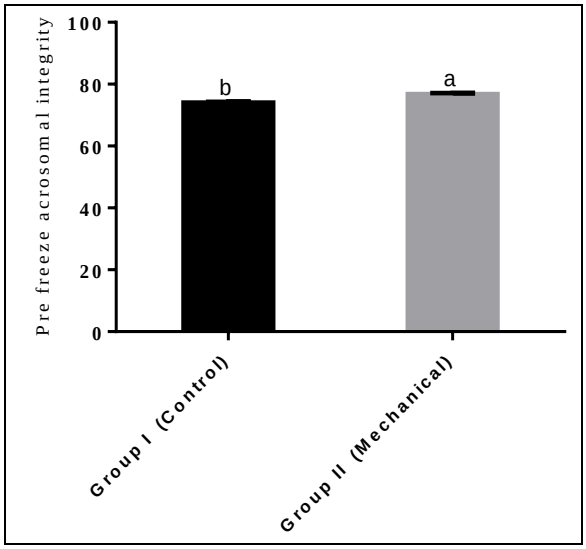

Fig 4: Pre freeze acrosomal integrity in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

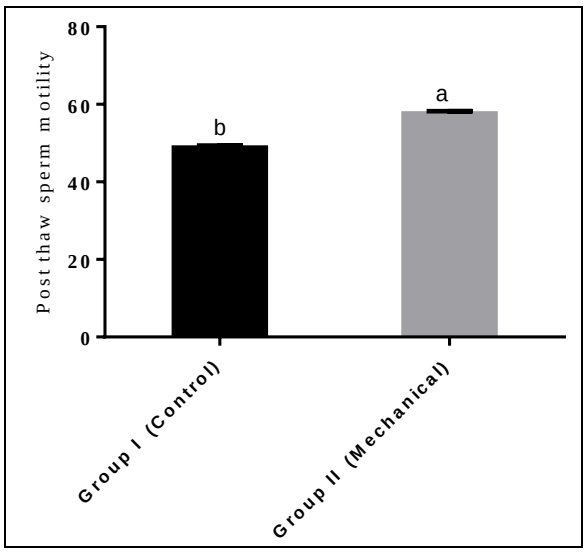

Fig 5: Post thaw sperm motility in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

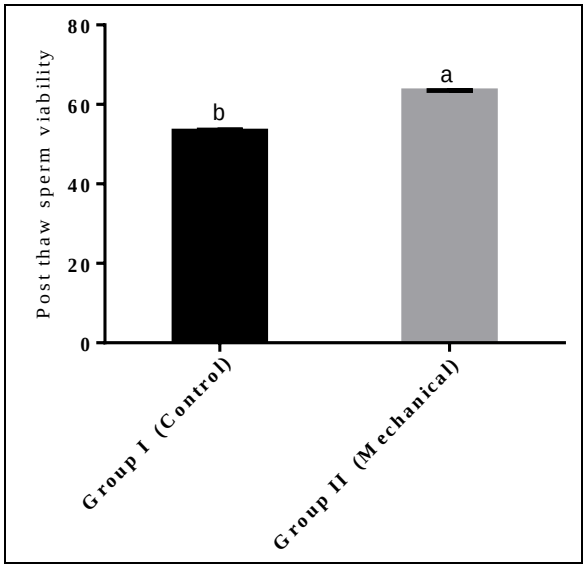

Fig 6: Post thaw sperm viability in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

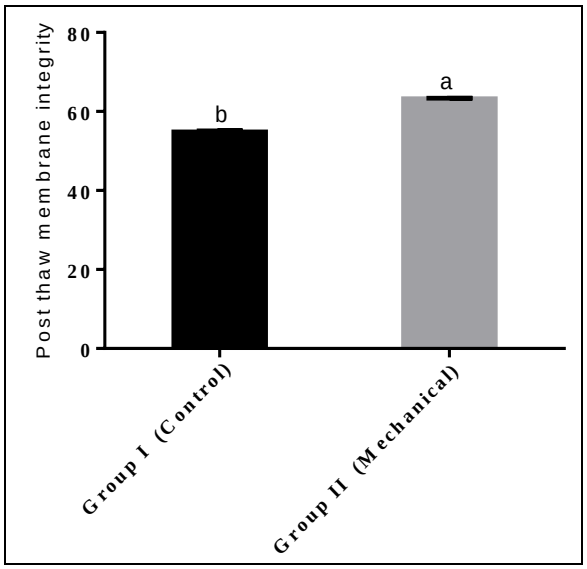

Fig 7: Post thaw membrane integrity in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

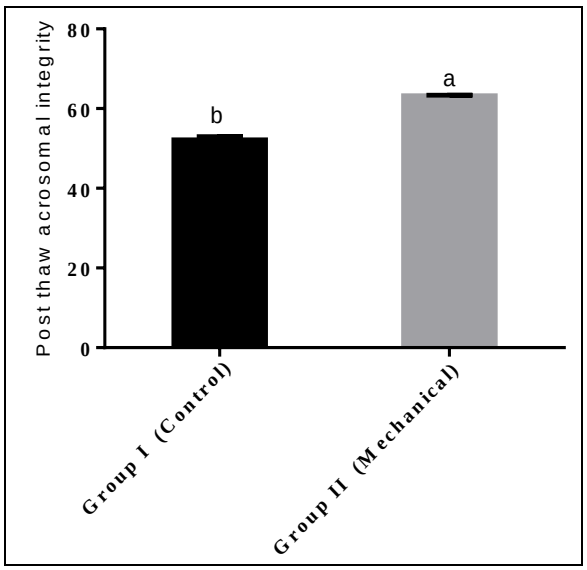

Fig 8: Post thaw acrosomal integrity in group I and group II (Mean ± SEM, N = 30) Values bearing different superscripts (a and b) differ significantly (P<0.05)

Table 1: Seminal antioxidant profile of buffalo at Pre freeze and Post thaw stage (Mean ± SEM, N = 30)

		Group I (Control)	Group II
SOD	Pre freeze	0.391 ± 0.01^{bA}	0.422 ± 0.01^{aA}
	Post thaw	0.193 ± 0.00^{bB}	0.215 ± 0.00^{aB}
GPx	Pre freeze	107.90 ± 6.58^{bA}	112.30 ± 6.66^{aA}
	Post thaw	59.22 ± 3.60^{bB}	67.24 ± 3.38^{aB}
TAC	Pre freeze	1.731 ± 0.05^{bA}	1.80 ± 0.02^{aA}
	Post thaw	1.42 ± 0.02^{aB}	1.57 ± 0.01^{aB}

SOD: Superoxide dismutase (Units/mg protein); GPx: Glutathione peroxidase (nmol min^{-1} ml^{-1}); TAC: Total antioxidant capacity (mM) Means bearing different superscripts in lower case letters (a and b) in row and upper case letters (A and B) in column differ significantly (P<0.05).

The mean seminal antioxidant profile at pre freeze and post thaw stage has been presented in Table 1. At pre freeze stage mean levels of SOD were significantly (P<0.05) higher in group II (0.422 ± 0.01) as compared to group I (0.391 ± 0.01). The mean SOD levels at post thaw stage were significantly

(P<0.05) higher in group II (0.215 ± 0.00) as compared to group I (0.193 ± 0.00). There was significant (P<0.05) reduction in average levels of SOD from pre freeze to post thaw stage in control (50.32%) and group II (42.64%). The mean GPx levels were significantly (P<0.05) higher in group II (112.60 ± 6.64) than control (107.90 ± 6.58) at pre freeze stage. At post thaw stage the mean GPx levels in group II (67.24 ± 3.32) were significantly (P<0.05) higher as compared to group I (59.22 ± 3.60). The percent reduction in GPx levels from pre freeze to post thaw stage were 44.11% and 39.55%, respectively, in group I and group II. The mean TAC levels at pre freeze stage were significantly (P<0.05) higher in group II (1.80 ± 0.02) than group I (1.731 ± 0.05). The mean TAC levels at post thaw stage were significantly (P<0.05) higher in group II (1.57 ± 0.01) as compared to group I (1.42 ±0.02). The percentage reduction in TAC levels from pre freeze to post thaw stage were 16.91% and 8.12%, in group I and group II, respectively.

Discussion

In buffaloes, cryopreservation of sperm for artificial insemination (AI) has played a crucial role in dissemination of its germplasm. One of the most potent causes of sperm damage in buffalo during cryopreservation is oxidative stress, which is primarily due to abundant amounts of membrane polyunsaturated fatty acids (PUFA), which are attacked by free radicals leading to lipid peroxidation cascades [10, 16]. Besides this cryopreservation damages sperm DNA [18], reduces seminal antioxidant profile [21], that in turn may reduce sperm freezability and fertility [6]. To combat with the deleterious effects of oxidative stress, mammalian semen has been endowed with a variety of antioxidants. The best known seminal antioxidants include superoxide dismutase (SOD) and glutathione peroxidase (GPx) [19]. SOD protects sperms from harmful effects of oxygen and lipid per oxidation by neutralizing superoxide anion ($O_2^{-\bullet}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2) [7]. The enhanced sperm motility, viability, membrane integrity and acrosomal integrity in partially deoxygenated by mechanical group (group II) was in agreement to the reports of Balamurugan *et al*. [20] who reported that flushing extender with liquid nitrogen improved sperm freezability in murrah buffalo bull. The reason may be significantly reduced DO levels in the partially deoxygenated extender (4.47 ± 0.03) as compared to control (7.54 ± 0.06), that in turn may have lead to reduced generation of reactive oxygen species (ROS). The higher average levels of SOD in group II may be because of reduced production of ROS due to lower initial DO levels partially deoxygenated extender. Although there was reduced activity of SOD from pre freeze to post thaw stage, which was in agreement to the reports of Lone *et al.* [21] in buffalo and Rajoriya *et al.* [22] in Tharparkar bull, who revealed reduced SOD activity from pre freeze to post thaw stage. During cryopreservation, the activity of activity of SOD was reduced to 50% in Holstein Friesian bulls [23]. The reduced GPx and TAC levels from pre freeze to post thaw stage was in line to the findings of Lone *et al.* [21] who reported reduced GPx and TAC levels in frozen thawed seminal plasma. The higher production of ROS in control group than treatment group may be responsible for reduced levels of GPx in control.

Conclusion

It is concluded that partial deoxygenation of the extender prior to its addition to semen enhances sperm quality in terms of sperm motility, live sperm count, acrosomal integrity, and hypo-osmotic swelling response and also improves seminal antioxidant profile (superoxide dismutase glutathione peroxidase and total antioxidant capacity.

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