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Determination of the Effect of Epigallocatechin Gallate on Oxidative Stress, Apoptosis and Sperm Quality in Rabbit Semen

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Abstract: In this study, it was aimed at determining the effect of Epigallocatechin gallate (EGCG) on the cryopreservation of rabbit semen. In the study, semen was collected from six male New Zealand rabbits using an artificial vagina. Collected semen control was divided into 5 groups as 25 µM, 50 µM, 100 µM and 200 µM. After adding EGCG to the extender, semen samples were frozen and thawed. After freezing and thawing, total motility, progressive motility and the rate of fast and medium speed spermatozoon were found to be statistically high in the 50 µM group. The static sperm ratio was found to be lower in the 50 µM group than in the other groups. When the flow cytometry results were examined, there was no statistical difference between the groups in terms of the ratio of dead and live sperm. However, the rate of dead sperm with acrosome damage was found to be the lowest in the 50 µM group. The high mitochondrial membrane potential sperm ratio was found to be higher in 50 µM and 100 µM groups compared to other groups ($p<0.05$). The apoptosis rate was numerically the lowest in the 25 µM and 50 µM groups. While the rate of ROS was the highest in the control group, it was significantly reduced in all EGCG support groups. In conclusion, the addition of EGCG to the dilution of rabbit semen improved semen quality in a dose-dependent manner. ©2023 NTMS.

Keywords: Antioxidants; Cryopreservation; Epigallocatechin Gallate; Rabbit; Semen.

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1. Introduction

Rabbits are widely used as animal models, as they have a similar lipid metabolism to humans [1]. Therefore, it is important to preserve rabbit genes as a biological resource and semen must be cryopreserved for long-term storage [2]. Since semen can be obtained from rabbits without euthanasia, the method of freezing rabbit semen is frequently used in experimental studies. However, the motility of spermatozoa decreases after freezing and thawing [3]. The rate of decrease in sperm motility after freezing is a major disadvantage for the use of frozen semen [4]. Cryopreservation of rabbit semen causes oxidative stress, decreased motility percentage, and decreased life span of spermatozoa [5]. This has been proven in several animal species and is supported by reduced DNA integrity as well as reduced sperm motility, damaged sperm cell membranes and premature acrosome reactions. [6]. Oxidative stress also causes losses in morphological integrity, and fertilization ability and induces sperm apoptosis [7]. To protect sperm cells against cryo-damage, extenders should be supplemented with cryoprotective and antioxidant substances [8].

The major polyphenolic compound in green tea is epigallocatechin-3-gallate (EGCG) [9]. EGCG is a natural antioxidant that can be used to treat problems related to male infertility, the fertilization rate of oocytes and maturation rate of embryos [10]. EGCG increases endogenous antioxidant activity and eliminates free radicals by reacting with hydrogen, alkoxyl or peroxyl radicals. [11],[12]. Sperm mitochondria are organelles damaged by cryopreservation [13]. EGCG is accumulated in mitochondria and maintains catalase activity [14]. In buffalo semen, 200 µM and 300 µM doses of EGCG were added to the cryo-diluent medium, and it was stated that total motility and progressive motility ratio, rapid velocity value, plasma membrane integrity and function were positively affected [15]. Doses of EGCG have been noted to increase cholesterol efflux and tyrosine phosphorylation through the estrogen receptor in human semen. It has been stated that EGCG increases sperm motility, viability and proteins that control cell viability such as Bcl2, Akt and Src via the ER. In addition, a decrease in triglyceride content, lipase induction as well as a decrease in G6PDH activity were observed [16]. In horses, EGCG at concentrations of 10 µM and 20 µM caused a significant increase in the number of sperm bound to zona pellucida [17, 18]. In pigs, EGCG increased sperm oocyte penetration and the total fertilization rate was found to be high in IVF [19]. In another study, EGCG treated arsenic-induced disruptions in epididymal sperm concentration, structural membrane integrity in kinematic properties, functional membrane integrity, and improved the impaired antioxidant defense system [20].

In the literature review, no experimental study was found that investigated the effect of EGCG on semen cryopreservation in rabbits. Therefore, in this study, the cryopreservative effects of EGCG supplementation in rabbit semen were investigated by CASA parameters. After cryopreservation, the effects on dead sperm ratio, acrosomal damage, reactive oxygen species (ROS) ratio as a marker of oxidative stress, mitochondrial membrane potential and apoptosis were investigated by flow cytometry.

2. Material and Methods

2.1. Animals, semen collection and cryopreservation process

All experimental procedures were carried out under the guidance of the European Union Directives for the Care and Use of Laboratory Animals. Six male New Zealand rabbits were used in the study. Rabbits were housed in standard conditions throughout the study. Animals were given feed and water ad libitum throughout the study. Ejaculate was collected from animals once a week. The gel part was removed from the ejaculate and motility was determined. The ejaculates were diluted 1:1 with Tris egg yolk diluent and pooled. Pooled semen samples were divided into 5 equal volumes by adding EGCG according to the groups to be diluted with 1/1 tris-egg yolk containing 5% DMSO in the final volume. It was diluted with spermatozoa in a final volume of $170x10^6$/mL [17].

Semen samples separated into groups were gradually cooled to 4 °C in approximately 90 minutes [21]. Semen analysis at +4 °C was performed with the computer assisted semen analysis (CASA) system (ISAS 2, Proiser, Spain) [22]. Sperm samples were drawn into straws with a volume of 0.25 mL and frozen with the help of a sperm freezing device (Minidigitcool, IMV, Spain). Frozen semen samples were thawed at 37°C for 30 seconds [23], after 2 months and analyzed [21]. Then, 10 µl of sperm samples were taken from all groups and analyzed by CASA.

2.2. Flow Cytometric Analysis

2.2.1. The Rate of Viable Sperm

The dead-viable sperm ratio was determined by the SYBR-14/PI staining kit [24]. Diluted semen samples were mixed with 2.5 µL of SYBR-14 and 2.5 µL of PI and incubated for 10 minutes at 22°C and analyzed.

2.2.2. Acrosomal Status

It was determined by the FITC-PNA/PI staining method [25]. 860 µL of PBS was placed on 30 µL of semen sample. It was incubated for 15 minutes with 5 µL of PNA and 2.5 µL of PI. The cytometer's 488 nm argon ion laser was used to excite fluorochromes, PI and FICT-PNA.

2.2.3. Sperm Apoptosis Rate

Briefly, 100 µl of the sperm sample was taken and incubated in an eppendorf tube containing 5 µL of annexin V and 5 µL propidium iodide (PI). It was then analyzed by flow cytometry [26].

2.2.4. Mitochondria membrane potential assay
For mitochondrial membrane potential (MMP) spermatozoa percentage, semen samples were washed with phosphate buffer solution (PBS) and analyzed with the commercial JC-1 commercial kit according to the manufacturer's instructions[27].

2.2.5. Reactive Oxygen Species Analysis
Reactive oxygen species analysis was determined using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen) and double staining with PI.[28]

2.3. Statistical Analysis
Statistical analyses of the data were performed via the SPSS (Version 26, SPSS, Chicago, IL) program. Values are expressed as Mean±S.E.M. Post hoc Tukey test and one-way ANOVA method were used to determine the differences between the groups.

3. Results

3.1. Sperm analysis results 4 ºC degree
The analysis results for cooled semen samples are presented in Table 1. Accordingly, no statistical difference was found between the experimental groups of EGCG and the control group.

Table 1: Sperm analysis results with CASA at 4 oC degrees after equilibration.

	Control	**25 µM**	**50 µM**	**100 µM**	**200 µM**
Total Motility (%)	59.55±10.74	64.45±5.33	68.58±7.46	66.15±5.51	61.98±6.06
Progressive Motlility (%)	29.18±4.54	39.88±6.95	40.20±10.72	42.78±8.50	40.15±9.41
Rapid (%)	43.78±9.58	50.58±6.52	43.86±21.33	49.30±7.12	48.86±7.70
Medium (%)	8.11±3.79	7.76±4.51	10.6±3.40	7.40±2.36	9.58±3.86
Slow (%)	10.96±9.59	5.66±4.19	3.70±0.96	5.46±3.09	3.21±1.11
Static (%)	37.11±11.86	36.08±5.63	31.58±10.51	34.51±5.18	38.35±5.97
VCL (µm/s)	90.21±15.36	93.83±16.42	92.80±18.67	92.05±7.85	94.91±17.39
VSL (µm/s)	41.93±17.12	41.33±11.67	42.33±14.67	43.56±11.30	45.70±17.95
VAP (µm/s)	56.51±16.82	55.75±13.71	57.21±17.28	56.13±7.14	60.36±19.09
LIN (%)	45.10±11.73	44.76±9.73	45.05±9.35	47.61±17.13	47.43±13.43
STR (%)	72.13±9.36	73.86±9.12	72.76±8.82	76.65±11.90	74.71±12.55
WOB (%)	61.63±8.55	59.30±7.86	60.90±7.80	61.08±6.76	61.71±9.68
ALH (µm)	3.36±0.64	3.28±0.36	3.30±0.64	3.28±0.36	3.25±0.48
BCF (Hz)	8.84±0.78	9.50±0.40	9±0.44	9.58±0.73	9.20±0.60

No statistical difference was found in the data without superscript. VCL=Curvilinear velocity; VSL=Straight linear velocity; VAP=Average path velocity; LIN=Linearity; STR=sperm track straightness; WOB=Wobble; ALH=Amplitude of lateral head displacement; BCF=Beat cross-frequency.

3.2. Sperm analysis results after freezing and thawing
The CASA analysis results for cooled semen are shown in Table 2. Total motility and progressive motility percentage was highest in 50 µM EGCG group (P<0.05). While the highest percentage of rapid spermatozoa was found in the 50 µM group, a statistical difference was found between the control and 200 µM groups (P<0.05). The highest percentage of medium spermatozoa was found in the 50 µM group, while a statistical difference was found between the control group (P<0.05). The static sperm ratio had the lowest value in the 50 µM group (P<0.05).

3.3. Flow cytometry analysis results after freezing and thawing
Flow cytometry analysis results are shown in Table 3. There was no significant difference between the groups in terms of percentage of dead sperm ratio by flow cytometry. However, the lowest rate of acrosome-damaged dead sperm was observed in the 50 µM group. The high mitochondrial membrane potential (HMMP) spermatozoon value was higher in the 50 µM group (P<0.05). While the lowest percentage of apoptosis was seen in the 25 µM and 50 µM groups, there was no statistically significant difference between the groups.

Table 2: Sperm analysis results with CASA after the freezing thawing process.

	Control	25 µM	50 µM	100 µM	200 µM
Total Motility (%)	17.38±3.35^{a*}	22.73±4.68^{a}	31.11±5,46^{b*}	23.61±5.44^{ab}	21.23±2.47^{a}
Progressive Motlility (%)	10.08±0.39^{a}	14.61±3.50^{ab}	19.18±4.48^{b}	14.08±4.80^{ab}	13.60±2.17^{ab}
Rapid (%)	10.35±4.19^{a}	14.31±4.03^{ab}	19.36±3,36^{b}	13.45±2.24^{ab}	12.55±4^{a}
Medium (%)	3.16±2.24^{a*}	5.63±1.00^{abc}	8.03±1.24^{c*}	5.86±1.24^{bc}	4.53±1.77^{ab}
Slow (%)	3.85±3.52	2.78±0.87	4.73±0,93	5.05±3.01	3.23±0.94
Static (%)	82.61±3.35^{b}	77.26±4.68^{b}	67.88±5.25^{a}	76.55±5.52^{b}	78.76±2.47^{b}
VCL (µm/s)	76.83±25.92	74.21±16.70	79.01±13.07	71.01±6.40	67.66±37.53
VSL (µm/s)	38.56±20.51	38.76±12.53	39.51±17.07	37.23±10.42	40.46±13.30
VAP (µm/s)	48.06±23.52	45.40±14.91	48.08±13,62	43.76±10.02	48.35±13.37
LIN (%)	49.05±12.77	51.65±7.81	50.25±17.22	51.91±11.61	51.40±11.91
STR (%)	79.25±8.24	72.86±33,81	80.50±11.76	84.16±5.71	83.31±9.95
WOB (%)	61.13±11.11	60.33±8.26	61.03±12.78	61.16±10.24	61.23±9.39
ALH (µm)	3.01±0.36	2.86±0.33	2.93±0.40	3.15±0.33	3.06±0.30
BCF (Hz)	8.23±1.47	9.05±1.26	8.88±0.83	8.66±0.46	8.71±0.97

Different superscript letters (a, b, c, P < 0.05, *; P<0,001) in the same row display significant differences between the groups. VCL = Curvilinear velocity; VSL = Straight linear velocity; VAP = Average path velocity; LIN = Linearity; STR = sperm track straightness; WOB = Wobble; ALH = Amplitude of lateral head displacement; BCF = Beat cross-frequency.

Table 3: Flow cytometry analysis results in frozen thawed semen.

	Control	25 µM	50 µM	100 µM	200 µM
Rate of dead spermatozoa %	78.75±8.13	79.08±9.89	78.35±5.93	81.38±5.22	84.5±8.45
Rate of dead sperm with acrosomal damage %	32.87±13.30^{ab}	28,83±2.99^{ab}	24.89±6.15^{a}	35.05±4.13^{ab}	39.74±6.67^{b}
Rate of viable sperm with acrosomal damage %	0.33±0.24	0.55±0.31	0.20±0.18	0.39±0.41	0.40±0.31
High mitochondrial membrane potential rate %	20.33±2.72^{a}	19.56±1.55^{a}	26.82±2.89^{b}	26.25±3,53^{b}	20.02±2.70^{a}
Apoptosis %	11.21±3.82	9.53±1.40	9.30±2.02	12.47±2.54	13.44±3.54
ROS ratio %	39.77±10.59^{a*}	15.75±10.83^{b*}	14.63±3.04^{b*}	15.39±2.21^{b*}	14.80±6.27^{b*}

Different superscript letters (a, b: P<0.05, *: P<0,001)in the same row display significant differences between the groups.

4. Discussion

Sperm cryopreservation causes a decrease in viability, motility and MMP, while causing an increas in apoptosis, DNA damage and ROS levels [29]. Rabbits are well suited for biomedical research [30]. After the cryopreservation of rabbit semen, a decrease occurs in rabbit sperm quality [31]. Therefore, in this study, the effects of EGCG supplemention semen extenders in New Zealand rabbits after freeze thawing were investigated.

Sperm quality predicted by CASA is a marker of cryopreservation and fertilization capacity of spermatozoa in animals [32]. Sarıözkan et al.[33] in their study with L-carnitine, stated that sperm motility increased between 0.5 and 2 mM in 12 and 24 hours of storage. However, they could not find a statistical difference between 0 and 6 hours in their study. In a study, three important components of tea (caffeine, EGCG and L-theanine) were stored in rat epididymal sperm at room temperature for 3 days and it was stated that these three compounds were eneficial for sperm storage [34]. While there was no statistical difference between the experimental groups in the cooled semen samples in our study, total and progressive motility values were found to be high in the 50 µM EGCG group after freezing and thawing (P<0.05). For this reason, EGCG was thought to have cryoprotectant properties as well as antioxidant properties. It is reported that 300 µM EGCG added to the cryomedium in buffalo spermatozoa improved CASA parameters [15]. In human semen, 2 to 20 µM EGCG caused an increase in sperm motility [16]. In stallion semen, 10 and 20 µM EGCG caused an increase in sperm motility [18]. In another study, it is stated that 50 µM EGCG increased the total motility and progressive motility values of stallion semen after cryopreservation [35]. In our study, it was observed that sperm motility gradually decreased in rabbits at doses above 50 µM EGCG. It is thought that this situation is caused by the differences in spermatozoon structure and membrane composition of different species.

The major problem in sperm cryopreservation is the ice crystals produced during the freezing process and consequently the reduced viability of the sperm [36]. In our study, there was no statistical difference between the groups in terms of the percentage of dead sperm. The rate of dead with acrosomal damaged sperm was statistically low in the 50 µM group. This suggests that EGCG at a dose of 50 µM can be used as an effective energy source in semen.

Successful preservation of semen in rabbits depends on procedures in sperm cryopreservation [30]. The sperm cryopreservation process, which includes cooling, freezing and thawing, causes damage to the sperm

membrane and genome structures [37]. Sperm cryopreservation increases oxidative stress and decreases antioxidant capacity [38]. Spermatozoa are exposed to oxidative stress during cryopreservation [39], so antioxidant substances should be added to the cryopreservation medium to improve sperm quality [30]. ROS has been tried to be eliminated by adding various antioxidant substances to the cryomedium of rabbit semen [40]. Together with the cellular enzymatic defense system, including CAT, SOD1, SOD2 and GPx, EGCG can enhance the antioxidant defense system of germ cells [41]. It is stated that EGCG may also offer a protective role against cellular oxidative damage involved in the pathogenesis of male infertility [42]. EGCG administration prevents the increase of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) [43]. Physiological ROS levels drive tyrosine phosphorylation cascades during sperm capacitation [44]. In our study, the rate of ROS was lower in all EGCG supplement groups compared to the control groups. However, a significant increase in total motility and progressive motility values is observed at 50 µM dose ($P<0.05$). Although very small changes were observed in the rate of ROS in a dose-dependent manner, it was interpreted that the effect of EGCG on sperm motility was mostly associated with acrosomal damage and HMMP.

Apoptosis can be induced by oxidative stress due to damage to mitochondria, plasma membrane and core material [45]. Increased oxidative stress in cryopreservation causes apoptosis by damaging the nucleus, acrosome and spermatozoa membrane [46]. ROS-induced oxidative stress is considered as the main cause of apoptosis [47]. Spermatogenesis in the seminiferous epithelium is accompanied by germ cell apoptosis. Apoptosis of germ cells is required to maintain an optimal germ cell ratio and to remove abnormal germ cells [48]. It is stated that exogenous EGCG supplementation can provide protection against short-term germ cell loss through mitogen-activated protein kinase, Bcl2 family and caspase 3 pathway [10]. The apoptosis rate was the lowest in the 50 µM group among the experimental groups without statistical difference. It is thought that the use of agents with antioxidant effect, suppressing oxidative stress and thus inhibiting apoptosis in sperm cryopreservation is important in this respect.

5. Conclusions

This study was conducted to elucidate the possible mechanism of action of EGCG, the major phenolic compound found in green tea, in semen cryomedium in rabbits. In the study, semen cryopreservation was performed using New Zealand rabbits.

The results showed that EGCG dose-dependently increased sperm quality in semen cryomedium, decreased the total ROS ratio significantly at all doses of EGCG, increased the mitochondrial membrane potential, reduced acrosomal damage, and partially affected the apoptosis rate.

Limitations of the Study

None.

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Our study was presented in the summary statement at the CRYO2023 congress (Minneapolis, USA).

Conflict of Interests

The authors declare no competing interests.

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Author Contributions

SAA designed the study. ÇCA, RHK, TCA, İHG and SAA contributed to data collection and data analysis. GT, MS, SG and ADÖ read the draft and approved the final scenario. HEE ensured the project completion.

Ethical Approval

Approval for this study was obtained from the Fırat University Animal Experiments Local Ethics Committee (Protocol No. 2021/14).

Data sharing statement, Consent to participate and Informed Statement

None.

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