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Quantification of Enzymatic Antioxidants and Level of Lipid Peroxidation in Pantja Buck Seminal Plasma at Different Stages of Semen Freezing

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ABSTRACT

Present study was conducted to quantify the status of enzymatic antioxidants and level of lipid per-oxidation in Pantja buck seminal plasma at different stages of semen freezing. Thirty- two (32) ejaculates were collected from four (04) sexually mature Pantja bucks using artificial vagina method during breeding season. In order to eliminate the individual differences among bucks, the satisfactory semen ejaculates after initial examination were mixed in a pool (4 ejaculate = 1 pooled sample) to form eight pooled samples. All pooled samples were diluted in glycerinated egg yolk tris extender to obtain a final concentration of 240 million spermatozoa/ml. Thereafter, these samples were evaluated for the status of antioxidative enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in relation to lipid peroxidation (malondialdehyde estimation) of sperm plasma membrane at different stages (neat, post-dilution, post-equilibration and post-thaw) of semen freezing. The overall mean values of natural enzymatic antioxidants viz; superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase and malondialdehyde in the neat seminal plasma of eight pooled samples were 296.73±6.17 U/ml, 1.63±0.06 U/ml, 10.45±0.37 nmol/ml, 61.74±1.9 nmol/ml and 2.19±0.06 nmol/ml respectively. The mean values of above enzymes continuously decreased significantly (P< 0.05) in post-thawed semen samples and were 232.76±9.78 U/ml, 0.64±0.03 U/ml, 3.05±0.16 nmol/ml and 31.84±1.9 nmol/ml, respectively, while the mean value of malondialdehyde (8.02±0.10 nmol/ml) increased significantly indicating varying levels of oxidative stress during semen freezing. The findings of the present study conclude that maintaining a robust antioxidant defense in semen during the freezing and thawing process is essential for achieving optimal post-thaw semen quality

Keywords: Pantja buck, Seminal plasma, Antioxidative enzymes, Lipid peroxidation, Glycerinated egg yolk tris extender.

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INTRODUCTION

Cryopreservation of semen has several kind of detrimental effects in form of mechanical injuries, osmotic stress and oxidative stress resulted in to biochemical functional damage to spermatozoa and deterioration of sperm motility, liveability, integrity and fertilizing ability (Purdy, 2006). Oxidative stress is related to increased cellular damage triggered by oxygen and oxygen-derived free radical/nonradical molecules known as reactive oxygen species (Sikka *et al.,* 1996). Extensive efforts have been made to minimize cryogenic mechanical injuries (ice crystal formation) and osmotic injuries (solution effect) to improve post-thaw semen quality, however, there is limited research addressing management of oxidative stress during cryopreservation of semen. Superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase are important antioxidant enzymes that actively participate in the process of reducing oxidative stress in biological systems including semen. Their real time concentration in any biological tissue or fluid has been found to be an important indicator to assess oxidative stress. Spermatozoa are protected from oxidative stress mainly from these enzymatic antioxidants present in seminal plasma. The antioxidant activities of these enzymes have been identified in the seminal plasma of different species (Sikka *et al.,* 1996; Papas *et al.,* 2019; Bilodeau *et al.*, 2000). Pantja is a medium sized native goat breed of Uttarakhand, reared for meat and milk purpose (Rashmi *et al,* 2024). The breed has been registered by the Breed Registration Committee of Indian Council of Agricultural Research (ICAR) on January 6, 2015 through Accession Number "INDIA_GOAT_2420_PANTJA_06024. Pantja goats are well adapted to hot and harsh humid conditions of the Tarai region of Uttarakhand and Uttar Pradesh. They are resistant to many diseases compared to other breeds of goat. The body colour of the goat is brown/ fawn with a black coloured crest line and white streaks on each side of the face. They are quite active and morphologically resemble the deer. Traditionally, bucklings are castrated by the incision method at about 10 days of age and hence intact Pantja bucks are not commonly seen with small flocks. To augment and preserve this pure germplasm, cryopreservation of the semen is the best process. There is lack of much research on the cryopreservation of Pantja buck semen. To the best of our knowledge no report is available regarding quantification of major enzymatic antioxidants and level of lipid per-oxidation in Pantja buck seminal plasma during semen freezing. Therefore, in the present study we quantified the status of enzymatic antioxidants and level of lipid per-oxidation in Pantja buck seminal plasma at different stages of semen freezing and emphasized the importance of oxidants and antioxidants balance in semen for achieving better post-thaw semen quality.

MATERIALS AND METHODS

The study was conducted at Goat unit, Department of Livestock Production Management, C.V.A. Sc., G. B. Pant University of Agriculture and Technology, Pantnagar, U S Nagar, Uttarakhand, India after proper permission from college ethical committee. Thirty-two (32) ejaculates were collected from four (04) sexually mature Pantja bucks using artificial vagina method during breeding season. Immediately after collection the ejaculates were rushed to the laboratory and kept within a water bath at 18^0C during their initial evaluations following all the standard measures. To minimize individual variations among bucks, semen from four ejaculates was pooled (4 ejaculates = 1 pooled sample) to form eight pooled samples. All pooled samples were diluted in glycerinated egg yolk tris extender {tris buffer 3.9g, citric acid 1.6g, fructose 1g - yolk (15%) and glycerol (6%)} to obtain a final concentration of 240 million spermatozoa/ml (Gangwar *et al.,* 2015). Seminal plasma was harvested by centrifugation at 1800 rpm for 10 minutes. Thereafter, these samples were evaluated for the status of antioxidative enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in relation to lipid-peroxidation (malondialdehyde estimation) of sperm plasma membrane at different stages (neat, post-dilution, post-equilibration and post-thaw) of semen freezing. Estimation of malondialdehyde concentration and catalase activity was done as per the methods described by Ducha *et al.* (2020) and Bergmayer (1983), respectively. All the data pertaining to the underwent experiment were presented as mean ± SEM and analyzed statistically using one way ANOVA of OPSTAT (Sheoran *et al.,*1998) and Graph Pad Prism software (Swift, 1997) to determine significant difference between freezing stages.

RESULTS AND DISCUSSION

The results of the present study are shown in table 1 and the variance values between freezing stages are given in Table 2. Mean values of superoxide dismutase declined significantly ($p \leq 0.05$) at every stage of semen freezing except between neat and post-dilution stage, where it declined significantly. The value declined up to 21.54% at post-thaw stage compared to pre-dilution value. In contrast, the mean values of other enzymatic antioxidants (catalase, glutathione peroxidase, and glutathione reductase) significantly declined ($p \leq 0.05$) at each stage of semen freezing, with reductions of 60.73%, 78.81%, and 48.42% at the post-thaw stage compared to their neat values. This indicates a gradual consumption of these enzymes to neutralize the reactive oxygen species generated during semen freezing

(Table 2). On the other hand, mean value of malondialdehyde increased significantly (p≤0.05) at every stage of semen freezing and it was maximum (266.21 % compared to neat value) at post-thaw stage further indicating occurrence of lipid per-oxidation as a result of oxidative stress during semen freezing.

Table 1. Quantification of enzymatic antioxidants and level of lipid per-oxidation (Mean±SE) in Pantja buck semen at different stages of semen freezing (n=32)

Parameters (Unit)	Neat	Pre-diluted	Post-equilibrated	Post-thawed
Superoxide Dismutase (U/ml)	296.73±6.17^{A}	277.90±4.84^{A}	260.74±8.48^{AB}	232.76±9.78^{C}
Catalase (U/ml)	1.63±0.06^{A}	1.23±0.06^{B}	0.92±0.03^{C}	0.64±0.03^{D}
Glutathione Peroxidase (nmol/ ml)	10.45±0.37^{A}	9.10±0.39^{B}	6.74±0.30^{C}	3.05±0.16^{D}
Glutathione Reductase (nmol/ ml)	61.74±1.90^{A}	52.92±2.42^{B}	40.42±1.59^{C}	31.84±1.90^{D}
Lipid Per-oxidation MDA (nmol/ml)	2.19±0.06^{A}	2.77±0.14^{B}	5.99±0.17^{C}	8.02±0.01^{D}

#Mean values with at least one common superscript within row (A, B, C, D) for one character do not differ significantly (p≤0.05)

Table 2. Analysis of variance among freezing stages in antioxidant enzymes (n=32)

Superoxide dismutase

Between freezing stages	Mean Diff.	SE of diff.	Level of significance	p-value
Neat vs PD	18.82	9.393	ns	0.1948
Neat vs PE	35.99	9.393	**	0.0014
Neat vs PT	63.96	9.393	****	<0.0001
PD vs PE	17.16	9.393	ns	0.2679
PD vs PT	45.14	9.393	****	<0.0001
PE vs PT	27.98	9.393	*	0.0194

Catalase

Between freezing stages	Mean Diff.	SE of diff.	Level of significance	p-value
Neat vs PD	0.3976	0.07666	****	<0.0001
Neat vs PE	0.7093	0.07666	****	<0.0001
Neat vs PT	0.9843	0.07666	****	<0.0001
PD vs PE	0.3116	0.07666	***	0.0006
PD vs PT	0.5866	0.07666	****	<0.0001
PE vs PT	0.275	0.07666	**	0.0031

Glutathione peroxidase

Between freezing stages	Mean Diff.	SE of diff.	Level of significance	p-value
Neat vs PD	1.347	0.4521	*	0.0194
Neat vs PE	3.709	0.4521	****	<0.0001
Neat vs PT	7.401	0.4521	****	<0.0001
PD vs PE	2.362	0.4521	****	<0.0001
PD vs PT	6.054	0.4521	****	<0.0001
PE vs PT	3.691	0.4521	****	<0.0001

Glutathione reductase

Between freezing stages	Mean Diff.	SE of diff.	Level of significance	p-value
Neat vs PD	8.815	2.553	**	0.0047
Neat vs PE	21.31	2.553	****	<0.0001
Neat vs PT	29.9	2.553	****	<0.0001
PD vs PE	12.5	2.553	****	<0.0001
PD vs PT	21.08	2.553	****	<0.0001
PE vs PT	8.585	2.553	**	0.0063

Malondialdehyde

Between freezing stages	Mean Diff.	SE of diff.	Level of significance	p-value
Neat vs PD	-0.5771	0.1512	**	0.0014
Neat vs PE	-3.799	0.1512	****	<0.0001
Neat vs PT	-5.834	0.1512	****	<0.0001
PD vs PE	-3.222	0.1512	****	<0.0001
PD vs PT	-5.257	0.1512	****	<0.0001
PE vs PT	-2.035	0.1512	****	<0.0001

#PD: Post-diluted; PE: Post-equilibrated; PT: Post-thawed*

*show significance at p≤0.05

Superoxide dismutase is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes, and kidney. Superoxide dismutase are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism (Agarwal *et al.,* 2007). They also documented a range of 250-400U/ml of superoxide dismutase in plasma of various species. Catalase is a ubiquitous enzymatic antioxidant that is present in most aerobic cells. This enzyme is involved in the detoxification of hydrogen peroxide (H2O2), a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic reactive oxygen species production. This enzyme catalyzes the conversion of two molecules of H2O2 to molecular oxygen and two molecules of water. A range of 1.0-2.0 U/ml catalase have been reported in the seminal plasma of healthy man (Mora-Esteves and Shin, 2013). On the other hand, glutathione peroxidase and glutathione reductase are the main reducing agents in the body and act as scavenging antioxidants in the epididymis and testes (Mora-Esteves and Shin, 2013). Their modification of the spermatozoa membrane confers protection on the lipid constituents, thus preserving sperm viability and motility (Lanzafame *et al.,* 2009). Glutathione peroxidase catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione (GSH) and functions to protect the cell from oxidative damage. In male reproduction glutathione peroxidase not only protects the spermatozoa from lipid per-oxidation but also helps the spermatozoa maturation (Vernet *et al.,* 1997; Hsieh *et al.,* 2006). Plasma concentration of glutathione peroxidase has been found in a range of 5-15 nmol/ml in fertile men (Hsieh *et al.,* 2006). Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (Carlberg and Mannervik, 1985). They also reported a range of 30-80 nmol/ml glutathione reductase in seminal plasma of healthy man. Glutathione reductase is essential for the glutathione redox cycle, which maintains adequate levels of reduced cellular glutathione. A high GSH/GSSG ratio is essential for protection against oxidative stress.

Lipid per-oxidation is a well-established mechanism of cellular injury in both plants & animals and is used as an indicator of oxidative stress in cells and tissues (Armstrong *et al.,*1994). Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as malondialdehyde. Various authors have reported that increased levels of malondialdehyde are associated with decreased sperm motility and sperm-oocyte fusion (Agarwal and Parbakaran, 2005). In the same study a concentration range of 1.5-4.5 nmol/ml MDA has been reported in seminal plasma of fertile man.

Malondialdehyde is a naturally occurring product of lipid per-oxidation and can be measured via the thiobarbituric acid assay, which is one of the oldest and most widely used indirect direct assays for assessing sperm membrane oxidation.

Spermatozoa are compact cells that lack a rich cytoplasm and subsequently adequate intracellular antioxidants. Therefore, these gametes rely mainly on seminal plasma for providing them with adequate antioxidants against oxidative stress (Abdallah *et al.,* 2009). The enzymatic and non-enzymatic antioxidants in the semen have capability to neutralize the free radical formed (Lewis *et al.,* 1997), but because of dilution of semen these endogenous antioxidants may be inadequate. Further the high content of polyunsaturated fatty acids in sperm plasma membrane and low enzymatic antioxidants in sperm cytoplasm make the spermatozoa vulnerable to damage from free radicals (Bollwein *et al.,* 2008). Therefore, it seems essential to keep higher antioxidant defense in spermatozoa during freezing and thawing. Antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GSH-Px), glutathione reductase (GSH) etc. are found in seminal plasma and shown to have a protective role in sperm cell physiology (Sikka, 2004; Hu *et al.,* 2010; Anand *et al.,* 2016). These enzymatic antioxidants are important molecules that actively participate in the process of reducing oxidative stress in biological system including semen. The real time concentration of these enzymes in any biological tissue or fluid has been found to be an important indicator to assess oxidative stress.

CONCLUSION

The study concluded that oxidative stress is one of the major culprits behind poor post thaw semen quality hence, maintaining a robust antioxidant defense in semen during the freezing and thawing process is essential for achieving optimal post-thaw semen quality and subsequently fertilizing capability of spermatozoa. The study will offer further important implications for semen preservation protocols in Pantja bucks and related species too.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

REFERENCES

Abdallah, F.B., Dammak, I., Attia, H., Hentati, B. and Ammar-Keskes, L. (2009). Lipid peroxidation and Antioxidant Enzyme Activities in infertile Men: Correlation with semen parameter. *J. Clinical Lab. Analysis,* **23**: 99-104.

Agarwal, A. and Prabakaran, S. A. (2005). Mechanism, Measurement and Prevention of Oxidative Stress in Male Reproductive Physiology. *Indian J. Exp. Biol.*, **43**: 963-974.

Agarwal, A., Prabakaran, S. A. and Sikka, S.C. (2007). Clinical relevance of oxidative stress in patients with male factor infertility evidence-based analysis. *Amer. Urolo. Assoc., Edu. Res. Inc.,* **26:** 1-12.

Anand, M., Yadov, S., Vaswani, S., Sukla, P.K. and Madan, A. K. (2016). Assessment of Membrane Integrity and Antioxidative Enzyme in Fresh Ejaculated Barbari Buck Semen during Breeding Season. *Int. J. Sci. and Env. Tech.*, **5 (5):** 2935-2942.

Armstrong, D. and Browne, R. (1994). The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory. *Adv. Exp. Med. Biol.,* **366:** 43-58.

Bergmeyer H. U. (1983). Methods of Enzymatic Analysis. IIIrd. Weinheim Deer field Beach, Florida: Bansal; UV method of catalase assay. pp.273.

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

Bollwein, H., Fuchs, I. and Koess, C. (2008). Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. *Reprod. Domest. Anim.,* **43:** 189–195.

Carlberg, I. and Mannervik, B. (1985). Glutathione reductase. *Methods Enzymol*., **113:** 484-490.

Ducha, N., Budijastuti, W. and Rahayu, D.A. (2020). Effect of Addition of Different Egg Yolks in Basic Tris-Soya Diluent on Quality, Membrane Integrity of Senduro Goat Sperm and Free Radicals during Storage at Temperature of 4-5°C. *J. Physics: Conference Series*, **1569:** 042081.

Gangwar, C., Kharche, S.D., Ranjan, R., Kumar, S., Goel, A. K., Jindal, S. K. and Agarwal, S. K. (2015). Effect of vitamin C supplementation on freezability of Barbari buck semen. *Small Rum. Res.,***129:** 104-107.

Hu, J. H., Zhao, X. L., Tian, W. Q., Zan L.S. and Li. Q. W. (2010). Effects of vitamin E supplementation in the extender on frozen-thawed bovine semen preservation. *Animal*, **5(1):** 107–112.

Hsieh, Yao-Yuan, Chang, Chi-Chen and Lin, Chich-Sheng. (2006). Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. *Int. J. Biol. Sci*., **2(1):** 23–29.

Lanzafame, F.M., La Vignera, S., Vicari, E. and Calogero, A.E. (2009). Oxidative stress and medical antioxidant treatment in male infertility. *Reprod Biomed. Online,* **19:** 638–659.

Lewis, S. E., Sterling, E. S. L., Young, I.S. and Thompson, W. (1997). Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Ferti. Steril*., **67:** 142–147.

Mora-Esteves and Shin, D. (2013). Nutrient supplementation: improving male fertility four-fold. *Sem. Reprod. Med.,* **31(4):** 293-30.

Papes, M., Arroyo, L., Bassols, A., Catalan, J. and Gacem, S. (2019). Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses. *Theriogenology,* **140:** 180-187.

Purdy, P.H. (2006). A review on goat sperm cryopreservation. *Small Rum. Res*., **63**(3)**:** 215-225.

Rashmi, Sunil Kumar, Harihar Prasad Gupta and Shiv Prasad. (2024). Effect of Supplementation of Different Levels of Egg Yolk on Cryopreservation of Pantja Buck Semen in Tris Dilutor. *Indian J. Anim. Reprod.,* **45**(1): 25-28.

Sheoran, O. P., Tonk, D. S., Kaushik, L. S., Hasija, R.C. and Pannu, R. S. (1998). Statistical Software Package for Agricultural Research Workers. Recent Advances in information theory, Statistics & Computer Applications by D.S. Hooda and R.C. Hasija Department of Mathematics Statistics, CCSHAU, Hisar (139-143).

Sikka, S. C. (1996). Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Front. in Biosc*., **1**: 78–86.

Sikka, S.C., (2004). Role of oxidative stress and antioxidants in anthology and assisted reproductive technology. *J. Androl*., **25**: 5–18.

Swift M L. (1997). GraphPad Prism, Data Analysis and Scientific Graphing. *Chem. Inf. Computer Science***, 37**(2): 411–412.

Vernet, P., Faure, J., Dufaure, J.P. and Drevet, J.R. (1997). Tissue and developmental distribution, dependence upon testicular factors and attachment to spermatozoa of GPX5, a