Indian Journal of Animal Sciences **95** (6): 520–523, June 2025/Article
https://doi.org/10.56093/ijans.v95i6.159736

Effect of butylated hydroxytoluene on capacitation like changes in spermatozoa of Hariana bull during cryopreservation of semen

AKHIL PATEL[1], ATUL SAXENA[2], CHETNA GANGWAR[2,1]✉, DUSHYANT YADAV[3], SANJAY SINGH YADAV[4], VIKAS SACHAN[1] and VIJAY SINGH[1]

U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, Uttar Pradesh 281 001, India

Received: 08 November 2024; Accepted: 18 August 2025

ABSTRACT

The experiment was planned to analyse the effect of butylated hydroxytoluene (BHT) supplementation as an antioxidant on capacitation like changes in spermatozoa of Hariana bull during cryopreservation. A total 24 ejaculates were collected bi-weekly by artificial vagina method from four Hariana bulls. The ejaculates fulfilling the minimum criteria as laid down for cryopreservation were further processed. The ejaculated samples were extended upto 80 million spermatozoa/mL in a Glycerolated Egg Yolk Tris (GEYT) Extender. Different groups of diluted semen were prepared having different concentration of BHT. One part was left as such (Control) while other two parts contain 0.5 and 1.0 mM BHT were considered Group 1 (G1) and Group 2 (G2), respectively. Following the process of extension and equilibration (4 h), the semen was frozen in liquid nitrogen vapour in French mini straw (20 million spermatozoa/straw) using biological freezer. Following freezing and storage in LN_2 for 24 h, thawing was performed at 37°C for 45 sec for all the cryopreserved straws. Chlortetracycline (CTC) assay was conducted to evaluate capacitation like changes in samples at initial stage of dilution, equilibration and following thawing. BHT was found to prevent capacitation like changes at all the stages of semen cryopreservation process. Inclusion of 1.0 mM was found to be effective in preventing capacitation like changes in Hariana bull spermatozoa.

Keywords: Butylated hydroxytoluene, Capacitation, Chlortetracycline assay, Cryopreservation, Semen

Semen cryopreservation provides multiple advantages to livestock sector, particularly, in the wide distribution of quality genetic material by the use of artificial insemination (Gangwar *et al.* 2023). For successful fertilization, mammalian spermatozoa undergo structural and biochemical alterations while passing through the female genital tract for fertilization of an oocyte. This process of sperm acquiring the strength to fertilize the ovum, is known as sperm capacitation. Spermatozoa suffer with stresses (cold shock, osmotic imbalances, cryoinjury and generation of reactive oxygen species) during cryopreservation inflicting structural and biochemical alterations that resemble capacitation (Vadnais *et al.* 2005, Gangwar *et al.* 2020). Semen cryopreservation results in increased production of reactive oxygen species (ROS) beyond its physiological limit and the high content of unsaturated fatty acid in chemical makeup of plasma membrane of the spermatozoa (makes them prone to peroxidative damage leading to structural and functional deformation, affecting the fertilizing ability of spermatozoa (Forouzanfar *et al.* 2010). Addition of antioxidant to the extender have been suggested to neutralize the excessive ROS production (Sun *et al.* 2020).

Many studies have earlier reported the beneficial effects of inclusion of different antioxidants in the semen of different livestock species. However, few studies reported the use of butylated hydroxytoluene as an antioxidant in dog semen (Sun *et al.* 2020), in bull semen (Patel *et. al*. 2015, Khumran *et al.* 2020), in buffalo bull semen (Mostafa *et al.* 2019, Nain *et al.* 2023) and in buck semen (El-Khawagah *et al.* 2020, Tudu *et al.* 2023), which indicated that BHT has got influence on preventing damage to sperm during the process of cryopreservation. But the current study focused on the advantages of butylated hydroxytoluene on capacitation like alterations in Hariana bull semen and maintaining its fertilizing potential during cryopreservation.

MATERIALS AND METHODS

Experimental animals: The present study included four Hariana bulls, aging about 5.5 to 6.5 years with average body weight between 450 and 500 kg, at college of Veterinary Science and Animal Husbandry, Veterinary University, Mathura, located in the semiarid region of Uttar

Present address: [1]Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut-250110. [2]College of Veterinary Science & Animal Husbandry (DUVASU) Mathura 281 001. [3]Bihar Animal Science University, Patna 800 014 Bihar, India. [4]Department of Animal Husbandry, Govt. of Uttar Pradesh, India. ✉Corresponding author email: chetnaom82@gmail.com

Pradesh in north India. All the bulls (04) were kept under standard feeding and management conditions at Livestock Farm Complex. All the bulls included in the study were apparently healthy and were regularly vaccinated in accordance with the accredited semen laboratory's recommended manual.

Semen collection: Over the course of the research, which took place between January and March, semen was taken twice a week in the morning. An artificial vagina was used to collect twenty-four ejaculates from all 04 bulls. Semen-containing tubes were labelled as soon as they were collected, brought to the lab, and put in a water bath set between 34–37°C to continue processing.

Semen dilution: The collected semen was evaluated and those which fulfill the criteria (≥ 3.0 mass motility, ≥ 70% progressive motility, and 80% viable sperm) for cryopreservation were pooled and extended with glycerolated egg yolk tris (GEYT) extender added egg yolk (20%) and glycerol (7%) to make final concentration of 80 million spermatozoa/mL. This extended semen was further divided into 3 parts- Control (without BHT supplementation), G1 (containing 0.5 mM BHT) and G2 (containing 1.0 mM BHT)

Evaluation of seminal attributes: The sperm evaluation was carried out at three stages *i.e.* (i) after dilution with the semen extender, (ii) after equilibration (four hours at 4-5°C) and (iii) after 24 h of cryopreservation. Semen parameters as capacitation like changes and acrosomal integrity were assessed in all three stages.

Preparation of BHT: Using the methods outlined by Patel *et al.* (2015), different concentration of BHT (Product number W218405, CAS number 128-37-0, Sigma- Aldrich, Saint Louis, USA) i.e. 0.5 mM and 1.0 mM in extended semen (80 million spermatozoa/mL) were obtained.

Processing of semen for freezing: All the samples (Control, G1 and G2 groups) were processed for cryopreservation under the vapour of liquid nitrogen using biological freezer (IMV, Technologies France), completing the process of freezing in 7 min and 5 sec.

Capacitation and acrosome reaction status: The capacitated and acrosome reacted spermatozoa were evaluated by chlortetracycline (CTC) assay previously prescribed by Collin *et al*. (2000), with slight alterations. Accordingly, 0.5 mL of semen samples were washed thrice (centrifugation at 1000 rpm for 3 min) using Tyrode's Albumin Lactate Pyruvate (TALP) @ of 5 mL /0.5 mL semen sample. After final washing, sperm pellet was resuspended in the 0.5 mL of TALP. 250 µL of this sample was incubated at 37˚C for 20 min with 250 µL (750 µM stock solution) of CTC (Product number C4881 Sigma-Aldrich, Saint Louis, USA) made fresh each time. The semen sample was then centrifuged (1000 rpm for 3 min) and pellet was suspended with 500 µL of TALP. 3 µL of this resuspended sample was then placed on grease free clean glass slide and cover slip was pressed gently to drain excess fluid. This slide was then evaluated under a phase contrast microscope using epifluroscence optics with the exposure of blue-violet illumination having excitation and emission at 400–440 and 470 nm, respectively. Sperm were assessed using Fraser *et al*. (1995) method. It revealed 03 distinct patterns of CTC i.e.

F pattern (or full form): Uncapacitated sperm were confirmed by the even distribution of fluorescence over the entire head.

B pattern (or banded): Capacitated and acrosome intact spermatozoa were confirmed by fluorescence-free appearance of bands in area of post acrosome and clear fluorescence in anterior part of portion of the head.

AR pattern (or acrosome reacted): It revealed acrosome reacted spermatozoa with appearance of thin bright fluorescent banding pattern along the equatorial segment and fluorescence free other area of head.

Statistical analysis: The Statistical Package for Social Science (SPSS® Version 22.0 for Windows®, SPSS Inc., Chicago, USA) was used to conduct the statistical analysis. The mean and its standard error (mean±standard error of mean) were used to display the data. One-way analysis was used to examine the effects of varying BHT (antioxidant) inclusion levels. The significance and analysis of variance were examined at the 5% level ($p<0.05$). The treatment means were compared for different sperm characteristics; Duncan's multiple range test was employed.

RESULTS AND DISCUSSION

In total, 24 ejaculates from four bulls were taken. The data for the effects of different concentrations of BHT in a tris-based extender on capacitation like changes of post-thawed bull sperm are presented in Table 2 and 3. Capacitation like changes in the semen samples was evaluated using CTC assay. For observing the effect of two different concentrations of BHT, semen samples were grouped as control, G1 and G2. The fraction of uncapacitated sperm (F pattern) were significantly ($p<0.05$) higher in treatment group G2 compared to G1 and Control groups. This pattern was observed for all the stages (Table 1). The fraction of capacitated spermatozoa (B pattern) were significantly ($p<0.05$) lower in treatment groups G2 compared to G1 and control groups and the pattern was uniformly observed at all stages of cryopreservation (Table 2). Similar trends were observed for acrosome reacted (AR pattern) spermatozoa (Table 3).

Capacitation is an important step in the event of fertilization. *In vitro* evaluation of the ability of sperm to undergo capacitation has been used as a sperm function test to predict the fertilizing ability of sperm (Gangwar *et al.* 2020). CTC assay is one such technique of measuring capacitation like changes and has advantage of measuring directly the percentage of uncapacitated, capacitated and acrosome reacted spermatozoa in the same semen sample preparation. According to several researchers, adding BHT prior to freezing may enhance post-thaw sperm parameters in spermatozoa of various species. However, there are no available references on the effects of altering concentrations of BHT in extenders on bull semen cryopreservation on the

Table 1. Uncapacitated spermatozoa ('F' pattern) in the semen of Hariana bulls extended in GYET with BHT supplementation during different stages of cryopreservation (Mean±SEM=24)

Stage	Mean percentage of uncapacitated spermatozoa ('F' pattern)		
	Control	G1	G 2
After dilution	71.31^{a} ±0.75	75.78^{b} ±0.78	79.89^{c} ±0.65
Pre-freezing	65.47^{a}±1.07	70.12^{b}±0.85	74.74^{c}±0.86
Post thaw	52.63^{a}±0.78	57.68^{b}±0.79	62.90^{c}±0.78

Means with different superscript letters (a, b, c) differ significantly within a row. Control: without BHT supplementation G 1: 0.5 mM BHT, G2: 1.0 mM BHT, significance level: 5%. BHT: Butylated hydroxytoluene, GEYT: Glycerolated egg yolk tris, SEM: Standard error of Mean. This pattern has been followed in subsequent tables.

Table 2. Capacitated spermatozoa ('B' pattern) in the semen of Hariana bulls extended in GYET with BHT supplementation different stages of cryopreservation. (Mean±SEM=24)

Stage	Mean percentage of Capacitated spermatozoa ('F' pattern)		
	Control	G1	G2
After dilution	24.19^{a}±0.71	20.54^{b}±0.76	17.02^{c}±0.62
Pre-freezing	28.83^{a}±0.89	25.13^{b}±0.82	21.27^{c}±0.84
Post thaw	40.59^{a}±0.79	36.80^{b}±0.79	32.21^{c}±0.77

Table 3. Acrosome reacted spermatozoa ('AR' pattern) in the semen of Hariana bulls extended in GYET with BHT supplementation during different stages of cryopreservation. (Mean±SEM=24)

Stage	Mean percentage of acrosome reacted spermatozoa ('AR' pattern)		
	Control	G1	G 2
After dilution	4.25^{a}±0.15	3.70^{b}±0.09	3.17^{c}±0.11
Pre-freezing	5.40^{a}±0.21	4.72^{b}±0.14	3.99^{c}±0.12
Post thaw	6.73^{a}±0.17	5.56^{b}±0.13	4.88^{c}±0.11

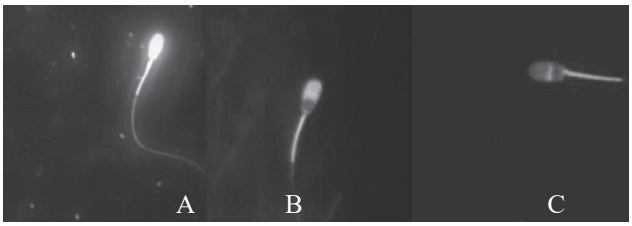

Fig. 1. A representing the F Pattern (Uncapacitated sperm), B representing the B pattern (capacitated, acrosome intact sperm), C representing the AR pattern (acrosome reacted sperm)

capacitation like changes. Thus, the goal of this experiment was to assess the capacitation status of bull semen extended with extenders having varying inclusion level of BHT and thus to improve the post thaw semen quality.

Cryopreserved sperm cells show a capacitation-like behaviour (cryo-capacitation) and it is considered as a major factor associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract, resulting in reduced fertility of frozen thawed semen (Nain *et al.* 2023). At present, it is generally accepted that poor survival of spermatozoa in the female reproductive tract is one of the most important consequences of sperm cryo-injury caused by cryopreservation (Gangwar *et al.* 2019).

In concurrence with our findings, Sun *et al.* (2020) and Nain *et al*. (2023) also revealed that 1.5 mM BHT is the ideal concentration for enhancing the post-thaw quality of buffalo and dog spermatozoa, respectively. However, Khumran *et al.* (2015) added BHT in lecithin-based Bioxcell®(BX) and two egg-yolk-based; Tris (TY) and citrate (CE) semen extenders and reported its beneficial effect on acrosome integrity and DNA integrity, when added in lower concentration (0.5–1.5 mM/mL). Similarly, earlier researcher reported the use of BHT in Tris egg yolk extender and soya lecithin extender in Boer and Zaraibi goats and reported that addition of BHT in 1.0 mM and 0.5mM concentration protect the sperm DNA during cryopreservation (El-Khawagah *et al.* 2020). However, Khumran *et al.* (2020) reported that addition of BHT in semen diluter protects bull sperm surface protein-P25b during cryopreservation. In similar line, Li *et al.* (2024) reported that BHT can effectively enhance the sperm quality parameters and fertility potential of post-thawed goat sperm and its cryoprotection may be encountered through regulation of sperm metabolism and antioxidative capability from the perspective of sperm proteomic modification.

The present study revealed that there was capacitation like changes during the cryopreservation and thawing processes. The addition of BHT significantly prevented the capacitation like changes and a BHT concentration of 1mM has proved better in preventing such changes compare with 0.5 mM. The present study also confirms cryo-capacitation and revealed that BHT supplementation prevented such changes significantly.

In this experiment, BHT to a level of 1.0 mM BHT (G2) in GEYT extender has been proved to be useful in preventing the capacitation like alterations during the event of cryopreservation. Further study is required to prove the fertility of frozen-thawed semen samples supplemented with 1.0 mM BHT by *in vivo* fertility tests. To demonstrate the fertility of frozen-thawed semen samples enriched with 1.0 mM BHT using more *in vivo* fertility testing is needed.

REFERENCES

Collin S, Sirard M, Dufour M & Bailey J. 2000. Sperm calcium levels and chlortetracycline fluorescence patterns are related to the *in vivo* fertility of cryopreserved bovine semen. *Journal of Andrology* **21**: 6.

El-Khawagah A R M, Rawash Z M, El-Badry D A, Kandiel M M M. 2020. Influence of butylated hydroxytoluene addition to cryodiluents on freezability and DNA integrity of Boer and Zaraibi buck spermatozoa. *Asian Pacific Journal of Reproduction* **9**(2): 96–103.

Forouzanfar M, Sharafi M, Hosseini S M, Ostadhosseini S,

Hajian M, Hosseini L, Abedi P, Nili N, Rahmani, H R and Nasr-Esfahani M H. 2010. *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology* **73**: 480–87.

Fraser L R, Abeydeera L R and Niwa K. 1995. Ca^{2+} regulating mechanisms that modulates bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Molecular Reproduction and Development* **40**: 233–41.

Gangwar C, Saxena A, Yadav S, Singh S P, Singh V and Patel A. 2019. Cryopreservation induced sperm cryoinjuries in hariana bull semen. *International Journal of Livestock Research* **9**(6): 136–47.

Gangwar C, Kharche S D, Mishra A K, Saraswat S, Kumar N and Sikarwar A K. 2020. Effect of diluent sugars on capacitation status and acrosome reaction of spermatozoa in buck semen at refrigerated temperature. *Tropical Animal Health and Production* **52**: 3409–15.

Gangwar C, Ranjan R, Kharche S D, Pourouchottamane R, Kharche S D and Rai B. 2023. Success of artificial insemination in goats: An overview. *Indian Journal of Small Ruminants* **29**(1): 1-10.

Khumran A M, Yimer N, Rosnina Y, Ariff M O, Wahid H, Kaka A, Ebrahimi M and Sarsaifi K. 2015. Butylated hydroxytoluene can reduce oxidative stress and improve quality of frozen-thawed bull semen processed in lecithin and egg yolk-based extenders. *Animal Reproduction Science* **163**: 128–34.

Khumran A M, Yimer N, Rosnina Y, Wahid H, Ariff M O, Homayoun H, Asmatullah K and Bello T K. 2020. Butylated hydroxytoluene protects bull sperm surface protein-P25b in different extenders following cryopreservation. *Veterinary World* **13**(4): 649–54.

Li C, Allai L, Liang J, Lv C, Zhao X, Ni X, Wu G, Deng W, Badaoui B and Quan G. 2024. The antioxidant effects of butylated hydroxytoluene on cryopreserved goat sperm from a proteomic perspective. *PeerJ* **12**: e17580 http://doi.org/10.7717/peerj.17580

Mostafa A A, El-Belely M S, Ismail S T, El-Sheshtawy R I and Shahba M I. 2019. Effect of butylated hydroxytoluene on quality of pre-frozen and frozen buffalo semen. *Asian Pacific Journal of Reproduction* **8**(1): 20–24.

Nain D, Mohanty T K, Dewry R K, Bhakat M, Nath S, Gupta V K and Parray M A. 2023. Butylated hydroxytoluene (BHT) improves the post-thaw semen quality in low-dose sperm cryopreservation in Murrah buffalo bull. *CryoLetters* **44**(1): 57–65.

Patel A, Saxena A, Swain D K, Yadav D, Yadav S S, Kumar A and Kumar A. 2015. Effect of supplementation of butylated hydroxytoluene on post-thaw sperm viability, motility and membrane integrity of Hariana bulls. *Veterinary World* **8**(6): 808–12.

Sun L, Wu C, Xu J, Zhang S, Dai J and Zhang D. 2020. Addition of butylated hydroxytoluene (BHT) in tris-based extender improves post-thaw quality and motion dynamics of dog spermatozoa. *Cryobiology* **97**: 71–75.

Tudu K C, Mandal A, Mondal M, Das S K, Ghosh M K, Rai S, Bhakat C and Karunakaran M 2023. Effect of butylated hydroxytoluene and tocopherol supplementation on in vitro sperm characters during cryopreservation of Black Bengal buck semen. *Indian Journal of Animal Research* **57**(5): 547–551. doi: 10.18805/IJAR.B-4349

Vadnais M L, Kirkwood R N, Specher D J and Chow K. 2005. Effects of extender, incubation temperature and added seminal plasma on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Animal Reproduction Science* **90**: 347–54.