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Effect of Butylated Hydroxytoluene on Post-thawed Semen Quality of Beetal Goat Buck, *Capra hircus*

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Abstract.- Present study was conducted to evaluate the effect butylated hydroxytoluene (BHT) on post-thawed semen quality of Beetal goat buck semen. Semen was obtained from six bucks using artificial vagina and cryopreserved in tris egg yolk extender and semen quality was assessed on the basis of post-thaw sperm motility, viability, plasma membrane integrity, and acrosomal membrane status. Ejaculates were pooled and extended to the concentration of 2×10^9 spermatozoa per mL in tris egg yolk extender (300 mOsmol/L). Tris egg yolk extender containing various concentrations of BHT (0.0, 2.0 and 5.0 mM) was prepared. French straws (0.5 mL) were manually filled with semen, gradually cooled from 39°C to 4°C and finally, cryopreserved in liquid nitrogen at -196°C. Five straws from each treatment [BHT (0.0, 2.0 and 5.0 mM)] were thawed and evaluated under phase-contrast microscope (40 ×) for sperm motility, whereas sperm viability, plasma membrane integrity and acrosomal integrity were assessed by the supravital staining, hypo-osmotic swelling test (HOST) and normal acrosomal reaction, respectively. The results showed that only acrosomal integrity was improved ($P < 0.05$) by the addition of BHT in semen extender. Motility was suppressed ($P < 0.05$) by increasing BHT concentration. The maximum motility of sperm was achieved with 0.0 mM BHT. The HOST response and viability of spermatozoa were increased by addition of 2 and 5 mM BHT, but this increment was not statistically significant. In conclusion, the addition of BHT to semen extender can partially improve semen quality of Beetal goat.

Keywords: Semen, butylated hydroxytoluene, goat buck, cryopreservation, semen quality

INTRODUCTION

Goats are very hardy and cope with harsh conditions of temperature and humidity (Gall, 1981). Less input, easy management, adaptability and above all ability to survive and produce on minimal fodder availability makes them as animals of choice in tropical and subtropical parts of the world. Due to this reason, perhaps, 60% of total world's goat population is raised in Asia especially in developing countries (FAO, 2007). Beetal breed is, dual purpose, mainly raised in tropical areas of Pakistan. Economic survey of Pakistan does prove that in the last two decades growth rate of goat population is 61% (37 to 59.7 millions heads) which is the highest among all domesticated livestock like cattle, buffalo and sheep. In this way, caprines are considered to play an important role in uplifting of socio-economic standards of common and poor income communities.

Currently, artificial insemination (AI) is getting more importance in all domestic animals. Genetically superior males can be used to improve genetic potential of the breed through intensive AI programs. Large scale AI program is essential in goat farming to meet ever increasing demand of milk, hair and meat. Artificial Insemination has been proved to be very useful in optimizing selection response and in spreading the genes of superior bucks, and has been used particularly in intensive production systems. Success in caprine AI is limited as conception rate depend on the proper collection and preservation of semen (Leboeuf *et al*., 2000), and influenced by cryopreservation induced lipid peroxidation (Bailey *et al*., 2000; Khalifa *et al*., 2008). Spermatozoa have only a short survival time outside the reproductive tract at ambient temperatures; however, for a large-scale AI program it is desired to preserve spermatozoa for an extended time. Fortunately, this could be achieved

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by the development and introduction of cryopreservation technique.

Post-thaw quality of cryopreserved semen is generally lower than fresh caprine semen (Ritar and Salamon, 1983) and requires eight times more spermatozoa number to achieve equivalent fertilization rate compared with fresh Bovine semen (Shannon and Vishwanath, 1995). It has been previously indicated that cryopreservation procedure may lead to some changes in morphology and biochemistry of spermatozoa such as reducing head size and altering membrane architecture, and lipid composition (Halliwell and Gutterridge, 1984; Gravance *et al*., 1998).

One of the most important factors resulting in decreased fertility in AI with cryopreserved semen is oxidative damage of spermatozoa during freeze and thaw processes. It is well known that reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and hydroxyl radical (OH^-) are physiologically produced in living cells during metabolism (Alvarez and Storey, 1992; Lasso *et al*., 1994). Moreover, it is well documented that cryopreservation of semen produces ROS which are detrimental to spermatozoa (Watson, 2000) due to the induction of lipid peroxidation (Chatterjee and Gagnon, 2000). The spermatozoa possess weak anti-oxidants and the process of cryopreservation increases the susceptibility to lipid peroxidation (Foote *et al*., 2002). The free radicals are known to be involved in lipid peroxidation as well as DNA and sperm membrane damages that may lead to decreased sperm motility or cell death (Uysal *et al*., 2007). However, it has been reported that cryopreservation reduces bull sperm glutathione and superoxide dismutase activity (Aitken, 1994; Bailey *et al*., 2000). Hydrogen peroxide and lipid peroxyl radicals are the lethal oxygen species that have ability to cross the plasma membranes freely, inhibit enzymatic activities and cellular functions, consequently decrease the antioxidant defenses of the spermatozoa (Aitken, 1994).

It has been clearly demonstrated that excess amount of ROS in semen arrests sperm motility, inhibits sperm-egg fusion and causes sperm DNA damage (Kessopoulou *et al*., 1995). Therefore, if an antioxidant system is involved in preservation process, an increasing semen quality would be expected after the addition of such antioxidant agents. Moreover, it has been stated that depicting use of antioxidant agents, such as vitamins, catalase, taurine, hypotaurine dimethylsulphoxide, N-acetylcysteine and butylated hydroxytoluene (BHT) in human (Alvarez and Storey, 1992), bovine (Bilodeau *et al*., 2001), rabbit (Abd-El-Salaam, 2002) and stallion (Ball *et al*., 2001) semen, revealed some controversial efficacies and successes. Besides the beneficial effect of BHT for the preservation of frozen sperm in ram (Watson and Anderson, 1983), boar (Roca *et al*., 2004), goat (Khalifa *et al*., 2008), cattle (Shoae and Zamiri 2008), buffalo bull (Ijaz *et al*., 2009), and dog (Neagu *et al*., 2010), the elucidating effect of BHT, a synthetic analogue of vitamin E which checks the auto-oxidation reaction by converting peroxy radicals to hydroperoxides, on goat buck spermatozoa, is still a debate. In this study, it was hypothesized that addition of BHT might have improved the oxidative defense system of sperm and therefore, this study was designed to elucidate the defensive role of BHT in goat semen cryopreservation extender.

MATERIALS AND METHODS

Preparation of extender

All chemicals were analytical graded and purchased from Sigma-Aldrich Co., Deisenhofen, Germany. Tris egg yolk extender (100 mL; 300 mOsmol/L) was prepared, which was composed of Tris (hydroxymethyl) amino methane (312.63 mM), fructose (34.69 mM), citric acid (103.36 mM), egg yolk (2.50 ml), glycerol (7 ml; 950.11 mM), benzyl penicillin (500,00 IU), streptomycin sulphate (0.1 g), and distilled water. The appropriate amount of BHT (0, 2.0 and 5.0 mM) was first dissolved in ethanol. The ethanol was allowed to evaporate so that a thin crystallized layer of BHT was deposited on the inner surface of the tubes and extender was added. Subsequently, semen was added and incubated to allow uptake of BHT by spermatozoa.

Semen collection and processing

Six beetal goat bucks were selected for semen collection using artificial vagina and adopting all hygienic measures. During the course of collection 2

bucks were replaced on the basis of post collection semen evaluation. Immediately after collection, semen was transferred to a water bath at 39°C. Ejaculates from each buck were subjected to the gross (volume, color) and microscopic evaluation (motility percentage, sperm cell concentration with haemocytometer) to discard the ejaculates having mass motility below 60 %. When the minimum standards of motility, morphology and concentration were achieved (Ijaz *et al*., 2009), ejaculates from all bucks were pooled and extended to concentration of 2×10^9 spermatozoa per mL. Extended semen contained 0.0, 2.0 or 5.0 mM BHT. French straws (0.5 mL) were manually filled with semen which contained increasing amounts of BHT [0 (control), 2.0 and 5.0 mM], gradually cooled from 39°C to 4°C, subjected to LN vapors and finally, cryopreserved in liquid nitrogen at –196°C.

Post-thaw semen evaluation

At the time of analysis, five straws of semen from each treatment were thawed at 39°C for 30 s and pooled to perform the following semen quality parameters. Eight replicates were used in each treatment.

Sperm motility

One drop of semen was placed on a pre-warmed glass slide and a cover slip was mounted over it. Percentage motility was observed under phase-contrast microscope (40 ×). The mean of three observations was considered as a single data point.

Acrosomal integrity

A 100 µL semen sample was fixed in 10 µL of 1–% formaldehyde citrate in tri-sodium citrate dehydrate solution (2.9% w/v). Two hundred spermatozoa were counted under a phase-contrast microscope (100 ×) for acrosomal integrity which was indexed as percentage of normal, ruffled, swollen or absent acrosomes (Ijaz *et al*., 2009).

Plasma membrane integrity

Hypo-osmotic swelling test (HOST) was performed to assess plasma membrane integrity. A hypo-osmotic solution (190 mOsm/L) was prepared by dissolving 0.735 g of tri-sodium citrate dihydrate and 1.351 g fructose in 100 µL of distilled water. A 50 µL semen sample was incubated with 500 µL of hypo-osmotic solution for 45 min at 39°C. One drop of the incubated semen was placed on a pre-warmed glass slide and examined under phase-contrast microscope (40×). Two hundred spermatozoa were counted, and the percentage of spermatozoa exhibiting tail curling was determined (Ijaz *et al*., 2009).

Sperm viability

One drop of semen was placed on a glass slide and mixed with a drop of iso molar supravital stain [eosin (1 % w/v), nigrosin (5 % w/v) in 3 % tri-sodium citrate dihydrate solution] to prepare a uniform air dried thin smear and was observed under phase-contrast microscope (100 ×). Totally two hundred spermatozoa either live (unstained heads) or dead (stained heads) were counted to find percentage viability (Ijaz *et al*., 2009).

Statistical analysis

Data were presented as mean ± SD. The data were subjected to ANOVA using SPSS-13.0, a statistical package (SPSS Inc., Chicago, IL, USA). Statistical differences among means ($P < 0.05$) were identified using Duncan's multiple range test (DMRT).

RESULTS

Data regarding post-thaw semen quality indices in response to various concentrations of BHT on Tris-based extender are presented in Table I. BHT inclusions negatively affect ($P<0.05$) sperm motility. The maximum motility of spermatozoa was achieved with 0.0 mM BHT treatment. Nevertheless, this difference in post-thaw progressive motility was not significant, statistically. Other two parameters used in this investigation were HOST-response and post-thawed sperm viability. Positive HOST response was noted in treated groups as in 2.0 mM (42.30±3.74) and 5.0 mM (42.80±2.78) addition of BHT as compared to control group (39.50±3.68). Similarly, viability response was also observed. Viability of spermatozoa containing 2.0 and 5.0 mM concentration of BHT was 59.00±3.74 and 59.90±2.78, respectively as compared to control

Table I.- Effect of BHT addition on post-thawed buck semen quality cryopreserved in a Tris egg yolk based extender.

BHT (mM)	Characteristics of spermatozoa (%)			
	Motility	Acrosomal integrity	HOST positive	Viability
0.0	36.90±2.42[a]	20.30±1.05[b]	39.50±3.68	54.40±3.68
2.0	34.00±2.78[ab]	27.00±0.47[a]	42.30±3.74	59.00±3.74
5.0	32.50±1.95[b]	27.70±1.76[a]	42.80±2.78	59.90±2.78

Data are expressed as mean ± S.D. (n = 8). [a,b] denote differences (P<0.05) within a column.

group having 54.40±3.68 viability. Although little improvement was observed in BHT treated groups, overall means of treated groups were improved. Moreover, neither HOST response nor viability of spermatozoa was significantly influenced by the inclusion of BHT.

Acrosomal integrity was improved ($P < 0.05$) by the addition of BHT (0.0, 2.0 and 5.0 mM) in Tris-based semen extender. This improvement in acrosomal integrity was gradual and was improved ($P<0.05$) by the addition of increasing concentrations of both 2.0 (27.00±0.47) and 5.0 (27.70±1.76) mM BHT in Tris-based semen extender as compared to control (20.30±1.05).

DISCUSSION

The ruminant sperm cell plasma membrane is particularly rich in poly unsaturated fatty acids (PUFA). This predominance of PUFAs renders spermatozoa highly susceptible to peroxidation because of the excessive production of ROS. ROS including H_2O_2, O^{2-} and OH^-, are strong oxidants that are physiologically produced in living cells during respiration (Alvarez and Storey, 1992; Bamba and Cran, 1992). Spermatozoa are vulnerable to ROS damage due to their high polyunsaturated fatty acid content. It is well documented that cryopreservation of semen produces ROS which are detrimental to spermatozoa (Watson, 2000) due to the induction of lipid peroxidation (Chatterjee and Gagnon, 2001). Further lipid peroxidation levels were found to have negative correlation with fertility. The spermatozoa possess weak anti-oxidants, and the process of cryopreservation increases the susceptibility to lipid peroxidation (Foote *et al*., 2002). The free radicals are known to be involved in lipid peroxidation as well as DNA and sperm membrane damages that may lead to decreased sperm motility or cell death (Uysal *et al*., 2007). Besides, during semen cryopreservation the antioxidant system of seminal plasma and spermatozoa is challenged by excessive ROS production (Leboeuf *et al*., 2000). Therefore, the addition of synthetic antioxidants in semen extender is generally practiced for semen cryopreservation to inhibit the peroxidation of spermatozoon phospholipids, particularly of polyunsaturated fatty acids.

Present study revealed that the inclusion of 5 mM BHT in semen extender could significantly improve acrosomal integrity only with mild improvement in viability and plasma membrane integrity. Proper acrosomal integrity is essential for the acrosome reaction, a prerequisite for successful fertilization and subsequent fertility. During acrosome reaction outer acrosomal membrane fuses with the plasma membrane of the spermatozoon resulting in hybrid membrane vesicle formation (Smith, 2001). During the process of freeze and thaw, production of ROS occurs due to inducing of acrosome damage, subsequently resulting in premature capacitation and acrosome reaction (Uysal *et al*., 2007). A proposed mechanism for BHT induced improvement in the integrity of acrosome and plasma membrane is that, BHT is incorporated into the cell membrane resulting in increased fluidity of the membranes and limited lipid peroxidation reactions (Shoae and Zamiri 2008). Conversely, Aitken and Clarkson (1988) proposed that BHT may convert lipid peroxyl radicals into hydro peroxides which may reduce spermatozoa damage. Our finding is in general agreement with previous reports (Anderson *et al*., 1994; Shoae and Zamiri, 2008; Ijaz *et al*., 2009). Concomitantly, added BHT negatively affected sperm motility. ROS produced during cryopreservation may suppress sperm motility and

fertility (Khalifa and El-Saidy, 2006).

Our finding is in contrast to the most of the previous published findings (Killian *et al*., 1989; Anderson *et al*., 1994; Khalifa *et al*., 2008; Shoae and Zamiri, 2008; Ijaz *et al*., 2009). It is difficult to argue poor motility rates in BHT treated groups, as several factors may be responsible like dose rate, breed differences, spermatozoa number per dose, application methods, thawing time and temperature, composition of extender and above all extent of cryo-damage itself. One major possible reason behind this is justifiable in the sense of protective effect of BHT in extender as it minimizes the production of ROS. Production of ROS may increase the motility and it might possible that this factor is responsible for decrease in motility. It is important to note that BHT is lipid soluble and possibility did exist that more BHT remained associated with egg yolk lipids, leaving a little concentration of free BHT molecules to penetrate spermatozoa membrane (Killian *et al*., 1989; Ijaz *et al*., 2009).

In a previous study (Graham and Hammerstedt, 1992), it was reported that inclusion of BHT analogues reduced the motility of the spermatozoa extended in extenders with no egg yolk and vice versa. This shows that BHT or its analogues may have a synergistic effect with egg yolk in protecting spermatozoa from cryo injury. This fact is further supported by Khalifa *et al*. (2008) who state that optimal goat semen cryopreservation was achieved at 5.0 mM BHT concentration in egg yolk based semen diluents and 0.3 mM BHT concentration in egg yolk-free diluents. In the current study, different concentrations of BHT (0–5.0 mM) were studied. It seems that optimal concentration of BHT depends upon animal species. For instance, the optimal concentration of BHT was 0.05–2.0 mM (Bamba and Cran, 1992) and 0.2– 1.6 mM for boar (Roca *et al*., 2004) and 0.5–1.0 mM for cattle bull (Shoae and Zamiri, 2008) and 2.0 mM for Nili Ravi buffalo bull (Ijaz *et al*., 2009). These results advocate that significant difficulties still exist with regard to species differences and technical abilities when using BHT to conserve sperm motility and viability.

Indeed, as reported elsewhere for BHT, our study concluded that addition of BHT in semen extender can partially improve semen quality of beetal goat buck. Still further studies are required to obtain more accurate dose rate and to identify molecular interactions between BHT, extender components and sperm membrane.

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