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Effect of quercetin or butylated hydroxytoluene on cooled or frozen-thawed ram sperm quality

Efeito da inclusão de quercetina ou butil-hidroxitolueno na qualidade do sêmen de carneiro resfriado ou congelado

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Highlights

The inclusion of quercetin did not affect sperm motility parameters.
The inclusion of BHT did not affect sperm motility parameters.
The ram's breed did not affect sperm volume and total sperm number.

Abstract

Cooling and freezing processes cause physical and chemical damage to sperm by cold shock and oxidative stress. This study aimed to evaluate the effect of two antioxidants on sperm parameters of cooled and frozen-thawed ram semen diluted in an egg yolk-based extender. Semen was collected from 30 rams and processed in two consecutive experiments to test the inclusion of different concentrations of quercetin and butylated hydroxytoluene (BHT) in an egg yolk-based semen extender. Dimethyl sulfoxide (DMSO) was added as a solvent to the semen extender in a ratio of 1 mL DMSO for 90 mg of quercetin and 1 mL DMSO for 880 mg of BHT. After collection, semen was diluted at 200 × 10^6 motile sperm/mL (control) and split into different groups in each experiment. In experiment 1, semen was diluted with the extender containing quercetin (Q5, 5 µg/mL; Q10, 10 µg/mL; Q15, 15 µg/mL) or DMSO alone (DMSO1, 0.055 µL DMSO per mL; DMSO2, 0.165 µL DMSO per mL). In experiment 2, semen was diluted with the extender with BHT (BHT1, 0.5 µg/mL; BHT2, 1 µg/mL; BHT3, 1.5 µg/mL) or DMSO alone (DMSO3, 0.375 µL DMSO per mL; DMSO4, 1.125 µL DMSO per mL). After dilution, the semen was divided into two aliquots. Treated ram sperm samples were also subjected to different storage methods. The first set of samples was cooled at 5 °C for 24 h, whereas the second set of samples was frozen-thawed. Sperm motility parameters and plasma membrane integrity (PMI) were evaluated immediately after dilution (0h) and 24 h after cooling and in the frozen-thawed samples

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via computer-assisted sperm analysis and epifluorescence microscopy, respectively. The inclusion of quercetin or BHT did not affect sperm motility parameters or PMI of fresh, cooled, or frozen-thawed sperm in this study (P < 0.05). However, further studies are needed to test the effects of these antioxidants on the fertility of cryopreserved ram semen.
Key words: Antioxidant. Cryopreservation. Oxidative damage. Lipid peroxidation. Small ruminants. Sheep.

Resumo

O resfriamento e o congelamento causam danos físicos e químicos aos espermatozoides por choque térmico e estresse oxidativo. Portanto, este estudo teve como objetivo avaliar o efeito da inclusão de dois antioxidantes em um diluente à base de gema de ovo sobre os parâmetros espermáticos do sêmen ovino resfriado e congelado. Trinta carneiros tiveram o sêmen coletado e processado em dois experimentos consecutivos para testar a inclusão de diferentes concentrações de quercetina e hidroxitolueno butilado (BHT) em diluente de sêmen à base de gema de ovo. O DMSO foi adicionado como solvente ao diluente de sêmen em uma proporção de 1 mL de DMSO parra 90 mg de quercetina e 1 Ml de DMSO para 880 mg de BHT. Após a coleta, o sêmen foi diluído a 200 × 10^6 espermatozoides móveis/mL (Controle) e dividido em diferentes grupos em cada experimento. Experimento 1, Quercetina (Q5, 5 µg / mL; Q10, 10 µg / mL; Q15, 15 µg / mL) ou DMSO (DMSO1, 0,055 µL de DMSO por ml; DMSO2, 0,165 µL de DMSO / mL) foram adicionados ao extensor. Experimento 2, BHT (BHT1, 0,5 µg / mL; BHT2, 1 µg / mL; BHT3, 1,5 µg / mL) ou DMSO (DMSO3, 0,375 µL de DMSO por ml; DMSO4, 1,125 µL de DMSO / mL) foram adicionados à o extensor. Após a diluição, o sêmen foi dividido em duas alíquotas. O primeiro foi resfriado a 5 ° C por 24h, enquanto o segundo foi congelado. Os parâmetros de motilidade espermática e integridade da membrana plasmática (PMI) foram avaliados, imediatamente após a diluição (0h) e 24h após o resfriamento e nas amostras congeladas, pelo CASA e microscopia de epifluorescência, respectivamente. A inclusão de quercetina ou BHT não afetou os parâmetros de motilidade espermática e PMI de espermatozoides frescos, resfriados ou congelados (P < 0,05). Portanto, a inclusão de quercetina e BHT não beneficiou os parâmetros espermáticos do sêmen ovino submetido a armazenamento líquido a 5 ° C por 24h ou protocolo de congelamento no presente estudo. No entanto, mais estudos são necessários para testar o efeito desses antioxidantes na fertilidade do sêmen ovino criopreservado.
Palavras-chave: Antioxidante. Criopreservação dano oxidativo. Peroxidação lipídica. Pequenos ruminantes. Ovinos.

Introduction

Semen cryopreservation plays an essential tool for conserving and distributing desirable genetic material over long distances. However, cooling and freezing cause physical and chemical damage to the sperm by cold shock and oxidative stress (Budai, Egerszegi, Olah, Javor, & Kovacs, 2014). Cold shock is associated with irreversible changes in the plasma membrane and acrosome, DNA fragmentation, and reduced fertilization capacity (Budai et al., 2014; Salamon, & Maxwell, 2000). During refrigeration and cryopreservation, sperms undergo a membrane phase transition and reorganization of the original lipid and protein composition, which causes a decrease in membrane fluidity and a higher

predisposition to ruptures, compromising cell function (Parks, & Lynch, 1992). Additionally, there is excessive production of oxygen-free radicals during sperm cooled-storage and freeze–thaw cycles (Budai et al., 2014). In rams, high reactive oxygen species (ROS) produced during cold storage affect sperm plasma membrane viability and mitochondrial function and reduce sperm motility (Allai et al., 2016; Amidi, Pazhohan, Shabani, Khodarahmian. & Nekoonam, 2016). The interaction between excessive reactive oxygen species (ROS) produced by sperm metabolism and dead sperm with polyunsaturated fatty acids in the sperm membrane induces lipid peroxidation and reduces sperm quality (Budai et al., 2014). Interestingly, the plasma membrane of small ruminant sperm is rich in polyunsaturated fatty acids, which renders the sperm highly vulnerable to oxidative damage (Bucak et al., 2010).

Several antioxidants have been tested to ameliorate the effects of oxidative stress in cryopreserved sperms or in cooled storage. Quercetin is a flavonoid compound found in fruits and vegetables with antimicrobial (Nitiema, Savadogo, Simpore, Dianou, & Traore, 2012), anti-inflammatory (Guardia, Rotelli, Juarez, & Pelzer, 2001), and antioxidative (Gibb, Butler, Morris, Maxwell, & Grupen, 2013; Ben, Zribi. & Ammar-Keskes, 2011) properties. Quercetin scavenges ROS and hydroxyl radicals (Boots, Haenen & Bast, 2008), and has been suggested as a potent antioxidant for the cryopreservation of semen from stallions, rats, rooster, and dogs (Gibb et al., 2013; Ben et al., 2011; Appiah et al., 2020; Kawasaki et al., 2020). Another antioxidant added to semen extenders to minimize sperm damage caused by oxidative stress and cold shock is butylated hydroxytoluene (BHT) (Jara, Merino, Sánchez, & Risopatrón, 2019; Merino et al., 2015). Butylated hydroxytoluene is a synthetic analog of vitamin E that reacts with peroxy radicals to transform them into hydroperoxides, interrupting the oxidative process (Seifi-Jamadi, Kohram, Zareh-Shahne, Dehghanizadeh, & Ahmad, 2016). In a previous study, the addition of BHT at a concentration of 5.0 mM improved sperm parameters of ram cryopreserved sperm (Palomo, García, & Tabarez, 2017). Therefore, the present study hypothesizes that the addition of quercetin or BHT to a commercially available egg yolk-based semen extender (BotuBov®) can increase the viability of ram sperm during the cooling and freezing processes. This study aimed to evaluate the effect of the inclusion of quercetin and BHT in a commercially available egg yolk-based extender on sperm parameters of cooled and frozen-thawed ram semen.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of São Paulo State University, Botucatu, São Paulo, Brazil, under protocol #0180/2014. The study was conducted from March to June 2015. Thirty rams (15 Santa Inês breed and 15 Texel breed) ranging from 2 to 5 years old, clinically healthy, with a good body condition score and satisfactory sperm parameters (Table 1) were enrolled in this study (Oliveira, Oliveira, Lima, Andrade, Abreu, & Oliveira, 2017; Mandiki, Derycke Bister, & Paquay, 1988). The rams were kept on a farm in laras city, SP, Brazil (22°52'15" S 49°09'46" W), and a total of 178 ejaculates were harvested and divided into two experiments, as described below. Rams were housed in 5 × 3 m stalls, fed with alfalfa grass hay, and free access to water and trace minerals.

Table 1
Seminal parameters of Santa Inês and Texel rams

Rams	Volume	SC (×10⁹/mL)	TM	PM	RAP	PMI
Santa Ines	1.0 ± 0.3	3.6 ± 0.8	88.4 ± 4.6	61.3 ± 10.6	81.2 ± 6.5	54.6 ± 14.3
Texel	1.0 ± 0.2	3.4 ± 0.4	88.1 ± 9.0	60.6 ± 10.2	79.6 ± 11.1	57.2 ± 13.1
Overall	1.0 ± 0.3	3.5 ± 0.6	88.2 ± 7.4	60.9 ± 10.2	80.2 ± 9.4	56.1 ± 13.4

SC, sperm concentration; TM, total motility; PM, progressive motility; RAP, rapid sperm; PMI, plasma membrane integrity. All data are presented as the mean ± SD.

Immediately before the study, all rams had two washout semen collections performed to deplete the old sperm reserves. Semen was harvested by electrostimulation (Eletrojet Premium, Eletrovet, Valinhos, SP, Brazil) The electroejaculation procedure involved a series of five pulses of similar voltage, each separated by an interval of 5 s. The initial stimulus was 1 V, and the maximum voltage was 9 V (Fidan, Yeni, Avdatek, Özçinar, & Hazman, 2018). Semen was then collected in a 15 mL plastic tube covered by a thermal protector. The semen volume was recorded, and sperm concentration was assessed with a hemocytometer (Optik Labor, Lancing, England) using a phase-contrast microscope (Jenamed 2: Carl Zeiss, Munich, Germany) at 200× magnification. An aliquot of raw semen was diluted (1:100) in 10% formol saline, and morphological characteristics were evaluated using a differential interference contrast microscope (Leica DM2500; Leica Microsystems, SP, Brazil) under 1000× magnification using oil immersion. Morphological characteristics were assessed in 100 sperms, and abnormalities of the head, midpiece, and tail were analyzed (Brito, 2007). Sperm motility parameters were evaluated using computer-assisted sperm analysis (CASA; IVOS 11, Hamilton Thorne, Inc., Beverly, MA, USA). Ejaculates with the following minimum criteria were selected for the study: volume, 0.75–2 mL; sperm concentration > 2.5 × 10^9 sperm/mL; percentage of motile sperm > 70%; less than 10% of morphologically abnormal cells.

After collection and initial analyses, semen was extended with a commercially available ram semen freezing extender (BotuBov®, Botupharma, Botucatu, SP, Brazil) at 200 × 10^6 motile sperm/mL and split into different groups in each experiment, as described below. After extension, the samples were stabilized for 20 min at room temperature (22 °C). Next, semen was loaded into 0.25 mL French straws (10 straws per group) and cooled (5 straws/group) or frozen (5 straws/group). For cooling, semen was stored in a passive semen cooling container (Botutainer, Botupharma, Brazil) at 5 °C for 24 h. For freezing, the straws were placed in a temperature-controlled refrigerator (Minitub do Brasil Ltda, Porto Alegre, Rio Grande do Sul, Brazil) at 5 °C for 240 min. Afterwards, the straws were placed 4 cm above liquid nitrogen for 20 min and then plunged into liquid nitrogen and stored at −196°C for further evaluation.

Sperm analyses

Sperm motility parameters were evaluated by CASA using customized settings for ram sperm (Additional file: S-Table 1). For each sample, the percentiles of total motility (TM), progressive motility (PM), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), and rapid spermatozoa (RAP) were evaluated. Each sample was incubated in a dry bath at 37 °C for 10 min before each evaluation. An aliquot of 10 μL was loaded into a Makler chamber (Irvine Scientific, Santa Ana, CA, USA) and a minimum of 1000 cells in five random fields were assessed.

The percentage of sperm with an intact plasma membrane (PMI) were assessed by epifluorescence microscopy (Leica Microsystems, DMLB, Germany) based on the association of fluorescence from propidium iodide (PI) and 6-carboxyfluorescein diacetate, as previously described (Harrison, R. A. P., & Vickers, S. E., 1990). Carboxyfluorescein diacetate–positive cells were considered sperm with intact plasma membrane and PI-positive sperm with damaged plasma membrane.

Preparation of the extenders

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). A commercially available egg yolk-based semen freezing extender (BotuBov®) was used for all tests, and quercetin or BHT were added according to the specified concentration. Since quercetin and BHT are poorly soluble in aqueous media, the addition of dimethyl sulfoxide (DMSO) as a solvent was required. DMSO was added at a ratio of 1 mL of DMSO to 90 mg of quercetin and 1 mL of DMSO for 880 mg of BHT (Neagu et al., 2010).

***Experiment 1** – Effect of quercetin on sperm parameters of ram semen subjected to cooling or freezing*

Eighty-eight ejaculates (three of 28 rams and two of two rams) were harvested and used in this experiment. Each ejaculate was split into six aliquots of equal sperm concentration and extended at 200 × 10^6 motile sperm/mL in BotuBov® (Control) according to the concentration of quercetin (Q5, 5 μg/mL; Q10, 10 μg/mL; Q15, 15 μg/mL) or DMSO (DMSO1, 0.055 μL of DMSO per ml; DMSO2, 0.165 μL of DMSO/mL) added to the extender. Groups DMSO1 and DMSO2 correspond to the amount of DMSO used as a vehicle for quercetin in groups Q5 and Q15, respectively.

***Experiment 2** – Effect of BHT on sperm parameters of ram semen subjected to cooling or freezing*

Ninety ejaculates (three per ram) were harvested and used in this experiment. Each ejaculate was divided into six aliquots of equal concentration, and extended at 200 × 106 motile sperm/mL in BotuBov® (Control) according to the concentration of BHT (BHT1, 0.5 μg/mL; BHT2, 1 μg/mL; BHT3, 1.5 μg/mL) or DMSO (DMSO3, 0.375 μL of DMSO per ml; DMSO4, 1.125 μL of DMSO/mL) that was added to the extender. Groups DMSO3 and DMSO4 correspond to the amount of DMSO used as a vehicle for BHT in groups BHT1 and BHT3, respectively.

Statistical analysis

Data analyses were performed using the GraphPad Prism 8.0.1 (GraphPad Software, San Diego, California, USA). The Gaussian distribution was evaluated using the Shapiro-Wilk normality test. Semen parameters were assessed using ANOVA and Tukey's post-hoc test. Ram was accounted as a random effect. Ejaculate order and time of storage in the extender were considered fixed effects. Significance was set at $P \leq 0.05$. All data are presented as the mean ± SD.

Results and Discussion

The overall ejaculated volume, concentration, and total sperm ejaculation obtained in the present study are shown in Table 1. There were no effects of breed (Santa Ines vs. Texel) or ejaculate order ($P > 0.05$), and rams displayed minimal variation in raw semen parameters (Table 1).

Experiment 1 – Effect of quercetin on sperm parameters of ram semen subjected to cooling or freezing

Sperm parameters were not different at 0 h among the groups ($P > 0.05$, Table S1). In addition, there were no differences in motility parameters and PMI between fresh semen and semen cooled at 5 °C for 24 h ($P > 0.05$). Sperm motility parameters and PMI in cooled and post-thawed semen extended with different concentrations of quercetin are shown in Tables 2 and 3, respectively. There was no effect of supplementation with quercetin or DMSO on the sperm parameters evaluated in the present experiment after cooling or freezing ram sperm using an egg yolk-based extender. There was a reduction in sperm parameters of frozen-thawed semen in all groups compared with pre-freezing sperm characteristics ($P < 0.05$).

Table 2
Parameters of ram sperm cooled for 24 h at 5 ºC in BotuBov® containing different concentrations of quercetin and DMSO

	Control	Q5	Q10	Q15	DMSO1	DMSO2	P value
TM (%)	89.8±5.6	89.6±5.6	89.6±5.9	89.7±4.0	89.7±6.4	88.9±6.8	0.985
PM (%)	62.1±9.5	60.2±10.5	61.3±9.4	62.3±9.6	61.9±10.0	61.7±9.0	0.978
RAP (%)	80.4±9.4	80.1±8.8	79.3±9.5	80.1±8.5	80.8±10.0	79.6±9.2	0.979
VAP (µm/s)	128.2±9.9	126.7±9.2	127.2±11.2	128.4±10.8	127.1±10.0	125.2±9.3	0.895
VSL (µm/s)	106.9±22.4	104.5±21.8	109.9±11.9	110.9±12.2	109.9±10.1	108.1±10.2	0.867
VCL (µm/s)	198.0±26.1	200.8±25.9	199.6±24.9	201.1±24.4	199.0±26.1	194.8±25.9	0.862
PMI (%)	63.4±13.4	58.7±14.8	63.1±14.4	57.3±19.5	57.7±16.5	55.6±13.9	0.442

TM, total motility; PM, progressive motility; VAP, average path velocity; VSL, velocity straight line; VCL, velocity curvilinear; RAP, rapid sperm; PMI, plasma membrane integrity. Control, egg yolk-based extender without quercetin (BotuBov®); Q5, BotuBov® containing 5 µg of quercetin/mL + 0.055 µL of DMSO/mL; Q10, BotuBov® containing 10 µg of quercetin/mL + 0.110 µL of DMSO/mL; Q15, BotuBov® containing 15 µg of quercetin/mL + 0.165 µL of DMSO/mL; DMSO1, BotuBov® containing 0.055 µL of DMSO/mL; DMSO2, BotuBov® containing 0.165 µL of DMSO/mL. All data are presented as the mean ± SD.

Table 3
Sperm parameters of post-thawed ram sperm extended and frozen in BotuBov® containing different concentrations of quercetin and DMSO

	Control	Q5	Q10	Q15	DMSO1	DMSO2	P value
TM (%)	69.5±10.4	68.5±14.9	71.2±12.8	69.2±10.2	63.0±15.9	65.0±15.5	0.420
PM (%)	38.9±9.4	41.9±11.7	41.9±11.7	41.3±9.3	37.1±10.0	37.1±10.5	0.451
RAP (%)	50.6±11.6	52.4±15.5	53.2±15.2	51.6±12.5	46.4±14.3	48.2±13.8	0.619
VAP (μm/s)	112.5±11.3	108.4±24.5	111.5±11.3	110.6±12.0	111.1±10.8	109.9±10.6	0.971
VSL (μm/s)	97.8±11.8	100.1±11.1	97.5±10.6	96.8±12.3	97.9±9.9	95.2±10.0	0.839
VCL (μm/s)	162.3±17.3	164.2±14.2	163.5±10.1	164.4±15.2	163.1±16.9	164.6±14.7	0.995
PMI (%)	25.8±9.8	25.9±12.8	27.4±11.6	24.1±8.1	24.5±13.3	20.3±10.2	0.384

TM, total motility; PM, progressive motility; VAP, average path velocity; VSL, velocity straight line; VCL, velocity curvilinear; RAP, rapid sperm; PMI, plasma membrane integrity. Control, egg yolk-based extender without quercetin (BotuBov®); Q5, BotuBov® containing 5 μg of quercetin/mL + 0.055 μL of DMSO/mL; Q10, BotuBov® containing 10 μg of quercetin/mL + 0.110 μL of DMSO/mL; Q15, BotuBov® containing 15 μg of quercetin/mL + 0.165 μL of DMSO/mL; DMSO1, BotuBov® containing 0.055 μL of DMSO/mL; DMSO2, BotuBov® containing 0.165 μL of DMSO/mL. All data are presented as the mean ± SD.

Experiment 2 – Effect of BHT on sperm parameters of ram semen subjected to cooling or freezing

Sperm parameters were not different at 0 h among the groups (P > 0.05, Table S2). In addition, there were no differences in motility parameters and PMI between fresh semen and semen cooled at 5 °C for 24 h (P > 0.05). However, there was a reduction in sperm parameters of frozen-thawed semen in all groups compared with the pre-freezing sperm characteristics (P < 0.05). Sperm motility parameters and PMI in cooled and post-thawed semen extended with different concentrations of BHT are highlighted in Tables 4 and 5, respectively. There was no effect of supplementation with BHT or DMSO on the sperm parameters evaluated in the present experiment after cooling or freezing ram sperm using an egg yolk-based extender.

The overall ejaculated volume, sperm concentration, total sperm ejaculated, and sperm motility of the rams used in this study were consistent with those of other studies on Santa Ines and Texel sheep breeds (Oliveira et al., 2017; Mandiki et al., 1998). The inclusion of quercetin or BHT in the semen with a freezing egg yolk-based extender was tested for sperm motility and viability of either cooled or frozen-thawed ram semen. Although quercetin has been reported to improve post-thawed sperm parameters of bulls (Avdatek et al., 2018; Tvrdá, Tušimová, Kováčik, Paál, & Lukáč, 2016), horses (Seifi-Jamadi et al., 2016), roosters (Appiah et al., 2020), rams (Banday, Lone, Rasool, Rashid & Shikari, 2017), buffalo (El-Khawagah, Kandiel, & Samir, 2020), and dogs (Kawasaki et al., 2020), and the inclusion of BHT improved cryopreserved sperm parameters in cats (Jara et al., 2019), dogs (Sun et al., 2020), boars (Trzcińska, Bryła, Gajda, & Gogol, 2015), goats (Memon et al., 2011), and bulls (Shoae, & Zamiri, 2008), in the present study, these substances were not able to produce better sperm motility parameters or PMI in ram semen stored at 5 °C or frozen-thawed.

Table 4
Sperm parameters of ram sperm cooled for 24 h at 5 ºC in BotuBov® containing different concentrations of BHT and DMSO

	Control	BHT1	BHT2	BHT3	DMSO3	DMSO4	P value
TM (%)	88.5±6.4	88.6±7.9	88.3±6.9	88.3±7.5	87.6±8.2	87.2±8.9	0.904
PM (%)	59.4±10.6	62.3±8.9	60.3±9.2	59.7±9.2	58.6±9.0	59.5±8.6	0.752
RAP (%)	77.9±9.7	79.9±9.7	78.0±8.6	78.1±9.8	77.6±9.6	76.9±10.4	0.871
VAP (µm/s)	126.7±10.6	127.0±11.3	123.3±10.1	125.5±12.5	124.4±9.7	124.3±9.5	0.744
VSL (µm/s)	109.0±11.5	109.8±11.3	106.5±9.6	108.2±11.9	106.9±10.1	107.6±8.5	0.842
VCL (µm/s)	195.6±26.1	196.4±26.3	192.3±25.7	189.5±46.0	194.5±28.1	192.1±26.9	0.960
PMI (%)	57.4±18.2	56.5±18.7	56.8±17.6	57.4±16.9	53.9±20.2	55.8±18.3	0.985

TM, total motility; PM, progressive motility; VAP, average path velocity; VSL, velocity straight line; VCL, velocity curvilinear; RAP, rapid sperm; PMI, plasma membrane integrity. Control, egg yolk-based extender without BHT (BotuBov®); BHT1, BotuBov® containing 0.5 µg of BHT/mL + 0.375 µL of DMSO/mL; BHT2, BotuBov® containing 1.0 µg of BHT/mL + 0.750 µL of DMSO/mL; BHT3, BotuBov® containing 1.5 µg of BHT/mL + 1.125 µL of DMSO/mL; DMSO3, BotuBov® containing 0.375 µL of DMSO/mL; DMSO4, BotuBov® containing 1.125 µL of DMSO/mL. All data are presented as the mean ± SD.

Table 5
Sperm parameters of post-thawed ram sperm extended and frozen in BotuBov® containing different concentrations of BHT and DMSO

	Control	BHT1	BHT2	BHT3	DMSO3	DMSO4	P value
TM (%)	68.7±13.9	69.8±11.4	68.9±13.8	70.3±12.3	68.8±14.7	64.9±14.3	0.849
PM (%)	36.8±10.2	35.6±9.4	36.2±9.6	37.2±9.8	36.3±11.2	33.6±9.7	0.849
RAP (%)	47.7±14.0	47.9±12.9	47.5±13.6	49.1±14.4	47.6±14.4	43.9±13.6	0.841
VAP (µm/s)	106.5±15.2	106.7±13.8	106.6±14.4	107.1±16.4	106.7±17.1	103.9±12.2	0.972
VSL (µm/s)	92.0±14.4	90.4±12.7	91.7±14.5	91.6±15.3	90.3±13.2	89.7±12.3	0.993
VCL (µm/s)	159.4±23.1	163.9±19.3	160.1±19.4	163.5±22.9	160.8±20.1	160.9±17.7	0.909
PMI (%)	33.1±16.9	32.6±16.3	28.5±14.3	29.4±15.3	30.9±15.6	28.9±13.6	0.842

TM, total motility; PM, progressive motility; VAP, average path velocity; VSL, velocity straight line; TM, total motility; PM, progressive motility; VAP, average path velocity; VSL, velocity straight line; VCL, velocity curvilinear; RAP, rapid sperm; PMI, plasma membrane integrity. Control, egg yolk-based extender without BHT (BotuBov®); BHT1, BotuBov® containing 0.5 µg of BHT/mL + 0.375 µL of DMSO/mL; BHT2, BotuBov® containing 1.0 µg of BHT/mL + 0.750 µL of DMSO/mL; BHT3, BotuBov® containing 1.5 µg of BHT/mL + 1.125 µL of DMSO/mL; DMSO3, BotuBov® containing 0.375 µL of DMSO/mL; DMSO4, BotuBov® containing 1.125 µL of DMSO/mL. All data are presented as the mean ± SD.

It is well known that sperm motility parameters and fertility are negatively affected by the overproduction of ROS and membrane lipid peroxidation, and findings from previous studies demonstrated that these antioxidants prevent sperm damage caused by oxidative stress (Guthrie & Welch, 2012). However, similar to our results, other studies have also shown no beneficial effect of including quercetin or BHT on motility parameters of cryopreserved

semen from bulls, rabbits, buffalo (Avdatek et al., 2018; Johinke & Bathgate, 2014; Ijaz, Hussain, Aleem, Yousaf, & Rehman, 2009), and ram sperm (Silva, Cajueiro, Silva, Soares, & Guerra, 2012; Watson, & Anderson, 1983). It is important to note that the inclusion of DMSO was tested at the lowest and highest concentrations used as a solvent for both quercetin and BHT in the present study and it did not produce any beneficial or adverse effects on sperm parameters. Although DMSO has been used for semen cryopreservation in other species (e.g., turkey, pheasant, red-tailed hawk, sandhill crane, Indian red jungle fowl, Peregrine falcon) (Rakha et al., 2018; Iaffaldano et al., 2016; Herrera, Quintana, Lopez, Betancourt, & Fierro, 2005; Gee, Baskt, & Sexton, 1985), higher concentrations of DMSO can lead to ultrastructural damage of the sperm membrane (Gurtovenko & Anwar, 2007). Therefore, the doses tested in the present study were safe for ram sperm.

Contradictory results using antioxidants for sperm cryopreservation are common in the literature (Banday et al., 2017; Silva et al., 2012; Sikka, 2004). However, the authors assumed that the differences in experimental methodology, the composition of the extender, and animal species may have affected these results. Interestingly, ram sperm is highly vulnerable to oxidative stress because of the high polyunsaturated fatty acid content in the plasma membrane (Bucak et al., 2010). Therefore, the antioxidants tested in the present study may not have sufficient action to overcome oxidative stress in this species. Quercetin inhibits ROS formation by enzymatic and non-enzymatic systems (e.g., NADPH oxidase, NADH-dependent oxidoreductase) (Li, 2011; Delmas, Jannin, & Latruffe., 2005), while BHT converts the peroxy radicals to hydroperoxides, thereby interrupting the development of oxidative processes (Seifi-Jamadi et al., 2016). Although Banday et al. (2017) reported a beneficial effect of quercetin on sperm motility in ram cryopreserved semen diluted in Tris extender, these authors reported that taurine at 40 mM produced better results than the other tested antioxidants (e.g., quercetin and glutathione). Interestingly, taurine is neither a classical scavenger nor a regulator of antioxidative defenses; it protects the mitochondria against excessive superoxide generation by regulating mitochondrial protein synthesis (Holt, 1997). Therefore, different antioxidant mechanisms of action or a synergistic effect may improve the regulation of oxidative stress in this species and should be tested in future studies.

Another difference that should be noted is that an egg yolk-based extender with an undefined composition (BotuBov®) was used for processing ram semen in the present study. In contrast, quercetin and BHT were added to a Tris extender in previous studies. The composition of the extender is a factor that could impact the results presented here, because sperm parameters were not affected after cooling at 5 °C for 24 h, even in the control group. However, it is not possible to discern the composition of the extender used in the present study because it is a commercially available extender with a protected composition.

Conclusions

These results suggest that the commercially available extender BotuBov® effectively protects ram sperm against cold

shock during liquid storage at 5 °C for up to 24 h. However, the inclusion of quercetin or BHT in BotuBov® does not enhance sperm motility and PMI of ram sperm subjected to cooling at 5 °C for 24 h or freezing processes. Further studies are needed to assess the fertility rates of cooled or frozen-thawed ram semen with the inclusion of BHT and quercetin.

Conflicts of interest

The authors declare no conflict of interest.

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