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Red pine (*Pinus brutia Ten*) bark tree extract preserves sperm quality by reducing oxidative stress and preventing chromatin damage

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Abstract

This study aimed to investigate the effectiveness of using red pine bark tree extract (P; *Pinus brutia Ten*) as a TRIS extender in an attempt to prevent oxidative stress in bull spermatozoa after freezing. Semen specimens were obtained from Simmental bulls via an artificial vagina and pooled. They were separated into five specimens and diluted with Tris extender consisting of P (200, 100, 50 and 25 μg/ml) and P free (control; C) up to a final concentration of 16×10^6 per straw. All specimens were equilibrated for a period of 4 hr at a temperature of 4°C, following which they were filled in 0.25-ml French straws and frozen. Addition of P resulted in favourable tail length in comparison with C ($p < .05$). The lowest malondialdehyde levels and the highest glutathione levels were detected in all P groups ($p < .05$). Supplementation with P did not show advanced results in terms of total, progressive sperm motility and total abnormality in comparison with C ($p > .05$). In conclusion, it has been shown that although P added to a Tris extender does not have a positive effect on sperm motility, it prevents chromatin damage by reducing oxidative stress, in addition to reducing head abnormalities when used at the amount of 50 μg/ml.

KEYWORDS

antioxidant, chromatin damage, cryopreservation, oxidative stress, spermatozoa

1 | INTRODUCTION

Although semen cryopreservation has unfavourable effects on mammalian spermatozoa (Yeste, 2016), using antioxidants prior to these freezing procedures has significant advantages (Büyükleblebici, Büyükleblebici, Taşdemir, & Tuncer, 2016). Prior to chilling and freezing spermatozoa, treatment at room temperature may produce toxic oxidants and reactive oxygen species (ROS) in the extender (Aitken, Irvine, & Wu, 1991). ROS are unstable, chemically reactive species that contain hydrogen peroxide and superoxide radicals. They attack and damage biological molecules, such as lipids and proteins, resulting in oxidative stress (Shin, Yoo, Min, Lee, & Choim, 2010). Therefore, ROS may have negative effects by breaking the membrane of the sperm cell, thus damaging the energy sources necessary for its survival (Guthrie & Welch, 2012), while oxidative stress may also result in chromatin damage (Taşdemir et al., 2013).

The effects of several plant extracts on fertility have been demonstrated in many animal species (Sharma, Goyal, & Bhat, 2013). However, it is known that the antioxidant property of these extracts is correlated with their free radical scavenging properties (Laura et al., 2011). The results of a recent study suggested that plant extracts may play an important role in enhancing endogenous antioxidant defence capacity (Zhang, Luo, Chang, Jiao, & Liu, 2017). Most herbs and herb-derived products offer a large variety of one or more active compounds (D'Cruz, Vaithinathan, Jubendradass, & Mathur, 2010). Recently, evaluation of plants to identify efficient and safe

substances for semen cryopreservation has increased around the world (Attia & Kamel, 2012). Antioxidants obtained from plants are considered to be a good alternative to chemically derived antioxidants due to the lack of or fewer side effects (Gupta & Sharma, 2006).

Pine bark tree extract has phenolic compounds, which consist of catechin, epicatechin, taxifolin and phenolic acids capable of producing diverse, potentially protective effects against hypertension, cancer and degenerative and chronic diseases (Packer, Rimbach, & Virgili, 1999; Rohdewald, 2002). Moreover, these substances have antioxidant activities, and they increase plasma antioxidant ability while decreasing plasma lipid peroxidation and malondialdehyde (MDA) levels (Scalbert & Williamson, 2000). They might be a potential alternative for pharmaceutical practices (Gulati, 2015; Iravani & Zolfaghari, 2011). To the best of our knowledge, the effects of red pine bark tree extract (P; *Pinus brutia Ten*) on freezing semen from bulls have not yet been studied. Thus, this is the first report on using P for semen cryopreservation.

This study aimed to investigate the effectiveness of P as a Tris (hydroxymethyl)-aminomethane (TRIS) extender in an attempt to prevent oxidative stress in bull spermatozoa after freezing.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Three Simmental breed bulls at the age of 3–5 years, with proven fertility from the Sultansuyu Agribusiness (Sultansuyu), were utilised as a semen source. Semen samples were obtained via an artificial vagina once per seven days, and pooling of ejaculates was performed for the purpose of eliminating variability among the assessed specimens. This study was repeated nine times per each group in the same season (from December to March). All specimens were kept in a water bath at a temperature of 37°C to evaluate concentration and motility in the future. The study was conducted according to the approval obtained from the Afyon Kocatepe University Faculty of Veterinary Medicine Animal Care Committee in terms of ethics, with the authorisation number of 49533702/29.

2.2 | Freezing of semen

A graded collection tube was utilised to determine the fresh semen volume, while a photometer (Minitube GmbH) was used to calculate concentrations. All ejaculates with mass activity ≥ +++ 3 [scale of 1–5], sperm concentration ≥ 0.8 × 10^9/ml, volume ≥ 5 ml and initial motility ≥ 80% were utilised. In this study, a Tris medium was utilised as the primary extender. P was collected in Muğla, Turkey (voucher number: 2017/1). It was botanically identified. The processes of collecting the P, making a sample of the particle size and keeping it until the time of studying were carried out. Besides, carbon dioxide (99%), ethanol (Merck, 99%) and distilled water were used for extraction testing as explained by Braga et al. (2008). Gallic acid (≥98.5%) from Sigma-Aldrich Chemical (Interlab Ltd.) was used for standardisation. CO_2 was delivered using a high-pressure liquid compressor at 15 MPa, and ethanol was delivered by a high-pressure liquid pump (L-6200A, Hitachi, Merck). The temperature and flow rates were chosen as 30°C and 7.5 ± 2.5 kg/s respectively. After the preliminary studies, the most suitable amounts of P to be added to the extender were determined. 10 mg of the P extract was mixed with ethanol (1 ml; Merck, 99%) to generate the P holding solution. The semen samples were divided into five and diluted with the Tris medium consisting of control (no-additive; C) and P (200, 100, 50 and 25 μg/ml; the osmolarity of all solutions was adjusted to 310 mOsm) up to a concentration of 16 × 10^6 sperm cells/straw, following which spermatozoon loading into French straws was performed. Cooling of the samples was carried out (4°C), and they were equilibrated for a period of 4 hr. Afterwards, each group was cryopreserved using a semen cryopreservation machine (SY LAB Gerate GmbH) according to the protocol of Avdatek et al. (2018). In conclusion, the straws were put into liquid nitrogen at a temperature of −196°C for 6 months. The frozen straws were individually thawed for post-thawed spermological evaluations at 37°C for a period of 30 s in a water bath.

2.3 | Determination of sperm motility and kinetic parameters

The Computer-Assisted Semen Analyser (CASA) system (Microptic S.L.) was used to evaluate sperm motilities, as stated by Avdatek et al. (2018). A 5-μl diluted semen sample was placed on a preheated slide, and the rates of total motility, nonprogressive motility and progressive motility were recorded. Furthermore, the motility movement characteristics of curvilinear velocity (VCL) μm/s, average path velocity (VAP) μm/s, straight linear velocity (VSL) μm/s, amplitude of lateral head displacement (ALH) μm, beat cross frequency (BCF), wobble (WOB, [VAP/VCL] ×100), straightness (STR, [VSL/VAP] ×100) and linearity (LIN, [VSL/VCL] ×100) were found. The sperm motilities were set as fast >80 μm/s, medium >60 μm/s, slow >20 μm/s and static protocols. For every trial, 230–380 sperm cells were analysed in six different fields by microscopy.

2.4 | Assessment of sperm morphology

The assessment of sperm morphologies was performed according to the method reported by Schafer and Holzmann (2000). Hancock's solution, containing double-distilled water (500 ml) with saline solution (150 ml), buffer solution (150 ml) and formalin (62.5 ml of 37%), was utilised. A 5 μl specimen was put into 500 μl of Hancock's solution for the purpose of investigating sperm morphological integrity. A 6 μl sperm mixture was put onto a slide and closed with a cover slide. The abnormal sperm rate (tail, head and total abnormality) was

assessed in a minimum of 200 spermatozoa by a phase-contrast microscope (1.000×).

2.5 | Evaluation of chromatin damage

Sperm chromatin damage was assessed using the single-cell gel electrophoresis method (COMET). The samples (slides) were investigated under a fluorescent microscope (Olympus CX31), and the images were analysed to perform scoring examination in the future by using the Comet Score software (TriTek, V. 1.5). On every specimen, one hundred cells of spermatozoa in total from six various fields were assessed for conducting the examination (Gundogan, Yeni, Avdatek, & Fidan, 2010).

2.6 | Evaluation of oxidative stress

A colorimetric commercial kit (Rel Assay®, Gaziantep, Turkey) was utilised to assess the total antioxidant (TA) status. Glutathione peroxidase (GPx) efficiency was determined by a GPx assay (OxisResearch™, Bioxytech® GPx-340™). A commercial kit (MDA-586; OxisResearch) was utilised to measure lipid peroxidation levels, created by MDA. The findings were determined in μmol/ml as identified by Kasimanickam, Pelzer, Kasimanickam, Swecker, and Thatcher (2006).

2.7 | Statistical analysis

Firstly, the normal distribution of the data was confirmed with a Shapiro–Wilk test. Then, the data were analysed by one-way analysis of variance (ANOVA) and Duncan's post hoc test to state the differences among the treatment groups in terms of all spermological and biochemical parameters. Independent statistical analyses were conducted for each end point for the frozen and thawed semen parameters. The data are presented as mean ± standard error of means (*SEM*). The degree of significance was set at $p < .05$. The SPSS/PC (version 10.0; SPSS) software was used for all analyses.

3 | RESULTS

As seen in Table 1, addition of P, as an antioxidant, did not provide the desired findings in the rate of total and progressive sperm motility after freeze-thawing. In particular, the 200 μg/ml P concentrations demonstrated unfavourable impacts in comparison with the C group ($p < .05$) on progressive motility. Besides, when the sperm movement traits were examined, the results demonstrated that the P treatments did not advance sperm movement characters (Table 1). As presented in Table 2, treatment with P50 and P200 resulted in decreased head abnormalities ($p < .05$). However, supplementation of P did not have a positive effect on total abnormality in comparison with the C group ($p > .05$).

As shown in Table 3, chromatin damage was significantly different among the implementation groups. Addition of P resulted in favourable tail length in comparison with the C group ($p < .05$). As seen in Table 4, the lowest malondialdehyde (MDA) levels (oxidant capacity) and the highest glutathione (GSH) levels (antioxidant capacity) were detected in all P groups ($p < .05$).

4 | DISCUSSION

This study was carried out to contemplate the effects of P—a natural extract—on prevention of possible negative effects of freezing and thawing on spermatozoa or on improvement of their harmful effects. As known, motility is one of the most precise

TABLE 1 Mean (±*SE*) sperm motility values in frozen–thawed bull semen

Analysis	Control	P25	P50	P100	P200	*p*
Nonprogressive motility (%)	22.14 ± 2.01^{b}	23.74 ± 3.32^{ab}	22.72 ± 1.61^{b}	28.96 ± 1.91^{ab}	30.03 ± 1.88^{a}	*
Progressive motility (%)	35.74 ± 1.63^{a}	32.08 ± 1.76^{a}	33.55 ± 1.49^{a}	33.59 ± 2.01^{a}	19.85 ± 1.57^{b}	*
Total motility (%)	57.88 ± 2.75^{ab}	55.82 ± 3.79^{ab}	56.27 ± 2.46^{ab}	62.55 ± 3.09^{a}	49.88 ± 2.08^{b}	*
VAP (μm/s)	84.73 ± 3.02^{a}	81.18 ± 4.15^{ab}	79.58 ± 1.84^{ab}	75.07 ± 2.71^{b}	60.71 ± 3.35^{c}	*
VSL (μm/s)	70.54 ± 2.98^{a}	65.74 ± 4.48^{ab}	65.28 ± 1.99^{ab}	59.57 ± 2.97^{b}	45.56 ± 3.28^{c}	*
VCL (μm/s)	113.97 ± 2.78^{a}	109.96 ± 3.64^{ab}	108.88 ± 1.69ab	104.87 ± 2.10^{b}	89.82 ± 3.47^{c}	*
ALH (μm/s)	4.13 ± 0.74	3.98 ± 0.98	4.08 ± 0.32	4.12 ± 0.05	4.04 ± 0.62	
BCF (Hz)	10.87 ± 0.13^{a}	10.51 ± 0.40^{a}	10.19 ± 0.17^{ab}	10.21 ± 0.11^{ab}	9.72 ± 0.21^{b}	*
LIN (%)	61.75 ± 1.64^{a}	59.38 ± 2.45^{a}	59.86 ± 0.96^{a}	56.58 ± 1.66^{a}	50.45 ± 2.13^{b}	*
STR (%)	83.07 ± 0.76^{a}	80.54 ± 1.80^{ab}	81.91 ± 0.63^{ab}	79.09 ± 1.06^{b}	74.58 ± 1.40^{c}	*
WOB μm/s	74.24 ± 1.35^{a}	73.52 ± 1.54^{a}	73.03 ± 0.69^{a}	71.41 ± 1.19^{a}	67.38 ± 1.57^{b}	*
Hyperactivity μm/s	39.41 ± 1.61^{a}	34.25 ± 2.51^{a}	33.99 ± 1.35^{a}	33.97 ± 1.95^{a}	22.12 ± 1.70^{b}	*

Note: Different superscripts letters (a, b, c) within the same row demonstrate significant differences (*$p < .05$). No significant difference ($p > .05$).

TABLE 2 Mean (±*SE*) sperm abnormality values in frozen–thawed bull semen

Analysis	Control	P25	P50	P100	P200	*p*
Head abnormalities (%)	3.88 ± 1.04^{a}	2.33 ± 0.38^{ab}	1.41 ± 0.59^{b}	3.16 ± 0.88^{ab}	1.54 ± 0.41^{b}	*
Mid-piece abnormalities (%)	5.72 ± 0.93^{a}	3.56 ± 0.71^{ab}	4.75 ± 0.84^{ab}	3.67 ± 0.96^{ab}	2.37 ± 0.46^{b}	*
Tail abnormalities (%)	2.03 ± 0.44	2.18 ± 0.67	3.83 ± 1.26	2.17 ± 0.84	3.06 ± 0.98	
Total abnormalities (%)	11.49 ± 1.42^{a}	8.05 ± 0.95^{ab}	10.11 ± 1.50^{ab}	9.06 ± 1.07^{ab}	6.97 ± 1.41^{b}	*

Note: Different superscript letters (a, b) within the same row demonstrate significant differences (*p < .05). No significant difference (p > .05).

TABLE 3 Mean (±*SE*) chromatin integrity values in frozen–thawed bull semen

Analysis	Control	P25	P50	P100	P200	*p*
Tail length (μm/s)	24.70 ± 4.20^{a}	15.71 ± 3.14^{b}	13.99 ± 0.79^{b}	10.29 ± 0.57^{b}	12.80 ± 0.71^{b}	*
Tail DNA (%)	28.80 ± 4.02^{a}	22.05 ± 1.01^{ab}	24.97 ± 2.72^{ab}	19.97 ± 0.71^{b}	22.66 ± 0.72^{ab}	*
Tail moment (μm/s)	20.06 ± 2.49^{ab}	20.91 ± 2.88^{a}	15.92 ± 1.71^{abc}	12.21 ± 1.41^{c}	14.13 ± 1.03^{bc}	*

Note: Different superscript letters (a, b, c) within the same row demonstrate significant differences (*p < .05). No significant difference (p > .05).

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TABLE 4 Mean (±*SE*) glutathione (GSH) and malondialdehyde (MDA) in frozen–thawed bull semen

Analysis	Control	P25	P50	P100	P200	*p*
GSH (mU/ml)	8.55 ± 0.38^{d}	10.03 ± 0.18^{c}	11.52 ± 0.17^{b}	13.25 ± 0.24^{a}	13.58 ± 0.28^{a}	*
MDA (μmol/ml)	7.61 ± 0.05^{a}	7.41 ± 0.05^{b}	7.15 ± 0.46^{c}	6.80 ± 0.33^{d}	6.74 ± 0.33^{d}	*

Note: Different superscript letters (a, b, c, d) within the same row demonstrate significant differences (*p < .05). No significant difference (p > .05).

characteristics used to estimate the capacity of fertility of spermatozoa (Verstegen, Iguer-Ouada, & Onclin, 2002). In this study, it was determined that P added to Tris semen extender had no positive effect on progressive and total sperm motility. It was determined that the dose of 100 μg/ml was the threshold value, and values above this dose (200 μg/ml) had a toxic effect on the spermatozoa (p < .05). Unlike the findings obtained, in a study conducted on bulls by Abdramanov et al. (2017), it was reported that *Sambucus nigra* extracts were used at concentrations of 5–10μg/ml, and motility was preserved even when there was a high amount of ROS. In another bull study, Losano et al. (2018) revealed that docosahexaenoic acid use has a healing effect on motility. In contrast to the results found in this study, in a study on buffalo bulls, it was stated that the liquid extract of *Nigella sativa* has extraordinary antioxidant properties and increases motility when added to the TRIS extender by 4% (Awan et al., 2018). In studies conducted in bulls, it was evaluated that the reason for obtaining different results in terms of motility is due to the different extraction methods, application of the extracts used in different doses and the different contents of the extracts. Contrary to the conclusions reached here, it was reported that the oral use of 200 mg pycnogenol (French maritime pine bark tree) for 90 days (Roseff, 2002) and a combination of L-arginine aspartate and pycnogenol for 4 weeks had a positive effect on sperm motility (Stanislavov, Nikolova, & Rohdewald, 2009). The differences between the results obtained from these studies in humans and our study were interpreted to be due to the difference in species and the way antioxidants were used.

In accordance with our results, Vichas et al. (2018) froze ram semen with the extender they prepared by using skim milk powder, α-tocopherol and quercetin. They concluded that this antioxidant mixture had no positive effect on sperm motility. Additionally, in the study, similar results were obtained to those in our previous studies where amino acid, fetuin, cysteine, quercetin and rosmarinic acid were used as antioxidants, and these antioxidants that were used did not have a curative effect on progressive sperm motility and motility movement characteristics (Avdatek et al., 2018; Taşdemir et al., 2014; Yeni et al., 2018). Considering the results, we maintain the opinion that the reason why the effects of addition of P on the total and progressive sperm motility and motility movement characteristics were not different from those of the control group was that ROS were eliminated by other substances added into the Tris extender, which resulted in positive, cryoprotective effects on spermatozoa.

The methods implemented during the freezing and thawing of spermatozoa cause a negative effect on sperm motility while also damaging spermatozoa morphologically (O'Connell, McClure, & Lewis, 2002). According to the results obtained, especially the addition of 50 μg/ml P had positive outcomes in preventing head abnormalities, excluding the amount of 200 μg/ml, which had a very negative effect on motility. In agreement with these findings, Taşdemir et al. (2014) reported that, although antioxidants have no positive effects on motility, they prevent sperm abnormality. Inconsistently with the abnormality results, researchers have reported that, while freezing bull semen, addition of quercetin and rosmarinic acid does not contribute positively to sperm head abnormalities (Avdatek et al., 2018; Yeni et al., 2018). It was interpreted that P demonstrated a favourable effect on head

abnormalities, especially by preserving the cell membrane rich in unsaturated fatty acids.

Sperm chromatin integrity is one of the best indicators of fertility capability (Barroso, Morshedi, & Oehninger, 2000), associated with fertilisation (Rathke et al., 2007). It was stated that damage to chromatin can not only reduce the fertilisation capacity of sperm but also cease the implantation and embryonic development of the embryo (Zini, San Gabriel, & Libman, 2010). Studies that report that antioxidants obtained from plants maintain chromatin integrity (Avdatek et al., 2018; Awan et al., 2018) and that there is a positive relationship between decreasing oxidative stress and decreasing chromatin damage (Losano et al., 2018) supported our findings. In another study conducted in rams, in accordance with our results, addition of 1 μM of coenzyme Q10, a lipophilic antioxidant, caused the sperm chromatin integrity to increase and MDA activity to decrease (Yousefian et al., 2018). On the contrary, there are studies expressing that quercetin addition has no healing effect on sperm chromatin damage (Seifi-Jamadi, Kohram, Shahneh, Ansari, & Macías-García, 2016). When the results of our study are compared to the findings reported in previous research, it is evident that P, when used as an antioxidant, has a direct relationship to a decrease in chromatin damage. It is thought that the positive effect caused by this antioxidant activity on chromatin damage occurs synergistically in conjunction with other antioxidants that are found in the extender.

Malondialdehyde is a marker that is widely used to detect oxidative stress (Zhang et al., 2017). GSH, however, is one of the most significant reagents with antioxidant characteristics (Kumar, Kumar, Sikka, & Singhbal, 2015). In this study, addition of P reduced the level of MDA and increased the level of GSH in all treatment groups. Oral application of 20 mg pycnogenol over 7 days was reported to reduce oxidative stress in rats in a study that was conducted to determine the effects of pycnogenol on spermatoxicity, which was in line with the findings obtained in this study (Kim et al., 2014). In another study, it was found that *Phellodendron amurense* and *Humulus japonicus* extracts administered before preimplantation of cattle embryos contributed positively to blastocyst development by decreasing ROS levels (Do et al., 2017). In studies that favour our results, Mao et al. (2018) stated that both MDA levels and oxidative stress would decrease by using plant-derived antioxidants, while İnanç et al. (2019) claimed that the antioxidant capacity of semen may be improved by utilising antioxidant supplements capable of reducing ROS levels in a semen extender. In a study on freezing of bull spermatozoa compatible with us, it was found that epicatechin, which is a type of natural antioxidant, relieves oxidative stress and improves sperm viability (Greifova, Tvrda, Jambor, & Lukac, 2017).

5 | CONCLUSIONS

In conclusion, it has been shown that although P added to a Tris extender does not have a positive effect on sperm motility, it prevents chromatin damage by reducing oxidative stress, in addition to reducing head abnormalities when used at the concentration of 50 μg/ml.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare.

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