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Effect of Butylated Hydroxytoluene and Tocopherol Supplementation on *In vitro* Sperm Characters during Cryopreservation of Black Bengal Buck Semen

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ABSTRACT

Background: Black Bengal goat is one of the important goat breeds of India. Cryopreservation of semen and artificial insemination are effective techniques for improving goat breeding programs. Various biochemical and functional changes that occur during freezing-thawing process results in poor post thaw sperm recovery. Supplementation of antioxidants to the extender has been reported to have positive effects on semen cryopreservation in various species. The present study was undertaken to assess the effect of antioxidants butylated hydroxyl toluene (BHT), α tocopherol on post thaw *in vitro* sperm characters, lipid peroxide level and superoxide dismutase (SOD) activity during cryo-preservation of Black Bengal buck semen.

Methods: Semen was collected from bucks by artificial vagina, antioxidant BHT was added to the Tris egg yolk extender @ 0, 1 and 2 mM/ml in control group (BHTC), treatment group 1 ($BHTT_1$) and treatment group 2 ($BHTT_2$), respectively. Similarly, tocopherol was added @ 0, 1 and 2 mg/ml in control group (TFC), in treatment group 1 (TFT_1) and treatment group 2 (TFT_2), respectively. Each antioxidant was tested with 20 semen ejaculates. Semen samples were frozen in liquid nitrogen and post freeze-thaw *in vitro* sperm characters, malondialdehyde (MDA) concentration and SOD activity were measured.

Result: Post thaw sperm motility, functional membrane integrity and viable count were significantly ($P<0.05$) higher in the BHT and α tocopherol supplemented groups than control groups. Significantly ($P<0.05$) higher acrosome intact cells were recovered in $BHTT_1$, $BHTT_2$ and TFT_2 groups than the control and TFT_1 groups. Further, BHT supplemented groups had significantly lower level of MDA than the control, while the supplementation of α tocopherol could not control the generation of MDA. Post thaw SOD activity was found significantly lower in the antioxidant treated groups than their respective controls. It is concluded that supplementation of BHT and α tocopherol were found to be more promising in conserving *in vitro* sperm characters from cryo-damages during freezing of Black Bengal bucks semen.

Key words: Antioxidants, Black bengal goat, Buck semen, *In vitro* characters.

INTRODUCTION

Black Bengal goat (*Capra hircus bengalensis*) belongs to the Bovidae family and found throughout West Bengal, throughout Eastern India and Bangladesh. Early maturity, high prolificacy, fertility and better adaptability to the adverse environmental condition are some of the special characters of Black Bengal goat. Besides, it produces delicious meat with low intramuscular fat and fine quality skin (Islam *et al*., 1991). Male goats of this breed are castrated at an earlier age to get better growth rate and to avoid the development goaty odour in the meat and are slaughtered at 12 to 15 months of age. This practice resulted in less availability of breeding males, the buck: doe ratio has become to 1.13: 88.7 against the recommended 1:20 (Nandi *et al*., 2011). The valuable traits of this breed are under threat of dilution due to indiscriminate breeding of Black Bengal does with available bucks of any breed or with larger breeds like Jamunapari for economic consideration. Black Bengal goat being one of the important goat germplasms of India and Bangladesh, needs to be protected from genetic dilution or loss due to infiltration of genes from other breed(s). To achieve this, selective breeding of pure Black Bengal goat to be practiced, semen preservation and artificial insemination (AI) may be the satisfactory way for faster dissemination of quality pure germplasm to larger population. The largest impediment to the exploitation of frozen semen is the significant loss of motile and viable sperm cells during freezing and

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thawing process of goat sperm (Alcay *et al*., 2016). A variety of biochemical changes occur in the spermatozoa during freezing-thawing process results in oxidative stress, which leads to decrease in sperm motility, viability and fertilizing ability. The sperm cells are protected by antioxidant systems present in the seminal plasma and in the cytoplasm, but this system is partly removed and severely altered during cryopreservation. Addition of antioxidants to the extender to counteract oxidative stress may have positive effects on semen cryopreservation (Najafi *et al*., 2014).

Vitamin E is a group of eight lipid soluble compounds which include four tocopherols and four tocotrienols and they present in α, β, γ and δ forms. Vitamin E is a highly powerful chain-breaking lipophilic antioxidant, intermingles with free radicals and neutralizes them to form tocopheroxyl radical. Vitamin E breaks the covalent bonds formed between fatty acid side chains in membrane lipids. α tocopherol is a vital membrane protecting agent against the lipid peroxidation damages (Jeong *et al*., 2009). Breininger *et al*. (2005) reported that addition of α-tocopherol in the freezing extender would control the development of lipid compound and preserve the intracellular conditions that were essential for the conservation of sperm motility. Butylated hydroxy toluene (BHT), a synthetic analogue of vitamin E, protects sperm membrane from reactive oxygen species (ROS) attack by interfering with the auto-oxidation chain reaction through the act of donating a hydrogen molecule to the lipid radical, thereby producing a product that is stable (Papas, 1993). BHT also reacts with ROS and converts it into hydroperoxides. Supplementation of BHT has been reported to improve the cryo-preservability of turkey, buffaloes and dogs semen samples (Memon *et al*., 2011). Considering the existing demand and future scope of preserved semen of Black Bengal goat, the current study was carried out to assess the beneficial effects of supplementing vitamin E in the form of α tocopherol and BHT on the improvement of cryopreservability of Black Bengal buck semen.

MATERIALS AND METHODS

The present study was carried out at ICAR-National Dairy Research Institute (NDRI), Eastern Regional Station, Kalyani, West Bengal, India. The experiment was conducted on Black Bengal bucks (n=8) of 1.5 to 3.5 years of age during the months of July, 2019 to January, 2020. The experiment was approved by the Institute Research Council of ICAR-National Dairy Research Institute, Karnal, India.

Semen ejaculates were collected from the bucks once a week. Semen samples with concentration less than 2500×10^6 spermatozoa/ml, mass activity less than 3 + and individual motility less than 70% were discarded. The basic extender was prepared by mixing 300 mM Tris, 28 mM glucose, 95 mM citric acid, egg yolk 20% (v/v) and 500 µg/ml gentamicin in distilled water respectively (Konyak *et al*., 2018). A single step addition of glycerol (5% v/v) into the extender was performed. The effect of each antioxidant on buck semen cryopreservation was tested separately with 20 numbers of ejaculates. To evaluate the effect of BHT, each semen ejaculate was divided into three aliquots and BHT was added to the semen extender @ 0 mM/ml in control group (BHTC), @ 1 mM/ml in treatment group 1 ($BHTT_1$) and @ 2 mM/ml in treatment group 2 ($BHTT_2$). Similarly, to study the effect of α tocopherol, it was added @ 0 mg/ml in control group (TFC), @ 1 mg/ml in treatment group 1 (TFT_1) and @ 2.0 mg/ml in treatment group 2 (TFT_2). Semen dilution with extender was done in such a way that final extended semen contained at least 300×10^6 sperm cells per ml. Filling and sealing of straws were done manually with 0.25 ml French mini straws and equilibrated for 3 hours at refrigeration temperature, followed by vapour freezing in liquid nitrogen (Karunakaran *et al*., 2019). Semen samples were evaluated for post thaw *in vitro* characters *viz.* sperm motility, viability, functional membrane integrity, acrosome membrane integrity, concentration of lipid peroxide compound malondialdehyde (MDA) and superoxide dismutase activity. Superoxide dismutase (SOD) activity in the post thawed semen samples were estimated using kits as per the instructions of the manufacturer (Immuotag SOD assay kit, catalog # ITFA1010) using spectrophotometer.

Prior to analysis, all percentage data were subjected to arc sine transformation to overcome the scale effects and for normalization of data and one-way ANOVA was applied on these transformed data. The means for arc sine percentile values of all seminal traits were back- transformed and presented as mean percentage. Comparisons among means of different treatments were done by Duncan's multiple comparisons test. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

The study revealed that post thaw *in vitro* sperm characters were significantly ($P<0.05$) higher in the BHT and α tocopherol supplemented groups ($BHTT_1$, $BHTT_2$ and TFT_1, TFT_2) than their respective untreated control groups (BHTC and TFC; Table 1 and 2). Significantly ($P<0.05$) higher number of sperm cells with intact acrosome were recovered in $BHTT_1$, $BHTT_2$ and TFT_2 groups than the BHTC, TFT_1 and TFC groups. In respect to the control of lipid peroxide compound generation during cryopreservation, BHT supplemented groups $BHTT_1$ and $BHTT_2$ had significantly lower level of MDA than BHTC, while the supplementation of α tocopherol could not control the generation of lipid peroxides and the tocopherol supplemented TFT_2 group had significantly higher level of MDA. SOD enzyme activity was significantly reduced during cryopreservation in both the antioxidant supplemented groups as compared to their respective controls.

Supplementation of vitamin E compounds BHT/ tocopherol to the semen extender had helped to preserve the vital characters of the sperm cells during cryopreservation. Lipophilic properties of vitamin E compounds make them possible to dissolve in to sperm

Table 1: *In vitro* sperm characters (Mean±SEM) in post thawed semen of Black Bengal bucks supplemented with Butylated hydroxy toluene.

Parameters	BHT @ 0 mM/ml (BHTC)	BHT @ 1 mM/ml ($BHTT_1$)	BHT @ 2 mM/ml ($BHTT_2$)
Progressive forward motility (%)	32.9 ± 2.62^{aA}	41.75 ± 3.22^{b}	44.65 ± 1.96^{bB}
Functional membrane integrity (%)	33.7 ± 1.93^{aA}	41.55 ± 1.95^{bB}	45.25 ± 1.58^{bB}
Sperm viability (%)	34.65 ± 1.53^{aA}	39.75 ± 2.35^{bB}	44.95 ± 1.63^{bB}
Acrosome integrity (%)	61.2 ± 1.4^{aA}	67.1 ± 2.00^{b}	69.5 ± 1.70^{bB}
MDA (µmol/ml)	0.75 ± 0.013^{aA}	0.721 ± 0.015^{bB}	0.625 ± 0.011^{cC}
SOD (U/mg of protein)	0.333 ± 0.004^{aA}	0.169 ± 0.003^{bB}	0.121 ± 0.003^{cC}

Rows with different superscripts a, b, c / A, B, C differ significantly @ P<0.05 and P<0.01, respectively (n=20).

Table 2: *In vitro* sperm characters (Mean±SEM) in post thawed semen of Black Bengal bucks supplemented with α tocopherol.

Parameters	α-tocopherol @ 0 mg/ml (TFC)	α-tocopherol @ 1 mg/ml (TFT_1)	α-tocopherol @ 1 mg/ml (TFT_2)
Progressive forward motility (%)	34.4 ± 4.43^{a}	43.5 ± 4.02^{b}	43.75 ± 3.89^{b}
Functional membrane integrity (%)	38.1 ± 1.43^{aA}	42.2 ± 1.73^{bB}	42.75 ± 1.61^{bB}
Sperm viability (%)	37.1 ± 1.72^{aA}	41.5 ± 2.07^{b}	42.65 ± 1.98^{bB}
Acrosome integrity (%)	63.95 ± 2.24^{aA}	67.1 ± 1.8^{aA}	68.85 ± 1.95^{bB}
MDA (µmol/ml)	0.509 ± 0.086^{aA}	0.769 ± 0.062^{bB}	0.634 ± 0.022^{aA}
SOD (U/mg of protein)	0.339 ± 0.006^{aA}	0.125 ± 0.006^{bB}	0.125 ± 0.002^{bB}

Rows with different superscripts a, b, c / A, B, C differed significantly @ P<0.05 and P<0.01, respectively (n=20).

cytoplasm, increasing the intra-cytoplasmic fluidity and then exert its effects from within and outside the cells. Further, they interfere with the auto-oxidation chain reaction by donating a hydrogen molecule to the lipid radical, thereby producing a product that is stable (Papas, 1993). Addition of BHT at lower concentrations (0.5-2 mM) had been reported to improve post thaw sperm motility (Mostafa *et al*., 2019), functional membrane integrity, viability (Memon *et al*., 2011) and acrosome integrity in different livestock species. Similarly supplementation of tocopherol to the extender was also reported to improve post thaw sperm motility (Ullah *et al*., 2019), functional membrane integrity; sperm cell viability (Jeong *et al*., 2009) and acrosome integrity in several species.

Anderson *et al*. (1994) stated that BHT improved sperm viability during preservation by increasing fluidity, hence rendering them less susceptible to cold shock. Hammerstedt *et al*. (1990) opined that supplementation of BHT prevents loss of phospholipid content of spermatozoa membrane, increases the fluidity of membrane, protects sperm cells from ROS attack and prevents membrane damage and cryo-capaciatation. Addition of α-tocopherol could improve freezing capability *via* changing the lipid composition of sperm cell. Perhaps phospholipids of the sperm cell membrane displayed different phase transition temperatures, bringing the transition to the gel phase in other molecules, which in turn influenced its diffusion coefficient (Maia *et al*., 2009) and fusion capacity of the membrane.

Supplementation of BHT significantly (P<0.05) reduced the development of lipid peroxide compound MDA during freeze - thawing of sperm cells in this present study. Similar to the present observation, Ghorbani *et al*. (2015) reported that the supplementation of BHT decreased ROS content, malondialdehyde formation in human semen samples. Mostafa *et al*. (2019) opined that addition of BHT in the levels of 0.5 and 2.0 mM/mL were the most protective for freezing of buffalo bull semen. BHT converts peroxyl radicals to hydroperoxides, thus acts as an antioxidant to the cryopreserved sperms. SOD is one among the crucial enzymes to control the oxidative stress in sperm. SOD plays an important role in decreasing LPO and protecting spermatozoa under oxidative damages (Du Plessis *et al*., 2008). In the present study, SOD activity was significantly reduced in the treatment groups supplemented with BHT/ tocopherol after freezing thawing when compared to their control groups. Marti *et al*. (2008) reported that SOD is the enzyme most affected by cryoinjury, with a decrease of 65% after freezing/thawing, which might be due to a partial inactivation of the enzyme. Bansal *et al*. (2014) reported a significant ($p\leq0.05$) decrease in SOD activity in pre-freeze and post thaw samples of buffalo bulls. Further, they indicated that freezing-thawing produces more oxidative stress/LPO and to neutralize the detrimental effects of ROS/LPO, there was a drop in the level of SOD. Orzołek *et al*. (2013) also found a significant increase in LPO level after cryopreservation of boar semen, which was moderately intervened by the loss of SOD activity. The decrease in SOD activity after freezing-thawing was also found in bull sperm (Bilodeau *et al*., 2000). Similar to the current observation, Bucak *et al*. (2010) reported that supplementation of the antioxidants hypotaurine and cysteamine decreased SOD activity when compared to the controls (p<0.001) post freeze thawing of Angora goat semen. It is generally accepted that cryopreservation provokes loss of antioxidant

defense in the semen (Bilodeau *et al*., 2000). In contrary, Marti *et al*. (2008) found that the addition of seminal plasma proteins, mixture of oleic/linoleic acid and vitamin E, accounted for an increase in the enzyme activity levels, not only in the fresh sample but also after freezing/thawing.

CONCLUSION

In conclusion, the present study showed that the supplementation of BHT and α-tocopherol antioxidants to the extender for freezing in Black Bengal buck semen extender had a positive effect on post-thawed sperm survivability. Specifically, BHT supplementation could protect the sperm against excessive ROS generation by reducing lipid peroxidation. However, further studies on conception rate and kidding size employing BHT and α-tocopherol antioxidants supplemented frozen thawed sperm in artificial insemination would add interest and significant credibility to the goat farming in Eastern India and Bangladesh in order to maximize the fertility rates as well as for protecting the Black Bengal goats from indiscriminate breeding.

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Ethics approval

The work was carried out with the approval of Institute Research Council of ICAR- National Dairy Research Institute, Karnal, India.

Conflicts of interest

The authors declare that there is no conflict of interest.

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