Research Article

Effect of EGCG Supplementation on Post-Thaw Sperm Quality and DNA Integrity of Cryopreserved Kacang Buck Semen

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Abstract | Cryopreservation is an important reproductive biotechnology for preserving the genetic resources of indigenous livestock, including the Kacang goat (*Capra hircus*), which plays a significant role in smallholder farming systems in Indonesia. However, the freeze–thaw process induces oxidative stress that can impair sperm motility, compromise plasma membrane integrity, and increase DNA fragmentation. This study evaluated the effect of epigallocatechin gallate (EGCG) supplementation in a skim milk–egg yolk semen extender on post-thaw sperm quality and DNA integrity of cryopreserved Kacang buck semen. Ejaculates meeting initial quality criteria were allocated into three treatment groups: A control without EGCG, EGCG supplementation at 0.05 mg/100 mL, and EGCG supplementation at 0.10 mg/100 mL. Post-thaw sperm quality was assessed based on progressive motility, antioxidant enzyme activity, and DNA fragmentation. EGCG supplementation, particularly at a concentration of 0.10 mg/100 mL, was associated with higher post-thaw sperm motility, increased catalase activity, and reduced DNA fragmentation compared with the control group. These findings suggest that EGCG supplementation enhances the resistance of Kacang buck spermatozoa to cryopreservation-induced oxidative stress and contributes to improved post-thaw sperm quality and nuclear integrity.

Keywords | EGCG, Kacang goat, Cryopreservation, Post-thaw sperm quality, DNA fragmentation

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INTRODUCTION

Sperm cryopreservation is a key reproductive biotechnology for conserving genetic resources in indigenous livestock, including the Kacang goat (*Capra hircus*), which plays an important role in smallholder farming systems in Indonesia. Preservation of local goat germplasm is essential for maintaining genetic diversity and supporting sustainable livestock production in tropical regions (Susilowati *et al.*, 2022, 2025). Despite its advantages, the freeze–thaw process during cryopreservation remains a major challenge because it adversely affects sperm quality, primarily through the induction of oxidative stress (Gungor *et al.*, 2021; Mustofa *et al.*, 2022).

Oxidative stress during cryopreservation is mainly associated with excessive production of reactive oxygen

species (ROS), which can impair sperm motility and viability, disrupt plasma membrane integrity, and compromise nuclear stability. Post-thaw spermatozoa are particularly susceptible to lipid peroxidation and oxidative damage to nuclear components, resulting in increased DNA fragmentation and reduced fertilizing potential (Agarwal *et al*., 2019; Abdelnour *et al*., 2020). Previous studies on Kacang buck semen have demonstrated that oxidative stress is a critical limiting factor affecting post-thaw semen quality and the success of artificial insemination programs (Mustofa *et al*., 2022; Susilowati *et al*., 2025).

One widely applied strategy to mitigate oxidative damage during cryopreservation is the supplementation of semen extenders with antioxidants. Epigallocatechin-3-gallate (EGCG), a major polyphenolic compound derived from green tea (*Camellia sinensis*), has attracted considerable attention due to its strong antioxidant and free radical scavenging properties. Numerous studies have reported that EGCG supplementation improves post-thaw sperm motility, mitochondrial function, and antioxidant status in bovine, buffalo, and goat semen (Bucak *et al*., 2019; Tuncer *et al*., 2020; Abdelnour *et al*., 2020). In Kacang goats, green tea extract and EGCG-based additives have also been shown to enhance semen quality and reduce oxidative stress during cryopreservation (Susilowati *et al*., 2022).

However, the effectiveness of EGCG in conventional semen extenders may be limited by its chemical stability and functional availability during the freezing–thawing process. In skim milk–egg yolk extenders, milk proteins and egg yolk lipoproteins may provide a protective colloidal matrix that stabilizes antioxidant compounds and facilitates their interaction with sperm membranes during cryopreservation. Such extender components have been reported to buffer oxidative stress and protect bioactive molecules during cooling and freezing, thereby potentially enhancing the functional efficacy of antioxidants such as EGCG without the need for complex delivery systems. Nevertheless, the extent to which EGCG remains biologically effective within this widely used extender system requires empirical validation.

Although improvements in conventional sperm quality parameters have been widely reported, studies focusing specifically on sperm DNA integrity in cryopreserved Kacang buck semen remain limited. Most available studies on antioxidant supplementation in small ruminants primarily emphasize motility, viability, and membrane integrity, while nuclear parameters such as chromatin stability and DNA fragmentation have received comparatively less attention, particularly in indigenous goat breeds. In the context of semen cryopreservation, DNA integrity refers to the preservation of chromatin structure and the reduction of DNA fragmentation rather than sequence-level genetic mutations. Maintaining sperm nuclear integrity is crucial because increased DNA fragmentation has been associated with reduced fertilization capacity and impaired early embryonic development.

Therefore, the present study aimed to evaluate the effect of EGCG supplementation in a skim milk–egg yolk extender on post-thaw sperm quality and DNA integrity of cryopreserved Kacang buck semen. It was hypothesized that EGCG supplementation would enhance antioxidant defense, reduce DNA fragmentation, and improve post-thaw sperm characteristics compared with a conventional extender without EGCG. The findings of this study are expected to contribute to the optimization of semen cryopreservation protocols for indigenous goat breeds and support the development of more effective strategies for germplasm conservation.

MATERIALS AND METHODS

Animal care and ethical approval

All procedures involving animals were reviewed and approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Ethical Clearance Certificate No. 1.KEH.024.02.2024). Animal handling and experimental procedures were conducted in accordance with institutional animal care guidelines and the standards of the American Veterinary Medical Association.

Animals and semen collection

Fifty (50) clinically healthy Kacang bucks (Capra hircus), aged 2–3 years and weighing 25–30 kg, were obtained from a local breeder cooperative in East Java, Indonesia. The animals were maintained under standardized feeding, housing, and management conditions throughout the study. Semen was collected twice weekly using an artificial vagina (IMV Technologies, France) pre-warmed to 42–45°C. Immediately after collection, ejaculates were maintained at 37 °C for initial evaluation.

Initial semen evaluation and selection

Macroscopic evaluation included semen volume, color, odor, consistency, and pH. Microscopic evaluation consisted of mass motility (scored on a scale of 0–5), progressive motility (%), sperm viability assessed by eosin–nigrosin staining, and sperm concentration measured using a spectrophotometer at 546 nm. Only ejaculates exhibiting ≥70% progressive motility and ≥70% viability were included in the experiment.

Experimental design and pooling strategy

Qualified ejaculates collected on the same collection day were pooled to minimize ejaculate-to-ejaculate variability

among individual bucks. Each pooled semen batch was considered one biological replicate. Three independent pooled semen batches were prepared on three different collection days, resulting in three biological replicates (n = 3).

Each pooled semen batch was divided into three treatment groups: Control (C), EGCG 0.05 mg/100 mL (T1), and EGCG 0.10 mg/100 mL (T2). The experiment followed a completely randomized design. Within each biological replicate, multiple straws were produced per treatment group and treated as technical subsamples, while the pooled semen batch served as the experimental unit to maintain statistical independence.

Source of epigallocatechin gallate (EGCG)

Epigallocatechin gallate (EGCG) was isolated from dried green tea (*Camellia sinensis*) leaves using ethanol extraction followed by polyamide-based column chromatography. EGCG identity was confirmed by high-performance liquid chromatography (HPLC; Shimadzu LC-20AD, Japan) and liquid chromatography–tandem mass spectrometry (LC–MS/MS; AB Sciex QTRAP 6500+, USA) through comparison with an authentic EGCG standard (Sigma-Aldrich, USA). Purified EGCG was used for semen extender supplementation.

Preparation of semen extender and EGCG treatments

A skim milk–egg yolk extender was prepared by dissolving skim milk powder (HiMedia Laboratories, India) in 100 mL of distilled water and heating at 92–95°C for 10 min, followed by cooling to room temperature. Egg yolk was added to achieve a final concentration of 5% (v/v). Penicillin (1000 IU/mL) and streptomycin (1 mg/mL) were added to prevent bacterial contamination. Glycerol was added as a cryoprotectant to reach a final concentration of 7% (v/v) in the extended semen.

The treatment groups were as follows: C (Control), extender without EGCG; T1, EGCG at 0.05 mg/100 mL; and T2, EGCG at 0.10 mg/100 mL. Semen was diluted at a ratio of 1:10, and the final sperm concentration was adjusted to 100×10^6 sperm/mL, providing approximately 25×10^6 sperm per 0.25 mL straw.

Cryopreservation procedure

After dilution at 37°C, semen samples were gradually cooled to 5°C over approximately 2 h and equilibrated at 5°C for an additional 2 h to allow glycerol penetration. Semen was then packaged into 0.25 mL French straws (IMV Technologies, France) and sealed. Freezing was performed using liquid nitrogen vapor by placing straws horizontally on a rack positioned approximately 4 cm above the surface of liquid nitrogen for 10 min, followed by direct plunging into liquid nitrogen (−196 °C) for storage. Semen samples were stored for at least 24 h before evaluation. For post-thaw analysis, straws were thawed in a water bath at 37°C for 30 s, wiped dry, and immediately evaluated.

Assessment of post-thaw semen quality

For each biological replicate and treatment group, at least three straws were randomly selected for post-thaw evaluation. Sperm motility was assessed under a light microscope at 400× magnification and expressed as the percentage of progressively motile spermatozoa. For each straw, motility was evaluated across multiple microscopic fields to minimize observer bias.

Sperm viability was evaluated using eosin–nigrosin staining by counting at least 200 sperm cells per sample; unstained sperm were classified as viable. Plasma membrane integrity was assessed using the hypoosmotic swelling test (HOST), where sperm exhibiting swollen or coiled tails were considered membrane intact.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) concentration using the thiobarbituric acid reactive substances (TBARS) assay. Antioxidant enzyme activity, including superoxide dismutase (SOD) and catalase, was measured using commercial assay kits (Randox Laboratories, UK) and expressed per milligram of protein.

DNA integrity assessment

DNA integrity was evaluated using toluidine blue staining and examined under a bright-field light microscope. Spermatozoa displaying dark blue or purple nuclear staining were classified as having abnormal chromatin or DNA fragmentation. At least 200 sperm cells per sample were evaluated across multiple microscopic fields. Chromatin maturity was assessed using aniline blue staining; sperm nuclei-stained blue were considered to exhibit immature chromatin. All microscopic evaluations were performed by a trained observer blinded to the treatment groups to reduce subjective bias.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA). Prior to analysis, data were examined for normality and homogeneity of variance. When significant differences were detected ($p < 0.05$), mean comparisons among treatment groups were performed using Tukey's post hoc test. Results are presented as mean ± standard deviation. The pooled semen batch was considered the experimental unit (n = 3), while individual straws served as technical subsamples.

RESULTS

Macroscopic characteristics of fresh kacang goat semen

Fresh semen collected from Kacang bucks maintained in three pens exhibited uniform macroscopic characteristics (Table 1). Ejaculate volume ranged from 1.10 to 1.20 mL, with slightly higher volumes observed in Pens I and III compared with Pen II. All semen samples displayed a normal seminal odor and a yellowish-white color, which are typical characteristics of semen from reproductively healthy bucks.

Table 1: Macroscopic characteristics of fresh Kacang buck semen used for cryopreservation.

Parameter	Pen I	Pen II	Pen III
Volume (mL)	1.20	1.10	1.20
Odor	Typical	Typical	Typical
Color	Yellowish white	Yellowish-white	Yellowish white
pH	6.8	6.8	6.8
Consistency	Thick	Thick	Thick

Note: Values represent representative baseline observations obtained during routine semen evaluation prior to pooling. These data are presented to demonstrate uniformity of fresh semen quality and were not subjected to statistical analysis.

Semen pH was consistent across pens (pH 6.8), and semen consistency was classified as thick in all samples, indicating adequate sperm concentration and viscosity for cryopreservation processing. The values presented in Table 1 represent representative baseline measurements obtained during routine semen evaluation prior to pooling and are intended to demonstrate uniformity of fresh semen quality rather than to serve as variables for statistical comparison.

Microscopic characteristics of fresh Kacang goat semen

Microscopic evaluation revealed consistently high semen quality among bucks from the three pens (Table 2). Mass motility was uniformly scored as +++, indicating vigorous collective sperm movement. Progressive motility values ranged from 90% to 92%, while sperm viability exceeded 94% across all pens. Plasma membrane integrity ranged from 81% to 84%.

These baseline microscopic parameters confirm that only ejaculates of high initial quality were selected for pooling and cryopreservation, thereby minimizing the influence of initial semen variability on subsequent post-thaw evaluations. As with macroscopic parameters, the values presented reflect baseline screening data rather than outcomes subjected to inferential statistical analysis.

Post-thaw sperm motility

Post-thaw evaluation revealed marked differences in sperm motility among treatment groups (Table 3). The control group exhibited the lowest progressive motility (38.2 ± 5.72%). Supplementation with EGCG resulted in higher post-thaw motility compared with the control group. Semen treated with EGCG at 0.05 mg/100 mL (T1) showed a mean progressive motility of 49.8 ± 2.39%, while the highest post-thaw motility was observed in semen supplemented with EGCG at 0.10 mg/100 mL (T2), reaching 64.2 ± 2.77%.

Table 2: Microscopic characteristics of fresh Kacang buck semen prior to cryopreservation.

Parameter	Pen I	Pen II	Pen III
Mass motility	+++	+++	+++
Progressive motility (%)	92	90	90
Viability (%)	95	94	95
Intact plasma membrane (%)	84	81	82

Note: Values indicate baseline microscopic semen quality prior to cryopreservation and pooling. These parameters were used for ejaculate selection and were not included in inferential statistical analysis.

Table 3: Post-thaw progressive motility of cryopreserved Kacang buck semen.

Treatment	Progressive motility (%)
C: Control (extender without EGCG)	38.2 ± 5.72
T1: EGCG 0.05 mg/100 mL	49.8 ± 2.39
T2: EGCG 0.10 mg/100 mL	64.2 ± 2.77

Note: Values are expressed as mean ± standard deviation of three independent biological replicates (n = 3). The pooled semen batch prepared on each collection day was considered the experimental unit.

Statistical analysis indicated significant differences among treatment groups, with EGCG supplementation producing a concentration-dependent increase in post-thaw sperm motility.

DNA fragmentation

The percentage of sperm exhibiting DNA fragmentation differed among treatment groups (Table 4). The control group showed the highest level of DNA fragmentation (23.62 ± 1.93%). EGCG supplementation was associated with lower levels of DNA fragmentation. Treatment with EGCG at 0.05 mg/100 mL (T1) resulted in a moderate reduction in DNA fragmentation (21.30 ± 1.29%), whereas the EGCG 0.10 mg/100 mL group (T2) exhibited the lowest DNA fragmentation value (14.68 ± 1.12%).

In this study, DNA integrity refers to reduced DNA fragmentation and improved chromatin stability as

assessed by toluidine blue staining, rather than sequence-level genetic alterations.

Table 4: DNA fragmentation of post-thaw cryopreserved Kacang buck semen.

Treatment	DNA fragmentation (%)
C: Control (extender without EGCG)	23.62 ± 1.93
T1: EGCG 0.05 mg/100 mL	21.30 ± 1.29
T2: EGCG 0.10 mg/100 mL	14.68 ± 1.12

Note: DNA fragmentation was assessed using toluidine blue staining. Values are expressed as mean ± standard deviation of three independent biological replicates (n = 3).

Catalase activity

Catalase activity varied among treatment groups following thawing (Table 5). The lowest catalase activity was observed in the control group (0.045 ± 0.006 U/mg). Semen supplemented with EGCG at 0.05 mg/100 mL (T1) showed a higher catalase activity (0.058 ± 0.008 U/mg), while the highest catalase activity was recorded in the EGCG 0.10 mg/100 mL group (T2), reaching 0.181 ± 0.027 U/mg.

Table 5: Catalase activity of post-thaw cryopreserved Kacang buck semen.

Treatment	Catalase activity (U/mg protein)
C: Control (extender without EGCG)	0.045 ± 0.006
T1: EGCG 0.05 mg/100 mL	0.058 ± 0.008
T2: EGCG 0.10 mg/100 mL	0.181 ± 0.027

Note: Catalase activity was measured using a commercial assay kit and expressed per milligram of protein. Values represent mean ± standard deviation of three independent biological replicates (n = 3).

Statistical analysis demonstrated significant differences in catalase activity among treatment groups, with higher EGCG concentrations associated with increased post-thaw catalase activity.

DISCUSSION

Cryopreservation is an essential technique in reproductive biotechnology for preserving the genetic resources of indigenous goats such as the Kacang goat (*Capra hircus*). However, the freezing–thawing process exposes spermatozoa to substantial physiological stress, primarily through oxidative mechanisms, which can damage plasma membranes, mitochondria, and nuclear components. Numerous studies have demonstrated that oxidative stress is a major contributor to post-thaw deterioration of sperm quality, including reduced motility, compromised membrane integrity, and increased DNA fragmentation (Gungor *et al*., 2021; Mustofa *et al*., 2022; Susilowati *et al*., 2025). The present study demonstrates that supplementation of a skim milk–egg yolk semen extender with EGCG mitigates several adverse effects of cryopreservation and improves post-thaw sperm quality of Kacang buck semen.

Post-thaw sperm motility is a key indicator of sperm functional competence and is closely associated with fertilizing potential. In the present study, cryopreservation markedly reduced sperm motility in the control group, consistent with previous reports describing the detrimental effects of oxidative stress on flagellar function and mitochondrial activity. Supplementation with EGCG significantly improved post-thaw motility in a concentration-dependent manner, with the highest motility observed in semen treated with EGCG at 0.10 mg/100 mL. These findings are in agreement with earlier studies reporting that EGCG enhances sperm motility by protecting mitochondrial membranes, preserving ATP production, and reducing oxidative damage during cryopreservation (Bucak *et al*., 2019; Abdelnour *et al*., 2020). Similar improvements in post-thaw motility of Kacang buck semen following EGCG supplementation have been reported in previous studies (Mustofa *et al*., 2022; Susilowati *et al*., 2025). From a practical perspective, post-thaw motility values exceeding 60% are generally considered acceptable for artificial insemination in goats, suggesting that EGCG supplementation at the higher concentration may have practical relevance, although fertility trials are required for confirmation.

In addition to improvements in motility, EGCG supplementation resulted in a significant reduction in sperm DNA fragmentation. The control group exhibited the highest level of DNA fragmentation, reflecting cryopreservation-induced chromatin damage associated with excessive production of reactive oxygen species. DNA fragmentation is a critical indicator of sperm nuclear integrity and has been linked to reduced fertilization capacity and impaired early embryonic development. In contrast, semen supplemented with EGCG particularly at the higher concentration showed substantially lower levels of DNA fragmentation. Although definitive threshold values for DNA fragmentation in goats have not been fully established, studies in small ruminants and other livestock species suggest that fragmentation levels below approximately 15–20% are generally compatible with normal fertilizing capacity, whereas values exceeding 20–25% are more consistently associated with reduced fertility (Bucak *et al*., 2019; Gungor *et al*., 2021; El-Nattat *et al*., 2021). In this context, the reduction of DNA fragmentation to approximately 15% observed in the EGCG 0.10 mg/100 mL group represents a biologically meaningful improvement in nuclear integrity. In this study, DNA integrity refers to the preservation of chromatin

structure and reduced DNA fragmentation rather than sequence-level genetic alterations.

Enhanced antioxidant defense appears to play a central role in the protective effects of EGCG observed in this study. Catalase activity was significantly higher in EGCG-treated groups compared with the control group, with the greatest increase observed at the higher EGCG concentration. Catalase is a key antioxidant enzyme responsible for detoxifying hydrogen peroxide and limiting oxidative damage to cellular and nuclear structures. The magnitude of increase in catalase activity observed in the present study is biologically plausible, as EGCG has been shown to preserve antioxidant enzyme functionality and upregulate endogenous antioxidant defenses under oxidative stress conditions (Abdelnour *et al*., 2020; Zhao *et al*., 2021). As catalase activity was measured after removal of extender components, direct chemical interference of EGCG with the assay is unlikely.

Despite these positive findings, several limitations should be acknowledged. The number of biological replicates was limited (n= 3 pooled semen batches), which inherently restricts statistical power and may increase uncertainty in variance estimates. This approach was adopted to reduce individual ejaculate variability and is commonly used in exploratory cryopreservation studies. In addition, oxidative stress was inferred indirectly through antioxidant enzyme activity and lipid peroxidation markers, as direct intracellular ROS levels were not measured. Future studies incorporating direct ROS measurements, larger numbers of biological replicates, and in vivo fertility assessments are required to confirm and extend the present findings.

Overall, the combined improvements in post-thaw sperm motility, antioxidant enzyme activity, and DNA fragmentation observed in this study indicate that EGCG supplementation enhances sperm resistance to cryopreservation-induced oxidative stress. These results support the potential use of EGCG as a functional antioxidant additive in semen extenders for cryopreservation of Kacang buck semen, while emphasizing the need for further validation under practical reproductive conditions.

CONCLUSION

This study demonstrates that supplementation of a skim milk egg yolk semen extender with epigallocatechin gallate (EGCG) improves post-thaw sperm quality of cryopreserved Kacang buck semen. EGCG supplementation, particularly at a concentration of 0.10 mg/100 mL, was associated with higher progressive motility, increased catalase activity, and reduced DNA fragmentation compared with the control and lower EGCG concentration. These findings suggest that EGCG enhances sperm resistance to cryopreservation-induced oxidative stress and contributes to the preservation of sperm nuclear integrity. Overall, EGCG shows potential as a functional antioxidant additive in semen extenders for improving post-thaw semen quality of indigenous goats. However, given the limited number of biological replicates and the absence of fertility trials in the present study, further research incorporating in vivo reproductive performance and fertility outcomes is required to confirm its practical application in artificial insemination and germplasm conservation programs.

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NOVELTY STATEMENT

This study is the first to evaluate the effect of epigallocatechin gallate (EGCG) supplementation in a skim milk–egg yolk semen extender on both post-thaw sperm quality and DNA integrity of cryopreserved Kacang buck semen. The findings demonstrate that EGCG, particularly at 0.10 mg/100 mL, significantly enhances post-thaw sperm motility, increases catalase activity, and reduces DNA fragmentation, providing a novel approach to improve semen cryopreservation protocols for indigenous goat breeds.

AUTHOR'S CONTRIBUTION

SS conceived and designed the study and supervised the research. IM and TH performed the experiments and collected the data. TWS and YO conducted data analysis and interpretation. S assisted with sample preparation and provided technical support. All authors reviewed, revised, and approved the final version of the manuscript.

Generative AI and AI-assisted technology statement

No generative AI was used for content creation. AI-assisted tools were used solely for improving English grammar and language clarity.

Conflict of interest

The authors have declared no conflict of interest.

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