Journal of Animal Research: v.6 n.3, p. 405-410. June 2016

DOI: 10.5958/2277-940X.2016.00038.3

Activity of Enzymatic Antioxidants and Total Antioxidant Capacity in Seminal Plasma of Murrah Bulls during Cryopreservation

S.A. Lone[1*], J.K. Prasad[2], S.K. Ghosh[2], G.K. Das[2], Balamurugan B.[2], A.A. Sheikh[3], R. Katiyar[2] and M.R. Verma[4]

[1]*Animal Reproduction, Gynecology & Obstetrics, NDRI, Karnal, Haryana, INDIA*
[2]*Germ Plasm Centre, Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, INDIA*
[3]*Dairy Cattle Physiology Division, NDRI, Karnal, Haryana, INDIA*
[4]*Division of Livestock Economics and Statistics, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, INDIA*

*Corresponding author: SA Lone; Email: drloneshabir@gmail.com

Received: 03 February, 2016 **Accepted:** 12 March, 2016

ABSTRACT

Semen samples with mass motility of 4+ or more and an initial progressive motility ≥ 80% collected from murrah buffalo bulls were utilized for study. Semen samples were evaluated for various seminal attributes, enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase) and total antioxidant capacity at fresh, pre-freeze and post-thaw stage. Seminal attributes (progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response) were significantly ($p<0.05$) higher in bull II as compared to bulls (I & III) at pre-freeze and post-thaw stage. Superoxide dismutase was significantly higher in bulls (II & III) than bull I at fresh stage ($p<0.05$) and significantly ($p<0.01$) higher in bull II as compared to bulls (I & III) at pre-freeze and post-thaw stage. Catalase was significantly higher in bull II than bulls (I & III) at fresh ($p<0.05$) and pre-freeze ($p<0.01$) stage. Significantly, higher total antioxidant capacity was noticed at pre-freeze ($p<0.05$) and post-thaw ($p<0.01$) stage in bull II and bulls (II & III), than bulls (I & III) and bull I, respectively. On the basis of our findings, it could be concluded that seminal enzymatic antioxidants (superoxide dismutase & catalase) and total antioxidant capacity varied among bulls at various stages of cryopreservation. There was progressive reduction in activity of enzymatic antioxidants and total antioxidant capacity from fresh to post-thaw stage.

Keywords: Enzymatic antioxidants, total antioxidant capacity, seminal plasma, murrah bulls

Exposure of spermatozoa to oxygen during cryopreservation and artificial insemination procedures leads to generation of reactive oxygen species (ROS), resulting in oxidative damage to spermatozoa (Chatterjee and Gagnon, 2001). Mammalian spermatozoa have evolved a defense system of enzymatic antioxidants to protect themselves against oxidative stress. The best known enzymatic antioxidants in seminal plasma are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The activity of these enzymatic antioxidants varies among species both in seminal plasma and spermatozoa (Asadpour and Nasrabadi, 2012; Cassani *et al.* 2005; Partyka *et al.* 2012). The balance between ROS production and their detoxification by antioxidants is essential for sperm stability and function (Cassani *et al.* 2005; Kothari *et al.* 2010; Guthrie and Welch, 2012). Frozen thawed bull spermatozoa are more easily peroxidized than fresh spermatozoa (Bansal and Bilaspuri, 2011). Higher activity of enzymatic antioxidants was observed in bovine semen samples with highest sperm viability after freezing and thawing (Chatterjee and Gagnon, 2001). No reports could be traced regarding the activity of enzymatic antioxidants and total antioxidant capacity in murrah buffalo's seminal plasma at various stages of cryopreservation. Therefore, the present study was conducted to investigate the activity of enzymatic antioxidants and total antioxidant capacity in seminal plasma of murrah bulls during cryopreservation.

MATERIALS AND METHODS

Climatic conditions and experimental animals

Geographical location of Bareilly is at an altitude of 169 m above mean sea level and at the latitude of 28.22°N and longitude of 79.22°E. Bareilly is known to have a moderate climate. Summer season goes up to 40°C, whereas winter goes down up to 8°C. The rainy season starts in June and extends up to September with humid and warm conditions. Three healthy Murrah buffalo bulls (4-6 years age) with good body condition (score 5-6) maintained under uniform feeding, housing, lighting and management conditions, maintained at Germ-plasm Centre, Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly were utilized for the study. Each experimental animal was offered ad libitum drinking water and concentrate: 1 kg/100 kg BW, green fodder: 25 kg, dry roughage: 6 kg. Concentrate mixture consists of 30 parts of maize, 30 parts of soy bean meal, 37 parts of wheat bran which are fortified with mineral mixture and salt daily.

Collection of semen and its processing

Semen was collected by using an artificial vagina as per the standard method. A total of 24 ejaculates, eight from each bull (8 × 3 = 24) were collected. Only ejaculates with mass motility ≥4+ were used in the study. Each ejaculate was divided into three aliquots; one aliquot was used for fresh semen studies, second aliquot was used for pre-freeze studies and third aliquot was for cryopreserved at -196ºC in liquid nitrogen.

Semen processing and preservation: Immediately after collection of semen, a fraction of each ejaculate was evaluated for various seminal attributes (progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response) and enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase) and (total antioxidant capacity). Rest ejaculate was diluted with Tris-egg yolk-glycerol dilutor up to 60 × 10^6 sperm/ml.

Extended semen samples were filled in french medium (0.5ml) straw contained 30 million progressively motile spermatozoa. Filled straws were then subjected to a combined cooling with an equilibration period of 3 h at 5°C. The rack along with the straws was transferred to biological cell freezer for automated freezing. The freezing in biological cell freezer was carried @ 5°C/min for 4 to –10°C; 40°C/min for –10 to –100°C and 20°C/min for –100 to –140°C. Straws were then plunged into liquid nitrogen (–196°C) for storage until assayed. Semen was evaluated before freezing and after thawing for seminal attributes (progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response) and enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) and total antioxidant capacity.

Semen evaluation

Seminal attributes

A drop of the diluted semen was kept on a clean, grease free, pre-warmed glass slide, cover slip was placed and progressive motility was assessed under high power magnification (Nikon, Eclipse 80i; 400 × magnification) of a phase contrast microscope. The live sperm percentage was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.* 1953). Pink stained sperms were counted as dead and colourless as live. Acrosomal intactness was determined by Giemsa stain (Watson, 1975). Hypo-osmotic swelling test (HOST) was carried out according to the method described by (Jeyendran *et al.* 1984) to access the functional integrity of sperm tail membrane which gives an idea of sperm membrane integrity. The percentages of sperm with swollen and curved tail were recorded. A total of 200 sperm were counted in at least five different fields in each smear for percentage live sperm, acrosome integrity and HOS response.

Activity of enzymatic antioxidants and total antioxidant capacity in seminal plasma

Preparation of Seminal Plasma

Fresh and pre-freeze 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 (−20ºC) until further analyses. Post-thaw seminal plasma was separated on the day of analysis. The supernatant was used for the estimation of glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (TAC).

Estimation superoxide dismutase activity (SOD)

Superoxide dismutase in seminal plasma was estimated as per the method of Madesh and Balasubramanian (1997) with some modifications. 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 1.266mg of pyrogallol in 10 ml phosphate buffer saline (PBS) containing 3.72mg of EDTA. MTT was prepared by dissolving 20.64mg 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 catalase activity (CAT)

Activity of catalase was estimated by spectrophotometric method as described by Bergmeyer (1983) and was expressed as mM H_2O_2 utilized /min / mg protein. Briefly in a test tube, 2 ml phosphate buffer was added to 10 µL seminal plasma (1:10 dilution); the contents were mixed and transferred to the cuvette. 1 ml of H_2O_2 was added directly into the cuvette and optical density was recorded every 30 sec for 2 min at 240 nm against distilled water taken as blank.

Catalase activity was calculated using following formula:

$$\frac{\Delta OD / time}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \frac{1}{\text{mg of protein}}$$

ΔOD: change in optical density per unit time, and 0.067—volume fraction

Estimation of glutathione peroxidase (GPx) activity

Glutathione peroxidase **(**GPx) activity was estimated in the seminal plasma by using GPx kit supplied by Cayman chemical company. Briefly, 100µL of assay buffer, 50µL of co-substrate mixture and 20µL standards/samples were added in each designated wells on the plate. The reaction was initiated by adding 20µL of cumene hydroperoxide to all the wells being used. The absorbances were taken in every min at 340 nm using plate reader to obtain at least five points. The standard curve was plot-ted using the GPx standards, and the activity of GPx for each sample was calculated from the standard curve and expressed as nmol/min/mL.

Estimation of total antioxidant capacity (TAC)

Total antioxidant capacity **(**TAC) capacity was estimated in seminal plasma by using antioxidant assay kit supplied by Cayman chemical company. Briefly standards in seven clean tubes were prepared and marked them A–G. Trolox and Assay buffer were added to each tube as shown in Table 1. Add 10µL of Trolox standard (tubes A–G) or sample in duplicate + 10 µL of metmyoglobin + 150 µL of chromogen per well. Reaction was initiated by adding 40 µL of hydrogen peroxide working solution using a multichannel pipette. Plate was covered with the cover and incubated on at room temperature for 5 min. Cover was removed and absorbance was read at 750 nm using a plate reader.

Antioxidant concentration of the samples was calculated by using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the following equation:

Antioxidant (µM) = (Unknown average absorbance intercept – y intercept × dilution) / (Slope)

Table 1: Preparation of the Trolox standards

Tube	Reconstituted Trolox (µL)	Assay buffer (µL)	Final concentration (mM) Trolox)
A	0	1000	0
B	30	970	0.044
C	60	940	0.088
D	90	910	0.135
E	120	880	0.18
F	150	850	0.225
G	220	780	0.330

Statistical analysis

Data collected were analyzed using two way ANOVA by Stastical Analysis System (SAS, 2011) Software Programme, version 9.3 and and results were expressed as mean±SE. Duncan's multiple range test was used to compare mean percentages of seminal attributes and seminal antioxidants. 5% (P<0.05) and1% (P<0.01) were considered statistically significant.

RESULTS AND DISCUSSION

Seminal attributes

The percentage of various seminal attributes (progressive motility, viability, acrosomal integrity and HOS response) is presented in Table 2.

Table 2: Mean±SE of seminal attributes of buffalo bulls at fresh, pre-freeze and post-thaw stage

Seminal attributes	Bull No.	Stage of semen preservation		
		Fresh	Pre-freeze	Post-thaw
Progressive motility (%)	I	88.25±0.67^{Aa}	77.50±0.94^{Bb}	51.87±0.91^{Bc}
	II	89.37±0.32^{Aa}	79.37±0.62^{Ab}	54.37±1.13^{Ac}
	III	87.12±0.63^{Ba}	76.87±0.91^{Bb}	51.25±0.81^{Bc}
Viability (%)	I	91.37±0.80^{a}	80.12±0.78^{Bb}	57.37±0.96^{Bc}
	II	92.62±0.41^{a}	83.12±0.39^{Ab}	59.50±0.42^{Ac}
	III	90.37±0.62^{a}	80.87±0.78^{Bb}	56.62±0.59^{Bc}
Acrosomal integrity (%)	I	87.00±0.56^{a}	79.12±1.12^{Bb}	58.37±1.05^{Bc}
	II	87.25±0.59^{a}	82.87±0.78^{Ab}	60.75±0.36^{Ac}
	III	86.62±0.77^{a}	80.12±0.95^{Bb}	58.25±0.61^{Bc}
HOS (%)	I	77.75±1.19^{a}	69.12±1.44^{Bb}	51.75±2.14^{Bc}
	II	78.12±1.23^{a}	72.12±1.31^{Ab}	53.62±2.29^{Ac}
	III	76.25±0.95^{a}	70.37±2.17^{Bb}	50.62±2.03^{Bc}

% = Percentage; HOS = Hypo-osmotic swelling

Mean showing different superscript in upper case letters ($^{A\ \&\ B}$) and in lower case letters ($^{a\ ,b\ \&\ c}$) respectively, in a column among bulls and row differ significantly at 5% (P<0.05) and 1% (P<0.01).

The initial progressive motility of a semen sample gives a good indication of the fertility of the bull and ability of spermatozoa to withstand the stress of cryopreservation process (Bishop *et al.* 1954). The initial progressive motility was significantly (p<0.05) higher in bulls (I & II) as compared to bull I at fresh stage. At pre-freeze and post-thaw stage, percent initial progressive motility, viability, acrosomal integrity and HOS response was significantly (p<0.05) higher in bull II as compared to bulls (I & III). Among fresh, pre-freeze and post-thaw stage, significant (p<0.01) difference in progressive motility, viability, acrosomal integrity and HOS response was noticed. The reduction in percentage of seminal attributes from fresh to post-thaw stage was in agreement to (Rajoriya *et al.* 2013).

Activity of enzymatic antioxidants and total antioxidant capacity in seminal plasma

The activity of enzymatic antioxidants (SOD, CAT and GPx) and total antioxidant capacity (TAC) is presented in Table 3.

Table 3: Mean±SE activity of enzymatic antioxidants (SOD, CAT & GPx) and total antioxidant capacity (TAC) in seminal plasma of buffalo bulls at fresh, pre-freeze and post-thaw stage

Parameter	Bull No.	Stage of semen preservation		
		Fresh	Pre-freeze	Post-thaw
SOD (U/mg)	I	0.45±0.01^{Ba}	0.34±0.01^{Bb}	0.17±0.00^{Bc}
	II	0.57±0.01^{Aa}	0.44±0.01^{Ab}	0.22±0.01^{Ac}
	III	0.52±0.08^{Aa}	0.38±0.01^{Bb}	0.19±0.00^{Bc}
GPx (nmol/ min/ml)	I	85.55±14.95^{b}	103.94±16.53^{a}	50.92±6.59^{c}
	II	95.41±13.43^{b}	116.08±14.40^{a}	63.94±8.14^{c}
	III	84.30±12.54^{b}	115.43±11.75^{a}	61.23±8.48^{c}
CAT (U/mg)	I	0.27±0.01^{Ba}	0.03±0.00^{bb}	0.00±0.00^{c}
	II	0.38±0.01^{Aa}	0.05±0.00^{ab}	0.00±0.00^{c}
	III	0.26±0.01^{Ba}	0.03±0.00^{bb}	0.00±0.00^{c}
TAC (Mm)	I	1.76±0.06^{a}	1.63±0.05^{Bb}	1.30±0.04^{bc}
	II	1.89±0.02^{a}	1.78±0.02^{Ab}	1.49±0.01^{ac}
	III	1.81±0.02^{a}	1.68±0.02^{Bb}	1.45±0.01^{ac}

SOD=Superoxide dismutase; GPx=Glutathione peroxidase; CAT=Catalase; TAC=Total antioxidant capacity; U/mg=Units per milligram of protein; Mm= milli mol; n=nano, min= minute and ml= milliliter

Mean showing different superscript in upper case letters ($^{A\ \&\ B}$) and in lower case letters ($^{a\ \&\ b}$) respectively, in a column among bulls differ significantly at 5% (P<0.05) and 1% (P<0.01).

Mean showing different superscript in lower case letters ($^{a\ ,b\ \&\ c}$) in row differ significantly at 1% (P<0.01).

Superoxide dismutase (SOD)

In fresh seminal plasma, mean activity of superoxide dismutase (SOD) was 0.45±0.01, 0.57±0.01 and 0.52±0.08, respectively, in bull I, bull II & bull III, being significantly ($p<0.05$) higher in bulls (II & III) as compared to bull I. At, pre-freeze and post-thaw stage, activity of superoxide dismutase was significantly higher in bull II as compared to bulls (I & III). There was significant ($p<0.01$) reduction in activity of superoxide dismutase at pre-freeze and post-thaw stage. This decline in activity of superoxide dismutase during equilibration period may be due to utilization of superoxide dismutase in neutralizing superoxides. Our results were in agreement with Rajoriya (2011) who reported decline in levels of superoxide dismutase during equilibration. The percent reduction in superoxide dismutase activity from fresh to post-thaw was 63.63. Mayuri, 2006 noticed absence of superoxide dismutase in post-thaw seminal plasma. In HF bulls also, cryopreservation reduces SOD activity by 50 % (Bilodeau *et al.* 2000). The loss of SOD activity after freezing may be due to altered membrane integrity and increased permeability as a consequence of cryopreservation (Watson, 1995 and Holt, 2000).

Glutathione peroxidase (GPx)

The average activity of glutathione peroxidase (GPx) in fresh seminal plasma was 85.55±14.95, 95.41±13.43 and 84.30±12.54, respectively, in bull I, bull II and bull III. At fresh, pre-freeze and post-thaw stage, no significant difference in activity of glutathione peroxidase was recorded among bulls. There was significant ($p<0.01$) reduction in activity of glutathione peroxidase from pre-freeze to post-thaw stage. The activity of glutathione peroxidase was reduced in frozen-thawed seminal plasma. Around 32.85% reduction in activity of glutathione peroxidase was recorded fresh to post-thaw stage. Kadervel *et al.* 2014 reported reduced glutathione peroxidase activity in frozen-thawed semen. The reduced activity of glutathione peroxidase may be due to utilization of glutathione peroxidase in destroying free radicals.

Catalase (CAT)

Average fresh seminal catalase (CAT) activity was 0.27±0.01, 0.38±0.01 and 0.26±0.01 in bull I, bull II & bull III, respectively, which was significantly ($p<0.05$) higher in bull II as compared to other two bulls. The average activity of catalase was significantly ($p<0.01$) higher in bull II at pre-freeze stage as compared to other two bulls. No, significant difference in activity of catalase was noticed among bulls at post-thaw stage. There was significant ($p<0.01$) reduction in activity of catalase from fresh to pre-freeze and pre-freeze to post-thaw stage in all bulls. There was reduction of 99.96% catalase activity from fresh to post-thaw stage. This was in agreement with Kadervel *et al.* 2014, who reported nil catalase activity in frozen-thawed semen. Catalase (CAT) activity is poor or absent in mammalian spermatozoa (Mann, 1964; Mennella and jones, 1980).

Total antioxidant capacity (TAC)

Mean fresh seminal total antioxidant capacity (TAC) of seminal plasma was 1.76±0.06, 1.89±0.02 and 1.81±0.02, respectively, in bull I, bull II & bull III. No significant difference in total antioxidant capacity was recorded among bulls in fresh seminal plasma. At pre-freeze stage, total antioxidant capacity was significantly ($p<0.05$) higher in bull II as compared to bulls (I & III) while as post-thaw total antioxidant capacity was significantly ($p<0.01$) higher in bulls (II & III) as compared to bull I. There was significant ($p<0.01$) reduction in total antioxidant capacity at pre-freeze and post-thaw stage. Total antioxidant capacity of fresh seminal plasma was higher than reports of Eghbali *et al.* 2010 and Raey *et al.* 2014. The variation may be due to different methodology and units of expression. Although, there was reduction in total antioxidant capacity during equilibration. The reduction may be due to consumption of antioxidants by the reactive oxygen species during equilibration. No literature could be traced to compare our finding at pre-freeze and post-thaw stage.

CONCLUSION

The present study concluded that activity seminal enzymatic antioxidant (SOD & CAT) and total antioxidant capacity (TAC) varied among murrah bulls at various stages of semen preservation. There was reduction in activity of enzymatic antioxidants and total antioxidant capacity in frozen-thawed seminal plasma.

REFERENCES

Asadpour, R. and Tayefi-Nasrabadi, H. 2012. Seasonal variation in antioxidant enzyme activity in seminal plasma in Holstein bulls. *Comp. Clin. Pathol*., **21**(2): 173-176.

Bansal, A.K. and Bilaspuri, G.S. 2011. Impacts of oxidative stress and antioxidants on semen functions: review. *Vet. Med. Int.,* 1-7.

Bergmeyer, H.U.J. and Grabl, M. 1983. Methods of enzymatic analysis. 3rd ed. *Weinheim Verlag chemie.,* 273-302.

Bilodeau, J.F., Chatterjee. S., Sirard, M.A. and Gagnon, C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol. Reprod. Dev.,* **55**: 282-288.

Bishop, M.W.H., Campbell, R.C., Hancock, J.L. and Watson, A. 1954. Semen characteristics and fertility in bulls. *J. Agric. Sci*., **44**: 227-248.

Campbell, R.G., Hancock, J.L. and Rothschild, L. 1953. Counting live and dead bull spermatozoa. *J. Expt. Biol.,* **30**: 44-45.

Cassani, P., Beconi, M.T. and Flaherty, C. 2005. Relationship between total superoxide dismutase activity with lipid peroxidation, dynamics and morphological parameters in canine semen. *Anim. Reprod. Sci.,* **86**: 163-173.

Chatterjee, S. and Gagnon, C. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol. Reprod. Dev*., **59**: 451-458.

Eghbali, M., Alavi, S.M., Rezaii, S.A. and Ansari, M. 2010. Calcium, magnesium and total antioxidant capacity (TAC) in seminal plasma of water buffalo (*Bubalus bubalis*) bulls and their relationships with semen characteristics. *Vet. Res. Forum*., **1**: 12-20.

Guthrie, H.D. and Welch, G.R. 2012. Effects of reactive oxygen species on sperm function. *Theriogenology*., **78**: 1700-1708.

Holt, V.W. 2000. Basic aspects of frozen storage of semen. *Animal Reprod Science*., **62**: 3-22.

Jeyendran, R.S., Vander., Ven, H.H., Parez, M., Crabo, B.G and Zaneweld, L.J.D. 1984. Development of an assay to assess the functional integrity of the human membrane and its relationship to other semen characteristics. *J. Reprod. Fert.,* **70**: 219-228.

Kadirvel, G., Kumar, S., Ghosh. S.K. and Perumal, P. 2014. Activity of antioxidative enzymes in fresh and frozen thawed buffalo (*Bubalus bubalis*) spermatozoa in relation to lipid peroxidation and semen quality. *Asian. Pacific. J. Reprod*. **3**(3): 210-217.

Kothari, S., Thompson, A., Agarwal, A. and Plessis, S.S.D. 2010. Free radicals: Their beneficial and detrimental effects on sperm function. *Indian. J. Exp. Biol*., **48**(5): 526 – 435.

Mann, T. 1964. Catalase. *In*: The Biochemistry of Semen and of the Male Reproductive Tract. *London Methuen and Co*., 130-131.

Madesh, M. and Balasubramanian, A.K. 1997. Micro titre assay for superoxide dismutase using

MTT reduction by superoxide. *Indian. J. Biochem. Biophy.,* **35**: 184-188.

Mayuri, R. 2006. Studies on oxidative damages and effects of certain antioxidants on buffalo spermatozoa during cryopreservation. M.V. Sc. Thesis submitted to Deemed University, IVRI, Izatnagar, U.P. India.

Mennella, M.R.F. and Jones, R. 1980. Properties of spermatozoa1 superoxide dismutase and lack of involvement of superoxides in metal-ion-catalyzed lipid-peroxidation reactions in semen. *Biochem J*., **19**: 1989- 297.

Partyka, A., Łukaszewicz, E and Niżański, W. 2012. Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. *Theriogenology*., **77**(8): 1497-1504.

Raey, E.I., Mohamed., Badr, M.R., Rawash, Z.M. and Darwish, G.M. 2014. Evidences for the role of melatonin as a protective additive during buffalo semen freezing. *American journal of animal and veterinary sciences.,* **9**(4): 252.262.

Rajoriya, J.S. 2011. Effects of seasons on enzymatic changes and cholesterol efflux in relation to freezability in Tharparkar bull semen. M.V.Sc thesis submitted to Deemed University, IVRI, Izatnagar, U.P. India.

Rajoriya, J.S., Prasad, J.K., Ghosh, S.K., Perumal, P., Kumar, A., Kaushal, S. and Singh, M. 2013. Effects of seasons on enzymatic changes and cholesterol efflux in relation to freezability in Tharparkar bull semen. *Asian. Pacific. J. of. Reprod.* **2**(4): 280-288.

S.A.S, Institute Inc. 2011. SAS® 9.3 System Options: Reference, Second Edition. Cary, NC: *SAS Institute Inc*.

Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Re-prod. Fertil. Dev*., **7**: 871–891.

Watson, P.F. 1975. Use of Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. *Vet Record.,* **97**: 12-15.