



Effect of Butylated Hydroxytoluene on Sperm Viability and Acrosomal Integrity in Cryopreserved Haryana Bull Semen

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ABSTRACT

The present study was aimed to see the effect of butylated hydroxytoluene (BHT) as semen additives during cryopreservation of Haryana Bull semen. Twenty four ejaculates from two Haryana bulls extended with glycerolated egg yolk tris (GEYT). Extended samples were split into three aliquots. One aliquot was kept as control while other two aliquots were supplemented with 0.5 mM (T1) and 1.0 mM (T2) of BHT. All semen samples were equilibrated and cryopreserved in liquid nitrogen vapours and subsequently plunged in liquid nitrogen for preservation. The effect of BHT was determined by assessment of sperm viability and acrosomal integrity at three stages i.e. after dilution, pre-freeze and post –thaw. Our study revealed that addition of 1.0 mM of BHT results in a significant ($p < 0.05$) improvement in sperm viability and acrosomal integrity during cryopreservation.

Keywords: acrosomal integrity, butylated hydroxytoluene, Haryana bull, sperm and viability

Artificial insemination is the most widely used reproductive biotechnological tool for rapid genetic improvement of livestock. Cryopreservation of sperm is the integral part of a successful artificial insemination. In spite of lots of development in cryopreservation and cryopreservation protocols, the spermatozoa suffer from irreversible damage both at structural and functional level leading to a reduction in viability, motility and fertility (Thomas *et al.*, 2006).

Free radicals generated from the spermatozoa (immature/defective/damaged/dead spermatozoa) as well as from the extenders, contain the molecular oxygen (Thomson *et al.*, 2009) that has adverse effect on spermatozoa. Antioxidants are usually supplemented in the semen extender to overcome the effect of free radicals (Bucak *et al.*, 2007; Bucak *et al.*, 2009b; Tuncer *et al.*, 2010b; Ansari *et al.*, 2011; Najjian *et al.*, 2013; Patel *et al.*, 2015).

Butylated hydroxytoluene (synthetic analogue of vitamin-E) possesses antioxidant property by converting the peroxy radicals to hydroperoxides (Fujisawa *et al.*, 2004). However, reports of its use in semen extender are scanty.

Present study was designed to observe the effect of BHT addition in the extended semen for its antioxidant effect during cryopreservation of semen.

MATERIALS AND METHODS

Experimental animals

The present study was conducted on two Haryana bulls of 5.5 and 6.5 years age and weighing as 450 and 500 kg. The bulls were reared at the University Instructional Livestock Farm Complex of the College of Veterinary Sciences & Animal Husbandry, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura, in the state of Uttar Pradesh. Each bull was fed 21.15 kg of greens, 4.83 kg of concentrate including service ration and 5.0 kg of wheat straw per day. Bulls were apparently free of infection and were regularly vaccinated as per Minimum Standard Protocol (MSP), GoI, for quality semen production.

Semen collection

The semen was collected biweekly & single ejaculate was collected at each time during entire study. Immediately after collection, tubes containing semen were marked, transferred to the laboratory and placed in the water bath at 34°C for physico-morphological studies.

Evaluation of seminal attributes

The collected semen was evaluated and those which fulfill the criteria (3.5 mass motility, 70% progressive motility and 80% viable sperm, 1ml volume and 500 millions spermatozoa/ml) for cryopreservation were extended with glycerolated egg yolk tris (GEYT). The extended semen was divided into three aliquots- control (without BHT supplementation), T1 (containing 0.5 mM BHT), T2 (containing 1.0 mM BHT). Sperm evaluation (viability and acrosomal integrity) was carried out after dilution with the extender, at pre-freeze (after equilibration at 4-5°C for 4 h) and at post-thaw (37°C for 45 s) stages.

Preparation of butylated hydroxytoluene

0.5 mM and 1.0 mM concentration of BHT (Product number W218405, CAS number 128-37-0, Sigma-Aldrich, Saint Louis, USA) in extended semen (80 millions spermatozoa/ml) were achieved adopting method described by Patel *et.al.* (2015).

Processing of semen for freezing

All the semen samples (control, T1 & T2 groups) were processed for cryopreservation under the vapour of liquid nitrogen using biological freezer (IMV, Technologies France), completing the process of freezing (lowering of temperature from 4°C to -10°C @ 5°C/min, -10°C to -100°C @ 40°C/min, -100 to -140°C @ 20°C/min) in 7 minutes and 5 second.

Evaluation of spermatozoa

Trypan blue-Neutral red-Giemsa Staining was carried out as described by Kovacs & Foote (1992) for evaluation of sperm viability and acrosomal integrity. Microscopic evaluation was carried out at 100x oil immersion magnification and sperm cells were differentiated as Acrosome intact live (AIL), Acrosome intact dead (AID), Acrosome lost live (ALL) and Acrosome lost dead (ALD).

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS® Version 22.0 for Windows®, SPSS Inc., Chicago, USA). Data are presented as mean and their standard error (Mean±standard error of mean). Effect of different inclusion levels of BHT (antioxidant) were analyzed using one-way analysis of variance and significance was tested at 5% level (p<0.05). Duncan's multiple range test was used to compare the treatment means for various sperm attributes.

RESULTS AND DISCUSSION

The mean percentage of live spermatozoa with intact acrosome was significantly (p<0.05) higher in treatment group T2 as compared to T1 and control groups. This pattern was observed at after dilution, pre-freeze and post-thaw stages (Table 1 & Fig. 1 a).

Table 1: Sperm viability and acrosomal status at different stages of cryopreservation in GEYT extended and BHT supplemented semen of Haryana bulls. (Mean±SEM, n=24)

Stages of semen cryopreservation	Mean percentage of viability and acrosomal status			
	Control			
	AIL	ALL	AID	ALD
After dilution	78.14±0.55 ^c	1.52±0.09 ^a	19.59±0.51 ^a	0.78±0.06 ^a
Pre-freeze	70.76±0.54 ^c	2.47±0.19 ^a	25.74±0.60 ^a	1.28±0.08 ^a
Post-thaw	56.18±0.64 ^c	3.05±0.11 ^a	38.79±0.57 ^a	1.98±0.13 ^a
	T1			
After dilution	81.52±0.52 ^b	1.26±0.07 ^b	16.51±0.49 ^b	0.67±0.05 ^{ab}
Pre-freeze	75.21±0.66 ^b	1.85±0.10 ^{ab}	21.90±0.66 ^b	1.02±0.06 ^b
Post-thaw	63.31±0.67 ^b	2.28±0.53 ^b	34.28±0.54 ^b	1.38±0.08 ^b
	T2			
After dilution	86.37±0.62 ^a	0.69±0.06 ^c	12.65±0.71 ^c	0.51±0.02 ^b
Pre-freeze	80.15±0.62 ^a	1.43±0.10 ^b	17.90±0.62 ^c	0.70±0.05 ^c
Post-thaw	67.93±0.72 ^a	1.80±0.08 ^c	28.86±0.76 ^c	1.01±0.06 ^c

Means with different superscripts (a, b, c) differed significantly within a column at respective stage of semen cryopreservation. Control=without addition of BHT, T1=0.5 mM BHT, T2=1.0 mM BHT, significance level=5%. BHT=Butylated hydroxytoluene, GEYT=Glycerolated egg yolk tris, SEM=Standard error of Mean. AIL: Acrosome intact live, AID: Acrosome intact dead, ALL: Acrosome lost live, ALD: Acrosome lost dead

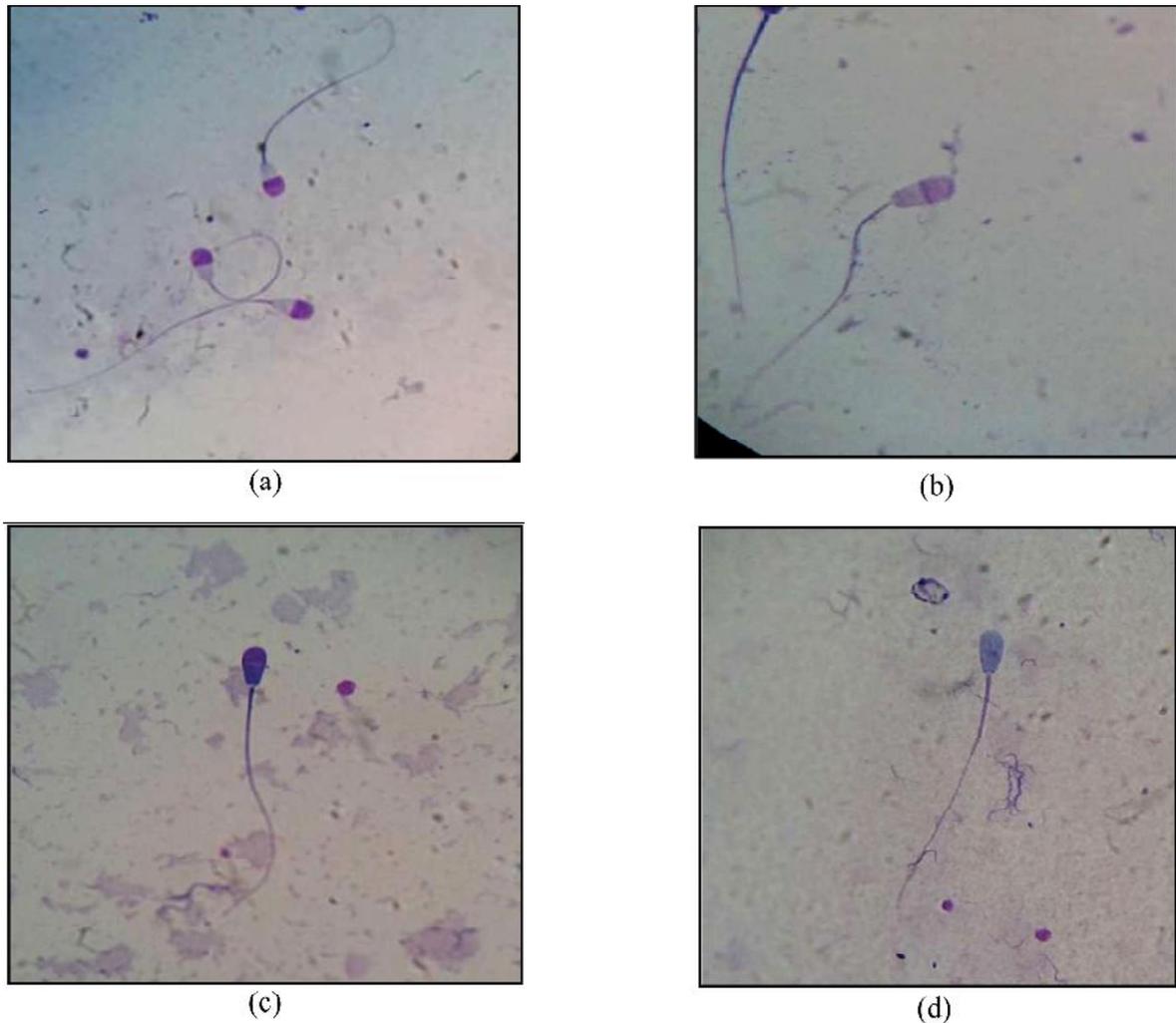


Fig. 1: Photograph showing stained spermatozoa (Trypan blue-Neutral red-Giemsa staining (Magnification 100x). (a) Live spermatozoa with intact acrosome (AIL); (b) Live spermatozoa with lost acrosome (ALL); (c) Dead spermatozoa with intact acrosome (AID) and (d) Dead spermatozoa with lost acrosome (ALD)

Further, the lost acrosome in the live spermatozoa were significantly ($p < 0.05$) lower in T2 compared to T1 and control groups and this pattern was observed for all stages of evaluation (Table 1 & Fig. 1 b).

The mean percentage of dead spermatozoa with intact acrosome was significantly ($P < 0.05$) lower in treatment groups T2 as compared to T1 and control. This pattern was observed at after dilution, pre-freeze and Post-thaw stages (Table 1 & Fig. 1 c).

Further, the spermatozoa with lost acrosome in dead spermatozoa was significantly ($p < 0.05$) lower in T2 as compared to T1 and control groups and this pattern was

observed for all stages of semen evaluation (Table 1, Fig. 1d).

The study was an attempt to evaluate the effect of BHT as antioxidant additive in the semen of Haryana bulls. The addition of BHT has been claimed to increase the antioxidant defence in the semen of other species (Bull, Ram, Goat, Turkey) which resulted in increase in the percent viable sperm (Farsad *et al.*, 2010; Neagu *et al.*, 2010; Suttotyotin *et al.*, 2011; Ogretmena and Inanan, 2014). In the present study, 1.0 mM of BHT has shown beneficial effect in term of sperm viability and acrosomal integrity. Other workers have used the BHT concentration in the



range of 0.5 mM to 4 mM and reported a beneficial effect at 0.5mM and 1.0 mM. Further, higher concentrations of BHT were found detrimental for bull spermatozoa (Shoae and Zamiri, 2008; Ijaz *et al.*, 2009). Farshad *et al.*, (2010) reported the beneficial effect of BHT at 2.0 mM concentration for ram semen. The other concentrations (0.5 & 1.0 mM) did not had a significant effect, however, differed significantly ($P<0.05$) with control.

The survival of sperm during cryopreservation is dependent on several factors which can affect the post-thaw outcome (Purdy, 2006). Watson (2000) speculated that under the best experimental conditions about 50% of motile sperm can survive the freeze-thaw process. Oxidative stress associated with decline in fertility during semen storage is one of the important factors (Stradaioli *et al.*, 2007). Antioxidants eliminate free radicals, which are detrimental to sperm (Watson, 2000). The present study investigated whether the presence of BHT would improve the quality of Harijana bull sperm after dilution, cooling and freezing. The results observed demonstrated a significant differences in improving sperm viability and acrosomal integrity after dilution, pre-freeze and post-thaw stages.

Our observations were in agreement with findings of Donoghue and Donoghue (1997) for turkey, Anderson *et al.* (1994) and Shoae and Zamiri (2008) for bull and Khalifa *et al.* (2008) for goat spermatozoa. These researchers reported that inclusion of BHT in semen extender improved the characteristics of spermatozoa. Our results are in agreement with the findings of Anderson *et al.* (1994) and Shoae and Zamiri (2008) reported that inclusion of BHT in semen dilution gave the highest post-thaw quality of cryopreserved bull spermatozoa. Ijaz *et al.* (2009) suggested that cryopreservation of buffalo sperm in extender containing BHT was better than extender without BHT (control group). Moreover, Roca *et al.* (2004) and Khalifa *et al.* (2008) reported that freezing of boar and 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. However, the potential effect of BHT in preventing damage to the spermatozoa depends on different parameters, such as species, added BHT concentration, cell membrane composition, incubation time and the composition of basic

diluents (Watson and Anderson, 1983; Killian *et al.*, 1989; Ball *et al.*, 2001; Roca *et al.*, 2004).

Based on our results and those of other researchers, we can hypothesize that supplementation of BHT to freezing media positively affects the post-thaw characteristics of spermatozoa. Furthermore, in our study we showed 1.0mM as best however, other concentrations (2.0 - 3.0mM) of BHT should have been done to further strengthen the beneficial and detrimental limit of BHT for Harijana bull semen. This is because of the fact that other workers have opined that optimal concentration of BHT depends upon the species of animals and ranges between 0.05-2.0 mM (Roca *et al.*, 2004; Shoae and Zamiri, 2008; Ijaz *et al.*, 2009).

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