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RESEARCH ARTICLE

The Effect of Gallic Acid Addition to Tris-Based Extender on Frozen Bull Semen

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Abstract

This study aimed to determine the probable protective effect of gallic acid (GA) on the spermatological parameters of frozen-thawed bull semen. Ejaculates were collected from four Holstein bulls. The mixed ejaculate was divided into four equal groups as control (0 µg/mL GA), 50, 100 and 200 µg/mL GA with a Tris-based extender. All extended groups were cooled, equilibrated into a +4°C cold cabinet and loaded into straws. The straws were then frozen and stored in a liquid nitrogen container (-196°C). Subsequently, samples were thawed in a water bath for analyzing motility and kinematic parameters, morphological integrity, DNA damage and biochemical alterations. GA50 (28.76±0.51%) and GA100 (29.32±0.31%) had improved progressive motility in comparison to the other groups (P<0.05). The highest total motility (69.71±0.52%) was detected in GA100 (P<0.05). Besides, the lowest DNA damage and total abnormality values were detected in the GA100 group (P<0.05). GA100 had the highest total antioxidant capacity, as well as the lowest malondialdehyde (MDA) level (P<0.05). In conclusion, GA 50 and 100 µg/mL protects the progressive motility, morphological and DNA integrity by improving the total antioxidant status from the harmful effects of the freezing and thawing protocol.

Keywords: *Antioxidant, Bull semen, DNA damage, Gallic acid*

Boğa Sperması Dondurulması Üzerine Tris Sperma Sulandırıcısına İlave Edilen Gallik Asidin Etkisi

Öz

Bu çalışmada, gallik asitin (GA) dondurulmuş çözdürülmüş boğa sperması spermatolojik parametreler üzerine olası koruyucu etkisinin belirlenmesi amaçlandı. Ejakulatlar dört adet Siyah ala boğadan alındı. Birleştirilen ejekulatlar kontrol (0 µg/mL GA), 50, 100 ve 200 µg/mL GA olmak üzere Tris bazlı sulandırıcı ile dört eşit gruba ayrıldı, Sulandırılan gruplar +4°C sıcaklığındaki soğuk kabinde soğutularak ekilibre edildi, payetlendi ve dondurularak sıvı azot tankında (-196°C) depo edildi. Daha sonra örnekler motilite ve kinematik parametreler, morfolojik bütünlük, DNA hasarı ve biyokimyasal analizler için çözdürüldü. Diğer gruplar ile karşılaştırıldığında GA 50 (%28.76±0.51) ve GA 100 (%29.32±0.31) progresif motiliteyi artırdı (P<0.05). En yüksek total motilite %69.71±0.52 ile GA100 grubunda tespit edildi (P<0.05). Bunun yanı sıra, en düşük DNA hasarı ve total anormal spermatozoa oranı GA 100 grubunda belirlendi (P<0.05). GA100 grubunda en yüksek total antioksidan kapasitenin yanında en düşük malondialdehit seviyesi (MDA) tespit edildi (P<0.05). Sonuç olarak, 50 ve 100 µg/mL dozlarındaki GA'nın ilavesi progresif motilite, total motilite, morfolojik ve DNA bütünlüğünü total antioksidan kapasiteyi artırarak dondurma ve çözdürmenin zararlı etkisinden koruduğu belirlendi.

Anahtar sözcükler: *Antioksidan, Boğa sperması, DNA hasarı, Gallik asit*

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Introduction

Semen freezing and artificial insemination (AI) have a potential to increase cattle production. Through the use of AI with frozen semen, semen from high-quality bulls may be used to inseminate thousands of cattle around the world [1]. The frozen semen use is the most suitable method for controlling diseases in high-yielding growing areas, as well as transfer of genetic information to other generations and spreading superior productive breeds [2-4]. Besides, semen freezing technology is very important for the success of AI, which has become the prior technique of breeding in the cattle industry [5]. In addition to these positive effects, the semen freezing protocol induces some irreversible damage to spermatozoa and reduces the live spermatozoa rate, as well as increasing aneuploidy and DNA damage [6-9]. The live spermatozoa rate and DNA integrity have a crucial role in sperm survival in frozen-thawed semen during AI for transmission of genetic material and early embryonic development [10,11]. During the freezing of sperm, the increase in reactive oxygen species (ROS) results in an inability to perform cell functions, and consequently, a gradual decrease in morphological integrity and motility reduces fertilization ability with sperm apoptosis [12]. There is a strong relationship between reduced sperm motility and oxidative stress by ROS [13]. Malondialdehyde (MDA) markers are commonly used to detect oxidative stress [14]. Antioxidant compounds control the effects of ROS and protect spermatozoa against oxidative stress. Non-enzymatic antioxidants also decrease ROS and indirectly protect the plasma membrane from lipid peroxidation. These oxidations and antioxidation levels can be determined based on MDA, which is formed upon peroxidation of lipids [15]. Due to these problems that arise during freezing, researchers have focused on semen diluents to develop an optimum diluent and freezing protocol by introducing new additives. The triphenolic compound gallic acid (GA) (3,4,5-trihydroxy benzoic acid) exists in some plants with organic acids such as lemons, grape seeds, bananas, carobs, sumac, apples, strawberries, gallnuts, and pineapples [16-18]. It is produced as a secondary metabolite pathway shikimic acid in plants [16]. Researchers have reported that GA has antioxidant, antiviral, anti fungal, anti-inflammatory, antitumor and antimutagenic effects [18-21]. Bello and Idris [22] reported that GA is an anti oxidant, and it is formed sometimes as a part of a free molecule or sometimes as a part of a tannin molecule.

This study aimed to determine the effects of GA on the *in vitro* spermatological parameters of motility and kinetic parameters, DNA integrity, morphological integrity and certain biochemical alterations of frozen-thawed bull semen.

Materials and Methods

Ethical Approval

Ethical permission for the study was given by the Afyon Kocatepe University, School of Veterinary Medicine, Animal Experiments Ethics Committee (with the authorization number of 49533702/29).

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co., unless otherwise stated.

Animals and Study Design

Forty ejaculates were collected at different times during the same seasonal period (from January to March) with an artificial vagina from four 2-4-year-old healthy Holstein bulls that were raised in the Sultansuyu Agriculture Business (Sultansuyu, Malatya, Turkey) affiliated with the General Directorate of Agricultural Enterprises. After collection, the semen was left in a water bath at 37°C, and the mass activity of the fresh semen was scored on a hot plate [on a scale of 1 to 5]. Semen volume was recorded with a graded collection tube. The concentration (mL/spermatozoa) of semen was recorded with the photometrical method (Minitube GmbH, Tiefenbach, Germany). The ejaculates with satisfactory characteristics (mass activity ≥3, concentration ≥800×10^6/mL and volume ≥5 mL) were used. The semen was mixed for minimizing differences from the bulls and volume.

Semen Processing and Freezing

The main Tris-based extender (248.22 mM Tris [T1503], 85.36 mM citric acid [C0759], 69.93 mM fructose [F0127], egg yolk 20% v/v and 6% glycerol, 310 mOsm) was prepared with the protocol described by Taşdemir et al.[23]. A 10 mg of GA [G7384] was dissolved with 1 mL of ethanol (Merck, 99%), and the GA stock solution was prepared. The mixed semen was divided into four groups and diluted with the Tris extender containing 0 µg/mL GA (control), 50 µg/mL GA (GA50), 100 µg/mL GA (GA100) and 200 µg/mL GA (GA200). All extended groups were cooled slowly in a water bath (22°C) and equilibrated for 3 h into a +4°C cold cabinet. After equilibration, the diluted semen groups were loaded into straws (0.25 mL) with 16×10^6 sperm cells in each straw, and then, frozen by using automated semen freezing machine (SY LAB Gerate GmbH, Neupurkersdorf, Austria) based on the guidelines reported by Avdatek et al.[24]. After storage (at least 3 mounts), the sample was thawed in a water bath (37°C/30 s for spermatological evaluations.

Spermatozoa Motility and Motility Motion Characters

A computer assisted semen analyzer (SCA, Sperm Class Analyzer, Microptics, Spain) was used to analyze various kinetic parameters and sperm motility after thawing (37°C, 30 s). The sperm motility properties were set as fast (>80 µm/s), medium (>60 µm/s), slow (>20 µm/s) and static. The semen sample (5 µL) was placed onto the slide, covered with a coverslip and analyzed with a 10× lens on

a preheated microscope stage (37°C). Total motility (%), progressive motility (%), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm/s), beat-cross frequency (BCF, Hz), linearity (LIN%) [(VSL/VCL)x100], wobble (WOB%) [(VAP/VCL)x100], straightness (STR%) [(VSL/VAP)x100], and hyperactivation [(VCL/VSL)xALH] were determined. A total of 200-400 spermatozoa per sample were evaluated in five microscopic zones [25].

Abnormal Spermatozoa Rate

The abnormal spermatozoa rate was determined via the fixation method using a Hancock solution under a phase-contrast microscope at 1000x magnification. The Hancock's fixation solution was prepared to contain a saline solution (150 mL), buffer solution (150 mL), distilled water (500 mL) and formalin (62.5 mL of 37%). For spermatozoa fixation, a 1/200 working solution (semen/Hancock solution) was prepared. A 6 µL of the working solution was placed on a slide and covered with a cover slide, and abnormal spermatozoa were determined by counting 300 spermatozoa in a 100x phase-contrast microscope through an immersion lens. The percentages of the normal and abnormal spermatozoa (head, midpiece and tail anomalies) were recorded [26].

Sperm DNA Integrity

Sperm chromatin damage was assessed using the alkaline single-cell gel electrophoresis (COMET Assay) method. The samples (slides) were examined under a fluorescent microscope (Olympus CX31), and the resultant images were scored using the Comet Score software (TriTek, V. 1.5). DNA integrity determination was held on basic protocol; eliminating captures not corresponding to Comets (i.e., debris); removing overlapping Comets; or correcting the head and tail detection for those Comets that were misanalysed. When the final number of correctly analyzed Comets was less than 50, more images from slides were captured and the process was repeated until obtaining at least 50 correctly analyzed images. Tail DNA, tail moment and tail length are relative measurement of the amount of DNA in the Comet tail in relation to the total DNA amount, expressed in percentage and calculated as: (tail intensity)/(comet intensity). Tail length was calculated as: (tail main intensity + head mean intensity)/2. Tail moment takes into account the amount of tail DNA, but also the differences of optical intensities between the Comet head and the Comet tail. This parameter was calculated as: (tail mean intensity-head mean intensity)xtail DNA/100. In every specimen, a total of two hundred spermatozoa observed in six different zones were assessed [27].

Biochemical Alterations

Total antioxidant capacities (TAC) were calculated according to Erel's procedure, spectrophotometrically at 660 nm [28]. A TAC Rel Assay brand kit (Rel Assay Diagnostics, Gaziantep, Turkey) was used. The discoloration of antioxidant molecules was used to make the calculation. As a calibration, Trolox was used. The results are presented as mmol Trolox Eq/L. The reference range for human serum is given by the manufacturer as 1.20-1.50 mmol/L, no data being available for seminal plasma. Measurements in duplicate were used to calculate intra-assay variability.

A MDA commercial kit (MDA586; OxisResearch, Portland, OR, USA) was used to assess MDA as a lipid peroxidation marker. The reaction of a chromogenic reagent, N-methyl-2-phenylindole (NMPI), with MDA at 45°C was used to make the measurement. A spectrophotometer was used to calculate the absorbance at 586 nm (Shimadzu UV-1601). MDA values were determined in µmol/mL [28]. The absorbance at 586 nm is a linear function of the MDA concentration over the range from 0.5 µM to 20 µM.

Glutathione peroxidase (GPx) enzyme activity in the semen was assessed using an Oxis Research test kit (Bioxytech® GPx340™, Portland, Oregon, USA). The GPx-340™ assay is an indirect indicator of cellular glutathione peroxidase (cGPx) activity. To test for cGPx, the sample was mixed with glutathione, glutathione reductase and NADPH in a solution. The tertbutyl hydroperoxide enzyme reaction was started by adding the substrate. The absorbance at 340 nm was measured every 15 sec for 3 min. The GPx activity in the sample was directly proportional to the rate of the decrease in absorbance at 340 nm. The findings are expressed in mU/mL [28]. While samples with a change in absorbance of less than 0.035 A340/min can be accurately assayed if the protocol is modified (longer reaction times), OXIS recommends that the researcher obtain values from 0.035 to 0.15 A340/min using the stated protocol. This corresponds to approximately 5.6 to 24 mU/mL enzyme activity.

Statistical Analysis

Before the significance tests, the obtained data were tested for normal distribution by Shapiro Wilk test. The homogeneity of the variances was examined with Levene's test. The statistical analysis of the difference between the variables was achieved with analysis of variance (ANOVA). Tukey's test was used to evaluate the differences between the groups. Descriptive statistics for each variable were calculated and are presented as "Mean ± Standard Mean Deviation" (Mean ± SD). All statistical analyses were interpreted with a minimum error of 5%. Using the SPSS 22.0 package program, $P<0.05$ was considered statistically significant.

Results

The results on semen CASA motility and kinetic parameters are given in *Table 1*. The GA50 (28.76±0.51%) and GA100 (29.32±0.31%) groups had improved motility in comparison to the other groups ($P<0.05$). Additionally,

Table 1. *CASA motility and kinetic parameters values in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
Progressive motility (%)	26.61±0.65^{a}	28.76±0.51^{b}	29.32±0.31^{b}	27.07±0.41^{a}	*
Total motility (%)	66.25±0.39^{b}	66.13±0.51^{b}	69.71±0.52^{a}	64.90±0.52^{b}	*
VAP (µm/s)	50.54±0.53^{b}	49.02±0.37^{ab}	47.83±0.77^{a}	52.60±0.66^{c}	*
VSL (µm/s)	35.74±0.28^{a}	39.82±0.40^{c}	37.77±0.26^{b}	36.94±0.51^{b}	*
VCL (µm/s)	80.54±0.54^{a}	86.77±0.89^{b}	86.37±0.74^{b}	85.64±1.19^{b}	*
ALH (µm/s)	3.89±0.09^{a}	3.89±0.06^{a}	4.15± 0.05^{b}	4.18±0.04^{b}	*
BCF (Hz)	12.27±0.29	12.82±0.23	12.42±0.22	12.34±0.34	-
LIN (%)	44.30±0.46^{ab}	45.87±0.88^{b}	43.70±0.81^{ab}	42.93±0.73^{a}	*
STR (%)	74.56±0.96	75.73±0.83	74.61±0.79	74.83±0.51	-
WOB (µm/s)	59.38±0.65^{ab}	58.51±0.89^{ab}	60.56±0.68^{b}	57.35±0.90^{a}	*
Hyperactivity (%)	27.31±2.20	29.66±2.93	30.64±1.89	27.47±0.62	-

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

Table 2. *Spermatozoa abnormality values in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
Head (%)	8.90±0.39^{b}	7.88±0.70^{ab}	7.11±0.26^{a}	7.91±0.49^{ab}	*
Mid-piece (%)	10.54±0.33	10.17±0.17	9.68±0.50	10.22±0.72	-
Tail (%)	6.92±0.28	6.45±0.30	6.24±0.16	6.56±0.11	-
Total (%)	26.36±0.94^{c}	24.51±0.51^{b}	23.03±0.44^{a}	24.69±0.31^{b}	*

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

Table 3. *Chromatin damage values in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
Tail lenght (µm/s)	21.16±0.29^{c}	14.81±0.18^{b}	11.95±0.29^{a}	15.6±0.30^{b}	*
Tail DNA (%)	34.15±0.46^{d}	21.96±0.30^{b}	19.86±0.97^{a}	27.78±0.41^{c}	*
Tail moment (µm/s)	20.03±0.20^{d}	10.96±0.26^{b}	7.20±0.10^{a}	17.25±0.37^{c}	*

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

the highest total motility (69.71±0.52%) was detected in the GA100 group (P<0.05). The spermatozoa were evaluated in terms of kinetic properties, where the highest VAP (µm/s) was detected in GA200, while the lowest VSL (µm/s), VCL (µm/s) and ALH were detected in the control group (P<0.05). There were differences between the GA50 (45.87±0.88%) and GA200 (42.93±0.73%) groups in terms of their LIN parameters (P<0.05). There were no differences among the groups based on their BCF (Hz) and hyperactivity (%) values (P>0.05).

The results of the morphological evaluation (spermatozoa abnormality) are given in *Table 2*. There were no significant differences between the groups based on their midpiece and tail abnormality rates (P>0.05). The lowest head (7.11±0.26%) and total abnormality (23.03±0.44%) rates were in the GA100 group (P<0.05).

DNA damage was significantly different among the treatment groups (P<0.05) as shown in *Table 3*. The lowest comet evaluation parameters of tail length (µm/s), tail DNA (%) and tail moment (%) were detected in GA100. In control group chromatin damage values [tail length (µm/s), tail DNA (%) and tail moment (%)] were higher than the treatment groups (P<0.05). The biochemical alterations of the frozen-thawed bull semen parameters are shown in *Table 4*. The GA100 groups displayed the highest TAC (0.81±0.03 Trolox equiv./L) and GPx activity (11.14±0.33 mU/mL) (P<0.05), as well as the lowest MDA level (7.05±0.38 µmol/mL) (P<0.05). Highest MDA level and lowest GPx activity were shown in the control group (P<0.05).

Discussion

At artificial insemination and *in vitro* fertilization organizations/procedure/application, spermatozoa motility is great importance for successful fertilization [29]. During fertilization, it has been shown that the spermatozoa are transported to the fertilization area to combine with the ovum [30]. Frozen

Table 4. *Total antioxidant capacities (TAC), malondialdehyde (MDA) levels and glutathione peroxidase (GPx) activities in frozen thawed bull semen*

Analysis	Control	GA 50 µg/mL	GA 100 µg/mL	GA 200 µg/mL	P
TAC (mmol Trolox Eq/L)	0.36±0.01[a]	0.40±0.02[a]	0.81±0.03[d]	0.59±0.03[c]	*
MDA (µmol/mL)	12.92±0.36[c]	7.99±0.43[a]	7.05±0.38[a]	9.30±0.15[b]	*
GPx (mU/mL)	8.62±0.10[a]	9.66±0.13[b]	11.14±0.33[c]	9.86±0.21[b]	*

*[a,b,c,d] Different superscripts within the same row demonstrate significant differences (*P<0.05); - No significant difference(P>0.05)*

thawed bull semen is more easily peroxidized than fresh sperm. Additionally, intracellular antioxidant capacity in sperm decreases following the freeze-thawing process [31]. The axosome and associated dense fibers of the middle pieces in spermatozoa are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility. In this study, the highest total motility values were detected in the GA100 group (P<0.05). In comparison to the other groups, GA100 and GA50 had improved progressive motility values (P<0.05). The positive effects of GA addition (except for the GA200 group) on progressive motility were supported by other researchers. Gungor et al.[32] supplemented GA into tris a ram semen extender and observed improved levels of post-thawing motility. Tris extender with GA was seen to improve viability and plasma membrane integrity in ram semen [33]. Although the species whose sperms were frozen were different, it was interpreted that this positive effect of GA was due to its cryoprotective effect on spermatozoa in harmony with other components added to the diluent. Besides motility, CASA kinetic parameters are important for the evaluation of individual spermatozoa movements. Many researchers have investigated the relationship among kinetic parameters in bull spermatozoa and conducted in vitro oocyte binding ability and fertilization studies in the field [34,35]. Amann [36] examined motility kinetic parameters in frozen-thawed bull semen, and they determined the VCL, VSL, LIN, ALH and BCF parameters to be associated with the fertility index. Nagy et al.[37] found that VCL, VSL and VAP values among velocity parameters are more useful for reflecting the results of AI. In this study, there was no significant difference among the groups in terms of their spermatozoa kinetic parameters compared control except for VAP, ALH, VSL and VCL. Incompatibility with this study, Daghigh et al.[38] was detected an ameliorating effect on the VSL, VCL and progressive motility values of frozen-thawed bull semen by supplementation of Origanum vulgare, which contains natural antioxidants and high levels of phenolic compounds including gallic acid. These supporting results were also in line with the reports of Osawa [39], who discovered that GA inhibits the adverse effects of free radicals, metal ions and hydroxyl groups by binding them. Lozano et al.[40] reported that the pro-oxidant actions of polyphenols (PP), flavonoids, anthocyanin's and carotenoids are typically catalyzed by transition metals such as Cu and Fe within cells under certain O_2 and pH conditions. GA can act as antioxidant/pro-oxidant reactions associated with efficient electron transfer capacity. Thus, it inhibits the uncontrolled ROS production to prevent the cell damage [41]. Moreover, a positive correlation was found between this compound and the LIN parameter in a ram semen cervical mucus test [30]. However, the results of the treatment and control groups were not different in the aforementioned study.

There were no significant differences among the groups in terms of their midpiece and tail abnormality rates (P>0.05). The lowest head and total abnormality rates were in the GA100 group (P<0.05). This result was also supported by the lowest DNA damage in this group (P<0.05). Majorly morphologically abnormal spermatozoa have been associated with decreased fertility, and morphological evaluation is used to provide an indication of the potential fertility of spermatozoa in bulls, whereas sperm DNA damage has an adverse effect on embryo development and subsequently fertility [42]. Consistent with this study, Mehraban et al.[18] identified the protective effect of GA on cyclophosphamide (chemotherapy drugs) toxicity in terms of not only morphological and DNA integrity but also viability and concentration. GA was also found to reduce abnormal spermatozoa rates in mice sperm induced with exposure to opioid drugs with analgesic properties (morphine) [43]. In the present study, the lowest DNA damage was observed in the GA100 group. In support of our findings, Wen Weng et al.[44] reported that GA alleviated DNA damage by DNA repair-associated protein expression in the SCC-4 cell line, namely human oral cancer cells. This result showed that GA stimulates cytotoxic effects on human cancer cells by cell-cycle arrest and induction of apoptosis. Erol et al.[45] found that GA significantly reduced both mitochondrial DNA and nuclear DNA damages that were induced by H_2O_2 exposure. When GA is used at the optimum doses, it is thought that it improves DNA integrity and repairs morphological abnormalities thanks to its antioxidant and anti-mutagenic effects.

Cryopreservation reduces the functional and structural integrity of bull spermatozoa, and is associated with ROS production. Oxidative stress during freezing of semen can induce functional and structural damage to spermatozoa involving ROS-mediated pathways [7]. Although bull semen has a natural defense system against the ROS, it is considered insufficient in protecting spermatozoa under cryopreservation mediated stress [46]. Hence, GA's mitochondrial PP acts might be occurring combined effect of antiproliferative mechanism and antioxidant activity

from more than one triggered mechanism. Beyond their antioxidant activity, some PP may decrease mitochondrial membrane fluidity or have a molecular mechanism related to: mimicking of the Bcl-2 homology-3 (BH3) domains, hexokinase inhibition and thiol redox inhibition [47]. Nevertheless, despite the fact that not all PP have the same properties and mitochondrial-related mechanisms, all of them have ROS-scavenging actions either at the ROS-removing or ROS-formation levels [48]. Also, PP cellular environment and concentration may influence these actions, and whether PP acts as pro-oxidant molecules. Although the selective antiproliferative effects of pro-oxidant PP are not fully known, it is attributed to the formation of an unstable redox complex with a metal cation supported by an unstable radical aroxyl or pro-oxidant PP, as metal ions catalyze the formation of ROS via Fenton or Fenton-like reactions [49]. Therefore, the addition to semen extenders of suitable antioxidants is suggested to reduce oxidative damage during freeze–thawing of bull spermatozoa [50]. GPx and TAC indicative activity of antioxidant levels. In this study, the highest TAC and GPx activity were detected in the GA100 group ($P<0.05$), which also showed the lowest MDA (not different from GA50) level. In line with this study, Jofre et al.[51] stored boar semen with different concentrations of Murtilla (Ugni molinae Turcz (average phenolic content includes gallic acid) extract at 17°C for 168 h. The long-term analyses showed that the Murtilla extract had a protective role on semen motility decay and reduced ROS and membrane damage. GA is known to effects inflammatory bioactivities and exhibit antioxidant properties [19]. That could explain all these positive effects of GA by protection against oxidative stress.

In conclusion, it was determined that supplementation of GA by 50 and 100 µg/mL into tris-egg yolk semen extender protected progressive motility, total spermatozoa abnormality and DNA integrity from the harmful effects of the freezing and thawing protocol by the improved total antioxidant status.

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Conflict of Interest

There is no conflict to interest.

Author Contributions

ŞG examined abnormal spermatozoa rate, did the statistical analysis and prepared original manuscript draft, MEİ examined abnormal spermatozoa rate, determined semen motility and motility kinetic parameters, prepared the original manuscript draft, DY and FA specified DNA damage, RT analyzed biochemical alterations, UT designed the study, froze the semen, determined motility and motility kinetic parameters and edited the manuscript.

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