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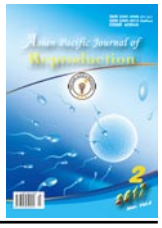

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Preservability of bull spermatozoa in Tris-egg yolk extender enriched with different concentrations of butylated hydroxytoluene

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ABSTRACT

Objective: To explore the effect of BHT on cattle spermatozoa during cooling and cryopreservation. **Methods**: Pooled bull semen were diluted by Tris-Citrate-Fructose egg yolk (TCFY) diluent considered as control (0 BHT) and different concentrations of BHT (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mM were prepared in ethanol in prewarmed (37 ℃) test tubes. The ethanol was allowed to evaporate so that, a thin crystallized layer of BHT was deposited on the inner surface of the tubes. Then extended semen was added into the tubes and incubated at 37 ℃ for 5 min to allow uptake of BHT by spermatozoa. The tubes were cooled slowly (approximately for 2 h) up to 5 ℃ and equilibrated for 4 h. After equilibration, semen freezing process was carried out. Extended semen was subjected to evaluation (motility, alive sperm, intact sperm membrane (HOST) % and acrosome integrity) in both cooled and cryopreserved semen. **Results**: The result revealed that sperm motility of post-cooled spermatozoa improved (P<0.05) by the use of BHT concentrations (1, 2 and 3 mM) in Tris semen extender if compared to the control (85.00±1.09), (83.33± 0.63), (81.67± 0.63) and (78.33± 0.63), respectively. Alive sperm percent was significantly higher in all concentrations of BHT. Sperm abnormalities percent were significantly lower in concentrations of BHT 1 and 2 (11.2±0.2), (11.8±0.2)and (13.4±0.4), respectively. Sperm membrane integrity were significantly higher in BHT concentrations (1, 2, 3, 4 and 5 mM). It is exhibited that improved sperm motility in post-thawed frozen semen in the concentrations of BHT (1, 2, 3 and 4 mM) if compared to the control. The sperm membrane integrity were significantly improved at all concentrations of BHT. Acrosome integrity was significantly higher at BHT concentration 1 mM (81.80±0.57) and (76.00±2.05), respectively. **Conclusions**: It could be concluded that some concentrations of BHT improved bull semen quality post-cooling and post-freezing.

1. Introduction

There are many advantages upon application of a breeding program using cryopreserved semen. The most important benefit is the long-term storage of genetic material. There are many factors affecting survival of cryopreserved spermatozoa[1]. Sperm freezing protocols differ from species to another. This species difference is related to variations in seminal plasma and membrane compositions between species[2]. Freeze thaw process has detrimental effects on spermatozoa as it induces lipid peroxidation[3] exerted by over accumulation of reactive oxygen species (ROS) causing oxidative damage to sperm membrane, acrosome and DNA, then finally loss of sperm viability[4-6]. Susceptibility to lipid peroxidation differs among species and also due to changes in cryopreservation protocol,

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extender formula and antioxidant concentration[7]. The spermatozoa membrane is the most susceptible part to the oxidative damage due to its high contents of polyunsaturated fatty acids and its deficiency of protecting enzymes[1,8-12], as GSH, CAT and SOD and metal chelators as transferring, lactoferrin and cerruloplasmin. Therefore, addition of antioxidants to the extender induce beneficial effects on spermatozoa via reducing the cryoinjury induced by ROS[13-20]. BHT is a synthetic analogue of vitamin E that has positive effects on semen quality of frozen bull semen[21], ram[22], boar[23], goat[24] and turkey[25].

BHT is one of the most important antioxidants that could be used in semen extenders as a cryoprotectant[1]. There is little literature with respect to its use in cattle bulls. So, the aim of the present study was to explore the effect of BHT on cattle spermatozoa during cooling and cryopreservation.

2. Material and methods

2.1. Semen collection and initial evaluation

Five mature genetically improved cattle-bulls with superior quality semen characteristics maintained at The Semen Freezing Center, General Organization for Vet. Services, Ministry of Agriculture, Abbasia, Egypt, were used for this study as semen source. Semen ejaculates were collected from bulls using an artificial vagina at weekly intervals for 5 wk. The semen samples were initially evaluated for volume (in graduated tube), concentration using Thoma rulling of the Neubaur haemocytometer and sperm motility. The neat semen samples with more than 70% motility and 80% morphologically normal spermatozoa were admitted to freezing procedure. The ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time for 10 min at 37 ℃ in a water bath before dilution to be evaluated for sperm motility, viability, total abnormalities, and acrosome and membrane integrities before processing.

2.2. Semen processing

Semen samples were extended (1:7 dilution rate) in a Tris-citrate egg yolk extender with 20% (v/v) egg yolk and 7% (v/v) glycerol at 37 ℃[26,27] to ensure 60 million motile spermatozoa mL-1.

Different concentrations of BHT (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mM were prepared in ethanol in prewarmed (37 ℃) test tubes. The ethanol was allowed to evaporate so that a thin crystallized layer of BHT was deposited on the inner surface of the tubes. Then extended semen was added into the tubes and incubated at 37 ℃ for 5 min to allow uptake of BHT by spermatozoa[28]. The control tubes were Tris containing (0 BHT). The tubes were cooled slowly (approximately for 2 h) up to 5 ℃ and equilibrated for 4 h. Semen was packed into 0.25 mL polyvinyl French straws (IMV, France). After equilibration periods, the straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen (LN2) for 10 min and were then dipped stored in liquid nitrogen at -196 ℃.

2.3. Assessment of semen quality parameters

Frozen straws were thawed individually at 37 ℃ for 30 s in a water bath for microscopic evaluation[29]. The parameters studied were sperm motility, sperm viability, sperm abnormality, sperm membrane integrity (HOST), percent of normal intact acrosome in cooled and frozen-thawed semen.

2.3.1. Sperm motility

Subjective motility was observed using phase contrast microscope (Olympus Optical Co. Ltd., Japan). Visual motility was assessed microscopically with closed circuit television[30].

2.3.2. Live and abnormal spermatozoa (%)

The viability and abnormalities% of sperm were evaluated using eosin-Nigrosin stained smear as described by Sidhu and Guraya[31].

2.3.3. Sperm membrane integrity

Sperm membrane integrity was assessed using the hypoosmotic swelling test[32]. Two hundred spermatozoa were assessed and the percentage of spermatozoa with curled tails (swollen/intact plasma membrane) was calculated.

2.3.4. Intact normal acrosome percent

Acrosome integrity was evaluated using giemsa stain as described by Watson[33]. The % intact acrosome was recorded for 200 spermatozoa that were randomly examined under an immersion objective (× 1 000 magnification) using phase contract microscope.

2.3.6. Statistical analysis

Output data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan test to determine significant differences in all the parameters among all groups, with SPSS Version 14.0 for Windows[34]. Differences with values of $P<0.05$ were considered to be statistically significant.

3. Results

Table 1 revealed that sperm motility of post-cooled spermatozoa improved ($P<0.05$) by the use of BHT concentrations (1, 2 and 3 mM) in Tris semen extender if compared to the control (85.00±1.09), (83.33±0.63), (81.67±0.63) and (78.33±0.63), respectively. Alive sperm percent was significantly higher ($P< 0.05$) in all concentrations of BHT if compared to the control. Sperm abnormalities percent were significantly lower in concentrations of BHT 1 and 2 with respect to the control (11.2±0.2), (11.8±0.2) and (13.4±0.4), respectively. Sperm membrane integrity (HOST %) were

significant (P<0.05) higher in BHT concentrations (1, 2, 3, 4 and 5 mM) as compared to the control.

Table 1

Effect of Tris extender enriched with BHT on the cooled extended bull semen.

Parameter(%)	Motility	Alive	Abnormality	HOST
Control	78.33±0.63^{c}	79.67±1.24^{c}	13.40±0.40^{c}	71.42±0.10^{d}
BHT 1	85.00±1.09^{a}	91.20±0.39^{ab}	11.20±0.20^{d}	79.67±0.33^{a}
2	83.33±0.63^{ab}	91.00±0.24^{ab}	11.80±0.20^{d}	77.67±0.55^{b}
3	81.67±0.63^{b}	92.00±0.38^{a}	15.80±0.52^{b}	75.33±0.67^{c}
4	78.33±0.63^{c}	91.60±0.58^{ab}	16.40±0.36^{b}	75.33±0.55^{c}
5	78.33±0.63^{c}	91.40±0.27^{ab}	16.20±0.31^{b}	75.33±0.77^{c}
6	73.33±0.63^{d}	90.20±0.26^{b}	19.40±0.40^{a}	70.00±0.44^{e}
F-cal	29.81	57.42	65.73	40.15
P<	0.000 1	0.000 1	0.000 1	0.000 1

The different superscripts within rows indicate significant differences between means compared with the Duncan's multiple range test (P<0.05).

Table 2 exhibited improved sperm motility in post-thawed frozen semen in the concentrations of BHT (1, 2, 3 and 4 mM) if compared to the control. The sperm membrane integrity (HOST%) were significantly (P<0.05) improved at all concentrations of BHT if compared to the control.

Acrosome integrity was significantly (P<0.05) higher at BHT concentration 1mM as compared to the control (81.80±0.57) and (76.00±2.05), respectively.

Table 2

Effect of Tris extender enriched with BHT on the freezed thawed extended bull semen.

Parameter (%)	Motility	Alive	Abnormality	HOST	Acrosome integrity
Control	41.00±1.00^{d}	80.60±0.95^{a}	18.50±1.09^{a}	71.20±0.62^{e}	76.00±2.05^{ab}
BHT 1	54.00±1.31^{b}	73.00±2.20^{b}	17.50±1.53^{a}	84.00±0.79^{a}	81.80±0.57^{a}
2	58.00±1.07^{a}	71.80±0.80^{b}	18.67±0.65^{a}	83.33±0.77^{a}	74.60±3.41^{b}
3	45.00±1.46^{c}	60.80±2.26^{cd}	19.67±1.05^{a}	84.67±0.77^{a}	75.20±2.67^{b}
4	44.00±1.31^{c}	64.00±2.12^{c}	18.33±1.64^{a}	80.67±0.45^{b}	60.80±2.40^{c}
5	37.00±1.07^{e}	58.80±2.30^{d}	17.78±0.40^{a}	78.67±0.77^{c}	59.00±1.66^{c}
6	25.00±0.85^{f}	45.67±0.96^{e}	17.82±0.35^{a}	74.33±0.98^{d}	59.40±2.23^{c}
F-cal	87.00	41.33	0.47	47.18	17.15
P<	0.000 1	0.831 7	0.000 1	0.000 1	0.000 1

The different superscripts within rows indicate significant differences between means compared with the Duncan's multiple range test (P<0.05).

4. Discussion

The generation of ROS resulting from cryopreservation[14] and the reduced activity of the antioxidant enzymes in semen post-freezing[11,35,36] bay be the causative factor of sperm cryoinjury, therefore, the addition of BHT as an antioxidant in buffalo semen extenders[28] in ram[37], in goat[7] and in canine[1] can improve post-thawed semen quality.

The results of the present study showed improved post-cooling and post-thawing semen quality in terms of motility, viability, acrosomal and sperm membrane integrities in Tris extender enriched with BHT. Our results are in agreement with those obtained in buffalo bulls[28], in bulls[5,21,38], ram[22], goat[24,39] and boar[23,40]. Also, our results are in accordance with Shoae and Zamir[21] who stated improved post-freezing semen quality with concentrations 0.5-1.0 mM BHT in bulls. However, they concluded that higher concentrations of BHT have detrimental effects. The improved post-thawed sperm motility in this study may be attributed to the protective antioxidant effect of BHT on morphological sperm integrity especially the integrity of the axosoma and mitochondria of the middle piece[7]. The improvement in post-cooling and post-freezing semen quality in our study may be due to the incorporation of BHT into the cell membrane increasing its integrity and fluidity[21]. This effect could be conducted by the antilipid peroxidation action of BHT[41–44]. In contrast to this, Ball *et al.*[45] observed detrimental effects of BHT supplement on stallion spermatozoa.

These findings could be related to species varieties in susceptibility to oxidative stress[46,47]. It could be concluded that some concentrations of BHT improved bull semen quality post-cooling and post-freezing.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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