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 strong 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 dose 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) had 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 moment, 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 in the treatment of sperm abnormalities and prevention of DNA damage in post-thawed bull sperm.

Introduction

Artificial insemination is a widely used biotechnological method in cattle to fertilize large numbers of females by taking a single ejaculate and also to increase 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). High levels of polyunsaturated fatty acids in the bovine sperm membrane make it vulnerable to attack by reactive oxygen species (ROS) and lipid peroxidation during freezing and dissolving processes (Holt 2000). In addition, ROS can affect fertility in mammalian organisms negatively, damaging DNA and protein-important biomolecules. It can even cause cell death (Farber et al. 1990). Enzymatic and non-enzymatic antioxidant mechanisms are used to detoxify ROS in bovine sperm (Meister 1994).

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 (Büyükleblebici et al. 2016; Gulhan 2019; 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 that have various pharmaceutical and antioxidant properties due to its rich natural bioactive components is 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 parts of plants to close the crevices and cracks of the hive in order to prevent microbial contamination (Hashem et al. 2021). Raw propolis 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 propolis 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 propolis 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 for minimize the differences between the samples. Semen samples were kept in a water bath (37°C) for observation of 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 propolis 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 according 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 recognize 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 amounts 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 as at least 200 spermatozoa in the phase-contrast microscope (1000 x).

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

In this study, 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 test of significance, the gathered 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 $P<0.05$.

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 significant effect 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 propolis applied to sperm samples in different doses are shown in the results in Table 3. The P25 and P50 antioxidant treatment groups achieved 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, were discussed 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 advantageous ($p<0.001$).

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). Thanks to its polyphenolic

compounds, P content can be effective in protecting reproductive health by preventing or minimizing stressful conditions that may arise from sudden temperature changes in the cell (Hashem et al. 2013). However, it is thought that P may cause toxic effects in high concentrations, as in all honey bee products (Fikri et al. 2019). Evaluating the results of the current study, 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). Tail abnormalities were significantly reduced in P25 and P50 treatments, while total abnormality values were not decreased in all P doses at the end of the freeze-thawing. Our results showed a gradual decrease in semen abnormalities upon the addition of high levels of P concentration.

There are some studies that examine motility changes by adding 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 (2%, 3%, 4%, and 5%) 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 (0, 0.05, 0.1, 0.2, 0.3, and 0.4%) added to bull semen did not show any change in sperm motility properties (Shahzad et al. 2016). Parallel to the conclusions reached here, the research findings of Inanç et al (2019) revealed that green tea extract supplemented to 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 at concentrations of 0.1%, 0.2%, 0.3, and 0.4%, had no advantage on sperm motility. In another study, powdered P extract added to ram semen extender decreased semen motility parameters during the 6 h incubation period compared to the 0. h (Mohamed and Zanouny 2017). The fact that 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 inability of the bee products used 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 breeds, some researchers have announced that P causes positive changes in sperm motility (Capucho et al. 2012; Hashem et al. 2013; Gabr 2013; Handayani and Gofur 2019). 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 goat semen after cold storage. Fakhrildin and Alsaadi (2014) reported that the addition of 10% honey as a cryoprotectant to the extender showed positive effects on spermatological parameters after thawing 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) and 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. In the current study, antioxidant dose groups P25 and P50 showed positive effects on DNA integrity, while toxic effects were detected at P100 and P200 doses. Following our data, different amounts of cysteine (0.0, 0.5, 1.0 mM) added to the frozen bull semen extender have been reported to have a positive effect on the preservation of DNA integrity (Ansari et al. 2016). Khalifa and Mohamed (2016) have proven that propolis is effective in protecting 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 groups in 6 hours 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 freezing-thawing (Soleimanzadeh et al. 2020). In our previous studies, we found that various plant extracts protect DNA integrity by reducing oxidative damage that may occur after freezing-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 as a result of 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 antioxidant substances (fetuin and cysteine) added 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 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 markers of oxidative stress (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 could eliminate ROS products as co-factors (Ugur et al. 2019). In our study, it is found that MDA levels increased in all P dose applications, with the highest levels in P100 and P200 groups ($p<0.001$) while GSH levels did not change in any of the P treatments ($p>0.05$). 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. The TAC level reached its highest level when P200 was applied ($p<0.001$). These results showed clearly that P has a stimulating effect by triggering intracellular enzymatic reactions. Possible 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 to us 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 hours. Soleimanzadeh et al. (2020) reported that caffeic acid reduced MDA levels at 50 and 100 µM after freezing-thawing in bull semen, increased TAC and GSH activities. In the study of Büyükleblebici et al. (2014), glycerol and ethylene glycol were added at 3, 5, and 7% concentrations. It was reported that 3% ethylene glycole+5 mM cysteine was the combination that reduced MDA levels in bull semen after freezing-thawing. 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 the addition of green tea extract 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 has positive effects on GSH and TAC levels in bull semen, especially at 25 µg/mL doses. 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.

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 the research conducted in this study, it can be clearly said that P25 and P50 doses are effective in maintaining some biochemical and physiological properties of bull sperm, while P200 doses cause toxic effects.

Declarations

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Conflict of Interest Statement (The authors confirm that they have no conflict of interest to declare.)

Consent to participate (Not applicable.)

Availability of data and material (Available with the permission of the authors.)

Code availability (Not applicable.)

Author contributions DY and FA determined DNA damage. MFG prepared propolis extraction and written the manuscript. MEİ determined semen motility and motility kinetic parameters, ŞG examined abnormal spermatozoa rate and did the statistical analysis. RT specified biochemical alterations. PBT obtained ejaculates and froze the semen. UT designed the study, froze the semen, determined motility and motility kinetic parameters and edited the manuscript. All authors read and approved the manuscript.

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Tables

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)

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).