Abstract

BACKGROUND: Many studies have been shown that freezing induced oxidative stress has detrimental effect on post-thaw sperm quality. OBJECTIVE: This study was conducted to investigate the effect of tert-butyl hydroquinone (tBHQ) on bull semen cryopreservation. MATERIALS AND METHODS: In this study, four different levels of tBHQ [Optidyl® containing zero (T0), 2.5 (T2.5), 5 (T5), and 7.5 µM (T7.5) tBHQ] was used to study the effect of tBHQ on freezability of bull semen. On each collection day, four ejaculates were collected (a total of 24 ejaculates from four bulls), pooled and divided to four equal parts. Each part was diluted with one of the above-mentioned extenders and frozen. After thawing, sperm motility, plasma membrane functionality and integrity, apoptosis status and mitochondrial activity were assessed. RESULTS: The results show that total sperm motility was significantly higher in T5 compared to other groups. The value of VSL was significantly lower in T5 compared to T0. Also, T5 resulted in lower LIN and STR versus T0 and T2.5 groups. All extenders containing tBHQ resulted in a significantly higher percentage of sperm with functional membrane compared to T0 groups. Finally, Apoptosis related parameters and mitochondrial activity were not significantly difference between the groups. CONCLUSION: adding 5 µM tBHQ to the bull semen extender can be beneficial for post-thaw sperm quality. Also, in vivo or in vitro fertility test is recommended to test fertilizing ability of tBHQ exposed sperm.

Keywords: antioxidant, bovine, freezing, sperm

INTRODUCTION

Since cryopreservation of sperm allows long-time storage and optimal distribution of superior germ plasm, it has become one of the most important biological technologies available in domestic animals industry (14). As a result of cryopreservation, availability of sperm regardless of time and location has led to the exploitation of reproductive techniques such as artificial insemination and in vitro fertilization. The wide use of artificial insemination has resulted in both acceleration in genetic selection and improvement in domestic animal production, mainly in dairy cows. Although
cryopreservation is beneficial for long-time storage of sperm, it can induce serious damages to spermatozoa, resulting in loss of their motility, viability, fertilization potential and damaged in acrosome and plasma membrane integrity. Moreover, it can also cause harmful effects on sperm DNA (1). Even with the best protocol of cryopreservation, approximately half of the sperm population may lose the initial motility they had in the raw semen. Furthermore, within the motile sperm population, some damages may impair the functional activity of the cryopreserved sperm in comparison with the fresh sample (23).

It is generally accepted that cryopreservation induces the production of reactive oxygen species (ROS). Although ROS play an important physiological role in sperm capacitation and acrosome reaction which are necessary for fertilization, they are harmful to sperm survival at high concentrations (7). Toxicity of ROS is related to the inactivation of proteins, DNA damage, and peroxidation of unsaturated lipids. Since spermatozoa have a high content of polyunsaturated phospholipids in the plasma membrane with low levels of antioxidants, they are particularly susceptible to oxidative damage (11). Detrimental effects of ROS are reduced by antioxidant enzymes such as superoxide dismutase, catalase, peroxidases and also reducing agents such as glutathione (GSH), ascorbic acid, taurine and hypotaurine, which are present in spermatozoa and seminal plasma (3, 13). In recent years, it has been shown that adding different antioxidants can protect sperm from the detrimental effects of ROS (6, 20). Some studies have shown that tert-butyl hydroquinone (tBHQ), a synthetic phenolic antioxidant, is effective in protecting cellular dysfunction caused by oxidative stress inducers such as alcohol, dopamine, hydrogen peroxide, and glutamate (15). In other tissues, tBHQ can activate transcription factors, resulting in the expression of certain detoxification and antioxidant enzymes (12).

Therefore, this study was conducted to investigate whether tBHQ addition to the bull semen extender can improve post-thawing quality.

**MATERIALS AND METHODS**

**Semen collection and cryopreservation**

Semen samples were collected from four Holstein bulls, using an artificial vagina, twice a week for three consecutive weeks. These bulls were kept at bull station of ZarGene Company, Firoozkooh, Tehran, Iran. Samples were immediately placed at 37°C and evaluated to make sure that they were suitable for cryopreservation (semen concentration: >1 × 10⁹ spermatozoa/mL; motility: >60%; abnormal morphology: <15%). To remove individual differences between bulls, the samples were pooled together and equally divided to for parts and each part was diluted with Optidyl® (Biovet, France) extender containing different levels of ethanol-dissolved tBHQ [0 (T0), 2.5 (T2.5), 5 (T5) and 7.5 (T7.5) µM tBHQ] to a final concentration of 100 × 10⁶ spermatozoa/mL. All treatments received same amount of ethanol (99.5%). Diluted semen samples were cooled at 4°C for 3 hours in a cool cabinet, packed in 0.25 mL straws (seven straws/treatment/replicate) (IMV Technologies, L’Aigle Cedex, France) and frozen using a computer controlled freezing system (Digit Cools, IMV Technologies), -3°C/min from 4°C to -10°C, -40°C/min from -10°C to -100°C, -20°C/min from -100°C to -140°C). Straws were stored in liquid nitrogen until assessment. Before starting any post-thaw evaluation, the samples were thawed in a 37°C water bath for 30 seconds.

**Semen assessments**

**Motility and kinematic parameters**

The thawed semen samples were loaded on a counting chamber (Leja, 20 µm depth, Nieuw-Vennep, The Netherlands) at 37°C. Sperm motility and kinematic parameters
(average path velocity (VAP, μm/s); straight line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); amplitude of lateral head displacement (ALH, μm); linearity (LIN, %); and straightness (STR, %)] were recorded using a computer-assisted sperm analysis system (CASA; CEROS version 12.3; Hamilton-Thorne Biosciences, Beverly, MA, USA).

**Plasma membrane functionality**

Plasma membrane functionality was evaluated by hypo-osmotic swelling (HOS) test (17) with minor modifications. For preparation of the HOS solution (100 mOsm/kg), fructose (9 g/L) and sodium citrate (4.9 g/L) were dissolved in distilled water. Briefly, 30 μL of semen samples was incubated with 300 μL of HOS solution at 37°C for 30-40 min. Afterward, a drop of the sample was placed on a microscope slide and covered with a coverslip. At least 200 spermatozoa were evaluated by phase-contrast microscope (Labomed, Lx 400, USA) at 400× magnification and sperm with swollen and coiled tails were recorded.

**Flowcytometry analysis**

Flow cytometry analyses were carried out with a BD-FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The fluorescent probes used in this study were excited by an argon ion 488 nm laser. Red fluorescence from Propidium iodide (PI) was detected with the FL3 photodetector (670 nm LP filter) and green fluorescence from Annexin-V/FITC and Rhodamine-123 fluorescence were detected with the FL1 photodetector (530/30 nm filter). For each sample, 10,000 events were collected. Acquired data were analyzed by FlowJo software (version 9.5.3). Also, an acquisition gate was applied to the FSC/SSC dot plot to restrict the analysis to sperm and to eliminate small debris and other particles.

**Mitochondrial activity**

Rhodamine-123 (R123; Invitrogen, Eugene, OR, USA) and PI were used to assess the mitochondrial activity of spermatozoa. Briefly, 10 μL of R123 solution (0.01 mg/mL in distilled water) were added to 0.5 mL of diluted semen samples and incubated for 30 min at room temperature (25°C) in the dark. Afterward, semen samples were centrifuged (500 g for 3 min), and sperm pellets were re-suspended in 0.5 mL of Tris buffer with 10 μL of PI (1 mg/mL) and analyzed by flowcytometry to determine the number of live spermatozoa with active mitochondria (positive for R123 and negative for PI) (9).

**Phosphatidylserine translocation**

To detect the membrane phosphatidylserine (PS) in the outer part of the sperm plasma membrane, PS translocation detection Commercial Kit (IQP, Groningen, the Netherlands) was used according to the manufacturers guide. In brief, spermatozoa were washed in calcium buffer and re-suspended to 1 × 10⁶ spermatozoa/mL. Thereafter, 10 μL of Annexin V-FITC and 100 μL of sperm suspension were mixed and incubated for 20 min at room temperature. Then, 5 μL of PI were added to the mixture and incubated for 15 min at room temperature in the dark. The samples were analyzed using flowcytometer. The analysis resulted in classifying spermatozoa in 4 distinct groups: 1) viable negative for both annexin-V and PI, 2) early apoptotic positive for annexin-V and negative for PI), 3) late apoptotic positive for both annexin-V and PI), and 4) necrotic (negative for annexin-V and positive for PI) spermatozoa.

**Statistical analysis**

Data were analyzed using MIXED procedure of the SAS software (SAS Institute, version 9.1, 2002, Cary, NC, USA). Results were represented as LS mean ± SEM. Tukey’s test was used to compare LS means differences. Differences with values of P<0.05 were considered to be statistically significant.
The statistical model used in the present study was as follow:

\[ Y_{ijk} = \mu + A_i + B_j + (AB)_{ijk} + e_{ijk} \]

where \( Y_{ijk} \) is the observed dependent variables, \( \mu \) is mean of population, \( A_i \) is the fix effect of \( i^{th} \) treatment \( (i = 1, 2, 3 \text{ and } 4) \), \( B_j \) is the random effect of \( j^{th} \) replicate \( (j= 1, 2, 3, 4, 5 \text{ and } 6) \), \( (AB)_{ijk} \) is the interaction of treatment and replicate and \( e_{ijk} \) is random residual error.

**RESULTS**

CASA results (total and progressive motility and kinematic parameters) are shown in Table 1. Total sperm motility was significantly higher in T5 compared to T0, T2.5, T7.5 groups. VSL was significantly lower in T5 compared to T0 group. The value of LIN and STR was lower for T5 \((P<0.05)\) compared to T0 and T2.5 groups.

The results of sperm plasma membrane functionality are represented in Table 2. All levels of tBHQ showed higher plasma membrane functionality than that T0 \((P<0.05)\).
Mitochondrial activity (Table 2), live, early apoptotic, late apoptotic and necrotic spermatozoa were non-significantly affected by tBHQ levels (Table 2).

### DISCUSSION

In order to improve the post-thaw quality of bull spermatozoa, tBHQ was used as a phenolic antioxidant. It was observed that the application of tBHQ at 5 µM resulted in higher total sperm motility compared to other groups containing 7.5 and 2.5 µM tBHQ or no tBHQ, although progressive motility was not affected by different levels of tBHQ. Also, among kinematic parameters, the group containing no tBHQ showed an increased velocity and linearity. This is in agreement with the findings of other researchers who evaluated the effect of BHT on quality parameters of post-thaw bull semen and showed that BHT (0.5-1 mM) had a beneficial effect on sperm quality (25). Like tBHQ, BHT is a synthetic phenolic antioxidant (22). A reason for tBHQ efficiency for protecting sperm motility could be its ability to keep ROS levels low (6) and to stop or delay lipid peroxidation (4). Moreover, tBHQ can avert or delay the beginning of lipid peroxidation (18). It seems that the improvement of total sperm motility in the current study is derived by reduction of ROS, which could lead to a decrease in lipid peroxidation and improvement in sperm plasma membrane function. Thus, the increase of total motility in the group containing 5 µM tBHQ may be partly due to the improvement of sperm plasma membrane functionality noticed in this study. It has been shown that sperm plasma membrane functionality has a direct relationship with sperm motility (10, 19).

Mitochondrial activity was not affected by tBHQ in this study. Whereas we could not find any study about the effect of tBHQ on the mitochondrial activity of sperm and other cells, our results were in agreement with others evaluating the effect of different antioxidants on spermatozoa. For example, it has been observed that using cysteine (5 and 10 mM) and GSH (1 and 5 mM) did not affect the mitochondrial activity of post-

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>tBHQ (µM)</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
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<tr>
<td>PMF (%)</td>
<td>67.7ᵇ</td>
<td>72.3ᵃ</td>
</tr>
<tr>
<td>MA (%)</td>
<td>66.7</td>
<td>63.9</td>
</tr>
<tr>
<td>Live (%)</td>
<td>68.5</td>
<td>66.8</td>
</tr>
<tr>
<td>Early apoptotic (%)</td>
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<td>13.2</td>
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<td>Late apoptotic (%)</td>
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<td>16.4</td>
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<tr>
<td>Necrotic (%)</td>
<td>3.9</td>
<td>4.4</td>
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</table>

ᵇᵃ Means with different superscript in each row differ significantly (** P < 0.01; NS: not significant).

Table 2. Effect of tBHQ levels on post-thaw sperm plasma membrane functionality (PMF), mitochondrial activity (MA) and apoptosis status (LS mean ± SEM).
thaw ram spermatozoa (16, 24). On the other hand, it has been reported (5) that GSH (1 and 5 mM) could improve mitochondrial activity of red deer spermatozoa especially after 6 h post-thaw incubation at 39ºC. Also, it has been shown that GSH at 7 mM had negative effects on some ram semen parameters, including mitochondrial ultrastructure (21). It is possible that GSH and other antioxidants at high concentrations could induce detrimental effects via decreasing the physiological levels of ROS in sperm. It has been well established that ROS at optimal level play necessary physiological roles in the spermatozoa (2). However, it seems that the tBHQ levels used in the current study were not high enough to affect mitochondrial activity.

Data in the present study showed that tBHQ treatment had no effect on the percentage of live spermatozoa and apoptosis status. However, several studies have reported a reduction in apoptosis of different kinds of cells which were treated with tBHQ (8, 26, 27). It has been reported that tBHQ suppressed oxidative stress-derived caspase-3 activation in NT2N neuronal cells (8). The reason why tBHQ could not affect viability and apoptosis status in the present study is not clear, but probably the pathway by which tBHQ can affect viability and apoptosis status does not occur in sperm or it is not activated at concentrations used in the present study.

In conclusion, our results demonstrate that adding 5 µM tBHQ to the bull semen extender can be beneficial for the post-thaw sperm quality. Although Optidyl® supplemented with tBHQ (5 µM) could improve some in vitro bull sperm quality parameters, but in vitro or in vivo fertility test is recommended.

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REFERENCES


