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Testing Usability of Butylated Hydroxytoluene in Conservation of Goat Semen

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Abstract: The definitive intent of this study was to investigate whether butylated hydroxytoluene (BHT) could be used as a suitable supporter or alternative of egg yolk during low-temperature manipulation of goat semen. Supplementation of Tris-based extenders containing low levels (2.50%) of egg yolk with 5.00 mM BHT resulted in a significant improvement in the viability of chilled-stored semen as well as in the motility (48.50%) and fertility (62.50%) of frozen-thawed spermatozoa. Whereas, instead of egg yolk, inclusion of 0.60 mM BHT in Tris-citric acid-glucose-glycerol extenders could sustain not only the viability of chilled-stored spermatozoa but also the post-thaw sperm motility (47.50%) and fertility (53.75%) of frozen goat semen.

Key words: Butylated hydroxytoluene · goat semen · kidding rate · egg yolk · motility

INTRODUCTION

Goat bulbourethral gland secretions are deleterious for the survival of goat sperm chilled or frozen in media containing high concentrations of milk or egg yolk [1].

Butylated hydroxytoluene (BHT), a phenolic antioxidant that has antiviral activity, was detected to have the ability to alleviate cold-induced membrane stress in spermatozoa from several animal species [2, 3].

The present study was initiated to investigate 1) whether BHT could interact synergistically with low levels of egg yolk to improve the preservability of goat semen; 2) whether BHT could be used as an alternative of egg yolk for hypothermic storage of goat semen and 3) to what extent BHT could affect the fertility of frozen-thawed goat spermatozoa.

MATERIALS AND METHODS

Chemical reagents and semen extenders: All chemicals were of the highest commercially available purity and were purchased from Sigma-Aldrich Co., Deisenhofen, Germany. Two types of diluents were used for preservation of goat semen:

- Egg yolk-based diluent [4], which was composed of Tris (hydroxymethyl) amino methane (3.786 g), glucose (0.625 g), citric acid monohydrate (2.172 g), fresh chicken egg yolk (2.50 ml), glycerol (5 ml), penicillin (100,000 IU), streptomycin (100 mg) and glass-distilled water to 100 ml.
- Egg yolk-free diluent which was composed of the same ingredients and concentrations of the above-mentioned diluent without egg yolk.

Semen collection and processing: Throughout a one-year period, semen samples were collected twice a week by means of an artificial vagina from 10 Damascus goat bucks (25-30 months old) belonging to Sakha Experimental Station, Animal Production Research Institute, Egypt. An anestrous doe was used as a mount animal for semen collection. Immediately after collection, the ejaculates were transferred to the laboratory and kept in a water bath at 30°C for the initial evaluation. Only ejaculates of at least 70% sperm progressive motility and 2000 x 10^6 sperm cells per ml were used in three in vitro experiments. In each experiment, 30 ejaculates (3 ejaculates per buck) were split and diluted (1:4) at 30°C with the following semen extenders:

Experiment 1: Egg yolk-based diluents supplemented with or without 0.30, 0.60, 2.00, 5.00 and 8.00 mM BHT. Since BHT is a fat soluble compound, it has been decided to dissolve these concentrations in 0.25% (v/v) dimethyl sulfoxide (DMSO) to achieve maximal permeation of sperm plasma membrane with BHT molecules.

Experiment 2: Egg yolk-free diluents supplemented with or without 0.30, 0.60, 2.00, 5.00 and 8.00 mM BHT dissolved in 0.25% (v/v) DMSO.

In experiment 1 and 2, to exclude any possible effects of DMSO on sperm viability, a second control diluent

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containing 0.25% (v/v) DMSO was used as a semen treatment. In addition, within 5 min after dilution, the extended semen in both experiments was placed in the refrigerator and incubated at 5°C for 168 h. The number of progressively motile spermatozoa per ml of diluted semen in both experiments was 300 to 400 x 10^6.

Experiment 3: Egg yolk-free extenders containing 0.30, 0.60, 0.90 mM BHT dissolved in 0.25% (v/v) DMSO; egg yolk-based extenders and egg yolk-based extenders containing 5.00 mM BHT dissolved in 0.25% (v/v) DMSO. The diluted semen was cooled to 5°C over a period of 4 h. The cooled semen was frozen in the form of pellets (0.30 ml containing 100 to 120 x 10^6 progressive motile spermatozoa per pellet) on a plate made of polytetrafluoroethylene that was cooled (-80 to -140° C) beforehand by immersing it in liquid nitrogen for 15 min and raising it up to be exposed to the vapour of liquid nitrogen [4]. After 2 to 3 min, the frozen pellets were immersed in liquid nitrogen, transferred into the liquid nitrogen container and stored for four weeks before thawing. Thawing of frozen semen was carried out by placing two pellets into a clean dry pre-warmed (40°C for 15 min) 10 ml glass test tube held in a water bath at 40°C. In order to ensure uniform thawing of the pellets, the test tube was shaken in the water bath until complete melting of the pellets.

Evaluation of preserved semen: Using a phase-contrast microscope (400 x) equipped with a thermal stage at 37°C, sperm progressive motility of chilled-stored semen in experiment 1 and 2 was subjectively assessed immediately after dilution as well as after 6, 24, 48, 72, 96, 120, 144 and 168 h of incubation period. The viability index of incubated semen was computed from the following formula [5]:

$$S = \Sigma\left[A \times \frac{T - R}{2}\right]$$

where; S is the viability index, Σ is a sign for the sum total, A is the percentage of sperm motility, T is the time of next determination of motility and R is the time of previous determination of motility.

For cryopreserved semen in experiment 3, sperm progressive motility was also assessed after dilution, before freezing and after thawing.

Fertility test: The purpose of the fertility test was to explore if the improvement in post-thaw sperm motility observed in experiment 3 due to treatment of semen with BHT would be reflected in an amelioration in the kidding rates. Hence, individual ejaculates, of at least 80% initial motility and 2500 x 10^6 sperm cells per ml, were collected from two bucks during the non-breeding season (April to August). Each ejaculate was then split and diluted (1:4) with egg yolk-free extender containing 0.60 mM BHT dissolved in 0.25% (v/v) DMSO; egg yolk-based extender and with egg yolk-based extender containing 5.00 mM BHT dissolved in 0.25% (v/v) DMSO. The diluted semen was cooled and frozen in the form of 0.30 ml pellets. After thawing, each pellet contained 60 to 70 x 10^6 progressively motile spermatozoa.

A total number of 230 goat does (15 nulliparous, 31 primiparous and 184 pluriparous) with an average body weight of 47.50 kg were used in the fertility test. Primiparous and pluriparous goats were lactating and were machine milked twice a day. Estrus was synchronized during the breeding season (September to December) at unknown stages of the estrous cycles using a double intramuscular injection of 125 μg (0.50 ml) per doe cloprostenol (Estrumate, Coopers Animal Health Limited, PHARMAGYPT TRADING) at a 12-day interval [6]. Estrus was detected thrice a day with two teaser bucks during the five days after the second cloprostenol injection. The does were considered to be in estrus when they stood to be mounted by teaser bucks.

At the time of insemination, two frozen pellets from each semen treatment were thawed as previously described in experiment 3, held in a water bath at 30°C and used for insemination within 15 min. An inseminate volume of 0.60 ml containing 120 to 140 x 10^6 progressively motile spermatozoa [7] was used for a single cervical insemination of the synchronized does after 12 h of estrus detection [8]. For insemination, the hindquarters of females were raised over a rail and the semen was deposited as deeply as possible into the cervical canal using a simple inseminating pipette with a bent tip and a duck-bill speculum. After 60 days of insemination, the does were screened for pregnancy by transabdominal ultrasonography. Fertility was defined as the number of kidding does over the number of inseminated does.

Statistical analyses: Using the general linear models procedures of the Statistical Analysis Systems [9], all data were subjected to analysis of variance (ANOVA) to clarify the effect of semen treatments on viability indices (Exp. 1 and 2) and sperm motility (Exp. 3). Treated means were compared by the least significant difference test (LSD) at

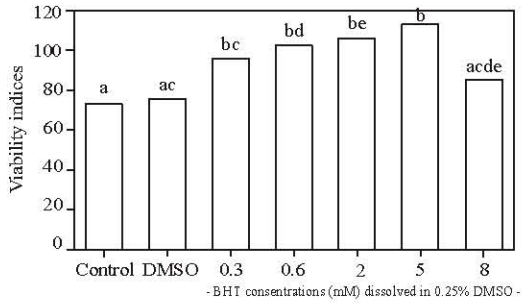

Fig. 1: Viability of chilled-stored semen in egg yolk-based extenders supplemented with or without BHT. Data are presented as mean values ± SEM of 30 measurements. Different letters above bars denote significance ($p<0.01$)

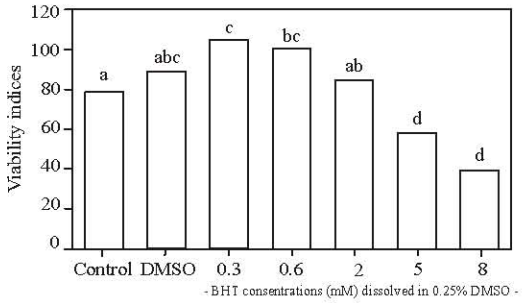

Fig. 2: Viability of chilled-stored semen in egg yolk-free extenders fortified with or without BHT. Data are presented as mean values ± SEM of 30 measurements. Different letters above bars denote significance ($p<0.01$)

1 and 5% levels of probability. The difference in kidding rates among semen treatments was analyzed by the Chi-square (χ^2) test at 1% and 5% levels of probability.

RESULTS

Figure 1 shows the impact of BHT on viability of chilled goat semen in egg yolk-based diluents. It was clear that the viability indices of stored spermatozoa were significantly ($p<0.01$) improved by inclusion of increasing concentrations (0.60, 2.00 and 5.00 mM) of BHT in semen extenders. The maximum value (114.51±4.25) of viability index was achieved after exposure of spermatozoa to 5.00 mM BHT.

Figure 2 explicates the influence of BHT on viability of chilled-stored semen in egg yolk-free extenders. Definitely, the viability indices of preserved spermatozoa were significantly ($p<0.01$) augmented by supplementation of diluents with 0.30 or 0.60 mM BHT. The superior value (106.17±7.84) of viability index was obtained after treatment of semen with 0.30 mM BHT. On the contrary, pre-storage addition of 5.00 or 8.00 mM BHT to the diluents significantly ($p<0.01$) elicited an explicit reduction in the viability indices of incubated spermatozoa. An intriguing observation during this experiment was the retention of a high percentage of motility in untreated spermatozoa (control) and in spermatozoa treated with 0.25% DMSO after cooling of goat semen in egg yolk-free extenders. The mean percentages of progressive motility of untreated spermatozoa (control) after dilution, 6, 72 and 168 h of incubation period were 75.00±2.24, 71.00±2.92, 52.00±2.83 and 16.00±2.45%, respectively. The corresponding values for spermatozoa treated with 0.25% DMSO were 75.00±2.24, 72.00±2.00, 55.00±2.06 and 25.00±2.48%, respectively.

As presented in Table 1, after freeze/thaw processing of goat semen in egg yolk-free diluents, the mean percentage of post-thaw motility of spermatozoa treated with 0.60 mM BHT was significantly ($p<0.05$) higher than that of spermatozoa treated with 0.30 or 0.90 mM BHT. Moreover, fortification of egg yolk-based extenders with 5.00 mM BHT induced a remarkable ($p<0.05$) increase in post-thaw sperm motility. Analysis of variance did not reveal any significant variation among post-thaw sperm motility percentages of semen processed in egg yolk-free diluents containing 0.60 mM BHT (47.50±2.39%) and in egg yolk-based diluents containing 5.00 mM BHT (48.50±2.97%).

Table 1: Effect of BHT on motility (%) of frozen-thawed spermatozoa

	Stages of semen processing		
Semen treatments	After dilution	Before freezing	After thawing
0.30 mM BHT in Egg yolk-free extenders	78.50±2.14	74.50±2.30	37.00±2.14^a
0.60 mM BHT in Egg yolk-free extenders	79.50±0.90	76.50±1.50	47.50±2.39^b
0.90 mM BHT in Egg yolk-free extenders	79.50±1.17	73.50±1.07	38.50±2.79^a
Egg yolk-based extenders	78.00±1.22	76.00±1.87	38.00±2.57^a
5.00 mM BHT in Egg yolk-based extenders	80.00±2.02	75.00±1.70	48.50±2.97^b

Means ± SEM with different superscripts in the same column are significantly different ($p<0.05$)

Table 2: Effect of BHT on fertility of frozen-thawed semen

Semen treatments	No. of does kidded/ No. of does inseminated	Kidding rates (%)
0.60 mM BHT in Egg yolk-free extenders	43/80	53.75^{ab}
Egg yolk-based extenders	27/70	38.57^{a}
5.00 mM BHT in Egg yolk-based extenders	50/80	62.50^{b}
Overall	120/230	52.17

Values with dissimilar superscripts are significantly different ($p<0.01$)

The outcomes of cervical insemination with frozen-thawed semen are delineated in Table 2. All females showed estrus after the second cloprostenol injection. The overall kidding rate was 52.17%. In spite of the kidding rate achieved with egg yolk-free extenders containing 0.60 mM BHT (53.75%) was superior to that achieved with egg yolk-based extenders (38.57%), this superiority did not reach to the level of statistical significance (χ^2=3.45). Likewise, while the kidding rate obtained with egg yolk-based extenders containing 5.00 mM BHT (62.50%) was significantly ($p<0.01$; χ^2=8.55)higher than that obtained with egg yolk-based extenders alone (38.57%), statistical analysis did not reveal any significant variation among the kidding rates of BHT containing extenders (χ^2=1.26).

DISCUSSION

After Ritar and Salamon [10] first reported that the coagulation reaction would not occur if the final concentration of egg yolk did not exceed 2.00% in diluted goat semen, the use of a low level (2.50%) of egg yolk as an extender ingredient has been suggested to eliminate the harmful effects of the sperm-deteriorating enzyme (phospholipase A_2 or egg yolk coagulating enzyme) in goat seminal plasma [4]. Nevertheless, the reduction in egg yolk concentrations may render semen extenders less effective in dulcifying the hypothermic stresses on sperm membranes with a subsequent decrease in the viability of preserved semen [11].

Supporting the foregoing assumption, the current investigation substantiated that supplementation of Tris-based extenders containing low levels of egg yolk with 5.00 mM BHT resulted in a significant amelioration not only in the viability of chilled-stored semen but also in the motility and fertility of frozen-thawed spermatozoa. In agreement with our findings, most workers in the former Soviet Union [12-14] and some others [15] recorded a clear improvement in sperm motility and fertility after processing and freezing of ram semen in egg yolk-based diluents containing BHT. Concomitantly, Killian *et al.* [16] found that the motility of cryopreserved bull spermatozoa at 0 hour after thawing was about 10% higher in whole milk extenders fortified with 0.50 or 0.75 mM BHT than that in extenders fortified with or without 1.00 mM BHT. As a consequence, Graham and Hammerstedt [11] and Anderson *et al.* [17] detected unique beneficial and synergistic effects on the motility and acrosome integrity of rapidly cooled bull spermatozoa when the concentrations of BHT and egg yolk were carefully controlled in semen extenders.

In the light of the above-mentioned argument, it seems that BHT could interact synergistically with lipid vesicles in egg yolk to improve the functional competence of stored goat spermatozoa. During slow cooling of semen, the biophysical properties of BHT render it an organic soluble quasi-spherical hydrocarbon membrane perturbant molecule that can partition into the sperm plasma membrane compartments and lower the effective phase transition temperature of their lipid domains by as much as 10°C with a concurrent decrease in membrane lipid viscosity as well as a subsequent maintenance of membrane fluidity which is a prerequisite for proper sperm functions [2, 11]. Also, considering the freezability of semen could be predicted based on its keeping quality at 5°C [18], it is perhaps not surprising from our data to find out that the concentration (5.00 mM) of BHT, which achieved the maximum value (114.51) of viability index, was able to attain an acceptable level (48.50%) of post-thaw motility.

As a common property of hydrophobic aromatic compounds, Bamba and Cran [19] claimed that the protective factors resided in egg yolk against loss of sperm viability during preservation of boar semen at 5°C could be substituted by pre-storage inclusion of 0.15 mM BHT in semen extenders. Likewise, while early investigators inferred that BHT was able to afford some protection for spermatozoa against the damaging effects of freezing [2] and lipid peroxidation [20], a comprehensive study demonstrated that the use of BHT might be one approach for the prevention of warm shock during rapid thawing of frozen boar semen [21]. In reality, the validity of the aforementioned observations was affirmed in the present study since the inclusion of 0.30 or 0.60 mM BHT in egg yolk-free extenders could sustain the viability of chilled-stored semen. Moreover, in terms of post-thaw sperm motility (47.50%) and fertility (53.75%), BHT at a concentration of 0.60 mM could be

utilized as an expedient alternative of egg yolk for preservation of goat semen in a frozen state. On the other hand, our study detected untoward effects of high concentrations (5.00 or 8.00 mM) of BHT on the viability of chilled semen in egg yolk-free extenders. Perhaps exposure of goat semen to high levels of BHT, particularly in lipid deficient extenders, caused a dramatic increase in the fluidity of sperm membranes beyond a critical limit, leading to an abrupt rupture in the membranes and a subsequent loss of sperm viability [19].

As can be seen from our investigation, the concentration of BHT that positively affected sperm viability was much more higher in egg yolk-based diluents (5.00 mM) than in egg yolk-free diluents (0.30 mM). Because BHT is a lipid-soluble antioxidant, its solubility in egg yolk-based diluents might be higher than in egg yolk-free diluents and it was possible that more BHT remained associated with egg yolk lipids, leaving a low concentration of free BHT molecules capable of permeating sperm plasma membrane [16]. Therefore, egg yolk-based extenders might require high concentrations of BHT to maximize its availability for sperm cells [19].

The current work indicated that goat spermatozoa could withstand cooling in the absence of egg yolk or BHT. Nearly similar observation was obtained by Abd Elhakeam [22], who harvested a high percentage of sperm motility after gradual cooling and preservation of freshly collected goat semen at 5°C. The author speculated that holding of fresh semen at 5°C allowed coating of sperm cells with seminal plasma proteins which might protect spermatozoa from cold shock injury during their preservation.

The overall kidding rate (52.17%) achieved in the present study was nearly comparable to that (53.40%) obtained by Karatzas *et al.* [23], who suggested that the fertility of frozen-thawed goat semen was significantly lower in Damascus bucks (47.40%) than in Alpine (57.70%) and Saanen (55.40%) bucks. Doubtless, the favourable influence of BHT on post-thaw sperm motility was amenable for the improvement in fertility of frozen semen either in egg yolk-free (53.75%) or egg yolk-based (62.50%) extenders.

In conclusion, BHT could be adopted as a reliable auxiliary or substitute of egg yolk for hypothermic preservation of goat semen.

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