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Original article

The cryoprotective potential of propolis supplemented in frozen-thawed bull semen; biochemical and physiological findings

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Abstract

In this study, the cryoprotective effect of different doses of propolis (P) on bull semen, which has solid pharmacological properties thanks to its rich phenolic components, was investigated biochemically and physiologically. Semen samples were collected from Simmental breed bulls via the artificial vagina and pooled. After dividing into five groups, control (C: no additive) and four different P (200, 100, 50, and 25 µg/mL) groups, the final concentration was diluted to 16×106 per straw. Semen samples were equilibrated at 4°C for approximately 4 hours, then placed in French straws and frozen. After thawing, sperm motility and kinetic parameters, DNA integrity by single-cell gel electrophoresis, sperm abnormalities by liquid fixation, and lipid peroxidation levels by the colorimetric method was analyzed by Computer-Assisted Semen Analyzer. P added to the diluent showed no effect on motility and kinetic parameters at P25 and P50 ($p>0.05$), while P100 and P200 had a negative effect ($p<0.001$). The addition of P (25 and 50) showed a treatment effect on tail abnormality compared to C ($p<0.05$). Especially P50 had a positive effect on tail length, tail DNA, and tail movement, while P100 and P200 caused DNA damage ($p<0.001$). MDA levels increased in all P dose groups compared to C ($p<0.001$). This study has clearly demonstrated that P25 and P50 supplements could be used therapeutically to treat sperm tail abnormalities and prevent DNA damage in post-thawed bull sperm.

Key words: cryopreservation, DNA damage, oxidative stress, propolis

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Introduction

Artificial insemination is a widely used biotechnological method in cattle to fertilize large numbers of females by taking a single ejaculate and increasing genetic abilities. For this method to be applied, the semen must be preserved for a long time. However, as a result of the freeze-thaw process, some handicaps may occur that may affect sperm motility, membrane integrity, and fertilization potential (Hu et al. 2010). The formation of ice crystals may cause damage to spermatozoa due to the increase in solute concentration in the insemination medium using freeze-thawed sperm (El-Harairy et al. 2011). Therefore, the success of cryopreservation depends not only on the preservation of sperm motility but also on the continuity of biochemical and physiological functions (Watson 2000). In studies conducted so far, the addition and removal of cryoprotectant substances have not been sufficient to eliminate toxicity due to osmotic damage and adverse effects on the genetic composition of semen (Gilmore et al. 1997). Antioxidant substances are added to the dilution medium to maintain sperm integrity during the freezing process (Singh et al. 1995). The amount and type of diluents affect the biochemical and physiological processes present in sperm during the freezing process (El-Harairy et al. 2011).

The application of natural antioxidant additives in sperm cryopreservation has been successfully tried by many researchers so far, and positive results have also been obtained (Kumar et al. 2019, Taşdemir et al. 2020). The use of natural products has come to the fore as an alternative to the possible toxic effects of synthetic prophylactic drugs on human and animal fertility. One of the natural products with various pharmaceutical and antioxidant properties due to its rich natural bioactive components in propolis (P; bee glue), a honey bee product (Hashem et al. 2013). P is a dark resinous substance produced by honey bees with salivary gland secretions from various plants to close the crevices and cracks of the hive to prevent microbial contamination (Hashem et al. 2021). Raw P mainly consists of wax, resin, water, essential oils, inorganic and phenolic compounds. The exact components vary depending on the botanical source and can be distinguished by various analytical methods. The phenolic variant of P extract contributes to antioxidant, antimicrobial, antiviral, anti-inflammatory, antifungal, cardioactive, and reproductive functions (Pasupuleti et al. 2017). It can also neutralize free radicals and can inhibit lipid peroxidation (Yousef and Salama 2009). No study has been found in the literature on the therapeutic use of P in cryopreservation of bull sperm.

Therefore, in this study, changes in the structure and function, DNA integrity, lipid peroxidation, and total antioxidant capacity of semen after thawing with P extract added to frozen bull semen using Tris diluent were investigated thoroughly.

Materials and Methods

Animals

Simmental breed (3-4 years) bulls from Sultansuyu Agricultural-Farm were selected as the semen source for fertility studies in this research. Fifty ejaculates were collected from 3 different bulls through an artificial vagina. Ejaculates were pooled to minimize the differences among the samples. Semen samples were kept in a water bath (37°C) to observe concentration and motility factors. Ethics committee approval of the study was given by Afyon Kocatepe University Faculty of Veterinary Medicine Animal Care Committee with the number 49533702/29.

Preparation of P extraction solution

P (30 g), collected from a bee farm in Muğla (Turkey), was extracted with 600 mL of 70% (v/v) ethanol at 60°C for 30 minutes. The resulting homogeneous mixture was then centrifuged and evaporated until the supernatant dried under vacuum at 40°C. The extracted product was stored at 4°C to be used for research purposes (Gulhan 2019). Same P samples were used in this study.

Semen processing and freezing

A volume-graded collection glass was used to determine the amount of semen. The concentration was calculated by a photometer (Minitube GmbH). Ejaculations were used to determine mass Activity ≥+++ 3 [scale 1-5], sperm concentration ≥ 0.8×10^9/mL, volume ≥ 5 mL, and initial motility 80%. Then, pooled ejaculates were divided into five groups, control (C; no additive), and four different dose P (200, 100, 50 and 25 µg/mL). The main extender in this study was a TRIS-based extender (TRIS 30.7 g, citric acid 16.4 g, fructose 12.6 g, distilled water 1000 mL, egg yolk 20% v/v and 6% glycerol; Taşdemir et al., 2013). In preliminary studies, 10 mg Propolis (70%) extract was mixed with ethanol (1 mL). The osmolarity of all solutions (310 mOsm) was adjusted to a maximum concentration of 16×10^6 sperm cells/straw with TRIS-containing media. Later, the sperm was stuffed into French straws. Samples were stored up to 4°C for about 4 hours to be brought to equilibrium temperature. After cooling, samples from each group were frozen in a cryopreservation device (SY LAB Gerate GmbH) for 6 months at -196°C accor-

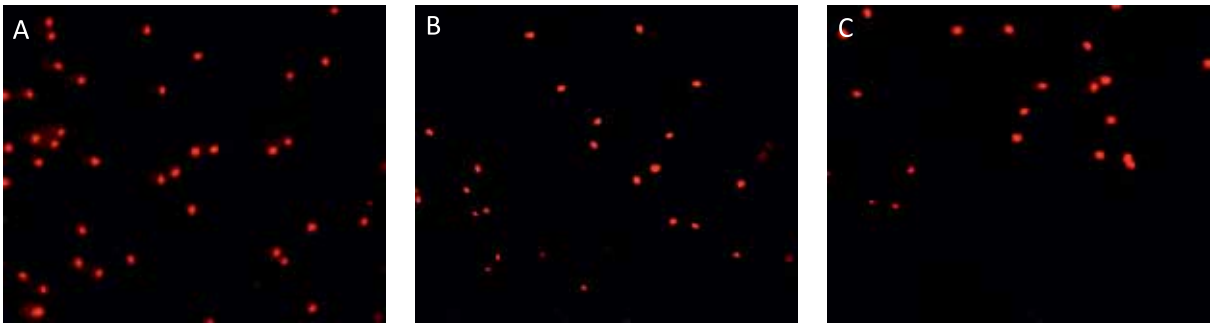

Fig. 1. Control (A), P25 (B) and P50 (C) comet analysis images. x1000

ding to the experimental protocol of Avdatek et al. (2018).

Sperm motility and kinetic parameters

Computer Assisted Semen Analyzer (CASA) system (Microptic S.L.) was used to assess sperm motility. For these measurements, 5 µL of diluted semen samples were placed in a pre-warmed plate, and total motility, progressive and non-progressive motility values were analyzed. Besides these parameters, the motility movement characteristics of curvilinear velocity (VCL) µm/s, average path velocity (VAP) µm/s, straight linear velocity (VSL) µm/s, the 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) data were also obtained. Sperm movement speed; fast (> 80 µm/sec), medium (> 60 µm / sec) and slow (> 20 µm/sec) in accordance with the static protocol. In each semen sample, 230-380 sperm cells were analyzed under the microscope in six different regions.

Sperm morphology

Sperm morphological assessment was performed according to the method determined by Schafer and Holzmann (2000). The Hancock solution used in the method consists of brine solution (150 mL), buffer (150 mL) and formalin (62.5 mL 37%), and double-distilled water (500 mL). After mixing with Hancock solution (500 µL) + semen sample (5 µL), 6 µL of the mixture was taken to the microscope table and covered with a slide to determine the morphological integrity. Abnormal sperm levels (middle part, tail, and total abnormality) were calculated for at least 200 spermatozoa in the phase-contrast microscope (x1000).

Assessment of DNA damage

DNA damage in semen was analyzed by the single-cell gel electrophoresis (COMET) assay method under intensely alkaline conditions. The samples on the slide were visualized with a fluorescence microscope (Olympus CX31) and scored using Comet Score software (TriTek, V.1.5). In order to make the assessment more accurate, at least a total of one hundred sperm cells were selected from six different regions in each sample (Gundogan et al. 2010).

Assessment of oxidative stress parameters

Evaluating the oxidative stress parameters after thawing, the spermatozoa were washed three times with phosphate-buffered saline (PBS) by being centrifuged at 800 g for 20 minutes with a refrigerated centrifuge to separate them from the diluent. Then the supernatant was made up to 0.5 mL with PBS. The samples were taken to falcon tubes in ice for homogenization. The sonication treatment was repeated six times by keeping them in the ice for 30 seconds after a ten second-sonication process (Akalın et al. 2016). After then, commercial kits were used to check oxidative stress parameters such as total antioxidant capacity (TAC) (Rel Assay®, Gaziantep, Turkey), Glutathione (GSH) (OxisResearch ™, Bioxytech® GPx-340 ™), and lipid peroxidation (MDA-586; OxisResearch) levels. All parametric findings were calculated as µmol/mL (Kasimanickam et al. 2006).

Statistical analysis

Before the significance test, the collected data were determined in terms of normality by Kurtosis, one of the parametric test assumptions. Descriptive statistics for each variable were calculated and presented as "Mean ± Standard Error" (Mean ± SE). Statistical analysis of data was performed by the general linear model (GLM) multivariate measures of SPSS 22.0 (SPSS Inc. Headquarters, Chicago, IL, USA). Also, we analyzed the data Related-Samples Friedman's Two-Way Analysis of Variance by Ranks. The significance of differences between the means was tested by Bonferroni test analysis and considered significant at $p<0.05$.

Table 1. Mean (±SE) sperm motility values in frozen-thawed bull semen after various doses of propolis treatment.

Parameters	C	P25	P50	P100	P200	*p*
Progressive motility (%)	31.20±2.00^{c}	33.04±2.23^{c}	33.05±3.32^{c}	17.39±1.84^{b}	0.77±0.24^{a}	0,001
Total motility (%)	55.59±2.28^{c}	58.80±1.95^{c}	59.37±4.46^{c}	34.37±3.71^{b}	3.19±0.28^{a}	0,001
VAP (µm/s)	76.48±4.23^{b}	78.03±2.45^{b}	78.73±3.28^{b}	73.80±2.16^{b}	42.55±3.86^{a}	0,009
VSL (µm/s)	60.84±4.10^{b}	61.83±2.66^{b}	62.61±3.11^{b}	56.21±1.99^{b}	24.05±3.05^{a}	0,009
VCL (µm/s)	105.51±4.65^{b}	110.20±2.88^{b}	107.08±3.55^{b}	103.31±3.51^{b}	71.38±7.06^{a}	0,010
ALH (µm/s)	3.80±0.23^{b}	4.13±0.07^{b}	3.76± 0.17^{b}	3.73±0.18^{b}	2.08±0.51^{a}	0,024
BCF (Hz)	10.97±0.36^{b}	11.57±0.28^{b}	11.73±0.47^{b}	11.76±0.52^{b}	5.10±1.34^{a}	0,008
LIN (%)	57.54±2.52^{b}	55.92±1.12^{b}	58.41±1.96^{b}	54.45±1.04^{b}	33.91±4.42^{a}	0,026
STR (%)	79.28±1.60^{b}	79.01±1.07^{b}	79.41±1.24^{b}	76.16±1.06^{b}	55.58±4.86^{a}	0,005
WOB (µm/s)	72.37±1.71	70.75±0.60	73.46±1.34	71.50±0.94	60.06±2.85	0,095
Hyperactivity (µm/s)	35.42±2.46^{c}	37.53±2.71^{c}	33.91±2.60^{c}	22.05±2.19^{b}	1.80±0.33^{a}	0,001

$^{a.b.c.d}$ Different superscripts within the same row demonstrate significant differences (** p<0.001)
$^{-}$ No significant difference (p>0.05)
$^{-}$ No significant difference (P>0.05)

Table 2. Mean (±SE) sperm abnormality values in frozen-thawed bull semen after various doses of propolis treatment.

Parameters	C	P25	P50	P100	P200	*p*
Head abnormalities (%)	3.60±0.66	2.27±0.29	2.34±0.95	1.66±0.85	0.49±0.49	0,178
Mid-piece abnormalities (%)	1.33±0.50	1.22±0.70	0.99±0.62	1.35±0.85	0.24±0.24	0,371
Tail abnormalities (%)	3.54±0.4^{bc}	1.26±0.60^{a}	0.99±0.62^{a}	1.96±0.57^{ab}	4.46±1.07^{c}	0,037
Total abnormalities (%)	8.48±0.83	4.75±0.90	4.34±1.41	4.98±0.63	5.20±1.37	0,251

$^{a.b.c.d}$ Different superscripts within the same row demonstrate significant differences (* p<0.05)
$^{-}$ No significant difference (p>0.05)

Table 3. Mean (±SE) chromatin damage values in frozen-thawed bull semen after various doses of propolis treatment.

Parameters	C	P25	P50	P100	P200	*p*
Tail lenght (µm/s)	15.41±0.54^{c}	10.36±0.59^{b}	4.44±0.42^{a}	31.22±1.98^{d}	33.13±0.70^{d}	0,001
Tail DNA (%)	19.02±1.08^{c}	9.72±0.19^{b}	4.37±0.23^{a}	27.75±1.56^{d}	35.36±1.42^{e}	0,005
Tail moment (µm/s)	15.00±0.97^{d}	3.51±0.48^{b}	0.77±0.15^{a}	9.28±0.259^{c}	18.10±0.42^{e}	0,019

$^{a.b.c.d.e}$ Different superscripts within the same row demonstrate significant differences (* p<0.001)
$^{-}$ No significant difference(p>0.05)

Table 4. Mean (±SE) malondialdehyde (MDA), glutathione (GSH) and total antioxidant capacity (TAC) level activities in frozen-thawed bull semen after various doses of propolis treatment.

Parameters	C	P25	P50	P100	P200	*p*
MDA (nmol/mL)	6.95±0.12^{a}	8.13±0.23^{b}	7.86±0.22^{ab}	10.68±0.47^{c}	10.84±0.53^{c}	0,007
GSH (nmol/mL)	5.39±0.07	5.47±0.14	5.39±0.78	5.12±0.14	5.20±0.05	0,348
TAC (mmol Trolox equiv/L)	0.46±0.01^{ab}	0.45±0.01^{a}	0.45±0.04^{a}	0.46±0.06^{ab}	0.47±0.04^{b}	0,035

$^{a.b.c.d}$ Different superscripts within the same row demonstrate significant differences (* p<0.05). (** p<0.001)
$^{-}$ No significant difference (p>0.05).

Results

Spermatozoa Microscopic Assessments

The results of spermatological parameters obtained compared to group C in bull semen after thawing various doses of P added to the diluent during freezing are shown in Table 1. VAP, VSL, VCL, ALH, BCF, LIN, and STR parameters show no difference compared to C (p>0.05). In the same parameters, the P200 treatment group reached a very low mean value compared to C and affected the kinetic parameters negatively p<0.001).

No statistically significant changes in progressive motility, total motility WOB and hyperactivity parameters were observed in antioxidant treatment groups compared to C at P25 and P50 doses ($p>0.05$). However, the P100 and P200 dose groups significantly decreased these parameters ($p<0.001$). At the end of the freeze-thaw period, mid-piece abnormalities and the total abnormality values in all antioxidant treatment groups did not change compared to group C ($p>0.05$). Tail abnormalities were significantly reduced in the P25 and P50 treatment groups compared to C ($p<0.001$, Table 2).

Assessment of DNA damage

The changes in DNA damage parameters after thawing P is applied to sperm samples in different doses are shown in Table 3. The P25 and P50 antioxidant treatment groups showed significant improvements in tail length and tail DNA. Even the P50 dose gave the best results in these parameters ($p<0.001$). The tail moment was significantly decreased in the P25, P50 and P100 treatment groups compared to C and increased in the P200 ($p<0.001$).

Oxidative stress parameters

The changes in oxidative stress parameters, MDA, GSH and TAC are shown in Table 4. MDA levels increased in all P dose applications compared to C, and even the highest levels were detected in the P100 and P200 groups ($p<0.001$). GSH levels did not change significantly in any of the P treatment groups ($p>0.05$). Although the TAC parameter was different among the groups, the results obtained in the treatment groups were not significant ($p<0.001$).

Discussion

The inability of low endogenous antioxidant levels in sperm to provide adequate protection against excessive ROS production during the cryopreservation process causes irreversible damage to the sperm (Mazzilli et al. 1995). Therefore, endogenous natural antioxidant compounds are needed to prevent or minimize the oxidative damage of sperm during the cryopreservation process (Yeste 2016). Evaluating the current study results, it can be easily said that antioxidant P doses do not cause any changes in sperm motility properties. On the contrary, it significantly limited the motility capability of semen in the highest dose (P200).

There are some studies that examine motility changes by added bee products and other antioxidant substances in various concentrations to the sperm diluent, and the results of these studies support our findings. Moraes et al. (2014) reported that 1.25 g powdered P added to the rabbit diet did not affect progressive spermatozoa motility and reduced spermatozoa tail abnormalities. Honey added to sperm at different concentrations did not show significant differences in sperm motility (El-Sheshtawy et al. 2016). Research results of Amini et al. (2019) revealed that royal jelly added to TRIS-egg yolk extender did not affect ram sperm motility after dilution. Royal jelly at various amounts added to bull semen did not show any effect on sperm motility (Shahzad et al. 2016). Parallel to the conclusions reached here, the research findings of Inanç et al. (2019) revealed that green tea extract supplemented with the TRIS diluent did not change the motility and kinetic parameters in bull semen. Considering the studies that support our results, it is thought that even if there are differences in mammalian species or antioxidant agents, the cause of similar effects on sperm motility may be antioxidant dose choices. In line with the current study, Malik's (2018) study showed that adding the honey solution to bull semen cryoprotective medium had no advantage on sperm motility. In another study, powdered P extract added to ram semen extender decreased semen motility parameters during the six hours incubation period compared to the 0 h (Mohamed and Zanouny 2017). The positive effects of the bee products used on motility were not different from the C in their studies. The first is the cryoprotective effect of the substances in the stock solution, the second is the bee products' inability to integrate well into the cell membrane phospholipid structure, and the last is the variety of stress factors in in-vitro conditions.

Contrary to our conclusions, in studies conducted in different species, some researchers have announced that P causes positive changes in sperm motility (Capucho et al. 2012, Hashem et al. 2013). El-Battawy and Brannas (2015) reported that 10% concentration provides the best motility from P added to mice semen. El-Sherbiny (2015) reported that royal jelly, honey and P significantly increased the percentage of sperm progressive motility in New Zealand White male rabbits, while the percentages of dead and abnormal sperm increased. Research results reported that royal jelly applied as a sperm protectant caused a significant increase in sperm motility of male mice (Karacal and Aral 2008), hamster sperm cell concentration (Kohguchi et al. 2004), and in sperm count and viable sperm ratios of adult male rats (Hassan 2009). Olayemi et al. (2011) showed that honey added to the egg yolk extender increases the motility of buck semen after cold storage. Fakhrildin and Alsaadi (2014) reported that adding 10% honey as a cryoprotectant to the extender showed positive effects on spermatological parameters after thaw-

ing in human sperm. We believe that the main reasons for obtaining different motility results in semen cryopreservation studies in different mammalian species may be due to differences in extraction methods, dose differences, active ingredient differences, and freezing times, even if the antioxidant components are the same. In addition, possible harmful effects of the cryopreservation process, including cooling, freezing and thawing, or the inability of the exogenous antioxidant agent to perform adequate treatment, can be argued.

Protection of sperm DNA damage is one of the most important factors affecting fertility capacity (Rathke et al. 2007). It also negatively affects the implantation ability and development of the embryo (Zini and Libman 2010). The lipid peroxidation reactions caused by an excessive increase of semen ROS cause sperm DNA damage, and eventually, its loss causes sperm dysfunctions (De Lamirande et al. 1997). Therefore, it is necessary to control ROS for normal acrosomal reactions, sperm capacity, and reproductive physiology. Following our data, different amounts of cysteine added to the frozen bull semen extender have been reported to positively affect the preservation of DNA integrity (Ansari et al. 2016). Khalifa and Mohamed (2016) have proven that P protects DNA integrity thanks to its antioxidant and antimicrobial properties by including it in the ram semen medium. Alcay et al. (2017) reported that 0.5 and 0.75% royal jelly administered in 6 h incubation significantly prevented cryodamage by preserving the DNA integrity of buck semen. Caffeic acid, one of the most effective components of P, was found to reduce DNA damage in bull semen at 50 and 100 µM doses after freez-thawing (Soleimanzadeh et al. 2020). Our previous studies found that various plant extracts protect DNA integrity by reducing oxidative damage that may occur after freez-thawing in bull semen (Avdatek et al. 2018, Taşdemir et al. 2020). Our results, and other studies supporting us, have revealed the positive effect of dose-dependent antioxidant supplements on DNA damage due to the synergistic effect with diluents and other substances in the intracellular endogenous antioxidant system. In contrast to the results obtained, Taşdemir et al. (2014) stated that adding antioxidant substances (fetuin and cysteine) to the bull semen did not affect DNA integrity. The reason for the conflicting findings with our study is that DNA damage may be related to not only to oxidative damage but also to osmotic damage.

Due to the high concentration of unsaturated fatty acids in the spermatozoa membrane, lipid peroxidation chain reactions can be induced. This situation plays a crucial role in the etiology of male infertility (Sharma and Agarwal 1996), causing decreased fluidity of the sperm plasma membrane and functional losses (Aitken 2002). MDA concentration levels are defined as one of the most basic and essential oxidative stress markers (Ottolenghi et al. 2019). High MDA concentration levels are indicative of the oxidative stress state of semen after thawing. Small molecules such as GSH assist endogenous antioxidants or eliminate ROS products as co-factors (Ugur et al. 2019). In our study, the reason for the high MDA levels in the treatment groups depending on the dose increase may be the oxidative reactions caused by the unstable molecules in the P content. Our study data revealed a significant increase in sperm TAC levels with P supplementation. These results showed clearly that P has a stimulating effect by triggering intracellular enzymatic reactions. Possible reactive oxygen species (ROS) damages in sperm cells after thawing may probably help to provide oxidative balance by increasing antioxidant defense capacity with P treatment. Studies with contrary findings have shown that antioxidants therapeutically applied to the bull semen diluent increase TAC and contribute to fertilization ability (Avdatek et al. 2018, Soleimanzadeh et al. 2020). El-Seadawy et al. (2017) reported that 0.8-2.0 mg of P/5 mL added to TRIS extender reduces lipid peroxidation in rabbit semen after chilling for up to 72 h. Soleimanzadeh et al. (2020) reported that caffeic acid reduced MDA levels at 50 and 100 µM after freez-thawing in bull semen and increased TAC and GSH activities. The study of Büyükleblebici et al. (2014) reported that 3% ethylene glycole+5 mM cysteine was the combination that reduced MDA levels in bull semen. Taşdemir et al. (2020) stated that Pinus brutia Ten extract, which they added as a cryoprotectant to Simmental bull semen, decreased MDA levels and increased GSH activity. Inanç et al. (2019) found that adding green tea extract to bull semen did not affect MDA levels and showed the highest total antioxidant activity in the 50 µg/mL green tea extract. Avdatek et al. (2018) reported that quercetin therapeutically reduces MDA levels and positively affects GSH and TAC levels in bull semen, especially at 25 µg/mL dose. The results of researchers have shown that bee products and other natural antioxidant agents reduce or prevent oxidative damage by having positive effects on some oxidative stress parameters depending on the concentration.

Conclusions

Due to its many positive effects, natural substances have been preferred instead of synthetic cryoprotectants in semen cryopreservation. It has also been shown that the phenolic, flavonoid, and terpenoid components of honey bee products are widely used among natural products. However, high doses of natural products can have adverse effects. Therefore, careful determination

of the dose of the antioxidant agent to be used therapeutically is highly critical. As a result, based on all research conducted in this study, it can be clearly said that P25 and P50 doses effectively maintain some biochemical and physiological properties of bull sperm, while P200 doses cause toxic effects.

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