

# The Effect of Green Tea Extract Supplementation in Bull Semen Cryopreservation

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## Abstract

This study aims to investigate the effect of catechin (CT), green tea extract, as a supplement to Tris extender on semen quality parameters in frozen-thawed bull sperm. Ejaculates were taken with artificial vagina from Holstein bulls and divided equal five aliquots, diluted to containing different amounts of CT (5, 10, 25 and 50 µg/mL) and no-additive (control). All samples were equilibrated at 4°C for 4 h and were frozen using a digital freezing machine. Post-thawed sperm motility and kinetic parameters were determined using the sperm analyser system. Spermatozoa DNA integrity was evaluated with the single cell gel electrophoresis, abnormal spermatozoa rate was evaluated by fluid fixation test and lipid peroxidation status was evaluated colorimetrically. CT supplementation did not improve motility and kinetic parameters. However, the higher morphological integrity was detected in CT10, 25 and 50 groups compared to control (P<0.05). Regarding chromatin integrity, positive effects of catechin were observed in the treatment groups while in CT 50 group adverse effects were found (P<0.05). Although there was no improvement in malondialdehyde levels, the highest total antioxidant activity was seen in the CT50 group (P<0.05). In conclusion, CT supplementation could be used the protection of morphological and DNA integrity from cryodamage and it has increased the total antioxidant activity depending of the dose in bull semen.

**Keywords:** Bull semen freezing, Catechin, DNA Integrity, Lipid peroxidation

## Yeşil Çay Ekstraktı İlavesinin Boğa Spermaları Dondurulmasına Etkisi

### Öz

Bu çalışmanın amacı yeşil çay ekstraktı olan kateşinin (CT) tris sulandırıcısına eklenerek boğa spermaları dondurulmasında dondurma çözündürme sonucu spermaları kalitesinin incelenmesidir. Ejakülatlar Holstein boğalardan alınarak eşit beş kısma ayrıldı ve farklı oranlarda (5, 10, 25 and 50 µg/mL) CT içeren ve içermeyen (kontrol) sulandırıcı ile sulandırıldı. Bütün örnekler 4°C'de 4 saat ekilibasyona bırakıldı ve daha sonra otomatik dondurma makinası ile donduruldu. Çözüm sonu spermatozoa motilitesi ve kinetik parametreler spermaları analiz sistemi ile değerlendirildi. DNA bütünlüğü tek hücre jel elektroforezi, anormal spermatozoa oranı sıvı fiksasyon testi ile, lipit peroksidasyon seviyesi ise kolorimetrik olarak ölçüldü. CT ilavesinin motilite ve kinetik parametreleri olumlu olarak etkilemediği görüldü. Fakat CT10 ve 25 ve 50 gruplarında kontrol grubuna göre daha yüksek morfolojik bütünlük tespit edildi (P<0.05). DNA bütünlüğü açısından CT'nin olumlu etkisi görülürken; CT 50 grubunda olumsuz etki tespit edildi (P<0.05). Malondialdehid seviyesi açısından herhangi bir iyileşme tespit edilememesine rağmen en yüksek total antioksidan kapasite CT50 grubunda görüldü. CT ilavesinin morfolojik ve DNA bütünlüğünü total antioksidan kapasitesini yükselterek soğuktan koruduğu tespit edildiği için boğa spermalarında doza bağlı olarak kullanılabileceği sonucuna varıldı.

**Anahtar sözcükler:** Boğa spermaları dondurulması, Kateşin, DNA bütünlüğü, Lipid peroksidasyon



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## INTRODUCTION

Sperm cryopreservation is essential for preserving the genetic diversity, conservation of wild and domestic species, worldwide dissemination of genetic progress and livestock management [1]. However, it causes detrimental effects on sperm quality parameters, such as motility, morphology, viability and DNA integrity through the cryoinjury and it may also lead to the production of reactive oxygen species (ROS) [2]. Notably, the cold shock and atmospheric oxygen exposition during the semen collection and freezing/thawing procedures render the semen vulnerable for lipid peroxidation and lead to further damage to spermatozoa [3]. The primary sources of ROS in semen are the immature spermatozoa and leucocytes. Although adequate levels of ROS are required for some cellular processes as the capacitation, hyperactivation and binding of spermatozoa to the zona pellucida, excess amounts of ROS can adversely affect the motility, morphology and concentration of sperm as well as it can cause DNA damage and lipid peroxidation in the sperm [4]. Thus, to prevent the damage caused by ROS on spermatozoa, there has been a growing interest in the use of plant-based substances as antioxidants in assisted reproductive technologies [5]. Up to now, many plant-derived compounds, notably including carotenoids and flavonoids, have been studied for their antioxidant capacity to improve the fertility as components of *in vitro* culture media and through their intake as dietary supplements [6]. Some polyphenols have been found to exhibit higher antioxidant activity and lower toxicity than synthetic antioxidants [7]. As one of the natural antioxidants, green tea polyphenols are water-soluble, phytochemical flavonoids and include epigallocatechin gallate, epicatechin gallate, epicatechin and epigallocatechin [8]. They are found in high density in a variety of plant-based beverages and foods such as apricots, strawberries, black grapes and broad beans [3,9]. Consumption of catechin (CT) has been associated with the increased plasma antioxidant activity (the ability of plasma to scavenge free radicals), the resistance of LDL to oxidation and fat oxidation, while decreasing the plasma lipid peroxide and malondialdehyde (MDA) concentrations [10]. Recently, various effects of CT buck, ram and boar semen [11,12] have been presented by several research groups however, there were very few studies regarding its effect on the cryopreservation process in bulls [13]. Thus, the current study aims to investigate the effect of CT addition into Tris extender on sperm quality parameters following the cryopreservation of bull sperm.

## MATERIAL and METHODS

### Animal Experiments and Semen Collection

Semen from five bulls (Holstein breed) with proven fertility, aged 3-5 years, from Sultansuyu Agribusiness (Sultansuyu, Malatya, Turkey) was used for this study. Ejaculates taken by an artificial vagina once a week, the ejaculates were

pooled to eliminate variability among the evaluated samples. This trial was replicated ten times for each group. All samples were kept in 37°C water bath for further evaluation of motility, concentration and progressive motility. All experiments were carried out in accordance with the approval of the Animal Care Committee of Afyon Kocatepe University Veterinary Medicine Faculty regarding ethics, with the authorisation number 49533702/29.

### Semen Processing and Freezing

Semen volume was determined via a graded collection tube, and concentration was calculated with a photometer (Minitube GmbH, Tiefenbach, Germany). Samples were showing a minimum of 80% progressive motile and of 80% morphologically normal spermatozoa were used. A Tris-based extender was used to the primary medium in this study [5]. Extracted CT (10 mg) was diluted with 1 mL ethanol (Merck, 99%) to create the CT stock solution. Ejaculates were divided into five aliquots and extended  $15 \times 10^6$  spermatozoa/straw with the Tris extender containing no-additive (control) and CT (5, 10, 25 and 50  $\mu\text{g}/\text{mL}$ ), and subsequently, sperm was loaded into mini straws. The experiment semen samples were cooled (4°C) and equilibrated for 4 h. After, every group was frozen with controlled semen freezing machine (SY LAB Gerate GmbH, Neupurkersdorf, Austria) with Avdatek et al. [5] protocol. Finally, the straws were immersed in liquid nitrogen at -196°C. Frozen straws were thawed individually at 37°C for 30 s in a water bath for post-thawed spermological evaluations.

### Assessment of Sperm Motility

Spermatozoa motilities were assessed using Computer-Assisted Sperm Analysis (CASA) system (Microptic S.L., Barcelona, Spain). A 5  $\mu\text{L}$  diluted semen sample was put onto a slide (pre-warmed) put on to cover slide and percentages of progressive and non-progressive motility, as well as total motility, were recorded. Besides, motility kinetic parameters (curvilinear velocity  $\mu\text{m}/\text{s}$  (VCL), Straight linear velocity  $\mu\text{m}/\text{s}$  (VSL), average path velocity  $\mu\text{m}/\text{s}$  (VAP), amplitude of lateral head displacement,  $\mu\text{m}$  (ALH), Wobble (WOB,  $[\text{VAP}/\text{VCL}] \times 100$ ), beat cross frequency (BCF), Linearity (LIN,  $[\text{VSL}/\text{VCL}] \times 100$ ) and Strainness (STR,  $[\text{VSL}/\text{VAP}] \times 100$ ) and were determined. The spermatozoa motilities were calculated set as static, slow  $>20 \mu\text{m}/\text{s}$ , medium  $>60 \mu\text{m}/\text{s}$  and fast  $>80 \mu\text{m}/\text{s}$  protocols. For each assessment, between 220 and 370 spermatozoa were analysed in six different fields in microscope [5].

### Evaluation of Sperm Morphology

Spermatozoa morphologies were evaluated Schafer and Holzmann [14] protocols. Hancock solution (500 mL double-distilled water with 150 mL buffer solution, 150 mL saline solution and 62.5 mL formalin 37%) was used. 10  $\mu\text{L}$  sample was added to 1000  $\mu\text{L}$  Hancock solution to examine spermatozoa morphological integrity. 5  $\mu\text{L}$  mix was put on a slide and mounted with a cover slide. The morphological

integrity (tail, acrosome, head and total abnormality) were evaluated under phase-contrast microscopy (1.000×) by evaluated minimum 200 spermatozoa.

### Evaluation of DNA Integrity

Spermatozoa DNA integrity was evaluated by the comet (single cell gel electrophoresis) assay kit using a (Trevigen, Gaithersburg, MD, USA). Slides were examined under fluorescent microscopy (Olympus CX31, Tokyo, Japan), and images were reflected for following scoring analysis with TriTek Comet Score software (V. 1.5). On each sample, a total of 100 spermatozoa cells from five different fields were evaluated for analysis [15].

### Assessment of Oxidative Stress

Total antioxidant (TA) status was evaluated by using a colourimetrically commercial kit (RelAssay®, Gaziantep, Turkey). Glutathione peroxidase (GPx) activity was identified using a GPx assay (OxisResearch™, Bioxytech® GPx-340™, Portland, USA). Levels of lipid peroxidation, which depends on MDA, were measured using a commercial kit (MDA-586; OxisResearch, Portland, USA). The results were indicated in  $\mu\text{mol/mL}$  [16].

### Statistical Analysis

One-way analysis of variance (ANOVA) and Duncan's post hoc test were used to state the differences among

the treatment groups in terms all spermatological and biochemical parameters. Data are presented as a mean  $\pm$  standard error of means (SEM). The degree of significance was set at  $P < 0.05$ . SPSS/PC (Version 10.0; SPSS, Chicago, IL) software package program was used for all analysis.

## RESULTS

As presented in *Table 1*, CT supplementation did not enhance the motility or the kinetic parameters of sperm. In other respects, CT50 had led to a significant decrease in motility and sperm motion characteristics ( $P < 0.05$ ). CT10, 25 and 50 concentrations have shown lower total abnormalities compared the control (*Table 2*;  $P < 0.05$ ) however, CT50 has produced unfavorable results regarding the chromatin integrity (*Table 3*;  $P < 0.05$ ). The other treatment groups had shown the lowest tail moment values, indicating the minimal DNA damage (*Table 3*;  $P < 0.05$ ). As shown in *Table 4*, the increased antioxidant activity of the CT was determined to start from the CT10 concentration ( $P < 0.05$ ). A dose-dependent positive effect was observed regarding the GPx and total antioxidant capacity values however, MDA values were increased as well within the higher dose groups.

## DISCUSSION

As various authors well documented, freezing and thawing steps of cryopreservation reduce the motility

**Table 1.** Mean ( $\pm$ SEM) sperm motility values in frozen thawed bull semen

| Analysis                     | Control                        | CT5                            | CT10                           | CT25                           | CT50                          | P |
|------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|-------------------------------|---|
| Non progressive motility (%) | 26.14 $\pm$ 3.23 <sup>a</sup>  | 37.00 $\pm$ 3.71 <sup>a</sup>  | 32.31 $\pm$ 3.26 <sup>a</sup>  | 36.88 $\pm$ 3.83 <sup>a</sup>  | 14.10 $\pm$ 3.51 <sup>b</sup> | * |
| Progressive motility (%)     | 21.71 $\pm$ 2.80 <sup>a</sup>  | 23.03 $\pm$ 2.44 <sup>a</sup>  | 19.17 $\pm$ 2.21 <sup>ab</sup> | 14.88 $\pm$ 1.90 <sup>b</sup>  | 3.47 $\pm$ 1.26 <sup>c</sup>  | * |
| Total motility (%)           | 47.88 $\pm$ 5.73 <sup>a</sup>  | 60.04 $\pm$ 5.91 <sup>a</sup>  | 51.49 $\pm$ 5.37 <sup>a</sup>  | 51.76 $\pm$ 5.34 <sup>a</sup>  | 17.55 $\pm$ 4.72 <sup>b</sup> | * |
| VAP ( $\mu\text{m/s}$ )      | 78.98 $\pm$ 2.65 <sup>a</sup>  | 73.55 $\pm$ 2.27 <sup>ab</sup> | 76.04 $\pm$ 2.42 <sup>ab</sup> | 67.11 $\pm$ 3.76 <sup>b</sup>  | 49.14 $\pm$ 3.58 <sup>c</sup> | * |
| VSL ( $\mu\text{m/s}$ )      | 55.84 $\pm$ 2.10 <sup>a</sup>  | 48.50 $\pm$ 1.47 <sup>b</sup>  | 47.60 $\pm$ 1.20 <sup>b</sup>  | 39.20 $\pm$ 2.42 <sup>c</sup>  | 28.16 $\pm$ 2.36 <sup>d</sup> | * |
| VCL ( $\mu\text{m/s}$ )      | 111.34 $\pm$ 2.78 <sup>a</sup> | 108.91 $\pm$ 2.45 <sup>a</sup> | 112.86 $\pm$ 2.98 <sup>a</sup> | 106.99 $\pm$ 4.50 <sup>a</sup> | 83.77 $\pm$ 5.95 <sup>b</sup> | * |
| ALH ( $\mu\text{m/s}$ )      | 4.10 $\pm$ 0.08                | 4.12 $\pm$ 0.06                | 4.04 $\pm$ 0.07                | 4.14 $\pm$ 0.05                | 3.52 $\pm$ 0.58               | - |
| BCF (Hz)                     | 11.86 $\pm$ 0.45 <sup>a</sup>  | 11.03 $\pm$ 0.23 <sup>a</sup>  | 11.56 $\pm$ 0.17 <sup>a</sup>  | 10.98 $\pm$ 0.34 <sup>a</sup>  | 8.21 $\pm$ 1.28 <sup>b</sup>  | * |
| LIN (%)                      | 50.16 $\pm$ 1.37 <sup>a</sup>  | 44.53 $\pm$ 0.86 <sup>b</sup>  | 42.31 $\pm$ 1.17 <sup>b</sup>  | 36.70 $\pm$ 1.72 <sup>c</sup>  | 33.82 $\pm$ 1.92 <sup>c</sup> | * |
| STR (%)                      | 70.68 $\pm$ 1.16 <sup>a</sup>  | 66.01 $\pm$ 1.01 <sup>b</sup>  | 62.87 $\pm$ 1.71 <sup>bc</sup> | 58.62 $\pm$ 1.97 <sup>cd</sup> | 57.01 $\pm$ 1.60 <sup>d</sup> | * |
| WOB $\mu\text{m s}^{-1}$     | 70.85 $\pm$ 1.01 <sup>a</sup>  | 67.47 $\pm$ 0.89 <sup>a</sup>  | 67.32 $\pm$ 0.68 <sup>a</sup>  | 62.51 $\pm$ 1.35 <sup>b</sup>  | 58.97 $\pm$ 1.88 <sup>b</sup> | * |

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

**Table 2.** Mean ( $\pm$ SEM) sperm abnormality values in frozen thawed bull semen

| Analysis                    | Control                       | CT5                            | CT10                            | CT25                           | CT50                         | P |
|-----------------------------|-------------------------------|--------------------------------|---------------------------------|--------------------------------|------------------------------|---|
| Head abnormalities (%)      | 2.95 $\pm$ 2.83               | 3.86 $\pm$ 2.41                | 1.75 $\pm$ 1.77                 | 2.59 $\pm$ 2.08                | 2.44 $\pm$ 1.73              | - |
| Mid-piece abnormalities (%) | 7.34 $\pm$ 4.86 <sup>a</sup>  | 5.63 $\pm$ 1.47 <sup>ab</sup>  | 3.89 $\pm$ 3.51 <sup>b</sup>    | 4.34 $\pm$ 3.39 <sup>ab</sup>  | 2.65 $\pm$ 1.83 <sup>b</sup> | * |
| Tail abnormalities (%)      | 6.21 $\pm$ 4.35 <sup>a</sup>  | 4.03 $\pm$ 4.16 <sup>ab</sup>  | 6.01 $\pm$ 4.95 <sup>a</sup>    | 3.62 $\pm$ 2.61 <sup>ab</sup>  | 1.97 $\pm$ 0.78 <sup>b</sup> | * |
| Total abnormalities (%)     | 16.50 $\pm$ 5.85 <sup>a</sup> | 13.53 $\pm$ 6.18 <sup>ab</sup> | 11.65 $\pm$ 5.96 <sup>abc</sup> | 10.56 $\pm$ 4.65 <sup>bc</sup> | 7.07 $\pm$ 3.96 <sup>c</sup> | * |

<sup>a,b,c</sup> Different superscripts within the same row demonstrate significant differences; \* $P < 0.05$ ; - No significant difference ( $P > 0.05$ )

**Table 3.** Mean ( $\pm$ SEM) chromatin integrity values in frozen thawed bull semen

| Analysis                 | Control                       | CT5                            | CT10                           | CT25                          | CT50                          | P |
|--------------------------|-------------------------------|--------------------------------|--------------------------------|-------------------------------|-------------------------------|---|
| Tail length ( $\mu$ m/s) | 19.27 $\pm$ 3.18 <sup>b</sup> | 14.26 $\pm$ 3.12 <sup>bc</sup> | 11.71 $\pm$ 3.97 <sup>bc</sup> | 8.75 $\pm$ 2.61 <sup>c</sup>  | 37.60 $\pm$ 4.07 <sup>a</sup> | * |
| Tail DNA (%)             | 19.11 $\pm$ 3.06 <sup>b</sup> | 12.36 $\pm$ 1.59 <sup>b</sup>  | 23.04 $\pm$ 5.58 <sup>b</sup>  | 17.85 $\pm$ 1.77 <sup>b</sup> | 45.10 $\pm$ 4.90 <sup>a</sup> | * |
| Tail moment ( $\mu$ m/s) | 29.01 $\pm$ 4.06 <sup>a</sup> | 12.59 $\pm$ 2.06 <sup>b</sup>  | 11.16 $\pm$ 2.04 <sup>b</sup>  | 7.43 $\pm$ 2.77 <sup>b</sup>  | 22.30 $\pm$ 3.17 <sup>a</sup> | * |

<sup>a,b,c</sup> Different superscripts within the same row demonstrate significant differences; \* P<0.05; · No significant difference (P>0.05)

**Table 4.** Mean ( $\pm$ SEM) glutathione peroxidase (GPx), malondialdehyde (MDA) and total antioxidant (TA) activities in frozen thawed bull semen

| Analysis  | Control                       | CT5                            | CT10                           | CT25                          | CT50                          | P |
|---|-------------------------------|--------------------------------|--------------------------------|-------------------------------|-------------------------------|---|
| GPx (mU/mL)   | 12.53 $\pm$ 0.12 <sup>d</sup> | 12.91 $\pm$ 0.15 <sup>cd</sup> | 13.22 $\pm$ 0.20 <sup>bc</sup> | 13.69 $\pm$ 0.29 <sup>b</sup> | 15.83 $\pm$ 0.14 <sup>a</sup> | * |
| MDA ( $\mu$ mol/mL)   | 2.51 $\pm$ 0.02 <sup>d</sup>  | 2.57 $\pm$ 0.02 <sup>d</sup>   | 2.67 $\pm$ 0.03 <sup>c</sup>   | 2.82 $\pm$ 0.02 <sup>b</sup>  | 3.12 $\pm$ 0.02 <sup>a</sup>  | * |
| Total antioxidant activities (mmol/trolox/mL $\cdot$ 10 <sup>9</sup> cell/mL) | 0.14 $\pm$ 0.01 <sup>d</sup>  | 0.15 $\pm$ 0.02 <sup>d</sup>   | 0.16 $\pm$ 0.02 <sup>c</sup>   | 0.24 $\pm$ 0.01 <sup>b</sup>  | 0.27 $\pm$ 0.01 <sup>a</sup>  | * |

<sup>a,b,c,d</sup> Different superscripts within the same row demonstrate significant differences; \* P<0.05; · No significant difference (P>0.05)

and fertilizing ability of spermatozoa leads to damage of plasma membrane [17], induce premature capacitation and nuclear decondensation [18]. ROS production during these cycles is considered to be one of the major cause that is accountable for the emerging impairment in the functionality of the sperm cell [19]. Whereas the problem is well-being pointed, the solution to prevent these damage is still being researched by many scientific groups. In recent years, numerous antioxidants have been introduced to the cryopreservation process on account of enhancing the post-thawed quality of sperm. Due to lack of carbonyl group in the epicatechin molecules, it is considered as a less potent antioxidant than other flavonoids such as quercetin or naringenin [12,20]. In the meantime, the emerging use of plant-based scavengers has resulted in controversial effects [7,21,22]. In the present study, CT supplementation did not enhance the post-thawed motility or kinetic parameters, but rather, adverse effects were observed in the highest dose group (P<0.05). In accordance with the delivered study, Gale et al. [23], the addition of green tea extract to the cryo-medium of boar semen extender did not produce any beneficial effects on motility, viability, acrosome integrity or membrane integrity. Another boar sperm study, in which, the toxicity of green tea extract on chilled spermatozoa was evaluated. Although no toxic effect was observed, sperm quality parameters did not differ between the control and different concentrations of green tea extract supplementation [11]. Additionally, in several studies on different species, the inclusion of natural antioxidants did not produce any positive effects [24-26]. On the other hand, several previous studies in different species generated results inconsistent to those found in current study, namely in chilled dog semen, the addition of green tea polyphenols into the extender has shown a significant protective effect on the motility and viability parameters up to four weeks of semen [8]. Also on boar semen [11] and at low concentration on human sperm [27] motility can be improved by green tea extract. Khan et

al. [28] have cryopreserved the bull semen with different rates of (0.25; 0.5; 0.75; 1.0%) green tea extract and evaluated *in vitro* spermological parameters (motility, viability and membrane integrity). They found the highest progress in 0.75% green tea groups. Besides, in bull semen cryopreservation, in which motility and membrane integrity were improved with the supplementation of green tea extract [13]. Considering our results, we can hypothesize that the discrepancy in the results may be due to the density of the other substances (Tris, egg yolk) used in the extender.

Since catechins are unstable molecules, ROS formation can occur due to auto-oxidation. The stability of these molecules can be altered depending on the environmental temperature, pH or oxygen level [29]. When the antioxidant capacity of the samples was evaluated, decreased lipid peroxidation level was observed in CT concentrations starting from the 10  $\mu$ g/mL (P<0.05). A dose-dependent positive effect was detected regarding the GPx and total antioxidant capacity; however, MDA values were increased as well within the higher dose groups. El-Seadawy et al. [30] have found that addition of pomegranate peel methanolic extract enriched with CT into chilled rabbit semen has decreased the lipid peroxidation level and increased the antioxidant activity. Similar results were observed in a rat [31], ram [12] and stallion [32] semen in which, were extended by CT supplementations. Also, Sugiyama et al. [33] reported that epigallocatechin gallate promotes protection against testicular ischemia-reperfusion injury due to its antioxidant activity since epigallocatechin gallate is the major CT compound of green tea extract with a 52% ratio of the total CT content [34]. The results demonstrated that polyphenols might interact with components of the spermatozoa and would have decreased the lipid peroxidation induced by free radicals [35]. On the contrary with the present study, Moretti et al. [20] supplemented swim-up selected human semen with 200  $\mu$ M epicatechin and they could not find any improvement regarding the antioxidant capacity. This

result might have seen due to the chemical structure of epicatechin which does not contain the carbonyl group.

The COMET assay is widely used for analysing DNA damages in multiple cell [36]. It is a suitable cell evaluation method while maintaining the integrity of genetic material in biological evaluations [37]. Zini et al. [38] reported that DNA damage of spermatozoa has a high impact on the fertilization rate, embryo quality, and the rate of miscarriages [39]. Green tea extract might also have a promoting effect on *in vitro* maturation and embryo development [40-42]. In the current study highest dose of CT had adversely affected the DNA integrity; however, the lower doses were able to protect the DNA integrity compared to control ( $P < 0.05$ ). Besides CT10, 25 and 50 concentrations have shown lower total abnormalities. This results might be due to phenolic compounds of CT that improve morphological and DNA integrity if an appropriate amount is used. Various research supports the present study with obtained improvement on morphological integrity in different animal models [11,32] in which, the effects were observed with the supplementation of different rates green tea extracts. On the contrary of our results, Bucci et al. [42] did not find any improvement in supplementing the thawing medium of boar semen with epigallocatechin gallate (50  $\mu\text{M}$ ). On the basis of our results, we might hypothesize that the differences among our results may be associated with the amounts and types of antioxidants that were used.

In conclusion, CT supplementation has provided the protection of morphological and DNA integrity from cryo-damage and it has increased the total antioxidant activity depending of the dose in bull semen. Addition of 25  $\mu\text{g}/\text{mL}$  CT concentration in Tris extender can be beneficial when the overall parameters are considered. Further research is required to understand the cellular mechanisms involved in antioxidant activity.

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## CONFLICT OF INTEREST

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

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