Indian Journal of Animal Sciences **85** (4): 352–355, April 2015/Article

Effect of butylated hydroxy toluene on the quality of frozen goat semen

R K DEWRY[1], B C DEKA[2], D BHUYAN[3], S SINHA[4], R K BISWAS[5], S B HAZARIKA[6], P BORAH[7], N C NATH[8] and A DAS[9]

Assam Agricultural University, Khanapara, Guwahati, Asom 781022 India

Received: 10 October 2014; Accepted: 19 November 2014

ABSTRACT

Ejaculates (40) collected from 5 Beetal bucks twice weekly were used to study the effect of 0 (control), 150, 200 and 300 µM butylated hydroxy toluene (BHT) on the quality of frozen semen by split sample technique. After removal of the seminal plasma, the semen was primarily extended with Tris extender (1: 5) considering the volume of semen prior to removal of seminal plasma and then split into four parts and finally extended with equal volume of Tris extender containing 0 (control), 300, 400 and 600 µM BHT. Consequent upon addition of equal volumes of Tris extender the rate of extension of semen rose to 1: 10 and eventual concentration of BHT in the extender was 0, 150, 200 and 300 µM. The extended semen was cooled gradually to 5°C @ 1°C/3 min and equilibrated in the cold handling cabinet for 4 h at 5°C. The extended semen was filled in French mini straws (0.25 ml) and polyvinyl alcohol powder of different colours were used for sealing the extended semen for identification of semen of different bucks processed with different concentrations of BHT. The freezing of semen was done by rapid horizontal vapour freezing technique using liquid nitrogen. The mean percent post thaw sperm motility, live sperm, live intact acrosome and HOST-reacted sperm were significantly higher in the extender with 200 µM BHT than with 150, 300 µM BHT and control. The release of ALT and AST from post thaw spermatozoa was also the lowest (33.75 ± 0.17 and 221.61 ± 0.73 U/L respectively) in the extender containing 200 µM BHT. Based on sperm motility, live sperm, live intact acrosome, HOST-reacted sperm, and ALT and AST release, 200 µM BHT in Tris extender was superior to 0, 150, and 300 µM BHT in freezing Beetal buck semen. It was concluded that BHT at 200 µM provided a better protection to the functional capacity of buck spermatozoa for cryopreservation.

Key words: Beetal goat, BHT, Freezing, Semen quality

Antioxidants naturally present in seminal plasma neutralize the normal amount of free radicals generated in semen *in vivo*. However, the dilution of natural antioxidants as a result of addition of extender during processing of semen for preservation and oxidative stress induced by cooling, freezing and thawing necessitates the need to supplement antioxidant in the extender. Butylated hydroxy toluene (BHT) is a lipid soluble antioxidant in sperm membrane systems and plays a major protective role against plasma membrane damage due to oxidative stress. It seems that optimal concentration of BHT depends upon the species of animals and ranges from 0.2 to 2.0 mM (Roca *et al*. 2004, Shoae and Zamiri 2008, Ijaz *et al*. 2009, Farshad *et al*. 2010). The present investigation was conducted to study the effect of concentration of BHT on quality of frozen goat semen.

Present address: [1]RA (dewryraju12@gmail.com), ICAR Research Complex for NEH Region, Umiam, Meghalaya. [2]Professor and Head, (bcdeka@gmail.com); [3,4,5]Professor, [6]JRF, NRC Mithun, ICAR, Jharnapani, Nagaland.[7]Junior Scientist, GRS, AAU, Burnihat, Asom. [8]Assistant Professor, Department of Veterinary Physiology, [9]Associate Professor, Department of Animal Genetics and Breeding.

MATERIALS AND METHODS

Five Beetal bucks 4–8 years of age maintained at Goat Research Station, Assam Agricultural University, Burnihat, Asom were used in the study. Semen samples were collected twice weekly in the morning from each buck with the help of artificial vagina. Immediately after collection, each ejaculate was evaluated for volume, mass activity (based on the numerical scale 0–4) and initial sperm motility. A total of 40 ejaculates having volume 0.8 ml or more, mass activity 3+ or more and initial sperm motility 70 % or more were used for the study. Each ejaculate was diluted (1: 5) with warm (35ºC) Tris buffer and centrifuged at room temperature for 5 min at 3,000 rpm. The clear supernatant was aspirated out using a pulled Pasteur pipette. The centrifugate was then extended (1: 5) using Tris extender. All the components of the extender, except egg yolk were mixed and the pH was adjusted to 6.8 using 5% citric acid solution. The extender was prepared afresh adding egg yolk just prior to collection of semen. The volume of fresh semen prior to washing was taken into consideration for extension of the centrifugate. The primarily extended semen was then split into 4 parts and finally extended with equal volume of Tris extender containing 0 (control), 300, 400 and 600 µM

of butylated hydroxy toluene (BHT) as antioxidant. After addition of equal volumes of Tris extender without BHT (for control) and with BHT, the rate of extension of semen rose to 1: 10 and eventual concentration of BHT in extended semen was 0, 150, 200 and 300 µM of BHT in 4 different parts respectively. The extended semen was cooled gradually to 5°C @ 1°C/3 min and equilibrated in the cold handling cabinet for 4 h at 5°C. The extended semen was filled in French mini straws (0.25 ml) and polyvinyl alcohol powder of different colours were used for sealing the straws. The straws were placed in a tray containing water (5ºC) inside the cold handling cabinet. Thirty minutes before the end of 4 h of equilibration the straws were taken out from water and wiped dry using pre-cooled (5ºC) towel kept inside the cold handling cabinet. The straws were arranged in a freezing rack and frozen in liquid nitrogen vapour (4 cm above liquid nitrogen) for 10 min. Immediately after vapour freezing, the frozen semen straws were collected in a goblet containing liquid nitrogen and transferred to liquid nitrogen container for storage. After 24 h of storage in liquid nitrogen, the frozen semen was thawed in warm water at 37°C for 30 sec. The semen was evaluated for sperm motility, live sperm, live intact acrosome (Tamuli and Watson 1994) and hypo osmotic swelling test (HOST)-reacted sperm (Revel and Mrode 1994) after equilibration and thawing. The frozen semen was also evaluated for extracellular release of alanine transaminase (ALT) and aspartate transaminase (AST). The statistical analysis of the data were done using SAS Enterprise Guide 4.2 version.

RESULTS AND DISCUSSION

The mean values of sperm motility, live sperm, live intact acrosome and HOST-reacted sperm after equilibration and after freezing, and extracellular release of ALT and AST after freezing of Beetal buck semen in Tris extender containing different concentrations of BHT are presented in Table 1.

The mean sperm motility after equilibration and after freezing of Beetal goat semen extended with Tris extender containing 0 (control), 150, 200 and 300 µM of BHT was 75.88 ± 0.43 and 59.13 ± 0.40, 72.93 ± 0.39 and 59.38 ± 0.32, 79.25 ± 0.29 and 60.63 ± 0.37, and 68.25 ± 0.42 and 52.25 ± 0.47 % respectively. The analysis of variance revealed that sperm motility differed significantly (P<0.01) between stages (equilibration and freezing), between concentrations of BHT and due to interaction. Critical difference test revealed that mean sperm motility both after equilibration and after freezing was significantly (P<0.05) higher in Tris extender containing 200 µM BHT than in that containing 150 and 300 µM BHT, and control (without BHT). The mean post thaw sperm motility after freezing in Tris extender containing 300 µM BHT was significantly lower than in that containing 150 µM BHT and without BHT (control), the difference between the latter 2 being nonsignificant (Table 1). The significant (P<0.01) variation in sperm motility after freezing in different concentrations of BHT in Tris extender recorded in the present study is in agreement with the observations of earlier workers in semen of goat (Khalifa *et al.* 2008, Memon *et al*. 2011, Naijian *et al*. 2013) and ram (Farshad *et al*. 2010). Khalifa *et al.* (2008) reported that in egg yolk free extender post thaw sperm motility in goat semen was significantly (P<0.05) higher in extender containing 0.6 mM BHT than in that containing 0, 0.3 or 0.9 mM BHT. On the other hand, Ijaz *et al.* (2009), Farshad *et al.* (2010), Memon *et al*. (2011) and Naijian *et al*. (2013) found significantly higher post thaw sperm motility with incorporation of 1.0 mM or 2.0 mM BHT and reduced sperm motility in Tris extender with 0, 0.5, 3.0 and 4.0 mM BHT. Sperm motility in Tris extender containing different concentrations of BHT dropped significantly (P<0.01) during freezing. In accordance with the present finding, significant drop in sperm motility due to freezing of goat semen was also reported earlier (Deka and Rao 1989, Deka *et al*. 2012 and Saraswat *et al*. 2012). In the present study, sperm motility varied significantly (P<0.01) due to BHT concentration × stage (equilibration and freezing) interaction which indicated that the main effects were not independent.

The mean percentage of live sperm after equilibration and after freezing of Beetal buck semen in Tris extender

Table 1. Percentage of sperm motility, live sperm, live intact acrosome and HOST- reacted sperm (mean*± SE) in Beetal goat semen after equilibration (AE) and after freezing (AF) and release of ALT and AST after freezing in Tris extender containing different concentrations of BHT

BHT concentration	Sperm motility (%)		Live sperm (%)		LIA (%)		HOST (%)		ALT (U/L)	AST (U/L)
	AE	AF	AE	AF	AE	AF	AE	AF	AF	AF
0 (Control)	75.88^{b} ± 0.43	59.13^{e} ± 0.40	80.38^{b} ± 0.26	60.91^{e} ± 0.18	70.95^{b} ± 0.15	43.11^{f} ± 0.16	70.85^{b} ± 0.10	49.79^{e} ± 0.20	34.85^{d} ± 0.35	229.28^{c} ± 0.79
150 µM	72.93^{c} ± 0.39	59.38^{e} ± 0.32	77.91^{c} ± 1.89	59.94^{f} ± 0.23	70.20^{c} ± 0.20	42.81^{f} ± 0.19	70.51^{b} ± 0.12	48.75^{f} ± 0.24	38.51^{a} ± 0.22	215.82^{a} ± 0.53
200 µM	79.25^{a} ± 0.29	60.63^{f} ± 0.37	82.58^{a} ± 0.27	64.13^{d} ± 0.38	72.44^{a} ± 0.15	44.58^{e} ± 0.18	71.98^{a} ± 0.13	51.41^{d} ± 0.14	33.75^{b} ± 0.17	211.61^{a} ± 0.73
300 µM	68.25^{d} ± 0.42	52.25^{g} ± 0.47	76.78^{c} ± 0.27	57.78^{g} ± 0.22	66.65^{d} ± 0.24	42.03^{g} ± 0.21	69.19^{c} ± 0.21	46.09^{g} ± 0.34	44.38^{c} ± 0.27	303.09^{b} ± 3.76

*40 observations; means bearing different superscripts within a parameter differ significantly (P< 0.05).

containing 0, 150, 200 and 300 µM BHT was 80.38 ± 0.26 and 60.91 ± 0.18, 77.91 ± 1.89 and 59.94 ± 0.23, 82.58 ± 0.27 and 64.13 ± 0.38, and 76.78 ± 0.27 and 57.78 ± 0.22 respectively. The analysis of variance revealed that the percentage of live sperm differed significantly (P<0.01) between stages and between concentrations of BHT but did not differ significantly due to interaction. Critical difference test revealed that mean percentage of live sperm after equilibration and after freezing was significantly (P<0.05) higher for 200 µM BHT than that for 150, 300 and 0 µM (Table 1). The mean percentage of live sperm, after equilibration and after freezing, differed significantly (P<0.05) between all concentrations of BHT except between 150 and 300 µM BHT after equilibration. Memon *et al*. (2011) observed a significant (P<0.05) improvement in viability of buck spermatozoa after equilibration in Tris extender supplemented with 1.0 mM BHT as compared to control. However, they failed to record any significant improvement in pre-freeze viability of sperm over that of control when the extender was supplemented with 0.5, 2.0 and 3.0 mM BHT. Farshad *et al.* (2010) observed significant increase in % live sperm after equilibration of ram semen in extender containing 2.0 mM BHT as compared to control but found no significant increase in percent live sperm in extender containing 0.5, 1.0 and 3.0 mM BHT. The significantly higher percent live sperm recorded in semen frozen with 200 µM BHT as compared to control recorded in the present study was in agreement with the findings of Roca *et al.* (2004); while 2.0 mM BHT improved the livability of frozen semen of Boer goat (Memon *et al*. 2011) and ram (Farshad *et al.* 2010). The discrepancy with the earlier workers might be due to the fact that optimum concentration of BHT in the extender might depend on several factors such as species, season of cryopreservation, extender and concentration of different components in the extender, processing and freezing technique used.

The mean percentage of live intact acrosome after equilibration and after freezing of buck semen in Tris extender containing 0 (control) 150, 200 and 300 µM BHT was 70.95 ± 0.15 and 43.11 ± 0.16, 70.20 ± 0.20 and 42.81 ± 0.19, 72.44 ± 0.15 and 44.58 ± 0.18, and 66.65 ± 0.24 and 42.03 ± 0.21 respectively. The analysis of variance revealed that the percentage of live intact acrosome differed significantly (P<0.01) between stages, between concentrations of BHT and due to interaction. Critical difference test revealed that mean live intact acrosome both after equilibration and after freezing was significantly (P<0.05) higher in Tris extender containing 200 µM BHT than in that containing 0, 150 and 300 µM BHT. The mean percentage of live intact acrosome after freezing with 300 µM BHT in extender was significantly (P<0.05) lower than that with 0 (control) and 150 µM BHT but the difference between the values for 0 and 150 µM BHT was not significant (Table 1). The significantly (P<0.05) higher percentage of live intact acrosome both after equilibration and after freezing with 200 µM BHT in the extender is in agreement with the findings of Roca *et al*. (2004) in boar semen. Memon *et al*. (2011) observed that acrosomal integrity in Boer goat spermatozoa before freezing and after freezing was significantly (P<0.05) higher with 0.5, 1.0, 2.0 or 3.0 mM BHT in Tris egg yolk extender than in control (0 mM BHT).

The mean HOST-reacted sperm in Beetal buck semen extended with Tris extender containing 0, 150, 200 and 300 µM BHT after equilibration and after freezing was 70.85 ± 0.10 and 49.79 ± 0.20, 70.51± 0.12 and 48.75 ± 0.24, 71.98 ± 0.13 and 51.41 ± 0.14, and 69.19 ± 0.21 and 46.09 ± 0.34 % respectively. The analysis of variance revealed that the percentage of HOST-reacted sperm differed significantly (P<0.01) between stages, between concentrations of BHT and due to interaction. Critical difference test revealed that mean HOST-reacted sperm both after equilibration and after freezing was significantly (P<0.05) higher in Tris extender containing 200 µM BHT than in that containing 0, 150 and 300 µM BHT. The HOST-reacted sperm after freezing differed significantly between the concentrations of BHT used and the values for 150 and 300 µM BHT were lower than that for control (Table 1). The significant (P<0.01) difference in percentage of HOST-reacted spermatozoa observed in the study between concentrations of BHT in Tris extender is in agreement with the findings of earlier workers in goat (Memon *et al*. 2011, Naijian *et al*. 2013) and ram (Farshad *et al*. 2010). The significant (P<0.05) improvement in the sperm membrane integrity with the addition of 200 µM BHT in Tris extender as compared to control observed in the present study is in agreement with the findings of Roca *et al.* (2004).

In Beetal buck semen the extracellular release of ALT after freezing was 34.85 ± 0.35, 38.51± 0.22, 33.75 ± 0.17 and 44.38 ± 0.27 U/L, and the extracellular release of AST was 229.28 ± 0.79, 215.82 ± 0.53, 211.61 ± 0.73 and 303.09 ± 3.76 U/L in Tris extender containing 0, 150, 200 and 300 µM BHT respectively. The extracellular ALT and AST activities in frozen semen differed significantly (P<0.01) between different concentrations of BHT. The more extracellular activities of ALT and AST indicated more sperm cell damage. In the present investigation the level of ALT was significantly (P<0.05) lower in the extender added with 200 µM BHT than with 150, 300 µM BHT and control. The level of AST was also significantly (P<0.05) lower with 200 µM BHT in comparison with that of 300 µM BHT and control. The finding could indicate that addition of 200 µM BHT entailed lower level of release of ALT and AST from spermatozoa post thaw as it could confer higher degree of protection against sperm damage. Lower values of GOT than that observed in the present study were reported in frozen thawed semen of Boer (Tuli and Holtz, 1994) and Black-Bengal × Beetal goats (Singh *et al*. 1996). The variations in leakage of ALT and AST could be attributed to differences in breeds and individuals within breed, extenders and concentrations of different ingredients of the extender and processing and freezing protocols (Singh *et al*. 1996, Deka 2008). Owing to lack of available literature the present values of ALT and AST in Tris extender

containing BHT could not be compared.

In the present study, BHT used at a concentration of 200 µM in Tris extender significantly improved the quality of frozen Beetal buck semen. Khalifa *et al.* (2008) reported that freezing of goat spermatozoa in extender containing exogenous antioxidants such as BHT may reduce the harmful effects of lipid peroxidation, thereby resulting in significantly greater post-thaw quality of spermatozoa. The BHT might break the covalent links that ROS form between fatty acid chains in membrane lipids and can inhibit the lipid peroxidation reaction in the sperm membrane by eliminating the free radicals (Roca *et al*. 2004), thereby protecting the spermatozoa by preventing endogenous oxidative DNA and membrane damage and helping them to overcome the oxidative attack.

ACKNOWLEDGEMENT

The authors are thankful to the Director of Research (Veterinary) and Principal Scientist, Goat Research Station, Assam Agricultural University for providing animals to carry out the study.

REFERENCES

Deka B C and Rao A R. 1989. Effect of glycerolization methods on the quality of frozen goat semen. *Indian Veterinary Journal* **66**: 135–37.

Deka B C. 2008. Studies on seminal attributes in Assam local, Beetal and crossbred bucks and evolving technology for improving the quality of frozen semen. *ICAR adhoc Project, Final Report*. Indian Council of Agricultural Research, New Delhi.

Deka B C, Biswas R K, Nath K C, and Barua P M. 2012. Effect of washing on quality of frozen semen in Assam local, Beetal and Beetal × Assam local goats. *Compendium*, *National Symposium "Addressing Animal Reprodructive Stressses through Biotechlogical Tools" and 28th Annual Convention of ISSAR,* P–187.

Farshad A, Khalili B and Jafaroghli M. 2010. Effects of butylated hydroxytoluene on freezability of ram spermatozoa. *Asian-Australasian Journal of Animal Science* **23**: 1276–81.

Ijaz A, Hussain A, Aleem M, Yousaf M S, and Rehman H. 2009. Butylated hydroxytoluene inclusion in semen extender improves the post-thawed semen quality of Nili-Ravi buffalo (*Bubalus bubalis*). *Theriogenology* **71**: 1326–29.

Khalifa T A A, Lymberopoulos A G and El-Saidy B E. 2008. Testing usability of butylated hydroxytoluene in conservation of goat semen. *Reproduction Domestic Anim*al **43**: 525–30.

Memon A A, Wahid H, Rosnina Y, Goh Y M, Ebrahimi M, Nadia F M and Audrey G. 2011. Effect of butylated hydroxyl toluene on cryopreservation of boer goat semen in Tris egg yolk extender. *Animal Reproduction Science* **129**: 44–49.

Naijian H R, Kohram H, Shahneh A Z, Sharafi M and Bucak M N. 2013. Effects of different concentrations of BHT on microscopic and oxidative parameters of Mahabadi goat semen following the freeze-thaw process. *Cryobiology* **66**: 151–55.

Revel S G and Mrode R A. 1994. An osmotic resistance test for bovine semen. *Animal Reproduction Science* **36**: 77–86.

Roca J, Gil A M, Hernandez M, Parrilla I, Vazquez J M and Martinez E A. 2004. Survival and fertility of boar spermatozoa after freeze thawing in extender supplemented with butylated hydroxy toluene. *Journal of Andrology* **25**: 397–405.

Saraswat S, Jindal S K, Ramachandran N, Yadav S and Priyadharsini R. 2012. Standardisation of antioxidant fortification in frozen buck semen. *Indian Journal of Small Ruminants* **18**: 47–51.

Shoae A and Zamiri M J. 2008. Effect of butylated hydroxy toluene on bull spermatozoa frozen in egg yolk-citrate extender. *Animal Reproduction Science* **104**: 414–18.

Singh M P, Sinha A K, Sinha B K and Prasad R L. 1996. Effect of cryoprotectants on release of various enzymes from buck spermatozoa during freezing. *Theriogenology* **45**: 405–16.

Tamuli M K and Watson P F. 1994. Use of a simple staining technique to distinguish acrosomal changes in the live sperm sub-population. *Animal Reproduction Sci*ence **35**: 247–50.

Tuli R K and Holtz W. 1994. Effect of glycerolization procedure and removal of seminal plasma on post-thaw survival and GOT-release from boer goat spermatozoa. *Theriogenology* **42**: 547–55.