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Epigallocatechin-3-gallate affects the quality of fresh and frozen-thawed semen of Simmental bull by two different cryopreservation methods

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Abstract

During the freezing process of semen, due to the generating of significant amounts of free radicals, the quality of sperm changes. Epigallocatechin-3-gallate (EGCG) is a green tea catechin, which in this study was applied to investigate its effect on the quality of bulls' sperm. We collected semen samples with an artificial vagina from 12 Simmental bulls to evaluate the effect of EGCG (10.00 and 20.00 µmol) in two cryopreserving methods on the quality parameters of semen. We designed six groups including two control groups (method one and two) and four treatments (EGCG 10.00 µmol + method one; EGCG 20.00 µmol + method one; EGCG 10.00 µmol + method two; EGCG 20.00 µmol + method two). The 20.00 µmol EGCG and a method two significantly affected the amending oxidative conditions as well as an increase in total antioxidant capacity and a decrease in malondialdehyde. The effect of EGCG in both concentrations was more on method two. The desired impact on sperm motility, viability, inhibition of lipid peroxidation and sperm DNA damage was observed in EGCG groups compared to control groups. Among the two methods, the method two had fewer adverse effects on the plasma membrane, motility parameters, viability and DNA of sperm. The EGCG in the semen extender yielded a favorable impact on thawed sperm. This effect was prompted in combination with the method two.

Introduction

In artificial insemination using frozen semen, the sperm of superior breeds of bulls are employed to generate progeny. Preservation of sperm until artificial insemination is a fundamental step in this process and freezing is one of the most common methods.[1] Semen freezing is done based on various methods as slow freezing, quick freezing and vitrification. These methods differ concerning time spent for freezing, appropriate equipment, preservation materials and possible costs.[2] The history of the use of frozen sperm dates back to when Spallanzani was able to preserve semen at – 15.00 ˚C and demonstrate the viability and motility of sperm after thawing. After that, researchers reported advances in sperm freezing.[3]

Today, sperm freezing is done in a multi-stage manner (including dilution, cooling, freezing, thawing, and equilibration times (0, 2, 4, and 8 hr) that causes damage to the sperm at each stage. Freezing is also associated with the generation of reactive oxygen species (ROS). Although the generation of ROS by sperm is a natural process, above the physiological level, it causes cell damage and male infertility. By inhibiting the oxidative chain reaction, antioxidants create a balance between oxidants and antioxidant levels and reduce oxidative stress.[4]

To minimize the effects of freezing on the quality parameters of sperm, preservatives are used. They include two categories permeable to sperm and non-permeable to sperm. The progress of the freezing process started with the accidental discovery of glycerol as a suitable preservative for bull sperms by Polge and colleagues in 1949.[5] The glycerol lowers the freezing point, gentle the freezing process or prevents the formation of ice crystals inside the sperms. Optimizing sperm storage methods by increasing sperm longevity without compromising sperm function is essential and helps boost fertilization rates in artificial insemination. Today, natural products of plant origin are used as an inexpensive and natural source of additives to preserve and enhance sperm function during

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semen storage. Most plant species are considered strong sources of antioxidants that can act as ROS scavengers to neutralize the harmful effects of oxidative stress on sperm function.[6] Nowadays, due to the safety, restrictions in use, high costs, and presence of carcinogenic and toxic compounds in some synthetic antioxidants, the interest in using of natural antioxidants has been increased significantly.[7] Polyphenol compounds of green tea are essential antioxidants in the field of treatment. Green tea is extracted from the camellia plant and contains many catechin compounds, a chemical antioxidant that can scavenge free radicals. Epigallocatechin-3-gallate (EGCG) is the most active and abundant catechin in green tea soluble in water, is a safe substance and can scavenge free radicals by breaking the oxidative chain reaction.[8] Due to the antioxidant properties of EGCG, it has been used as an antioxidant to improve sperm in various animals such as horse,[9] boar,[10] dog,[11] Mouse,[12] and pig.[13] One-step and two-step dilution methods for semen freezing has led to conflicting results in the previous researches. The one-step dilution method preserves the vitality and motility of sperm[14] and reduces oxidative stress.[15] On the other hand, the two-step dilution method has advantages such as time management, stability and stress resistance reducing the storage time of fresh semen and lowering the possible risk of mistakes due to fewer processing steps.[16]

The Simmental breed is preferred for crossbreeding due to its ability to grow well and yield better beef and milk to its crossbred progeny. therefore, this breed provides a good source of income for dairy farmers.[17] Today, although the technology of semen freezing and preserving for artificial insemination, especially in bulls, has advanced beyond other species, there are still prominent gaps in the foundation of this knowledge and technology. The percentage of sperm viability and motility after thawing is still significantly reduced and varies considerably among breeding bulls. These weaknesses are significant because they prevent progress in the fundamental science of reproductive biotechnology. This study aimed to investigate the impact of EGCG on the quality of frozen-thawed semen using two cryo-preservation methods in Simmental bulls.

Materials and Methods

Preparation of appropriate concentration of EGCG. The ready-to-use extract of EGCG (95.00%) was purchased from Sigma-Aldrich Co. (St. Louis, USA). To prepare the main stock, 0.458 g of EGCG was dissolved in 1.00 mL of 5.00% dimethylsulfoxide. We mixed one part of this solution with nine parts of distilled water to create the main stock.

Semen collection and study design. The procedure carried out in this study was approved by the ethics committee of Urmia University, Faculty of Veterinary Medicine (IR-UU-AEC-1383/DP/3). We randomly selected 12 healthy Simmental bulls (aged 4 - 8 years) from the Simmental cattle breeding center of Amol, Mazandaran Province, Iran (altitude: 47 meters, longitude: 52° 23′57.76″ east and latitude: 36° 30′ 18.55″ north) for collecting the semen sample. Sampling was performed during the usual weekly semen collection at 8 - 12 AM from late September 2021 to December 1st, 2021. The average humidity and temperature of the study period were 57.42 ± 3.62% and 9.37 ± 3.73 ˚C, respectively. The samples were examined for sperm quality andonly those with a concentration greater than 500 × 10^6 cells mL^{-1}, progressive motility (PM) greater than 60.00% and abnormal morphology less than 20.00% per ejaculation were considered normal and investigated. Each bull semen sample (three replicates for each bull) was collected with an artificial vagina preheated at 46.00 ˚C in an oven. Sexual preparation of the bull was done with three false mounts by standing for 10 min at the collection site. Semen concentration was measured by a photometer (photometer SDM1 Minitube, Tiefenbach, Germany) calibrated to count bull sperm cells. Each ejaculation was equally divided into six groups: Group 1 (C1: control of method one), Group 2 (C2: control of method two), Group 3 (10-method one: 10.00 µmol of EGCG in cryopreserving method one), Group 4 (10-method two: 10.00 µmol of EGCG in cryopreserving method two), Group five (20-method one: 20.00 µmol of EGCG in cryopreserving method one) and Group 6 (20-method one: 20.00 µmol of EGCG in cryopreserving method two).

Preparation of extender, pre-extender dilution, and final extender volume. Steridyl (Minitube) as an extender was applied for this study. The ingredients of this extender included Tris buffer, citric acid, fructose, glycerol, the purest water, sterilized egg yolk, and antibiotics (tylosin, gentamicin, spectinomycin, linco-mycin). To prepare the extender, 500 mL of Steridyl and 750 mL of double distilled water were incubated at 32.00 - 34.00 ˚C for 10 min). The distilled water was slowly added to the extender and the resulting solution was mixed with a magnetic stirrer gently. To prepare the pre-extender, the diluent used in the previous step was slowly added to the semen at a ratio of 1:1. The pre-extender was put in a water bath (34.00 ˚C, 10 min). The volume of the extender was calculated based on the following formula:

Number of doses = (semen volume × semen concentration × progressive motile sperm × morphologically normal sperm) / (sperm per dose [15.00 × 10^6]).

Semen freezing. Sperm freezing was done with two cryopreserving methods.

Cryopreserving method one. The flasks of samples containing the final volume of the diluent were placed in a plastic container in a water bath (34.00 ˚C) and kept at room temperature (20.00 ˚C for 15 min). Diluted semen was packed in 0.50 mL straws (Minitube) with the MPP

Uno automatic filling and sealing machine (Minitube). The straws were set in a refrigerator at 4.00 ˚C for 3 hr. Packed semen was frozen in an MT freezer freezing device (Minitube) according to the following method: From + 4.00 to – 12.00 ˚C at a rate of – 4.00 ˚C per min, from – 12.00 to – 40.00 ˚C at a rate of – 40.00 ˚C per min, and from – 40.00 up to – 140 ˚C at a rate of – 50.00 ˚C per min. Then, they were transferred into goblets floating in liquid nitrogen. To evaluate semen quality parameters, three semen samples from each ejaculation were thawed at 38.00 ˚C for 30 sec.

Cryopreserving method two. Before preparing the diluted semen, the pre-extender solution was slowly added to the bottle containing 20.00 mL of the prepared steridyl solution (at 32.00 ˚C) for 10 min. The resulting solution was put in a plastic container at 32.00 ˚C. After warming, the bottle was placed in a cold cabinet at 4.00 ˚C for 90 min. The final diluent was then prepared by adding the calculated residual diluent previously stored in a cold cabinet at 4.00 ˚C. Diluted semen was equilibrated for 5 hr in a cold and dark cabinet. They were packed in 0.50 mL straws at 4.00 ˚C in a cold cabinet with the MPP Uno automated filling and sealing machine

Evaluation of sperm motility. The PM using a light microscope equipped with a hot plate (samples were conserved at 37.00 ˚C) was evaluated.

Viability and morphology. To assess sperm morphology and vitality, the World Health Organization protocol for the eosin-nigrosin staining method was followed.[18] In addition, the proportion of abnormal sperm was determined using eosin-nigrosin staining. Sperm with discolored head, neck or tail sections were considered dead. Sperm that had significant abnormalities such as remnants of cytoplasm were marked as abnormal.[19]

Hypo-osmotic swelling test (HOST). The HOST is a test to check the functional integrity of the sperm membrane. The goal of this test is to estimate the percentage of sperms with twisted tails that are induced due to swelling of the flagella. According to the instruction of the HOST test, 50.00 µL of semen sample (fresh or frozen-thawed) was added to 450 µL of hypo-osmotic solution (prepared from trisodium citrate dehydrate and fructose),[20] after that, two drops of the final solution was added on the preheated glass slide and covered with a cover slip. Finally, we counted 200 spermatozoa with a binocular phase contrast microscope and estimated the percentage of sperm with the twisted tail.

Sperm DNA fragmentation analysis (SDFA). The SDFA was performed using the reagents of the SDFA kit of Ibn Sina Research Institute (Dhanmondi, Dhaka, Bangladesh).[21] The reagents were exposed to 50.00 µL of diluted semen with phosphate buffered saline. The reagents induce a blue halo around the heads of normal sperms (under the microscope view), however, unhealthy sperms remained without a halo (Fig. 1A). For estimating the percentage of unhealthy sperms in each group, 200 sperms in 10 microscopic fields were evaluated.

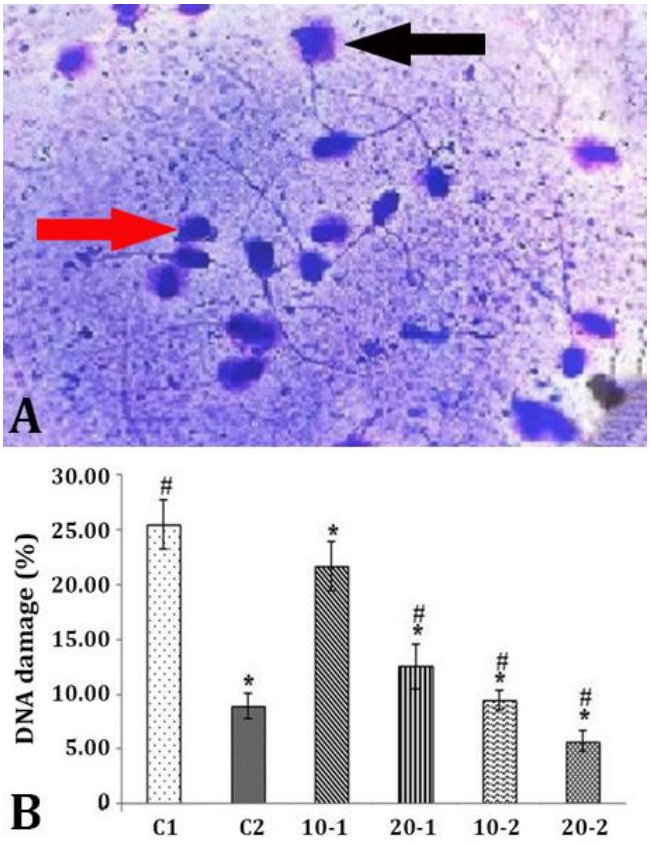

Fig. 1. A) Sperm with DNA damaged are swollen in the head (black arrow), and healthy sperm have a purple halo around the head (red arrow), (SDFA staining; 400×). **B)** DNA damaged results by different dilutions. C1: Control one-step semen dilution, C2: Control two-step semen dilution,10-1:10.00 µmol concentration of epigallo-catechin-3-gallate (EGCG) in one-step semen dilution, 10-2:10.00 µmol concentration of EGCG in two-step semen dilution, 20-1:20.00 µmol concentration of EGCG in one-step semen dilution and 20-2: 20.00 µmol concentration of EGCG in two-step semen dilution.* $p < 0.001$ versus C1; # $p < 0.001$ versus C2.

Total antioxidant capacity (TAC). The TAC of seminal plasma was measured according to the manufacturer's kit protocol (Naxifer™; Navand Salamat, Urmia, Iran). Diluted seminal plasma (1:10) was mixed with stock solutions step by step. Then, the TAC content in the final solution was measured by a Chromate microplate reader at 593 nm.[22]

Lipid peroxidations. The Malondialdehyde (MDA) status of seminal plasma was measured by thiobarbituric acid (TBA) protocol. After melting the straws, the semen was centrifuged twice (10 min, 3,000 rpm). According to the manufacturer's kit protocol (Navand Salamat), TBA reagent was added to the samples and the final reaction was read by a Chromate microplate reader at a wave-length of 586 nm.[23]

Statistical analysis. The data collected in the study were analyzed using SPSS software (version 26.0; IBM Corp., Armonk, USA). The ANOVA test was used to compare the mean variables in the control and EGCG groups before and after cryopreserving methods. Bonferroni's post hoc test was used to compare the group means two by two. To compare the average variables in each group before and after freezing, paired samples *t*-test was used. To compare the mean of variables in different groups, whether cryopreserving method one or cryopreserving method two was used, the multivariate two-way ANOVA analysis was applied.

Results

The study examined semen quality in two cryopreserving methods with and without EGCG (20.00 and 10.00 µmol) supplementation. The results indicated that the freezing procedure (method one or two), regardless of any intervention, did not have a significant effect (p = 0.536). However, the different concentrations of EGCG (0.00, 10.00, and 20.00 µmol) had a significant effect irrespective of the type of freezing procedure ($p < 0.05$). Furthermore, the pattern of changes in sperm variables at different EGCG doses in each cryopreserving protocol (interaction effect) was not significant indicating no interaction effect ($p < 0.05$). Based on our findings, sperm quality was better preserved using cryopreserving method two compared to method one ($p < 0.05$). Method two showed a significant increase in PM- frozen thawed semen (FT) compared to method one ($p < 0.05$). Additionally, PM was significantly increased with 10 and 20.00 µmol EGCG in method two compared to method one ($p < 0.05$). Furthermore, parameters such as sperm total abnormal morphology (Morph), sperm head abnormality (Head) and Mid-piece abnormality (Midp) were significantly lower compared to the control group ($p < 0.05$). There was no significant effect on tail abnormality and HOST parameters in cryopreserving methods one and two and different concentrations of EGCG (Table 1). In frozen-thawed semen, certain parameters like PM and viability showed significantly higher values in both cryopreserving methods compared to the control group ($p < 0.05$). However, only in cryopreserving method one, the addition of EGCG led to a significant decrease in Morph and Midp parameters compared to the control group ($p < 0.05$; Table 2).

Our study results indicated that cryopreserving method two had fewer adverse effects on sperm DNA compared to method one and the difference was statistically significant ($p < 0.05$). Additionally, when 20.00 µmol of EGCG was added to cryopreserving method two, it demonstrated better prevention of sperm DNA fragmentation during the freezing and thawing process compared to cryopreserving method one (Fig. 1; $p < 0.05$).

There was a significant difference in lipid peroxidation between the two cryopreserving methods as indicated by the MDA test results. Along with the increase in lipid peroxidation, the TAC was also increased. The highest level of TAC was observed with a concentration of 20.00 µmol EGCG and cryopreserving method two ($p < 0.05$). In frozen-thawed semen, there was a significant difference in MDA levels and TAC levels among different groups (Fig. 2). The cryopreserving method one showed a significantly higher rate of lipid peroxidation compared to cryopreserving method two ($p < 0.05$). Furthermore, there was a significant difference in lipid peroxidation rate among different concentrations of EGCG with a higher concentration (10.00 µmol) resulting in more lipid peroxidation ($p < 0.05$; Table 3).

Table 1. Comparison of different sperm parameters in frozen thawed semen using two cryopreservation methods with 10.00 and 20.00 µmol of Epigallocatechin-3-gallate (EGCG)

Parameters	Dilution method	EGCG 10.00 µmol		EGCG 20.00 µmol	
		Mean ± SD	Sig	Mean ± SD	Sig
Progressive motility (%)	1	50.37 ± 3.23	0.003	53.64 ± 2.39	0.013
	2	54.44 ± 2.65		57.27 ± 3.97	
Viability (%)	1	60.08 ± 4.20	0.236	62.17 ± 4.28	0.103
	2	61.93 ± 3.18		64.94 ± 3.67	
Abnormal morphology (%)	1	20.65 ± 7.99	0.679	19.06 ± 5.15	0.647
	2	19.38 ± 6.86		17.95 ± 6.53	
Head abnormality (%)	1	9.83 ± 6.61	0.641	10.06 ± 4.50	0.191
	2	8.70 ± 4.94		7.78 ± 3.71	
Mid-piece abnormality (%)	1	2.07 ± 0.55	0.675	1.46 ± 0.46	0.670
	2	1.96 ± 0.70		1.56 ± 0.71	
Cytoplasmic droplet (%)	1	0.25 ± 0.21	0.048	0.44 ± 0.57	0.461
	2	0.87 ± 0.99		0.29 ± 0.34	
Tail abnormality (%)	1	8.50 ± 2.42	0.542	7.09 ± 1.42	0.205
	2	7.84 ± 2.69		8.29 ± 2.82	
Hypo-osmotic swelling test (%)	1	27.38 ± 7.24	0.662	26.58 ± 5.30	0.208
	2	28.60 ± 6.22		29.40 ± 5.33	

Table 2. Comparison of different parameters of different group in two cryopreservation methods with control group.

	Parameters	Dilutions	Method one		Method two	
			Mean ± SD	*p*-value	Mean ± SD	*p*-value
Fresh semen	**Progressive motility (%)**	Control	68.50 ± 6.30	0.008	68.80 ± 5.91	<0.001
		10.00 µmol	69.79 ± 5.03		69.89 ± 3.71	
		20.00 µmol	74.73 ± 2.15**		77.40 ± 3.14**	
	Viability (%)	Control	75.02 ± 5.14	0.015	75.88 ± 5.55	0.002
		10.00 µmol	76.92 ± 4.81		80.57 ± 4.43*	
		20.00 µmol	80.30 ± 2.17*		82.64 ± 2.83**	
	Abnormal morphology (%)	control	21.91 ± 5.74	0.344	22.71 ± 6.43	0.015
		10.00 µmol	18.52 ± 7.41		19.02 ± 7.76	
		20.00 µmol	18.45 ± 6.32		14.63 ± 4.54*	
	Head abnormality (%)	Control	11.66 ± 5.50	0.374	11.67 ± 5.88	0.032
		10.00 µmol	8.55 ± 5.60		9.06 ± 6.75	
		20.00 µmol	9.71 ± 5.08		5.55 ± 2.88*	
	Mid-piece abnormality (%)	Control	1.73 ± 0.89	0.808	2.31 ± 0.87	0.011
		10.00 µmol	1.72 ± 0.88		1.53 ± 0.55*	
		20.00 µmol	1.54 ± 0.64		1.44 ± 0.72*	
	Cytoplasmic droplet (%)	Control	0.82 ± 0.54	0.031	0.77 ± 0.81	0.361
		10.00 µmol	0.38 ± 0.25*		0.43 ± 0.33	
		20.00 µmol	0.44 ± 0.43		0.54 ± 0.48	
	Tail abnormality (%)	Control	7.68 ± 1.66	0.461	7.90 ± 2.28	0.684
		10.00 µmol	7.86 ± 2.88		7.91 ± 3.56	
		20.00 µmol	6.75 ± 2.23		7.09 ± 2.45	
	Hypo osmotic swelling test (%)	Control	40.45 ± 8.15	0.141	40.80 ± 8.07	0.200
		10.00 µmol	39.78 ± 6.72		47.44 ± 9.26	
		20.00 µmol	45.22 ± 6.34		45.28 ± 9.56	
Frozen thawed semen	**Progressive motility (%)**	Control	42.18 ± 4.54	<0.001	48.77 ± 4.96	< 0.001
		10.00 µmol	50.37 ± 3.23**		54.44 ± 2.65**	
		20.00 µmol	53.64 ± 2.39**		57.27 ± 3.97**	
	Viability (%)	Control	56.15 ± 5.41	0.011	57.92 ± 4.21	< 0.001
		10.00 µmol	60.08 ± 4.20		61.93 ± 3.18*	
		20.00 µmol	62.17 ± 4.28*		64.94 ± 3.67**	
	Abnormal morphology (%)	Control	22.86 ± 5.08	0.038	24.09 ± 5.78	0.063
		10.00 µmol	20.66 ± 7.99		19.38 ± 6.85	
		20.00 µmol	19.06 ± 5.15		17.95 ± 6.53	
	Head abnormality (%)	control	10.74 ± 4.47	0.909	11.40 ± 4.51	0.129
		10.00 µmol	9.83 ± 6.61		8.70 ± 4.94	
		20.00 µmol	10.06 ±4.50		7.78 ± 3.71	
	Mid-piece abnormality (%)	control	2.30 ± 0.63	0.002	2.19 ± 0.72	0.105
		10.00 µmol	2.07 ± 0.55		1.96 ± 0.70	
		20.00 µmol	1.46 ± 0.46**		1.56 ± 0.70	
	Cytoplasmic droplet (%)	Control	0.31 ± 0.42	0.562	0.64 ± 0.47	0.121
		10.00 µmol	0.25 ± 0.21		0.87 ± 0.99	
		20.00 µmol	0.44 ± 0.57		0.29 ± 0.34	
	Tail abnormality (%)	control	9.51 ± 2.87	0.051	9.84 ±2.09	0.150
		10.00 µmol	8.49 ± 2.42		7.84 ± 2.69	
		20.00 µmol	7.09 ± 1.42		8.29 ± 2.82	
	Hypo osmotic swelling test (%)	Control	25.09 ± 5.62	0.654	27.70 ± 5.07	0.777
		10.00 µmol	27.38 ± 7.24		28.60 ± 6.22	
		20.00 µmol	26.58 ± 5.30		29.40 ± 5.33	

* Significant (p <0.05), ** Significant (p < 0.01) *versus* compared to the control group.

Table 3. Correlations coefficient between sperm parameters of frozen thawed semen and antioxidant level in seminal fluid

Variables	Malondialdehyde (mmol mL^{-1})		Total antioxidant capacity (mmol mL^{-1})	
	Rate	*p-value*	Rate	*p- value*
Sperm DNA fragmentation analysis (%)	0.794**	< 0.001	–0.524**	< 0.001
Progressive motility (%)	0.515**	< 0.001	0.441**	< 0.001
Viability (%)	0.302*	0.010	0.370**	0.001
Abnormal morphology (%)	–0.129	0.279	–0.201	0.091
Head abnormality (%)	–0.057	0.634	–0.176	0.140
Mid-piece abnormality (%)	–0.331**	0.004	–0.222	0.061
Cytoplasmic droplet (%)	–0.104	0.383	–0.041	0.734
Tail abnormality (%)	–0.106	0.374	–0.107	0.373
Hypo osmotic swelling test (%)	0.088	0.463	0.203	0.086

* Correlation is significant at the < 0.05 level, ** Correlation is significant at the < 0.01 level.

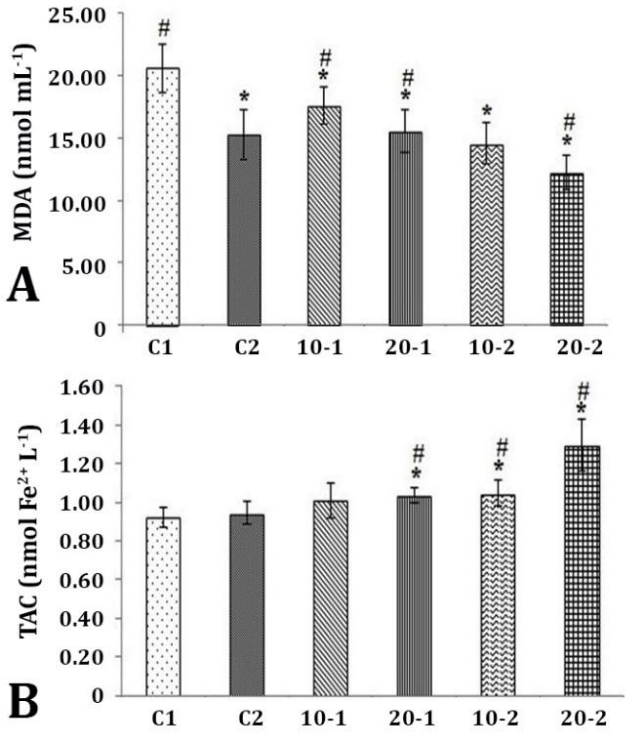

Fig. 2. Result of **A)** MDA and **B)** TAC in sperm cells from Simmental bulls in different groups. C1: Control one-step semen dilution, C2: Control two-step semen dilution, 10-1:10.00 µmol concentration of epigallocatechin-3-gallate (EGCG) in one-step semen dilution, 10-2:10.00 µmol concentration of EGCG in two-step semen dilution, 20-1:20.00 µmol concentration of EGCG in one-step semen dilution and 20-2: 20.00 µmol concentration of EGCG in two-step semen dilution.
* $p < 0.001$ versus C1; # $p < 0.001$ versus C2.

Discussion

In this study, we investigated the effect of EGCG (10.00, and 20.00 µmol) under two cryopreserving methods on the quality of semen obtained from Simmental bull. Considering that EGCG is one of the polyphenols of green tea and its antioxidant properties have been demonstrated,[8] we also investigated its antioxidant effect by measuring MDA and TAC tests. In general, the two-step dilution method had fewer adverse effects on the plasma membrane, motility parameters, viability and DNA of sperm. The concentration of 20.00 µmol EGCG and a two-step method significantly affected the amending oxidative conditions, with an increase in TAC and a decrease in MDA. Among the two dilutions methods, the favorable effect of EGCG in both concentrations was more on the cryopreserving method two.

According to our results, the sperm quality was preserved in the cryopreserving method two more than in the cryopreserving method one. In a study conducted on one, two, and three-step cryopreservation processes on Bangladeshi ram semen, no significant changes were observed in the parameters of survival, motility, acrosome and plasma membrane integrity in the three mentioned methods. Jha *et al*. reported that sperm viability and mobility were amended by the one-step dilution method more than the two-step dilution.[14] In a study conducted by Dwinofanto *et al.* on the quality of Bali sperm in different periods of being in cold conditions suggested that the quality of sperm, when subjected longer to 5.00 ˚C (22 hr), was kept more than the short period (4 hr). They cited the reason for this as the possibility of sperm compatibility with the extender in long cold periods.[23] In evaluating the quality of Holstein cattle sperm under freezing on different concentrations of glycerol with CASA software, it was shown that the use of 99.00% glycerol and a one-step cryopreserving method caused an increase in the quality of sperm.[24] In a comparative study of Limousin cattle sperm quality with different techniques of cryo-preservation (one-step, two-step, and three-step), there were no statistically significant differences in sperm motility parameters and DNA integrity in all three dilution methods, however, the parameters of viability and integrity of the membrane in the one-step method were more than the two-step and three-step methods. In general, they suggested that the one-step cryopreserving method could better maintain the quality of sperm after thawing.[15] These results were in contrary to the results of our study on maintaining the quality of sperm after thawing.

In a study by Schulze *et al*., factors on the quality of the seminal fluid were investigated in three cryopreserving methods (one, two, and three steps). They evaluated the quality of semen in terms of sperm motility, acrosome and

plasma membrane integrity, thermo-resistance, and mitochondrial function using specially programmed software SEQU. Their study showed that the one-step dilution process was better than other sperm cryopreserving methods in terms of time management, protective effect, stability and stress resistance. On the other hand, the one-step process reduced the storage time of raw ejaculates and reduced the possible risk of error due to the lower number of processing steps.[16] The results of their study which indicated the favorable effect of the cryopreserving process were in contrary to our results.

We also investigated the oxidative effect of EGCG on semen in two cryopreserving methods. The results indicated an increase in the level of MDA in cryopreserving method one compared to cryopreserving method two. Indeed, the addition of EGCG in both dilution methods caused a decrease in MDA compared to the control group. Endogenous antioxidants in bovine semen are not enough to ensure sperm integrity against oxidative stress during freezing. The addition of antioxidants is required to improve the viability of sperm cells after thawing. Plant extracts are sources of natural antioxidants with less cyto-toxicity compared to synthetic antioxidants. Khan *et al*. found that adding green tea extract to the semen extender protected the plasma membrane and increased the motility rate of frozen and thawed sperm.[25] Mizera *et al*. suggested that adding the Spirulina maxima extract and microalgae to the extender induced positive effects on post-thaw sperm parameters including sperm motility and morphology, and caused a significant reduction in ROS synthesis.[26] For example, trehalose is a sugar that acts as an antioxidant and protects sperm cell structure against oxidative damage and cold shock. Adding 100 mM trehalose to the seminal fluid extender improved sperm motility after thawing, membrane integrity and catalase and glutathione activity.[27] Employing potent antioxidants, such as selenium (dose of 2.00 μg mL^{-1}), with the seminal extender improves the morphology and integrity of frozen sperm.[28] In our study, EGCG improved sperm motility parameters, membrane integrity, viability and DNA fragmentation in two cryopreserving methods. Considering that green tea polyphenols can induce the expression of various antioxidant enzymes,[8] the effect of EGCG on sperm quality could be largely attributed to its antioxidant properties. Our study showed that concentrations of 10.00 and 20.00 μmol of EGCG in the cryopreserving method two activated the antioxidant capacity of the whole cell to neutralize the effect of stress caused by the freezing process in sperms. These results were consistent with some previous studies and contradicted some. In a study conducted by Plaza Dávila *et al.* on the effects of EGCG on the quality of horse sperm under the influence of rotenone (an inhibitor of the respiratory complex in mitochondria), the presence of EGCG in the sperm preserving process reduced the toxic effects of rotenone.[9] The effect of EGCG on the quality of boar sperm in the presence and absence of resveratrol after thawing showed that the sperms had higher fertility in terms of viability, acrosome integrity, mitochondrial function and lipid peroxidation in the presence of EGCG and resveratrol. Also, EGCG alone, without resveratrol, had a positive effect on sperm quality.[10] In a previous study, EGCG did not improve the quality of frozen dog sperm after thawing at any concentration and did not increase the ability to bind to ovarian zonaplacids.[11] The effect of EGCG on the motility and penetration of thawed porcine spermatozoa was investigated by Kaedei *et al*., EGCG (50.00, and 100 μmol) improved motility but did not significantly affect sperm viability. Also, EGCG was influential on the penetration power of sperms on the ovule.[13]

Among the indices of sperm quality, protection from DNA fragmentation and chromatin damage are critical for sperm ability to withstand the freezing process and support embryo development.[29] Our study showed that the cryopreserving method two better protected sperm DNA fragmentation than the cryopreserving method one. In this regard 20.00 μmol EGCG was better than 10.00 μmol EGCG. Freezing and thawing negatively affect DNA integrity and make DNA vulnerable and sensitive to molecular and epigenetic changes that affect fetal development.[30] Sperm freezing, as an adverse process has been shown to destabilize chromatin and lead to sperm DNA fragmentation in boars and birds.[31,32] The DNA damage is probably related to different mechanisms that occur during the freezing process. Double-stranded DNA breaks are due to high levels of ROS production, disruption of DNA repair enzymes and mechanical stress of DNA molecular genomic regions where chromatin density increases due to cell contraction.[33]

According to our results, there was a significant negative correlation between increased levels of TAC and DNA damage (Table 3). The increase in TAC has been accompanied by a decrease in DNA fragmentation. High concentration of polyunsaturated fatty acids (poly-unsaturated phospholipids or PUFA) in mammalian sperm makes them sensitive to oxidative damage. This consequence is due to the production of reactive α and β-unsaturated aldehydes and enals which can directly react with DNA or proteins to create pro-mutagenic cyclic adducts or protein carbonyls. The same in sperm can lead to DNA fragmentation, low sperm fertilization rate and embryo death. The EGCG effectively prevents the formation of both adducts over a wide range of concentrations.[27] Since mammalian spermatozoa lack part of the cytoplasm, their antioxidant capacity is not sufficient to resist lipid peroxidation by ROS induced by PUFA binding to sperm phospholipids. About 50.00% of the sperms head is lost due to freezing and thawing and as a result, the ability of insemination declines. Oxidative stress is the major cause of sperm dysfunction mainly due to the imbalance

between ROS and various endogenous anti-oxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase.[34] Addition of green tea extract to semen extender has improved the morphology, function, total antioxidant capacity and fertility of buffalo sperm (*Bubalus bubalis*).[35,36] Spermatozoa are incompetent to resynthesize their membrane components, therefore, the risk of lipid peroxidation by ROS is high. At lower concentrations, ROS has a vital role in sperm capacitation, acrosome reaction and sperm-oocyte fusion. However, at higher concentrations, ROS behaves as a genotoxic agent that affects the parameters of PM, viability, acrosome and plasma membrane integrity.[35]

In this study, we investigated the effect of EGCG (10.00 and 20.00 µmol) under two cryopreserving methods on the quality of semen obtained from Simmental bull. The cryopreserving method two had fewer adverse effects on the plasma membrane, motility parameters, viability and DNA of sperm. The concentration of 20.00 µmol EGCG and cryopreserving method two significantly affected the amending oxidative conditions with an increase in TAC and a decrease in MDA. Both concentrations of EGCG in the cryopreserving method two showed more favorable effects on some sperm quality parameters. This investigation was done only on the quality of sperm in laboratory conditions. To obtain the result of the effect of EGCG on fertilization power, a study on embryo production *in vitro* is needed.

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Conflict of interest

The authors do not have any financial or personal relationships with other people or organizations that might inappropriately influence or bias our work.

References

1. Ugur MR, Saber Abdelrahman A, Evans HC, et al. Advances in cryopreservation of bull sperm. Front Vet Sci 2019; 6: 268. doi: 10.3389/fvets.2019.00268.
2. Jang TH, Park SC, Yang JH, et al. Cryopreservation and its clinical applications. Integr Med Res 2017; 6(1): 12-18.
3. Yánez-Ortiz I, Catalán J, Rodríguez-Gil JE, et al. Advances in sperm cryopreservation in farm animals: cattle, horse, pig and sheep. Anim Reprod Sci 2022; 246: 106904. doi: 10.1016/j.anireprosci.2021.106904.
4. Galarza DA, López-Sebastián A, Woelders H, et al. Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols. Cryobiology 2019; 91: 84-89.
5. Isachenko V, Rahimi G, Mallmann P, et al. Technologies of cryoprotectant-free vitrification of human spermatozoa: asepticity as criterion of effectiveness. Andrology 2017; 5(6): 1055-1063.
6. Ros-Santaella JL, Pintus E. Plant extracts as alternative additives for sperm preservation. Antioxidants (Basel) 2021; 10(5): 772. doi: 10.3390/antiox10050772.
7. Lourenço SC, Moldão-Martins M, Alves VD. Antioxidants of natural plant origins: from sources to food industry applications. Molecules 2019; 24(22): 4132. doi: 10.3390/molecules24224132.
8. Prasanth MI, Sivamaruthi BS, Chaiyasut C, et al. A review of the role of green tea (*Camellia sinensis*) in antiphotoaging, stress resistance, neuroprotection, and autophagy. Nutrients 2019; 11(2): 474. doi: 10.3390/nu11020474.
9. Plaza Dávila M, Bucci D, Galeati G, et al. Epi-gallocatechin-3-gallate (EGCG) reduces rotenone effect on stallion sperm–zona pellucida heterologous binding. Reprod Domest Anim 2015; 50(6): 1011-1016.
10. Bucci D, Spinaci M, Yeste M, et al. Combined effects of resveratrol and epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters. Theriogenology 2018; 117: 16-25.
11. Bucci D, Cunto M, Gadani B, et al. Epigallocatechin-3-gallate added after thawing to frozen dog semen: effect on sperm parameters and ability to bind to oocytes' zona pellucida. Reprod Biol 2019; 19(1): 83-88.
12. Chen M, Liu W, Li Z, et al. Effect of epigallocatechin-3-gallate (EGCG) on embryos inseminated with oxidative stress-induced DNA damage sperm. Syst Biol Reprod Med 2020; 66(4): 244-254.
13. Kaedei Y, Naito M, Naoi H, et al. Effects of (-)-epi-gallocatechin gallate on the motility and penetrability of frozen-thawed boar spermatozoa incubated in the fertilization medium. Reprod Domest Anim 2012; 47(6): 880-886.
14. Jha PK, Shahi Alam MG, Mansur AA, et al. Cryo-preservation of Bangladeshi ram semen using different diluents and manual freezing techniques. Cryobiology 2019; 89: 35-41.
15. Arif AA, Maulana T, Kaiin EM, et al. Comparative analysis of various step-dilution techniques on the

quality of frozen Limousin bull semen. Vet World 2020; 13(11): 2422-2428.
16. Schulze M, Kuster C, Schäfer J, et al. Effect of production management on semen quality during long-term storage in different European boar studs. Anim Reprod Sci 2018; 190: 94-101.
17. Götz KU, Luntz B, Robeis, et al. Polled Fleckvieh (Simmental) cattle - current state of the breeding program. Livest Sci 2015; 179: 80-85.
18. Björndahl L, Kirkman Brown J; et al. The sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen: ensuring quality and standardization in basic examination of human ejaculates. Fertil Steril 2022; 117(2): 246-251.
19. Ramazani N, Mahd Gharebagh F, Soleimanzadeh A, et al. Reducing oxidative stress by κ-carrageenan and C60HyFn: The post-thaw quality and antioxidant status of Azari water buffalo bull semen. Cryobiology 2023; 111: 104-112.
20. Ijaz A, Hussain A, Aleem M, et al. Butylated hydroxy-toluene inclusion in semen extender improves the post-thawed semen quality of Nili-Ravi buffalo (*Bubalus bubalis*). Theriogenology 2009; 71(8): 1326-1329.
21. Kim GY. What should be done for men with sperm DNA fragmentation? Clin Exp Reprod Med 2018; 45(3): 101-109.
22. Khaki A, Araghi A, Lotfi M, et al. Differences between first and second ejaculations regarding seminal plasma calcium, magnesium and total antioxidant capacity in dual-purpose Fleckvieh bulls and their relationships with semen quality. Vet Res Forum 2019; 10(4): 333-341.
23. Dwinofanto H, Rimayanti R, Mustofa E, et al. The effect of duration of preservation on the quality, MDA level, and DNA damage of post-thawed Bali cattle bull sperm. Iraq J Vet Sci 2019; 32(2): 249-252.
24. Uavechanichkul R, Pongpeng J, Wirojwutthikul S, et al. Quality of bull deep frozen semen extended with different glycerol concentrations: evaluated by computer assisted semen analysis. J Kasetsart Vet 2010: 20: 53-62.
25. Khan H, Khan M, Qureshi MS, et al. Effect of green tea extract (*Camellia sinensis*) on fertility indicators of post-thawed bull spermatozoa. Pakistan J Zool 2017; 49(4): 1243-1249.
26. Mizera A, Kuczaj M, Szul A. Impact of the Spirulina maxima extract addition to semen extender on bovine sperm quality. Ital J Anim Sci 2019; 18(1): 601-607.
27. Nath RG, Wu MY, Emami A, et al. Effects of epigallocatechin gallate, L-ascorbic acid, alpha-tocopherol, and dihydrolipoic acid on the formation of deoxyguanosine adducts derived from lipid peroxidation. Nutr Cancer 2010; 62(5): 622-629.
28. Zubair M, Ali M, Ahmad M, et al. Effect of selenium and vitamin E on cryopreservation of semen and reproductive performance of animals (a review). J Entomol Zool Stud 2015; 3(1): 82-86.
29. Agarwal A, Varghese AC, Sharma RK. Markers of oxidative stress and sperm chromatin integrity. Methods Mol Biol 2009; 590: 377-402.
30. Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 2005; 322(1): 33-41.
31. Fraser L, Strzezek J. Is there a relationship between the chromatin status and DNA fragmentation of boar spermatozoa following freezing-thawing? Theriogenology 2007; 68(2): 248-257.
32. Gliozzi TM, Zaniboni L, Cerolini S. DNA fragmentation in chicken spermatozoa during cryopreservation. Theriogenology 2011; 75(9): 1613-1622.
33. Bogle OA, Kumar K, Attardo-Parrinello C, et al. Identification of protein changes in human spermatozoa throughout the cryopreservation process. Andrology 2017; 5(1): 10-22.
34. ChaithraShree AR, Ingole SD, Dighe VD, et al. Effect of melatonin on bovine sperm characteristics and ultrastructure changes following cryopreservation. Vet Med Sci 2020; 6(2): 177-186.
35. Ahmed H, Jahan S, Khan A, et al. Supplementation of green tea extract (GTE) in extender improves structural and functional characteristics, total antioxidant capacity and in vivo fertility of buffalo (*Bubalus bubalis*) bull spermatozoa. Theriogenology 2020; 145: 190-197.
36. Ahmed H, Jahan S, Riaz M, et al. Epigallocatechin-3-gallate (EGCG) addition as an antioxidant in a cryo-diluent media improves microscopic parameters, and fertility potential, and alleviates oxidative stress parameters of buffalo spermatozoa. Cryobiology 2020; 97: 101-109.