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Evaluation of the *in vitro* cryopreservative performance of Juniper berry oil (*Juniperus communis*) on frozen-thawed bull semen

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Abstract

This study aimed to reveal the effects of juniper (*Juniperus communis*) berry oil (JB) when added to the Tris extender, on bull (Holstein) sperm motility characteristics, abnormal sperm ratio, DNA fragmentation and some oxidative stress markers. The pooled ejaculate was divided into five equal parts as control (C) and Tris diluent containing 25, 50, 100, 200 µg/ml JB. Diluted semen was incubated at 4 °C for 4 h and then frozen in liquid nitrogen (−196 °C). After freezing-thawing, motility characteristics were determined using a sperm analyzer system. The results showed that the 25 µg/ml dose of JB had an improving effect on overall sperm motility ($P < 0.05$). The total abnormal spermatozoa ratio was not significantly different between the groups ($P > 0.05$). The JB25 and JB50 groups had higher DNA integrity than the other groups ($P < 0.05$). This finding was supported by low malondialdehyde levels ($P < 0.05$). Especially the 25 µg/ml dose of JB was found to have a remedial effect on DNA integrity, as well as increasing progressive and total motility rates. This study has revealed new information on the effects of JB on bull semen cryopreservation. Juniper berry oil may be used as an antioxidant in bull semen freezing.

Cattle, oxidative stress, cryopreservation, DNA damage, motility

Cryopreservation has detrimental effects on mammalian spermatozoa (Hafez 1987). The increase in reactive oxygen species (ROS) during the dilution, cooling, and freezing stages of semen reduce cell function, and eventually, the gradual decrease in motility and morphological integrity reduces sperm apoptosis and fertilization ability (O'Connell et al. 2002; Meyers 2005). It is estimated that about 50% of spermatozoa die or get damaged during the semen freezing phase. Therefore, studies have focused on either eliminating or mitigating unintended effects of the freezing and thawing procedure on spermatozoa (Watson 2000). Reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are the final products after the mitochondrial and enzymatic activities of cells. These reactive species that cause oxidative stress cause DNA fragmentations (Taşdemir et al. 2013), degeneration in protein and cell membrane lipids (Lubos et al. 2011), and changes in spermatozoon metabolism (Guthrie and Welch 2012). The adverse effects of pre-and post-freezing applications on spermatozoa are directly linked to the extender composition and antioxidant substances injected into the extender (Mocé and Vicente 2009). For this reason, research has focused on adjusting extender composition and exploring effective

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reliable alternative antioxidants. Due to the low toxicity of natural antioxidant products, their use as an additive to semen extenders has come to the fore compared to synthetic ones (Hashem et al. 2013). Juniper berry oil (JB) consists of monoterpene hydrocarbons such as β-pinene (5.0%), limonene (5.1%), sabinene (5.8%), myrcene (8.3%) and α-pinene (51.4%) (Höferl et al. 2014). It is anticarcinogenic, it is used in the treatment of a number of skin diseases and aromatically, and it shows antioxidant properties by removing free oxygen radicals and blocking the oxidation of cells (Medini et al. 2011). It was determined that its antioxidant property manifests itself by contributing to electron transfer and increasing the catalase activities, superoxide dismutase and glutathione peroxidase (Höferl et al. 2014). The hypothesis of this study was that JB reduces the negative effects of freezing on spermatozoa. Until today, no studies have shown the effects of JB on the semen quality after freezing. The objective of the study was to reveal the effects of JB on bull spermatozoa motility characteristics, abnormal spermatozoa ratio and DNA fragmentation when JB was added to the Tris extender.

Materials and Methods

Animals and the study design

The ejaculates used in the study were obtained from four healthy 2–4-year-old Holstein bulls. The sperm sample was obtained at two times a week via an artificial vagina and placed in a water bath at 37 °C. The total ejaculate volume was determined using graduated tubes. The sperm concentration was evaluated with the aid of a photometer (Minitube GmbH, Tiefenbach, Germany). Fresh semen was placed on a hot plate and the activity of mass was determined. The sperm source was a specimen of at least 4 ml, with material that had an activity of mass > 3 (grading 1–5) and a density > 800 × 10^6/ml. After that, all specimens were mixed to eliminate variations from increased volume and individual bulls. In an effort to avoid any standard errors, the study was replicated 10 different times along a similar seasonal period (from January to April). Ethical approval of the animal experiments was granted by the Animal Care Committee of Afyon Kocatepe University (approval no. 49533702/29).

Semen processing and freezing

The basic extender was used as a Tris-based extender (Tris 30.7 g, citric acid 16.4 g, fructose 12.6 g, purified water 1000 ml, yolk 20% v/v and 6 percent glycerol) as specified by Taşdemir et al. (2013); pH was set to 6.8. Juniper berry oil used in the study was collected from the forested land. Following the identification of its species by expert botanists, it was stored after a specimen number was assigned to it (voucher number: 2017/02). Extraction was performed with ethanol (99%, Merck, Darmstadt, Germany), carbon dioxide (99%) and distilled water by using gallic acid (≥ 98.5%, Sigma-Aldrich Chemical, Interlab Ltd., Ankara, Türkiye) as described by Braga et al. (2008) to ensure standardisation. At the time of extraction, carbon dioxide was applied using a high-pressure liquid compressor at a pressure of 15 MPa, while ethanol was applied using a high pressure liquid pump (L-6200A, Hitachi, Merck). During the application, the temperature was set at 30 °C, and the flow rate was set at 7.5 ± 2.5 kg/s (L-6200A, Hitachi, Merck). For the stock solution to be used for the experimental groups, 10 mg of JB was taken from the extracted JB and mixed with 1 ml of ethanol (Merck, 99%) to dissolve, and the JB stock solution was prepared. The prepared mixture was divided into five parts. One was used as a control group (without additive). The other four groups were used as experimental groups consisting of semen samples extended with the Tris extender, and contained 25, 50, 100 and 200 µg/ml JB, respectively, after preliminary studies. Osmolarities of all samples were set to 310 mOsm. The diluted semen was equilibrated at 4 °C in a cold air cabin for 4 h. It was then placed in 0.25 ml straws with 16×10^6 sperm cells in each straw, followed by freezing by using an automatic sperm-freezing machine (SY LAB Gerate GmbH, Neupurkersdorf, Austria) according to Avdatek et al. (2022). Frozen sperm specimen was stored in a liquid nitrogen tank for 12 weeks. After this procedure, the straws were thawed for 30 s in a 37 °C hot water bath and evaluated for spermatological parameters after thawing procedure.

Spermatozoon motility characteristics

Spermatozoon motility properties were assessed by the Computer-Assisted Semen Analyzer system (CASA) (Microptic S.L., Barcelona, Spain). Five µl semen samples diluted with Ringer's lactate at a ratio of 1:4 were placed on a pre-heated 20 mm slide in the device adjusted for bull semen samples (Leja 4, Leja Products BV, Nieuw-Vennep, The Netherlands). Total, progressive, and non-progressive motility of sperm was evaluated with the aid of a ×10 magnification microscope eyepiece at 37 °C. The spermatozoon motility features were set as fast (> 80 µm/s), medium (> 60 µm/s), slow (> 20 µm/s) and static protocols. Afterwards, the following motility characteristics were measured: Average path velocity (VAP) µm/s, curvilinear velocity (VCL) µm/s, straight linear velocity (VSL) µm/s, straightness (STR, [VSL/VAP]×100), linearity (LIN, [VSL/VCL]×100), beat cross frequency (BCF), amplitude of lateral head displacement (ALH) µm, wobble (WOB, [VAP/VCL] × 100), and hyperactivity µm·s^{-1}. During each measurement, an average of 300 spermatozoa were microscopically evaluated in 8 various places.

Abnormal spermatozoon rate

The abnormal spermatozoa rate in samples were assessed by the liquid fixation technique. One to two drops of semen sample were dropped into 1 ml of Hancock fluid for fixation. After thorough mixing, a spot of semen from the mixture was taken on a slide, covered with a coverslip, and 400 spermatozoa were counted under an immersion objective and a ×1000 phase-contrast microscope, and the rate of abnormal spermatozoa was recorded as %. Structures other than normal spermatozoa were accepted as abnormal, head, middle and tail of spermatozoa abnormality were evaluated (Schaäfer and Holzmann 2000).

Spermatozoon DNA integrity

The single-cell gel electrophoresis (COMET) method was used to determine the integrity of spermatozoon DNA. Straws taken from the nitrogen tank were thawed at 37 °C and transferred to Eppendorf tubes. They were diluted at a rate of 1:1 with a Ca^{2+} and Mg^{2+} free phosphate buffer solution (PBS) and washed by centrifuging for 10 min at 800 × g, where the temperature was set to 4 °C. This procedure was repeated twice. Then, the supernatant was added, and the mixtures were diluted with PBS to obtain 20×10^4 sperm cells per ml. One hundred twenty µl of 0.75% low-melting agarose (LMA) prepared in PBS were taken and dropped on specially frosted slides, and then spread in the form of smear and left to dry at room temperature. Five µl of the diluted sperm suspension was taken and mixed with 85 µl 1% LMA gel at 37 °C, and then layered on the first agarose layer and covered with a 24 × 60 mm cover slide and kept to fixate at 4 °C. After the solidification process, the cover slides were carefully lifted, and the bottom slides were prepared. The slides were incubated at 4 °C for about 1 h in a lysis solution in a CometAssayTM, Reagent Kit for Single Cell Gel Electrophoresis Assay, which includes high amounts of salt and detergent. After 1 h, 40 mM dithioerythritol were put into the lysis solution, and the slides were incubated at the same temperature and duration. Next, 100 µg/ml proteinase-K were added to the lysis solution and the slides were incubated for one night at 37 °C. The slides were left for 20 min in a freshly prepared and cooled electrophoresis buffer to separate DNA chains before they were subjected to electrophoresis. After the incubation of the spermatozoa embedded in agarose was completed in the electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 12.5), the DNAs were processed in this buffer solution with 300 mA and 20 V electric fields for 20 min. The slides were neutralised by washing three times with freshly prepared Tris buffer (40 mM Tris HCl, pH 7.4). Slides stained with ethidium bromide (5 µg/ml) were covered with cover slides, and 100 DNA images were determined by fluorescent microscope at ×100 magnification (Olympus CX-31). The analysis was conducted by using the Tritek Comet Score Software.

Spermatozoon oxidative stress markers

Glutathione (GSH) level was assessed via using the steps outlined by Sedlak and Lindsay (1968). The specimens were centrifuged at 1000 × *g* for 5 min after precipitating with 50% trichloroacetic acid. The concentration of the solution was determined by measuring it at 412 nm using a spectrophotometer. The reaction mixture included 2.0 ml Tris-EDTA buffer (0.2 mol/l, pH 8.9), 0.1 ml 0.01 mol/l 5,5′-dithiobis (2-nitrobenzoic acid) and 0.5 ml supernatant. The GSH values found as a result of the analysis are presented in units of nmol/ml. The malondialdehyde (MDA) level, and thus the lipid peroxidation level was measured using a commercial kit (MDA-586, OxisResearch, Portland, USA). The MDA values that were found are presented in units of µmol/ml. Before the analyses, the data gathered in the study were tested for distribution based on the kurtosis value, assumptions one of parametric tests. The spread of each variable was calculated and shown as mean ± standard error of the mean. The main effect of treatment, time and the interaction term of treatment × time upon the spermatological indices were analysed by the general linear model multivariate repeated measures of SPSS 13.0 (SPSS Inc. Headquarters, Chicago, Illinois, USA). The significance of the difference in the mean of the data was determined by Duncan *post hoc* test, and $P < 0.05$ was considered as significant. Duncan's *post hoc* test was performed using simple effect analysis of treatment groups (25, 50, 100 and 200 µg/ml JB). A total of 50 applications were tested in 10 replications, and each treatment prosedure was conducted independently with the pair considered as the experiment.

Results

The comparison of the treatment groups to the control group showed that the addition of JB to the Tris semen extender at a dose of 25 µg/ml had an ameliorative effect on both total (59.51 ± 2.75%) and progressive (36.40 ± 1.73%) sperm motility (Table 1; $P < 0.05$). It was found that a 100 µg/ml dose (44.61 ± 4.68%) was the limit value and administration of JB on 200 µg/ml (35.99 ± 3.56%) caused harmful effects on sperm cells ($P < 0.05$). Although significant differences were found between the groups when they were assessed in terms of motility characteristics other than the VAP, BCF and STR characteristics, no positive impact of the treatment on these characteristics could be found. In addition to this, it was observed that adverse effects arose due to toxicity above the threshold value.

On the other hand, the total abnormal spermatozoa ratio was not significantly different between the groups (Table 1; $P > 0.05$). The DNA integrity values were significantly different between the groups ($P < 0.05$). The JB25 (17.18 ± 0.91%) and JB50 (17.91 ± 1.87%) groups, in particular, were found to have greater DNA integrity values compared to the other treatment other groups and the control group (25.26 ± 1.66%) (Table 2; $P < 0.05$). Malondialdehyde levels were lower in all treatment groups compared to the control group ($P < 0.05$), but such low MDA levels were not supported by the results on GSH activity (Table 3).

Table 1. Sperm motility and kinetic indicators of freeze-thawing bull semen (mean ± SEM) after addition of different doses of juniper berry oil (JB).

	Group					
Indicator	Control	JB25	JB50	JB100	JB200	*P*
Non-progressive motility (%)	19.06 ± 3.45	23.10 ± 2.18	19.84 ± 2.60	17.82 ± 1.48	16.60 ± 1.41	–
Progressive motility (%)	29.51 ± 3.88^{b}	36.40 ± 1.73^{a}	32.62 ± 2.35^{ab}	26.77 ± 3.30^{bc}	19.38 ± 2.39^{c}	*
Total motility (%)	48.57 ± 6.40^{bc}	59.51 ± 2.75^{a}	52.46 ± 3.52^{ab}	44.61 ± 4.68^{bc}	35.99 ± 3.56^{c}	*
VAP (µm/s)	88.66 ± 4.94	88.07 ± 2.62	86.08 ± 2.85	84.87 ± 1.94	79.32 ± 2.61	–
VSL (µm/s)	73.44 ± 4.97^{a}	70.86 ± 2.71^{ab}	71.05 ± 2.76^{ab}	68.31 ± 1.87^{ab}	63.13 ± 3.09^{b}	*
VCL (µm/s)	120.02 ± 5.31	120.40 ± 2.86	118.63 ± 3.91	120.40 ± 2.26	112.58 ± 2.50	–
ALH (µm/s)	4.22 ± 0.14^{b}	4.40 ± 0.02^{ab}	4.40 ± 0.13^{ab}	4.62 ± 0.05^{a}	4.62 ± 0.02^{a}	*
BCF (Hz)	11. 68 ± 0.64	11. 30 ± 0.35	11.23 ± 0.31	11.14 ± 0.46	10.37 ± 0.23	–
LIN (%)	60.82 ± 1.96^{ab}	58.68 ± 1.03^{ab}	59.85 ± 0.97^{ab}	56.68 ± 0.85^{ab}	55.80 ± 1.53^{b}	*
STR (%)	82.46 ± 1.53	81.21 ± 0.90	82.42 ± 0.80	80.44 ± 0.59	79.26 ± 1.35	–
WOB (µm/s)	73.61 ± 1.13^{a}	72.22 ± 0.62^{ab}	72.58 ± 0.55^{ab}	70.47 ± 0.69^{c}	70.32 ± 1.35^{c}	*
Hyperactivity (µm/s)	39.10 ± 5.40^{ab}	45.68 ± 2.74^{a}	44.37 ± 3.97^{ab}	32.80 ± 4.12^{bc}	23.95 ± 2.51^{c}	*
Total abnormalities (%)	11.37 ± 2.83	6.44 ± 1.34	8.89 ± 0.85	10.10 ± 0.71	7.66 ± 1.72	–

SEM - standard error of the mean; VAP - average path velocity; VSL - straight linear velocity; VCL - curvilinear velocity; ALH - amplitude of lateral head displacement; BCF - beat cross frequency; LIN - linearity; STR - straightness; WOB - wobble

^{a,b,c} Different superscripts within the same row demonstrate significant differences (*$P < 0.05$); – - no significant difference ($P > 0.05$)

Table 2. Chromatin damage indicators in freeze-thawing bull semen (mean ± SEM) after addition of different doses of juniper berry oil (JB).

	Group					
Indicator	Control	JB25	JB50	JB100	JB200	*P*
Tail lenght (µm/s)	12.43 ± 2.70^{ab}	6.97 ± 0.74^{cd}	5.04 ± 0.44^{d}	9.52 ± 1.55^{bc}	15.65 ± 0.62^{a}	*
Tail DNA (%)	25.26 ± 1.66^{a}	17.18 ± 0.91^{b}	17.91 ± 1.87^{b}	24.18 ± 1.05^{a}	19.18 ± 1.23^{b}	*
Tail moment (µm/s)	14.94 ± 2.88^{b}	4.08 ± 0.88^{c}	5.36 ± 1.28^{c}	10.55 ± 1.62^{b}	26.42 ± 1.02^{a}	*

SEM - standard error of the mean

^{a,b,c,d} Different superscripts within the same row demonstrate significant differences (*$P < 0.05$)

Table 3. GSH and MDA levels in freeze-thawing bull semen (mean ± SEM) after addition of different doses of juniper berry oil (JB).

	Group					
Indicator	Control	JB25	JB50	JB100	JB200	*P*
GSH (nmol/ml)	70.32 ± 6.16^{ab}	56.27 ± 3.05^{bc}	63.57 ± 3.40^{bc}	79.51 ± 1.51^{a}	46.90 ± 3.21^{b}	*
MDA (µmol/ml)	1.84 ± 0.12^{a}	0.68 ± 0.04^{c}	0.96 ± 0.03^{bc}	1.25 ± 0.12^{b}	1.13 ± 0.07^{b}	*

GSH - glutathione; MDA - malondialdehyde; SEM - standard error of the mean

^{a,b,c} Different superscripts within the same row demonstrate significant differences (*$P < 0.05$)

Discussion

Motility, which is one of the notable characteristics in defining the fertilization ability of a sperm cell, is specified as the proportion of sperm cells moving in a straightforward direction to all sperm cells in the assessed area (Hafez 1987). In our study, JB added at a dose of 25 μg/ml had a remedial effect on both progressive and total sperm motility although there was no significant difference in the other treatment groups ($P < 0.05$). In line with these findings, Khan et al. (2017) reported that green tea had an advance effect on motility, and the best result was obtained from its addition to the extender at a rate of 0.75%. Similarly, Daghigh Kia et al. (2014) reported that the use of rosemary extract alone (10 g/l) and the use of it with glutathione (5 mM GSH with 5 or 10 g/l) increased motility in bull semen. However, the values of characteristics obtained in their study were found to be lower than those in our findings. Active substances such as α- and β-pinene are common in JB extracts, and thus, it was interpreted that JB showed similar results to both green tea and rosemary extracts by reducing lipid peroxidation. In studies on different species compatible with our results, it has been shown that the addition of astaxanthin to semen diluent increases the motility of pig spermatozoa, but there is no difference in terms of sperm motility characteristics (Lee and Kim 2018). In another study on the *in vitro* capacitation of ram spermatozoa, it was shown that the oregano (*Origanum vulgare*) extract, a natural antioxidant, had a healing effect on live spermatozoa and motility (Alenezy et al. 2019). A study on rams revealed that fennel (*Foeniculum vulgare*) extract does not have a promoting effect on the total abnormal spermatozoa ratio (Najafi et al. 2019). In accordance with our results, Taşdemir et al. (2020) stated in their study on bulls that the pine tree extract had no favourable effect on the total abnormal spermatozoa ratio. Yeni et al. (2018) reported that rosmarinic acid that they used as an antioxidant in their study did not make a positive contribution to the total abnormal spermatozoa ratio. Given the results, we believe that the reason why the progressive and total sperm motility values in the JB25 group in our study were different from those in the C group was that JB decreases the negative effects of oxidative stress measures by MDA levels on motility under *in vitro* conditions when used at appropriate doses.

In contrast to our study, it has been reported in a study on bull semen that when different doses of the spirulina (*Spirulina maxima*) extract were added to the BIOXcell extender and frozen, a 4 μg/ml dose of the spirulina extract had a therapeutic effect on spermatozoon motility characteristics except for ALH (Mizera et al. 2019). We believe that the cause of this difference was the antioxidants that were added as well as the fact that different extender compositions were used. In a study on bulls with results that were inconsistent with the results of our study, it was found that a Tris semen diluent enriched with silymarin had no favourable effect on motility (El-Sheshtawy and El-Nattat 2017). In another experiment, green tea extract was found to have no therapeutic effect on spermatozoon motility, but the findings in terms of spermatozoon motion characteristics were similar to those in our study (İnanç et al. 2019). Considering the findings of these researchers, we speculate that the main reason for the lack of positive effects of the antioxidant substances used in their studies on motility was the cryoprotective positive effect on spermatozoa as a result of the mitigation of ROS by other cryoprotectant substances added to the Tris extender.

At the stages of freezing and thawing spermatozoa, DNA integrity is damaged so much that it would even affect post-fertilisation embryo development by causing molecular and epigenetic changes (Lewis and Aitken 2005). DNA damage can be caused by damage to the genomic region due to osmotic stress (Kopeika et al. 2015), in addition to excessive ROS accumulation due to oxidative stress (Tirpak et al. 2021). Incompatible with the results of our study, where the DNA integrity levels in the JB25 and JB50 groups were

found to be higher than those in the other groups ($P < 0.05$), the date palm extract (Dwitya et al. 2019) and royal jelly (Shahzad et al. 2016) added to the Tris extender at different rates were reported to have no curative effect on spermatozoon DNA integrity values of bulls and water buffalo bulls, respectively. It was reported that the aloe vera extract added to the Tris extender (10% and 20%) during the freezing phase of semen taken from cat epididymis had no positive effect on spermatozoon DNA integrity, either (Barbosa et al. 2020). Although Tris extenders were used in these studies as was the case in our study, it was interpreted that the added antioxidant materials did not exhibit a positive activity on spermatozoon DNA integrity, compatible with the diluent composition. Supporting the DNA integrity result identified in the JB25 and JB50 groups in this study, it was demonstrated that 2, 4 and 6 µg/ml spirulina extract had a protective effect on spermatozoon DNA integrity, and a toxic effect when > 6 µg/ml of it was used (Mizera et al. 2019). The findings of this study were in favour of the findings of the studies conducted by our researcher group in recent years, where we have used various antioxidants (rosmarinic acid, pine bark tree extract, vaccenic acid, hesperidin, thymoquinone) due to their remedial effects on DNA integrity (Yeni et al. 2018; Taşdemir et al. 2020; Avdatek et al. 2022; İnanç et al. 2022; Yeni et al. 2022). It was concluded that as an antioxidant, JB at appropriate doses (25 and 50 µg/ml) maintains the integrity of DNA and this antioxidant property of JB manifests its healing effect on DNA damage by creating an additive effect along with other cryoprotectants in the extender. However, it should be noted that it will have a toxic effect if the amount used exceeds the threshold value.

Malondialdehyde markers are commonly used to determine lipid peroxidation (Zhang et al. 2017). In support of the results of our study where the MDA levels were determined to be low in all treatment groups compared to control, it was reported that 4 ml/dl oregano extract in a study on Holstein bulls (Daghigh Kia et al. 2016) and crocin in another study on bulls had positive effects on *in vitro* sperm indices by lowering the MDA levels (Sapanidou et al. 2015). Likewise, it was reported that 10 mg/ml fennel extract had a promoting effect on progressive and total motility by lowering the MDA levels in rams (Najafi et al. 2019). However, in this study, the low MDA levels were not supported by the GSH activity results. Glutathione has the ability to maintain the biological value of the germ cells. Its presence has been determined both intracellularly in spermatozoa and extracellularly in seminal plasma (Bisht et al. 2017). It was reported that the reduction in the level of GSH is around 80% due to freezing and thawing in bull semen (Bilodeau et al. 2000). Besides, GSH reduction is higher in the extender composition with egg yolk (Stradaioli et al. 2007). These results were considered likely to be because GSH activity decreased due to the egg yolk included in the extender, and this decrease was effective on progressive motility, except for the JB25 group.

In conclusion, it was demonstrated in this study that JB added to the Tris extender reduced MDA levels and when 25 µg/ml or 50 µg/ml of JB was added, it had a positive effect on DNA integrity. Especially the 25 µg/ml dose was found to have a remedial effect on DNA integrity, as well as increasing progressive and total motility rates.

Conflict of interest

The authors declare that they have no conflicts of interest.

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References

Alenezy ES, Barakat IAH, Al Musayeib NM 2019: Effect of wild marjoram (*Origanum vulgare*) plant extracts on capacitation of sheep spermatozoa *in vitro*. Adv Biosci Biotechnol **10**: 86-97

Avdatek F, Taşdemir U, İnanç ME, Yeni D, Gülhan MF, Güngör Ş, Türkmen R, Tuncer PB 2022: Cryoprotective effect of vaccenic acid supplemented in bull semen extender. Turkish J Vet Anim Sci **46**: 100-106

Barbosa BS, Santos FA, Macêdo LB, Izzo RG, Fernandes DP, Praxedes É. Silva AR, Bezerra MB 2020: Effect of supplementation of aloe vera extracts in cold storage media and cryopreservation of domestic cat epididymal spermatozoa. Anim Reprod **1**: 7-9

Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C 2000: Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev **55**: 282-288

Bisht S, Faiq M, Tolahunase M, Dada R 2017: Oxidative stress and male infertility. Nat Rev Urol **14**: 470-484

Braga MEM, Santos RMS, Seabra IJ, Facanali R, Márcia OM, Marques MOM, de Souza HC 2008: Fractioned SFE of antioxidants from maritime pine bark. J Supercrit Fluids **4**: 37-48

Daghigh Kia H, Farhadi R, Ashrafi I, Mehdipour M 2016: Anti-oxidative effects of ethanol extract of *Origanum vulgare* on kinetics, microscopic and oxidative parameters of cryopreserved Holstein bull spermatozoa. Iran J Appl Anim Sci **6**: 783-789

Daghigh Kia H, Olfati-Karaji R, Hoseinkhani A, Ashrafi I 2014: Effect of rosemary (*Rosmarinus officinalis*) extracts and glutathione antioxidants on bull semen quality after cryopreservation. Span J Agric Res **12**: 98-105

Dwitya Y, Eriani K, Saputra H, Azhar A, Rizal M 2019: Cryopreservation of Aceh cattle semen with date (*Phoenix dactylifera*) extract supplementation. Biosaintifika J Biol Educ **11**: 117-124

El-Sheshtawy RI, El-Nattat WS 2017: Impact of silymarin enriched semen extender on bull sperm preservability. Asian Pac J Reprod **6**: 81-84

Guthrie HD, Welch GR 2012: Effects of reactive oxygen species on sperm function. Theriogenology **78**: 1700-1708

Hafez ESE 1987: Semen evaluation. Reprod Farm Anim **5**: 455-480

Hashem NM, El-Hady AA, Hassan O 2013: Effect of vitamin E or propolis supplementation on semen quality, oxidative status and hemato-biochemical changes of rabbit bucks during hot season. Livest Sci **157**: 520-526

Höferl M, Stoilova I, Schmidt E, Wanner J, Jirovetz L, Trifonova D, Krastev L, Krastanov A 2014: Chemical composition and antioxidant properties of juniper berry (*Juniperus communis L.*) essential oil. Action of the essential oil on the antioxidant protection of *Saccharomyces cerevisiae* model organism. Antioxidants **3**: 81-98

İnanç ME, Çil B, Yeni D, Avdatek F, Orakçi D, Tuncer PB, Türkmen R, Taşdemir U 2019: The effect of green tea extract supplementation in bull semen cryopreservation. Kafkas Üniv Vet Fak Derg **25**: 703-708

İnanç ME, Güngör Ş, Avdatek F, Yeni D, Gülhan MF, Olğaç KT, Denk B, Taşdemir U 2022: Thymoquinone improves motility, plasma membrane integrity and DNA integrity of frozen–thawed ram semen. Andrologia **54**: e14547

Khan H, Khan M, Qureshi MS, Ahmad S, Gohar A, Ullah H, Hussain A, Khatri P, Shahn SSA, Rehman H, Khan A 2017: Effect of green tea extract (*Camellia sinensis*) on fertility indicators of post-thawed bull spermatozoa. Pakistan J Zool **49**: 1243-1249

Kopeika J, Thornhill A, Khalaf Y 2015: The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod **21**: 209-227

Lee E, Kim D 2018: Effects of astaxanthin on miniature pig sperm cryopreservation. Biomed Res Int **4**: 1-9

Lewis SEM, Aitken RJ 2005: DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res **322**: 33-41

Lubos E, Loscalzo J, Handy DE 2011: Glutathione peroxidase-1 in health and disease: From molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal **15**: 1957-1997

Medini H, Elaissi A, Khouja ML, Piras A, Porcedda S, Falconieri D, Marongiu B, Chemli R 2011: Chemical composition and antioxidant activity of the essential oil of *Juniperus phoenicea* L. berries. Nat Prod Res **25**: 1695-1706

Meyers S 2005: Spermatozoal response to osmotic stress. Anim Reprod Sci **89**: 57-64

Mizera A, Kuczaj M, Szul A 2019: Impact of the *Spirulina maxima* extract addition to semen extender on bovine sperm quality. Ital J Anim Sci **18**: 601-607

Mocé E, Vicente JS 2009: Rabbit sperm cryopreservation: A review. Anim Reprod Sci **110**: 1-24

Najafi A, Daghigh Kia H, Mehdipour M, Shamsollahi M, Miller DJ 2019: Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm? Cryobiology **87**: 47-51

O'Connell M, McClure N, Lewis SEM 2002: The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod **17**: 704-709

Sapanidou V, Taitzoglou I, Tsakmakidis I, Kourtzelis I, Fletouris D, Theodoridis A, Zerv, I, Tsantarliotou M 2015: Antioxidant effect of crocin on bovine sperm quality and *in vitro* fertilization. Theriogenology **84**: 1273-1282

Schaäfer S, Holzmann A 2000: The use of transmigration and spermac TM stain to evaluate epididymal cat spermatozoa. Anim Reprod Sci **59**: 201-211

Sedlak J, Lindsay RH 1968: Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem **25**: 192-205

Shahzad Q, Mehmood MU, Khan H, Husna AU, Qadeer S, Azam A, Naseer Z, Ahmad E, Safdar M, Ahmad M 2016: Royal jelly supplementation in semen extender enhances post-thaw quality and fertility of Nili-Ravi buffalo bull sperm. Anim Reprod Sci **167**: 83-88

Stradaioli G, Noro T, Sylla L, Monaci M 2007: Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: Comparison between two extenders. Theriogenology **67**: 1249-1255

Taşdemir U, Büyükleblebici S, Tuncer PB, Coşkun E, Özgürtaş T, Aydin FN, Büyükleblebici SO, Gürcan IS 2013: Effects of various cryoprotectants on bull sperm quality, DNA integrity and oxidative stress parameters. Cryobiology **66**: 38-42

Taşdemir U, Yeni D, İnanç ME, Avdatek F, Çil B, Türkmen R, Güngör Ş, Tuncer PB 2020: Red pine (*Pinus brutia ten*) bark tree extract preserves sperm quality by reducing oxidative stress and preventing chromatin damage. Andrologia **52**: e13603

Tirpak F, Greifova H, Lukac N, Stawarz R, Massanyi P 2021: Exogenous factors affecting the functional integrity of male reproduction. Life **1**: 213

Watson PF 2000: The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci **60-61**: 481-492

Yeni D, Avdatek F, Tuncer PB, Çil B, Türkmen R, Taşdemir U 2018: Supplementation of rosmarinic acid has reduced oxidative stress on bull spermatozoa following the freeze thawing process. Cryoletters **39**: 156-165

Yeni D, Güngör Ş, Avdatek F, Gülhan MF, Olğaç KT, İnanç ME, Denk B, Taşdemir U 2022: Investigation of changes in spermatozoon characteristics, chromatin structure, and antioxidant/oxidant parameters after freeze-thawing of hesperidin (vitamin P) doses added to ram semen. Life **12**: 1780

Zhang YW, Luo HL, Chang YF, Jiao LJ, Liu K 2017: Effects of liquorice extract on the activity and gene expression level of antioxidant enzymes in longissimus dorsi muscle of Tan lamb. Small Rumin Res **154**: 23-28