



## Effects of Butylated Hydroxytoluene on Freezability of Ram Spermatozoa

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**ABSTRACT :** The present study was conducted to investigate the effects of butylated hydroxytoluene (BHT) supplementation on diluted, cooled and frozen-thawed ram spermatozoa. After primary evaluation of collected ejaculates, only semen samples with motility of more than 70% and sperm concentration higher than  $3 \times 10^3$  sperm/ml were used for cryopreservation. The selected semen samples were then pooled and diluted 1:4 with Tris Citrate Fructose Yolk (TCFY) extender supplemented with different concentrations of BHT (0.5, 10, 2.0 and 3.0 mM). As the control, semen was diluted and frozen in the diluent without BHT. Motility, progressive motility, viability, membranes and acrosome integrity were evaluated after dilution (part 1), cooling (part 2) and freezing and thawing (part 3). The results of the first part of the experiment showed that there were no significant difference between treatments in the motility, progressive motility, viability, membranes and acrosome integrity of spermatozoa, but the results with 2.0 mM BHT were slightly better than obtained with other levels of BHT and control extender. Significantly better results ( $p < 0.05$ ) were observed in the second part of the experiment for cooled spermatozoa characteristics, when extender was supplemented with 2.0 and 3.0 mM BHT. Furthermore, the results obtained in the third part of the experiment indicated that, after freezing and thawing, all evaluated semen characteristics were improved significantly ( $p < 0.05$ ) by increasing BHT levels, with the best results obtained for extender containing 2 mM BHT. Comparison of these results with those of control diluent, the effects of supplementation were significantly ( $p < 0.01$ ) better. However, the higher concentration of BHT (3.0 mM) reduced the motility, acrosomal integrity, viability and hypo-osmotic swelling response of spermatozoa compared to extender containing 2.0 mM BHT. In conclusion, the results obtained in this study showed that the semen quality of rams was improved when BHT was added to extender used before the freezing process. (**Key Words :** Butylated Hydroxytoluene, Ram, Spermatozoa, Freezability)

### INTRODUCTION

Cryopreservation is an important and widely used element for long-term storage of sperm cells. However, this technique induces partially irreversible damages to sperm (Amann and Pickett, 1987; Purdy, 2006), which may decrease motility, viability and the fertilization rate after artificial insemination (Matsuoka et al., 2006). Therefore, considerable care must be exercised during semen freezing to avoid damaging the sperm unduly at this stage of the processing. It has been suggested that membrane is a primary target of chilling or freezing damage in cells (Morris et al., 1981) and occurs when cell membrane undergo the phase transition from liquid crystalline to a gel

phase (Darin-Bennett and White, 1977).

On the other hand, sperm cells are highly susceptible to lipid peroxidation (LPO) by free radicals such as  $O_2^-$  and  $H_2O_2$  (Alvarez et al., 1987; Sinha et al., 1996; Gadella et al., 2001), which lead to the structural damage of sperm membranes during cryopreservation (Alvarez et al., 1987; Sinha et al., 1996). However, differences among species in the sensitivity of their sperm to cooling are largely attributable to compositional variations of the sperm plasma membrane (Bailey et al., 2000). There is evidence that the ram sperm membrane has a higher polyunsaturated/saturated fatty acids ratio (Evans and Maxwell, 1990), which thereby makes the membrane of this species more sensitive to cold shock and peroxidative damage than that of other species such as bull, rabbit, or even the human with subsequent loss of membrane integrity of the acrosomal region and impaired cell function (Watson, 1981; Fiser and Fairfull, 1989; Aitken and Fisher, 1994).

Based on this information, Hammerstedt et al. (1990) and Curry et al. (1994) suggested that the composition of

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diluents and suitable cryoprotectants seem to play an important role in successful semen cryopreservation. Therefore, in recent years, different antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation by elimination of free radicals (Curry et al., 1994; Baumber et al., 2000; Purdy, 2006). One of the antioxidants supplemented to extender is butylated hydroxytoluene (BHT), and its beneficial effects have been reported in many studies (Hammerstedt et al., 1976; Pursel, 1979; Watson and Anderson, 1983; Killian et al., 1989; Bamba and Cran, 1992; Donoghue and Donoghue, 1997; Roca et al., 2004; Khalifa et al., 2008; Shoaie and Zamiri, 2008; Ijaz et al., 2009).

There is evidence that butylated hydroxytoluene, as a phenolic anti-oxidant, acted as a membrane lipid perturbant which prevented or substantially reduced the permeability changes of sperm plasma membrane when the cell was cold-shocked (Hammerstedt et al., 1976). Snipes et al. (1975) and Hammerstedt et al. (1976) suggested that BHT has antiviral activity and thereby it can inactivate lipid-containing viruses. Furthermore, it is important to note that butylated hydroxytoluene, as a synthetic analogue of vitamin E, has been used successfully to preserve liquid semen of different species, such as ram (Watson and Anderson, 1983), turkey tom (Donoghue and Donoghue, 1997), bull (Killian et al., 1989; Shoaie and Zamiri, 2008), boar (Roca et al., 2004), goat (Khalifa et al., 2008) and buffalo (Ijaz et al., 2009). However, there is evidence that extender containing BHT could not positively affect the motility of stallion spermatozoa at 5°C (Ball et al., 2001).

Despite years of research, there are few available reports in which the effects of BHT on post-thaw motility and viability of ram spermatozoa were studied. Therefore, within this study, an attempt was made to investigate the positive effects of BHT at three phases (after dilution, after cooling and after frozen-thawing) on different characteristics of ram spermatozoa.

## MATERIALS AND METHODS

### Location, animals and extender

This study was conducted on the testing station for Iranian Moghani sheep in Jafarabad, Province Ardebil, Iran. The animals were kept under natural photoperiod and maintained using conventional feeding, housing and lighting conditions. Five mature and fertile rams (2-4 years old) were used in this study. The base extender consisted of 3.786% Tris (hydroxymethyl-aminoethane, Merck 64271, Germany), 2.172% citric acid (BHD 1081, England), and 1% fructose (BDH 28433, England) in 100mL distilled water, containing 5.0% (v/v) glycerol (Merck, 2400 Germany) and 10% (v/v) egg yolk, Penicillin (100,000 IU), Streptomycin (100 g) at pH 6.8 (Evans and Maxwell, 1990).

### Semen collection and evaluation

Semen samples were collected twice weekly for 8 weeks using an artificial vagina (42-43°C). Immediately after collection, the fresh semen was transported to the laboratory and kept in a water bath at 37°C. Ejaculates were evaluated for initial quality from volume (ml, using a calibrated semen collection tube), sperm concentration ( $\times 10^9$  sperm/ml, using a Neubaur hemocytometer after 1:200 dilution of semen with 0.5% eosin solution), mass activity (%), undiluted semen), motility (%), diluted with normal saline), and progressive motility using an arbitrary scale of 1 (10 to 25%), 2 (25 to 50%), 3 (50 to 70%), 4 (70 to 90%), and 5 (90 to 100%) of the motile spermatozoa. To evaluate mass activity, motility and progressive motility, a sample of the diluted spermatozoa was placed under a cover slip in the centre of a pre-warmed (37°C) slide and transferred to a heated microscope stage set at 37°C and subjectively assessed by phase contrast microscopy ( $\times 400$  magnification). The assessment of live sperm used the eosin-nigrosin stain procedure described by Evans and Maxwell, 1990). A mixture of 10  $\mu$ l of diluted spermatozoa and 10  $\mu$ l eosin-nigrosin stain was smeared on a slide and allowed to air dry in a dust-free environment. Two hundred spermatozoa from different microscopic fields were examined under a bright-field microscope using a 400 $\times$  objective, and the number of non-stained (viable) spermatozoa was counted. The morphological acrosome abnormality was assessed by viewing a wet mount of diluted spermatozoa fixed in buffered Formalin-Citrate solution as described by Weitze (1977). A drop of the fixed spermatozoa was placed on a slide under a cover glass. The slides were examined by phase-contrast microscopy using a 400 $\times$  oil immersion objective and white light. Spermatozoa ( $n = 200$ /slide) were examined and the percentage with normal acrosomes determined. The hypo-osmotic swelling test (HOS-test) was used to evaluate the functional integrity of the sperm membrane, and was performed by incubating 20  $\mu$ l of semen with 200  $\mu$ l of a 100 mOsm hypo-osmotic solution (9.0 g fructose+4.9 g sodium citrate/L distilled water) at 37°C for 60 min. After incubation, 100  $\mu$ l of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm were counted in at least 5 different microscopic fields. The percentage of sperm with swollen and curled tails was then recorded (Revell and Mrode, 1994). The frozen-thawed semen was assessed for motility, progressive motility, viability, acrosome and membrane integrity.

### Experimental procedure and semen dilution

This study was conducted to investigate the influence of different concentrations of BHT at three different phases

**Table 1.** Macroscopic and microscopic characters of seminal plasma of Moghani ram spermatozoa

Seminal characters	n	Mean	SEM	Min-Max
Volume (ml)	64	1.2	0.05	0.8-2.5
Sperm concentration ( $\times 10^9$ ml <sup>-1</sup> )	64	4.5	0.10	3.5-5.0
Mass activity (1-5)	64	4.50	0.06	3-5
Motility (%)	64	88.7	0.74	70-95
Progressive motility (%)	64	82.1	0.85	65
Live sperm (%)	64	86.5	0.76	80
Normal acrosome (%)	64	87.3	0.65	84

(after addition, cooling and frozen-thawed steps) on diluted ram spermatozoa. Ejaculates showing >70% motility and having  $>3 \times 10^9$  sperm/ml concentration were pooled and used for freezing. After pooling, semen was diluted 1:4 with the basic extender containing different concentrations (0.5, 1.0, 2.0 or 3.0 mM, according to Ijaz et al. (2009) for freezing of buffalo sperm) of butylated hydroxytoluene (W218405, Sigma-Aldrich Co, Australia), which was dissolved in 0.25% (v/v) dimethyl sulfoxide (DMSO) before addition to extender. As a control, semen was frozen in the diluent without butylated hydroxytoluene. Diluted semen samples were cooled to 5°C over 2 h, transferred into 0.25-ml straws, equilibrated for 1.5 h at 5°C and frozen in liquid nitrogen vapor (4-5 cm from the LN<sub>2</sub> surface level) for 10 min. They were then stored in liquid nitrogen for 24 h. The straws were thawed in a water bath at 37°C for 2 min. Semen samples were evaluated after dilution, cooling and thawing for motility, progressive motility, viability, morphological acrosome abnormality and hypo-osmotic swelling test.

### Statistical analysis

The experiments were conducted as a completely randomized design, and statistical analysis of data was performed by the General Linear Model (GLM) procedure of SAS (1996). All percentage data were arcsine transformed before statistical analysis. Back-transformed data are reported as mean  $\pm$ SEM. A probability level of  $p \leq 0.05$  was considered as significant.

## RESULTS

The average macroscopic and microscopic seminal characteristics in primary evaluation are presented in Table 1. The results indicated that the average volume ( $1.22 \pm 0.05$  ml), sperm concentration ( $4.52 \pm 0.10 \times 10^9$  ml<sup>-1</sup>), mass activity ( $4.50 \pm 0.06$ ), motility ( $88.56 \pm 0.74\%$ ), progressive motility ( $82.09 \pm 0.85\%$ ), live sperm ( $86.54 \pm 0.76\%$ ) and rate of morphologically normal acrosome ( $87.34 \pm 0.65\%$ ) were in the ejaculates of Moghani ram spermatozoa.

Table 2 shows the effect of supplementation of Tris-citric acid-fructose-egg yolk extender with different concentrations of BHT (0.5, 1.0, 2.0 or 3.0 mM) on the motility, progressive motility, viability, membranes (hypo-osmotic swelling test) and acrosome integrity after dilution (first part of experiment) of ram spermatozoa. The obtained results showed no significant differences ( $p > 0.05$ ) between treatments and control extender. However, the results indicated that extenders containing 2.0 and 3.0 mM BHT were slightly better than other BHT concentrations and control diluent.

The result of the second part (effects of BHT levels after cooling) of this study is presented in Table 3. The data demonstrated significant ( $p < 0.01$ ) improvement by supplementation and increasing levels of BHT to extenders. The results showed that significantly ( $p < 0.01$ ) better values were achieved when extenders contained 2.0 and 3.0 mM BHT. Furthermore, the highest sperm motility ( $76.25 \pm 0.85$ ), progressive motility ( $70.25 \pm 0.96$ ), viability ( $80.31 \pm 0.84$ )

**Table 2.** Effect of different concentrations of BHT added to a basic TCEY extender on Moghani ram spermatozoa characteristics after dilution (mean $\pm$ SEM, n = 16)

Semen treatments (mM)	Characteristics of spermatozoa (%)				
	Motility (%)	Progressive motility (%)	Live sperm (%)	Acrosome abnormality (%)	Membrane integrity (%)
0.5	78.6 $\pm$ 0.69	73.8 $\pm$ 0.76	83.1 $\pm$ 0.62	5.9 $\pm$ 0.15	79.9 $\pm$ 0.76
1	78.9 $\pm$ 0.65	74.4 $\pm$ 0.78	83.4 $\pm$ 0.66	5.7 $\pm$ 0.16	80.6 $\pm$ 0.75
2	80.4 $\pm$ 0.78	76.1 $\pm$ 0.92	84.2 $\pm$ 0.74	5.7 $\pm$ 0.24	81.4 $\pm$ 0.89
3	79.3 $\pm$ 0.74	74.7 $\pm$ 0.82	83.8 $\pm$ 0.68	5.6 $\pm$ 0.18	81.0 $\pm$ 0.82
Control	79.0 $\pm$ 0.68	73.7 $\pm$ 0.76	82.3 $\pm$ 0.61	5.9 $\pm$ 0.16	79.2 $\pm$ 0.78

All data within each column are not statistically different (Duncan's multiple range test;  $p > 0.05$ ).

**Table 3.** Effect of different concentrations of BHT added to a basic TCEY extender on cooled Moghani ram spermatozoa characteristics (mean±SEM, n = 16)

Semen treatments (mM)	Characteristics of spermatozoa				
	Motility (%)	Progressive motility (%)	Live sperm (%)	Acrosome abnormality (%)	Membrane integrity (%)
0.5	72.3±0.75 <sup>b</sup>	64.6±0.88 <sup>c</sup>	75.4±0.72 <sup>b</sup>	8.7±0.60 <sup>b</sup>	72.9±1.05 <sup>bc</sup>
1	73.3±0.78 <sup>b</sup>	66.1±0.84 <sup>bc</sup>	76.2±0.69 <sup>b</sup>	8.3±0.58 <sup>b</sup>	74.7±1.09 <sup>ab</sup>
2	76.3±0.85 <sup>a</sup>	70.3±0.96 <sup>a</sup>	80.3±0.84 <sup>a</sup>	6.1±0.72 <sup>c</sup>	76.5±1.34 <sup>a</sup>
3	74.1±0.80 <sup>ab</sup>	68.5±0.89 <sup>ab</sup>	79.1±0.70 <sup>a</sup>	7.4±0.66 <sup>bc</sup>	74.9±1.25 <sup>ab</sup>
Control	69.4±0.72 <sup>c</sup>	64.3±0.92 <sup>c</sup>	75.1±0.65 <sup>b</sup>	10.5±0.62 <sup>a</sup>	70.5±1.06 <sup>c</sup>

<sup>a, b</sup> Within each column, means with similar letter (s) are not significantly different (Duncan's multiple range test;  $p < 0.05$ ).

and membrane integrity (76.47±1.34) were observed for 2 mM BHT and the lowest values for 0.5 mM BHT concentration (72.28±0.75, 64.56±0.88, 75.39±0.72 and 72.90±1.05, respectively). However, there was no significant differences ( $p > 0.05$ ) in the presence of 0.5 or 1.0 mM BHT.

Table 4 presents the results of different concentrations of BHT supplementation after freezing and thawing on evaluated spermatozoa characteristics. The results indicated significant ( $p < 0.01$ ) improvement by increasing of BHT concentrations. Comparison of these results with those of control diluent showed that the effects of supplementation were significantly ( $p < 0.01$ ) better. However, there was no significant difference ( $p > 0.05$ ) between extenders which contained 0.5 or 1.0 mM BHT. Furthermore, the highest post-thawed sperm motility (52.25±1.02), progressive motility (45.37±1.05), viability (59.43±1.24) and membrane integrity (54.36±1.32) were observed at 2 mM BHT and the lowest values for 0.5 mM BHT (41.37±0.76, 33.34±0.78, 47.12±0.86 and 38.58±0.82, respectively).

## DISCUSSION

The survival of sperm during cryopreservation is dependent on several factors which can affect the post-thawing outcome (Eiman and Terada, 2004, 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 (Sinha et al., 1996; Stradaoli et al., 2007). Alvarez et al. (1987) and Sinha et al. (1996) reported that sperm plasma membrane is rich in polyunsaturated fatty acids and is therefore highly susceptible to lipid peroxidative damage by  $O_2^-$  and  $H_2O_2$ , resulting from reactive oxygen species during aerobic incubation. Based on speculation by Evans and Maxwell (1990), ram sperm membrane has a higher polyunsaturated/ saturated fatty acids ratio and the sperm membrane of this species is more sensitive to cold shock and peroxidative damage than other species. Therefore, in recent years, antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation. Furthermore, antioxidants eliminate free radicals, which are detrimental to sperm due to the induction of lipid peroxidation (Baumber et al., 2000; Watson, 2000).

Concerning cryopreservation of ram spermatozoa, various extenders have been described (Salamon and Maxwell, 2000), but there are few available reports on the effects of BHT, as an antioxidant, on the characteristics of ram spermatozoa. The present study investigated whether the presence of BHT would improve the quality of Moghani ram sperm after dilution, cooling and freezing. The results observed in the first part of this study (after dilution) demonstrated no significant differences between the

**Table 4.** Effect of different concentrations of BHT added to a basic TCEY extender on post-thaw characteristics of Moghani ram spermatozoa (mean±SEM, n = 16)

Semen treatments (mM)	Characteristics of spermatozoa				
	Motility (%)	Progressive motility (%)	Live sperm (%)	Acrosome abnormality (%)	Membrane integrity (%)
0.5	41.4±0.76 <sup>c</sup>	33.3±0.78 <sup>c</sup>	47.1±0.86 <sup>c</sup>	11.2±0.45 <sup>b</sup>	38.6±0.82 <sup>c</sup>
1	42.6±0.78 <sup>c</sup>	34.6±0.76 <sup>c</sup>	48.1±0.92 <sup>c</sup>	11.8±0.48 <sup>b</sup>	39.5±0.98 <sup>c</sup>
2	52.3±1.02 <sup>a</sup>	45.4±1.05 <sup>a</sup>	59.4±1.24 <sup>a</sup>	8.1±0.68 <sup>d</sup>	54.4±1.32 <sup>a</sup>
3	45.4±0.82 <sup>b</sup>	38.1±0.84 <sup>b</sup>	53.1±0.94 <sup>b</sup>	9.9±0.54 <sup>c</sup>	48.4±1.02 <sup>b</sup>
Control	38.3±0.75 <sup>d</sup>	29.4±0.76 <sup>d</sup>	42.2±0.82 <sup>d</sup>	14.3±0.46 <sup>a</sup>	38.3±0.82 <sup>c</sup>

<sup>a, b</sup> Within each column, means with similar letter (s) are not significantly different (Duncan's multiple range test;  $p < 0.05$ ).

treatments, when BHT was added to the extenders used. However, the data observed after cooling presented significant improvement, with the highest motility, progressive motility, viability, acrosomal integrity and hypo-osmotic swelling response of spermatozoa observed for 2.0 and 3.0 mM BHT concentrations. These observations were in agreement with findings of Donoghue and Donoghue (1997) for turkey, Anderson et al. (1994) and Shoaie 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 evaluated after the cooling step. However, there is evidence that extender containing BHT could not positively affect the motility of stallion spermatozoa at 5°C (Ball et al., 2001). Furthermore, the frozen-thawed results in this study indicated that with addition of increasing BHT concentrations to basic TCEY-extender, all evaluated spermatozoa characteristics were improved significantly. In agreement with our finding, Anderson et al. (1994) and Shoaie 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 diluent (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. However, the exact mechanism by which the butylated hydroxytoluene causes improvement is not clearly understood. A variety of hypotheses and speculations have been proposed by various authors to explain the protective mechanism of BHT during cryopreservation. Snipes et al. (1975) and Hammerstedt et al. (1976) suggested that BHT has antiviral activity and thereby, it can inactivate lipid-containing viruses. Moreover, Hammerstedt et al. (1976) suggested that BHT, as a phenolic anti-oxidant, is incorporated into the spermatozoal membrane and thereby decreases the viscosity of membrane lipids. This may imply greater lipid fluidity at reduced temperatures and would prevent or substantially reduce the permeability changes of sperm plasma membrane when the cells were cold-shocked.

Killian et al. (1989), Aitken and Clarkson (1988) and Aitken, (1995) speculated that BHT serves as a scavenger of oxygen free radicals, which are associated with the extender and sperm, to minimize damage to sperm characteristics. However, Watson (1981) suggested that the membrane disruption which occurs in ram sperm during cold shock is extensive, and is difficult to account for by concepts of lipid-phase changes alone. Furthermore, the results in this study showed that optimal ram semen cryopreservation was achieved when 2-3 mM BHT was added to extender. It seems that optimal concentration of BHT depends upon the species of animals and ranges between 0.05-2.0 mM (Bamba and Cran, 1992; Roca et al., 2004; Shoaie and Zamiri, 2008; Ijaz et al., 2009). The exact mechanism by which the higher butylated hydroxytoluene concentrations negatively affected the frozen-thawed sperm characteristics, remains unclear. Shoaie and Zamiri (2008) speculated that fluidity of the plasma membrane can increase above the desired point, making spermatozoa more prone to acrosomal damage, when higher levels of BHT are used in extender. However, contrary to our results and those of other publications, Watson and Anderson, (1983) reported 2-4 mM BHT as the optimal concentration. Khalifa et al. (2008) reported that optimal goat sperm cryopreservation was achieved when 5.0 mM BHT was added to egg yolk-based semen extender. In conclusion, the present data indicated that 2.0-3.0 mM BHT may be suitable for freezing ram spermatozoa in the TCFY extender. However, further studies are still needed to establish the effect of such addition on *in vitro* and *in vivo* fertility in farm animals.

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