Original Research

Supplementation of Butylated Hydroxytoluene (BHT) Reduces Oxidative Stress and Improves Quality of Frozen–Thawed Frieswal Bull Spermatozoa

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Abstract

The present study was conducted to compare the effects of different concentrations of the antioxidant Butylated Hydroxytoluene (BHT) on semen quality parameters and oxidative stress (OS) of Frieswal bulls. The semen samples (22) were diluted in cryo-protective egg yolk Tris glycerol extender (EYTG) with different concentrations of BHT (2.5, 5.0 and 10 mM; Group I, II and III respectively). Motility, viability, abnormality, acrosome integrity and membrane stability tests were performed at post-dilution, post equilibration and post thaw stage. Subsequently, levels of lipid peroxidation along with superoxide dismutase (SOD) and catalase activities were evaluated following freezing-thawing. Group II with 5 mM BHT supplementation at post thaw stage, showed significant (p<0.05) increased sperm motility, viability, acrosomal intactness, plasma membrane integrity and a significant reduction in abnormal sperm cells. Likewise, all the parameters related to OS were also found to be significantly superior (p<0.05) in this group. Thus, the present data indicated that BHT at the concentration of 5 mM in EYTG extender is optimal for cryopreserving Frieswal bull spermatozoa by improving post-thaw sperm characteristics including acrosome intactness and plasma membrane integrity, and limiting oxidative stress.

Key words: Butylated Hydroxytoluene, Frieswal, Bull, Semen Quality, Oxidative Stress.

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Introduction

The artificial insemination coupled with cryopreservation is decades old, utmost applied and best opportune assisted reproductive technology in bovines. However, despite of the improvements, cryopreserved bull semen is often associated with decreased motility, viability and other spermatozoal defects (Watson, 2000). The reasons to this decreased spermatozoal functions following freezing-thawing have not been fully deciphered, however the oxidative stress (OS) is considered as one of the major detrimental factors affecting quality of spermatozoa (Chatterjee and Gagnon, 2001). Reactive oxygen species (ROS) when present in pathologically higher amount induces a number of irreversible structural and functional changes in sperm cell (Agarwal *et al.,* 2014). To counteract OS, semen itself has an in built arrangement that includes enzyme scavengers like reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) and antioxidants like β-mercaptoethanol, protein, vitamin E, vitamin C, cysteamine, cycteine, taurin and hypotaurin (Bucak *et al.,* 2010). However, the concentration of these naturally present antioxidants and scavengers is significantly reduced when semen is extended and cryopreserved. To make up with this loss, a series of different antioxidants have been tried, amongst which butylated hydroxytoluene (BHT), a synthetic antioxidant and analogue of vitamin E had shown beneficial effects in exotic bull (Khumran *et al.,* 2015), buffalo bull (Ijas *et al.,* 2009), ram (Naijian *et al.,* 2013) and boar semen (Trzcinska *et al.,* 2015).

Frieswal bulls (Holstein Friesian and Sahiwal cross; having 5/8 Holstein Friesian inheritance) suffer from poor freezability and more than 50% of its semen is rejected following freezing-thawing (Tyagi *et al.,* 2000). The benefits of BHT are well known in protecting the spermatozoa from actions of ROS in beef and exotic bulls (Khumran *et al.,* 2015). However, to the best our knowledge there is not any study which investigated the role of this or any other additive in the cryopreservation extender, against cryodamage of Frieswal bull semen. As the amount of antioxidant needs to be standardized with different breed of bulls and type of semen extenders used (Khumran *et al.,* 2015 and Singh *et al.,* 2017), this study was therefore conducted to evaluate and compare the effects of different concentrations of the antioxidant BHT on oxidative stress and semen quality parameters of Frieswal bulls when evaluated simultaneously under similar experimental conditions.

Materials and Methods

The study was conducted at Semen Freezing Laboratory, ICAR-Central Institute for Research on Cattle, Meerut Cantt., India. Twenty-two healthy Frieswal breeding bulls maintained at Bull rearing unit under uniform feeding and management were employed for the study. One semen sample from each bull (first ejaculate of the day) was collected for the experiment. Immediately after collection, samples were evaluated for determining fresh sperm quality (sperm motility, viability, plasma membrane/acrosome integrity and abnormality) and oxidative stress parameters (lipid peroxidation; LPO, catalase, CAT; and superoxide dismutase, SOD). Semen samples with a volume of ≥4 mL, initial progressive motility of ≥ 70% and concentration of above 500 million/mL were selected for further processing.

The semen samples were diluted in cryo-protective egg yolk Tris glycerol (EYTG) extender, composed of 3.028 g of Tris, 1.675 g of citric acid, 1.25 g of fructose, 7.0 mL of glycerol, 1 x 10^5 IU Penicillin G Sodium

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and 1 x 10^5 µg Streptomycin in 100 mL of Milli-Q® water, in such a way to yield approximately 20 million sperm cells/ 0.25 mL straw. BHT (PHR1117, Sigma–Aldrich) was incorporated in the samples as per the method described by Ijaz *et al*. (2009). Briefly, different concentrations of BHT (2.5, 5.0 and 10 mM, grouped as I, II and III respectively) were prepared in ethanol in pre-warmed (34 °C) test tubes. The ethanol was allowed to evaporate so that a thin crystallized layer of BHT was deposited on the inner surface of the tubes. Then extended semen was added into the tubes and incubated at 37 °C for 5 min to allow uptake of BHT by spermatozoa. Thereafter semen was cryopreserved. Subsequently viability, abnormality, acrosome integrity and membrane stability tests were performed for semen samples (Pande *et al.*, 2018). A minimum of 200 spermatozoa were counted per slide. The levels of lipid peroxidation (LPO) were determined by measuring the levels of malondialdehyde (MDA) which is an end product of lipid peroxidation. MDA was estimated using thiobarbituric acid (TBA) assay (Sanocka and Kurpisz, 2004). Superoxide dismutase (SOD) and catalase activity in sample were estimated as per the method of Madesh and Balasubramanian (1998) and Aebi (1984) respectively. The treatment groups I, II and III along with the control (where BHT was not added to EYTG) were thereafter packaged in 0.25 mL mini semen straws (IMV). After filling and sealing, straws were subjected to a combined cooling with an equilibration period of 4 h at 5°C. The rack along with the straws was transferred to Biological Cell Freezer (IMV- France) for automated freezing. Straws were then cryopreserved into liquid nitrogen (-196°C) for storage until assayed. The straws were stored at least 2 weeks before evaluation.

Following freezing, three straws were randomly collected from each group and thawed at 37°C for 30 s and tested for individual progressive motility, viability, acrosome and plasma membrane integrity, abnormality of spermatozoa and oxidative stress parameters. Statistical evaluations were carried out using the Statistical Analysis System for Windows, SAS Version 16.0; SAS Institute, Inc., Cary, NC, 2001.

Result and Discussion

The effect of different concentrations of BHT on semen quality parameters in fresh semen at post dilution (PD; period not more than 5 minutes after the addition of extender into the control group, and extender supplemented with different concentration of BHT into the treatment groups), post equilibration (PE; the equilibration time was 4 h in this study) and post thaw (PT; immediately after freezing-thawing) stages in Frieswal bull semen sample is shown in Table 1. The effect of BHT supplementation was significant ($p<0.05$) in Group II at post thaw stage, with improvement in all the SQP viz. sperm motility, viability, acrosomal intactness and plasma membrane integrity (Table 1) and a significant ($p<0.05$) reduction in abnormal sperm cells.

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Table 1: The effect of different concentrations of butylated hydroxytoluene (BHT) on semen quality parameter (SQP) at post dilution (PD), post equilibration (PE) and post thaw (PT) stages (N=22) in Frieswal bull semen sample (mean ± SEM)

SQP (%)	Stage	Control	I (2.5 mM)	II (5.0mM)	III (10 mM)
Motility	PD	70.82±2.16	73.25±2.15	75.55±2.74	68.84±2.75
	PE	65.25±2.85 [a]	68.58±2.75 [a]	73.25±2.85 [b]	66.20±2.25 [a]
	PT	45.52±2.35[a]	53.54±2.15[a]	60.25±2.75 [b]	50.55±2.90 [a]
Viability	PD	76.44±0.84	78.18±1.06	80.46±1.08	75.18±1.41
	PE	73.14±1.58 [a]	75.15±2.11 [a]	79.14±2.24 [b]	72.12±1.98 [a]
	PT	52.26±3.15 [a]	57.46±2.91 [a]	65.14±1.19 [b]	54.78±1.64 [a]
Acr-Int	PD	80.35±3.45	80.86±4.20	78.36±4.56	79.45±5.35
	PE	68.18±5.35	67.46±6.35	68.35±5.36	66.35±6.38
	PT	52.65±7.65 [a]	55.82±6.68 [a]	66.62±6.35 [b]	54.25±7.35 [a]
HOST	PD	70.45± 8.63	68.50±7.75	69.35±8.85	66.40±7.56
	PE	63.15±7.70	65.35±6.84	66.32±6.86	61.52±4.35
	PT	47.35±6.85 [a]	52.85±6.34 [a]	59.15±5.88 [b]	52.14±7.15 [a]
Abnormality	PD	9.85±2.12	10.14±1.02	9.34±2.11	9.24±0.98
	PE	12.12±1.11	12.45±0.88	10.04±2.31	11.24±1.02
	PT	15.12±1.75 [a]	14.45±0.26 [a]	11.04±2.14 [b]	14.71±1.11 [b]

N, number of samples; Acr-Int: acrosome integrity; HOST: Hypo-osmotic swelling test; [ab] Values bearing different superscripts in a row differ significantly ($p<0.05$).

Similarly, at PE stage the percent motility and viability were significantly higher ($p<0.05$) in Group II, however rest of the SQP remained unaffected. There were no significant differences observed in the SQPin the treatment and control group at PD stage. Significant ($p<0.05$) reduction in MDA levels and higher SOD activity in all the treatment groups (Table 2) suggested that supplementation of BHT may control the level of OS in frozen thawed semen samples. Again Group II appeared to be most certainly affected group wherein all the parameters studied related to OS were found to be significantly superior ($p<0.05$) than the control and other treatment groups.

Table 2: The effect of different concentrations of butylated hydroxytoluene (BHT) on malondialdehyde (MDA) concentration, superoxide dismutase (SOD) and catalase activity in Frieswal bull semen sample (mean ± SEM) at post-thaw stage

Semen Attributes	Control	I(2.5 mM)	II(5.0mM)	III(10 mM)
MDA (µmol/mL)	3.69±0.14 [c]	2.64±0.09 [b]	1.26±0.07 [a]	2.74±0.21 [b]
SOD (U/mL)	21.45±0.49 [a]	38.82±0.94 [b]	43.16±1.24 [c]	39.61±1.14 [b]
Catalase (U/mg)	0.002±0.0 [a]	0.004±0.0 [a]	0.02±0.0 [b]	0.005±0.0 [a]

[abc]Values bearing different superscripts in a row differ significantly ($p<0.05$).

Spermatozoa are considered highly vulnerable to oxidative damage because their membranes are rich in polyunsaturated fatty acids (Shoae and Zamiri, 2008). OS during freezing thawing further damages the sperm membrane and reduces the sperm viability (Chatterjee and Gagnon, 2001). As seminal and sperm antioxidants are reduced during semen processing exogenous antioxidants such as BHT may reduce the harmful effects of lipid peroxidation (Shoae and Zamiri, 2008). The potential effect of BHT in preventing

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damages to the spermatozoa depends on the species, BHT concentration, cell membrane composition, incubation time and the type of extender used (Shoae and Zamiri, 2008 and Naijian *et al.,* 2013).

In the present experiment in Frieswal bull, Group II resulted in notably increased post-thaw motility. Similarly, more number of sperms with intact acrosome and functional plasma membrane reflected that BHT supplementation at 5 mM concentration had significantly improved the semen quality following freezing-thawing. However, BHT at higher concentration (10 mM) showed no significant improvement than the control group. Our data are in agreement with the findings of Roca *et al.* (2004) which supposed that excessive levels of antioxidants would have led to redox imbalance and thus neutralizing the positive effects of BHT. Thus it is important to use an appropriate antioxidant level according to the type of extender and animal species.

The lipid peroxides, assayed in terms of MDA concentration cause membrane damage and reduced motility. In the present study the MDA concentration of post-thaw semen in control group was significantly higher as compared to all the treatment groups. This in agreement to previous findings which showed that BHT may modify the properties of lipid bilayer and membrane of sperm cell and serve as a scavenger of ROS associated with diluent and sperm (Killian *et al.,* 1989). BHT may directly annul ROS from surroundings of the sperm and convert these molecules into hydroperoxides (Merino *et al.*, 2015) thereby reducing their harmful effects.

Conclusion

In conclusion, the present data indicated that BHT at the concentration of 5 mM in egg yolk Tris glycerol extender, may be associated with better frozen-thawed semen quality parameters and reduced oxidative stress, thus making it optimal for cryopreserving Frieswal bull spermatozoa. However, whether the improved semen quality translates into better fertility or conception rates under field conditions remains to be determined.

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