

## Effect of green tea (*Camellia sinensis* L.) extract on the quality of Gaok rooster spermatozoa stored in a fructose-egg yolk-milk diluent at room temperature

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### ABSTRACT

This study evaluated the effect of a fructose-egg yolk-milk diluent supplemented with different concentration of green tea leaf extract (*Camellia sinensis* L.) on the quality of rooster semen stored at room temperature (25 °C). Semen was collected from five healthy Gaok roosters (1.5-2 years old), pooled and allocated into four treatment groups: T0 (diluent only, control), T1 (diluent + 0.005 mg green tea extract/10 mL), T2 (diluent + 0.010 mg/10 mL), and T3 (diluent + 0.015 mg/10 mL). Semen quality was evaluated over a 6-hour storage period, focusing on progressive motility, viability, plasma membrane integrity (assessed using the hypoosmotic swelling test), and morphological abnormalities. The addition of green tea extract significantly improved progressive motility, sperm viability, and membrane integrity compared to the control throughout the storage period ( $p < 0.05$ ). These improvements followed a dose–response trend, with the intermediate dose (T2, 0.010 mg/10 mL) showing the most stable and sustained protective effects. No significant differences were observed in spermatozoa morphological abnormalities among treatments ( $p > 0.05$ ), with values remaining low and comparable across all groups. Overall, the results suggest that incorporating green tea leaf extract into a fructose-egg yolk-milk diluent can enhance the short-term preservation of rooster semen by maintaining motility, viability, and membrane functionality at 25 °C, without adversely affecting morphology. The optimal dose identified in this study was 0.010 mg per 10 mL diluent (T2), which provided effective protection for up to 6 hours of storage. In conclusion, green tea extract shows promise as a practical antioxidant additive for improving the short-term handling and transport of rooster semen under field conditions.

**Keywords:** Gaok rooster, green tea extract, morphological abnormalities, motility, viability

### INTRODUCTION

The Gaok rooster is an indigenous Indonesian breed that originates from Puteran Island, Sumenep regency, Madura, East Java. The term “Gaok” in the Madurese language refers to a long drawn-out call similar to that of

the pelung rooster from Cianjur, West Java; therefore, this breed is often called the Madura pelung (Wulandari *et al.*, 2025). Gaok roosters have potential to be developed as meat producers, egg layers, or ornamental and pet birds. Their egg production is relatively good, averaging 30.2 eggs per 12 weeks with a mean

egg weight of 46.7 g (Komarudin *et al.*, 2020). However, despite this potential, the Gaok rooster remain relatively unknown and its distribution is still limited compared to other local breeds. In 2013, the estimated population of the Gaok roosters across Madura was only about 2,000 individuals (Kostaman *et al.*, 2013), and no further reports on their populations have been documented in subsequent years. Field observations suggest that the Gaok rooster population in Madura continues to decline. This decline is likely associated with low low productivity, limited management practices, and high genetic variation among individuals. Therefore, achieving optimal productivity requires genetic improvement, for which reproductive biotechnologies such as artificial insemination (AI) can be applied (Meel *et al.*, 2025).

Artificial insemination is an effective strategy to optimize the use of local roosters because it offers advantages over natural mating, for example by addressing reduced fertility caused by strong mate selectivity in hens (Getachew, 2016). Successful AI in poultry depends on high-quality semen; determinants of semen quality include the type and concentration of diluent, physico-chemical properties of the diluent (pH, osmotic pressure, electrolyte composition), storage temperature, and storage duration (Saniasi *et al.*, 2026). Fresh rooster semen remains viable for less than two hours and is typically of high spermatozoa concentration with small volume, appropriate dilution is required to maintain quality and to make efficient use of semen (Arif *et al.*, 2025).

Semen dilution aims to increase volume, reduce spermatozoa density, and maintain spermatozoa survival during storage, preserving motility, viability, and fertility (Chankitisakul *et al.*, 2022). A suitable diluent must maintain pH, protect spermatozoa from cold shock, and supply nutrients. Common diluent constituents used to prolong storage include skim milk, egg yolk, and fructose (Bustani and Baiee, 2021). Skim milk is relatively inexpensive and readily available; it provides nutrients as an energy source for spermatozoa and contains lipoproteins and lecithin that protect spermatozoa from cold shock. Milk also contains enzymes that, when heated above 80°C, release sulfhydryl (-SH) groups that act as

reducing agents in spermatozoa oxidative metabolism (Rodrigues *et al.*, 2020). Egg yolk is an extracellular cryoprotectant rich in lipoproteins and lecithin that preserve spermatozoa membrane integrity during storage; it also provides preservative properties, energy, and physico-chemical characteristics similar to semen (Bustani and Baiee, 2021). Fructose is incorporated as an additional energy substrate for spermatozoa; several studies report that fructose better sustains spermatozoa motility than glucose (Tsujii *et al.*, 2006).

Storage temperature critically influences rooster semen quality because spermatozoa are highly temperature sensitive. Low temperatures reduce motility, while high temperatures denature protective spermatozoa proteins and likewise decrease motility. The ambient storage temperature of approximately 25 °C is optimal for rooster semen, at which spermatozoa exhibit greater progressive motility, viability, and normal morphology compared with storage at 4 °C or 37 °C (Blank *et al.*, 2021). In addition to physical factors, oxidative stress from free radicals such as reactive oxygen species (ROS) affects spermatozoa quality. At low levels ROS play physiological roles in capacitation, hyperactivation, acrosome reaction, and oocyte fusion, but high ROS levels are detrimental to spermatozoa quality and function (Khan, 2011). Therefore, incorporating antioxidants into diluents can prevent membrane damage by free radicals, preserve spermatozoa viability and motility, and improve diluent composition (Ratchamak *et al.*, 2025). Green tea (*Camellia sinensis* L.) is a potent natural source of antioxidants, primarily catechins, most notably epigallocatechin gallate (EGCG), which have been reported to neutralize free radicals more effectively than vitamin C and, to a lesser extent, vitamin E (Capasso *et al.*, 2025). Therefore, green tea leaf extract has potential as an antioxidant supplement in semen diluents. Previous study reported that green tea extract addition in the diluent promising additive for semen preservation in buffalo bulls (Khattak *et al.*, 2022), Bali bulls (Prastiya *et al.*, 2023; Prastiya *et al.*, 2024), kacang bucks (Suprayogi *et al.*, 2022), and cats (Wittayarat *et al.*, 2022).

Based on the foregoing, this study aims to evaluate the effect of adding green tea leaf extract (*Camellia sinensis* L.) to a skim milk-egg

yolk-fructose diluent on the quality of Gaok rooster spermatozoa, assessed by motility, viability, abnormality, and plasma membrane integrity during storage at room temperature (25°C).

## MATERIALS AND METHODS

This research was approved by the Animal Research Ethics Commission, Faculty of Veterinary Medicine, Universitas Airlangga (No. KEH.071.05.2023, dated May 8, 2023).

### Green tea leaf extract

Green tea leaves were sourced from tea plantations in Bandung, West Java. The extract was prepared by first drying the leaves, followed by maceration using 96% ethanol as the solvent. The resulting concentrated extract was then freeze-dried to obtain a dry powder (Miranda *et al.*, 2023).

### Dosage of green tea leaf extract

The ethanol extracted green tea leaves was administered in three doses: 0.005, 0.010, and 0.015 mg per 10 mL of diluent. The doses were adapted from the study by Susilowati *et al.* (2022) which investigated the use of green tea leaf extract to kacang goat semen diluent, with adjustment made to maintain a comparable ratio.

### Fructose egg yolk skim milk diluent

The fructose egg yolk skim milk diluent was made by mixing 100 g of skim milk with 100 mL of distilled water, then heating it to 92 °C for 10 minutes. The skim milk was then cooled and filtered twice using sterile gauze, after which egg yolk was added at 5% of the total volume, fructose 0.75% of the total volume, Penicillin 1000 IU/mL and Streptomycin 1 mg/mL at 0.1% of the diluent volume, and then homogenized using a magnetic stirrer for 10 minutes (Miranda *et al.*, 2023).

### Semen collection

The cloaca was cleaned with tissue paper dipped in 70% alcohol before the collection. Semen was collected using the massage technique as described by Gitayana *et al.* (2023). The massage was applied from the back to the base of the tail and then upward to the tip of the tail following the direction of the tail feathers.

Semen was collected twice a week, with two ejaculations required in each session.

### Fresh semen evaluation

Examination of semen after collection was carried out to determine the quality of fresh semen and to ensure it met the requirements before treatment. There are two types of examination, namely macroscopic and microscopic. Macroscopic examination includes odor, color, volume, viscosity, and pH. Microscopic examination includes semen concentration, percentage of live and dead spermatozoa, percentage of abnormal spermatozoa, mass movement, and individual movement (Gitayana *et al.*, 2023).

### Treatment of semen

In this study, one control group and three treatments were used in semen diluents: control group (T0): rooster semen + fructose-egg yolk milk diluent; treatment group 1 (T1): rooster semen + fructose-egg yolk milk diluent + 0.005 mg/10 mL green tea leaf extract; treatment group 2 (T2): rooster semen + fructose-egg yolk milk diluent + 0.010 mg/10 mL green tea leaf extract; treatment group 3 (T3): rooster semen + fructose-egg yolk milk diluent + 0.015 mg/10 mL green tea leaf extract. The samples were then labeled and stored in a cupboard at room temperature (25°C). The samples were examined before and after being placed in the cupboard. Semen sample examinations were conducted every two hours. Samples were taken using a pipette while still in the cupboard. If the semen sample still met the AI standard requirements, it was examined again after two hours.

### Semen examination post-treatment

Semen from each treatment was evaluated every two hours until the motility of rooster spermatozoa reached 40% (Gitayana *et al.*, 2023). The parameters assessed included motility, viability, and plasma membrane integrity.

### Spermatozoa motility

A drop of the semen-diluent mixture from each treatment was placed on a glass slide, covered with cover slip, and examined under a light microscope at 400× magnification.

Progressive (forward) motility was recorded by counting motile (progressively moving) and non-motile spermatozoa until a total of 100 spermatozoa were evaluated in each replicate (Gitayana *et al.*, 2023).

### Spermatozoa viability

Fresh semen smears were prepared using Eosin Nigrosin stain, mixed until homogeneous, and air-dried quickly over a Bunsen flame. The smears were examined at 400× magnification. The percentages of live and dead spermatozoa were determined in triplicate by counting 100 spermatozoa in each replicate. Viable spermatozoa heads remained unstained or translucent, whereas non-viable spermatozoa absorbed the stain (red heads) due to increased membrane permeability (Gitayana *et al.*, 2023).

### Plasma membrane integrity

Plasma membrane integrity was assessed using the hypoosmotic swelling (HOS) test. The hypoosmotic solution was prepared by dissolving 2.7 g fructose in 100 mL of distilled water and 1.47 g sodium citrate in 100 mL distilled water; the two solutions were then combined to obtain a total of 200 mL hypoosmotic solution. For the test, 0.1 mL semen was added to 1 mL hypoosmotic solution and incubated at 25 °C for 30 minutes. A drop of the incubated sample was placed on a glass slide, covered with a cover slip, and observed at 400× magnification (Pitaloka *et al.*, 2023).

### Data analysis

Data were analyzed using Analysis of Variance (ANOVA) with IBM SPSS (Version 20.0). Significant differences were further examined using Duncan's Multiple Range Test at 95% significance level.

## RESULTS

Findings from the macroscopic examination of fresh semen from Gaok roosters, including volume, color, odor, consistency, and pH, are presented in Table 1. The semen volume was  $0.3 \pm 0.19$  mL, with a milky white color, a typical rooster odor, thick consistency, and a pH of  $7.00 \pm 0.00$ . The results of the microscopic

examination of fresh semen from Gaok roosters are presented in Table 2. The semen exhibited strong mass movement (+++), progressive individual motility, and a spermatozoa concentration of  $2.362 \pm 0.56 \times 10^6/\text{mL}$ . The percentages of motility and viability were  $89.2 \pm 1.64\%$  and  $90.4 \pm 1.14\%$ , respectively, while morphological abnormalities remained low ( $7.70 \pm 1.92\%$ ).

**Table 1** Macroscopic characteristics of fresh semen of Gaok rooster

|             |                  |
|-------------|------------------|
| volume (mL) | $0.3 \pm 0.19$   |
| color       | milky white      |
| odor        | specific rooster |
| consistency | thick            |
| pH          | $7.00 \pm 0.00$  |

**Table 2** Microscopic characteristics of fresh semen of Gaok rooster

|                                           |                  |
|-------------------------------------------|------------------|
| mass movement                             | +++              |
| individual movement                       | progressive      |
| concentration ( $\times 10^6/\text{mL}$ ) | $2.362 \pm 0.56$ |
| motility (%)                              | $89.2 \pm 1.64$  |
| viability (%)                             | $90.4 \pm 1.14$  |
| morphologic abnormality (%)               | $7.70 \pm 1.92$  |

Spermatozoa of the Gaok rooster showed decreased motility (Table 3) and viability (Table 4), increased morphological abnormalities (Table 5), and decreased plasma membrane integrity (Table 6) during storage in all treatment groups. In the treatment group, spermatozoa motility persisted for up to six hours, whereas in the control group, it remained above 40% for four hours. Group T2 had the highest spermatozoa motility (Table 3) and viability (Table 4) after six hours of storage ( $p < 0.05$ ). At all storage times, there was no significant difference ( $p > 0.05$ ) in the morphological defects of spermatozoa between treatments. In contrast to the other groups, plasma membrane integrity at six hours of storage had the lowest percentage ( $p < 0.05$ ) (Table 5). After six hours of storage, there was no significant difference ( $p > 0.05$ ) in the plasma membrane integrity of groups T1, T2, and T3 (Table 6).

**Table 3** Motility (%) of Gaok rooster spermatozoa during storage following treatment

|    | duration of storage        |                            |                            |                            |
|----|----------------------------|----------------------------|----------------------------|----------------------------|
|    | 0 hour                     | 2 hours                    | 4 hours                    | 6 hours                    |
| T0 | 78.80 ± 1.30 <sup>a</sup>  | 64.60 ± 2.40 <sup>a</sup>  | 49.60 ± 2.30 <sup>a</sup>  | 36.80 ± 4.08 <sup>a</sup>  |
| T1 | 81.60 ± 1.14 <sup>b</sup>  | 67.80 ± 3.19 <sup>ab</sup> | 54.20 ± 3.34 <sup>b</sup>  | 40.80 ± 2.77 <sup>ab</sup> |
| T2 | 83.80 ± 0.83 <sup>c</sup>  | 73.40 ± 2.40 <sup>c</sup>  | 60.00 ± 3.00 <sup>c</sup>  | 46.60 ± 2.40 <sup>c</sup>  |
| T3 | 83.20 ± 1.48 <sup>bc</sup> | 70.80 ± 1.64 <sup>bc</sup> | 57.00 ± 4.06 <sup>bc</sup> | 44.20 ± 2.38 <sup>bc</sup> |

Different superscripts in the same column indicate significant differences ( $p < 0.05$ ); T0: semen diluted with fructose-egg yolk-milk diluent, control; T1, T2 and T3: diluent supplemented with 0.005, 0.010, and 0.015 mg green tea leaf extract/10 mL, respectively.

**Table 4** Viability (%) of Gaok rooster spermatozoa during storage following treatment

|    | duration of storage        |                            |                            |                            |
|----|----------------------------|----------------------------|----------------------------|----------------------------|
|    | 0 hour                     | 2 hours                    | 4 hours                    | 6 hours                    |
| T0 | 82.60 ± 2.30 <sup>a</sup>  | 71.40 ± 1.67 <sup>a</sup>  | 61.40 ± 1.67 <sup>a</sup>  | 50.40 ± 2.70 <sup>a</sup>  |
| T1 | 85.00 ± 2.12 <sup>b</sup>  | 74.60 ± 2.07 <sup>ab</sup> | 64.40 ± 1.81 <sup>b</sup>  | 54.60 ± 2.70 <sup>ab</sup> |
| T2 | 90.00 ± 1.22 <sup>c</sup>  | 80.80 ± 3.70 <sup>c</sup>  | 68.20 ± 3.96 <sup>c</sup>  | 59.20 ± 3.42 <sup>c</sup>  |
| T3 | 87.20 ± 1.48 <sup>bc</sup> | 77.60 ± 1.94 <sup>bc</sup> | 66.00 ± 4.12 <sup>bc</sup> | 56.80 ± 3.11 <sup>bc</sup> |

Different superscripts in the same column indicate significant differences ( $p < 0.05$ ); T0: semen diluted with fructose-egg yolk-milk diluent, control; T1, T2 and T3: diluent supplemented with 0.005, 0.010, and 0.015 mg green tea leaf extract/10 mL, respectively.

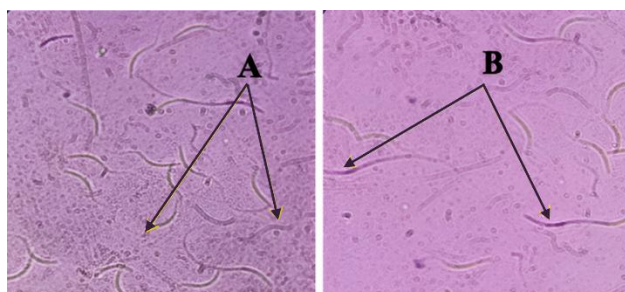


**Figure 1** Eosin nigrosin staining of Gaok rooster spermatozoa showing live (unstained/clear) spermatozoa (A) and dead spermatozoa with reddish heads (B); observed using a Nikon Eclipse E100 LED microscope at 400× magnification.

**Table 5** Morphological abnormalities (%) of Gaok rooster spermatozoa after treatment

|    | duration of storage |             |             |             |
|----|---------------------|-------------|-------------|-------------|
|    | 0 hour              | 2 hours     | 4 hours     | 6 hours     |
| T0 | 2.90 ± 0.54         | 3.70 ± 0.75 | 4.00 ± 0.61 | 4.40 ± 0.65 |
| T1 | 2.10 ± 1.08         | 3.10 ± 0.65 | 3.50 ± 0.50 | 4.30 ± 0.90 |
| T2 | 2.60 ± 0.96         | 2.90 ± 0.41 | 3.70 ± 1.71 | 3.90 ± 1.19 |
| T3 | 2.10 ± 0.65         | 3.40 ± 1.14 | 4.00 ± 1.45 | 4.80 ± 1.09 |

T0: semen diluted with fructose-egg yolk-milk diluent, control; T1, T2 and T3: diluent supplemented with 0.005, 0.010, and 0.015 mg green tea leaf extract/10 mL, respectively.

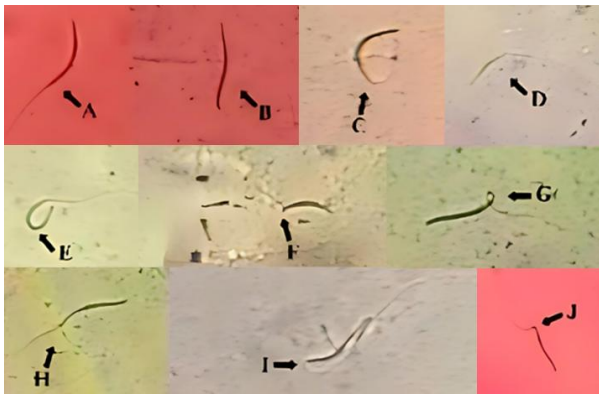


**Figure 2** Eosin–nigrosin staining of Gaok rooster spermatozoa showing normal spermatozoa (A) and various morphological abnormalities, including head without tail (B), folded tail (C), tail without head (D), bent head (E), protoplasmic droplet on the head (F), coiled neck (G), forked tail (H), bent neck (I), and midpiece defect (J); observed using a Nikon Eclipse E100 LED microscope at 400× magnification.

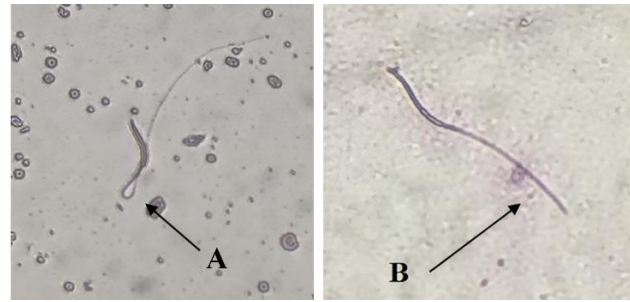


**Figure 3** Eosin nigrosin staining of Gaok rooster spermatozoa showing spermatozoa with intact plasma membranes, characterized by tails curling at the tip (A), whereas spermatozoa with damaged plasma membranes are characterized by straight tails (B); observed using a Nikon Eclipse E100 LED microscope at 400× magnification.

**Table 6** Plasma membrane integrity (%) of Gaok rooster spermatozoa after treatment

|    | duration of storage        |                            |                           |                           |
|----|----------------------------|----------------------------|---------------------------|---------------------------|
|    | 0 hour                     | 2 hours                    | 4 hours                   | 6 hours                   |
| T0 | 63.60 ± 3.50 <sup>a</sup>  | 41.00 ± 1.41 <sup>a</sup>  | 31.00 ± 1.41 <sup>a</sup> | 22.40 ± 1.14 <sup>a</sup> |
| T1 | 63.40 ± 3.04 <sup>a</sup>  | 43.00 ± 2.00 <sup>ab</sup> | 33.60 ± 0.89 <sup>b</sup> | 25.00 ± 1.58 <sup>b</sup> |
| T2 | 69.80 ± 3.11 <sup>b</sup>  | 46.80 ± 1.48 <sup>c</sup>  | 37.20 ± 0.83 <sup>c</sup> | 28.40 ± 2.07 <sup>b</sup> |
| T3 | 65.60 ± 3.04 <sup>ab</sup> | 44.60 ± 1.14 <sup>b</sup>  | 34.80 ± 0.83 <sup>b</sup> | 25.80 ± 1.30 <sup>b</sup> |

Different superscripts in the same column indicate significant differences ( $p < 0.05$ ); T0: semen diluted with fructose-egg yolk-milk diluent, control; T1, T2 and T3: diluent supplemented with 0.005, 0.010, and 0.015 mg green tea leaf extract/10 mL, respectively.

## DISCUSSION

The semen volume obtained from Gaok roosters in this study was  $0.30 \pm 0.19$  mL, which is higher than the  $0.12 \pm 0.03$  mL reported for indigenous roosters by Khaeruddin *et al.* (2020). Semen volume is influenced by several factors, including male age, level of stimulation, ejaculation frequency, diet quality, and overall health status (Oyeleye *et al.*, 2025).

Fresh Gaok semen in this study was milky white with a viscous consistency. Color and consistency indicate spermatozoa concentration: thick, milky semen generally corresponds to high concentration, whereas clear, watery semen indicates low concentration (Tribudi *et al.*, 2024). Observations indicated that the color and

consistency of Gaok semen were within normal ranges. Semen pH in this study was neutral (pH 7), comparable to the 7.16 reported by Komarudin *et al.* (2020). Semen pH strongly affects spermatozoa survival; lower pH values are associated with reduced spermatozoa viability (Khaeruddin *et al.*, 2016). Variation in pH may arise from ambient temperature microbial contamination, or increased numbers of dead spermatozoa in the ejaculate leading to ammonia formation (Komarudin *et al.*, 2020).

The concentration of fresh spermatozoa in Gaok semen was  $2.362 \times 10^6$ /mL, slightly lower than the  $2.460 \times 10^6$ /mL reported by Murcahyana *et al.* (2016). Spermatozoa concentration in rooster semen can vary depending on collection frequency, male libido, nutrition, temperature,

and season (Oyeleye *et al.*, 2025). Mass spermatozoa movement was rated very good (+++), indicating large, numerous, and rapid wave motion. Progressive individual motility was  $89.2 \pm 1.64\%$ , indicating that it is qualified for dilution and use in artificial insemination. According to Ardhani *et al.* (2025), fresh rooster semen should have motility  $\geq 80\%$  to be appropriate for further processing for artificial insemination.

Viability in this study was 90.4%, higher than the mean viability of 80.26% reported for various local rooster lines by Azizah *et al.* (2023). However, the motility/viability value of  $89.2 \pm 1.64\%$  is slightly lower than the  $92.61 \pm 1.93\%$  reported by Pratiwi *et al.* (2019). The percentage of spermatozoa abnormalities in fresh Gaok semen was 7.70%, lower than the 9.09% average abnormality reported by Khaeruddin and Kurniawan (2020) for indigenous rooster semen.

### **Spermatozoa motility of Gaok roosters after treatment**

In motility evaluation, the proportion of spermatozoa exhibiting forward progressive movement is recorded; spermatozoa showing circular movement or immobility are excluded. Motility assessment is a critical parameter for evaluating spermatozoa quality because only progressively motile spermatozoa are likely to fertilize the ovum (Oyeleye *et al.*, 2025). At the initial assessment (0 h), the control group (T0, no extract) exhibited the lowest motility, suggesting that the absence of antioxidant supplementation results in suboptimal motility at room temperature, although semen remained suitable for artificial insemination. The T2 group (0.010 mg extract) showed the highest motility (83.80%), demonstrating that green tea extract, as an antioxidant, can protect spermatozoa from oxidative damage. Previous studies have reported that antioxidants (vitamins, enzymes, and radical scavengers), including compounds found in green tea, protect spermatozoa from ROS-induced damage and improve post-dilution motility (Lopez *et al.*, 2015). Motility declined progressively during subsequent assessments (2–6 h), consistent with reports that spermatozoa motility and viability decrease significantly during storage at room temperature due to increased oxidative stress and deterioration of membrane quality (Tvrdá *et al.*, 2023).

The T1 group (0.005 mg extract) at 0 hours and 4 hours of storage showed higher motility than T0, confirming a protective effect of low-dose green tea extract; however, T1 motility remained lower than T2, indicating that 0.005 mg was insufficient to achieve optimal preservation at 25°C. The superior performance of T2 is attributed to bioactive compounds in green tea, such as flavonoids, phenolic acids, EGCG, which exert strong antioxidant activity by interrupting radical chain reactions and scavenging free radicals (Leão *et al.*, 2021). The T3 group (0.015 mg) exhibited lower motility than T2. Physiologically, low levels of ROS are necessary for normal spermatozoa function; excessive antioxidant dosing or disruption of redox balance can impair spermatozoa function (Onochie *et al.*, 2025). Therefore, supra-optimal extract doses may have negative effects.

Egg yolk content in the diluent and storage duration also influenced motility. Egg yolk provides lipoproteins and lecithin that help maintain spermatozoa membrane integrity and act as energy sources and protective agents during storage (Bustani and Baiee, 2021). In summary, spermatozoa motility declined with storage time at room temperature (25 °C) due to ongoing metabolism and increasing oxidative stress. The addition of green tea leaf extract at an optimal dose (0.010 mg) effectively mitigated motility decline, whereas lower doses were less effective and higher doses could be detrimental.

### **Spermatozoa viability of Gaok roosters after treatment**

The T2 group (0.010 mg extract) exhibited the highest viability (90%), indicating a protective antioxidant effect of green tea extract against oxidative damage in spermatozoa. Green tea polyphenols have been reported to reduce ROS production, lipid peroxidation, protein carbonylation, and DNA damage, thereby improving spermatozoa quality (Rahman *et al.*, 2018; Selvam *et al.*, 2018). In contrast, the control group (T0, no extract) showed the lowest viability, indicating that without antioxidant supplementation viability is difficult to maintain optimally at room temperature (25 °C).

Viability decreased from the second (2 h) to the fourth (6 h) assessment across all treatments. This decline is consistent with reports that storage at room temperature increases the

numbers of dead spermatozoa due to structural and functional damage during preservation (Blank *et al.*, 2021). Although viability declined up to 6 h, Gaok spermatozoa in this study still met the minimum requirement for artificial insemination, with a recommended viability of  $\geq 50\%$  (Arif *et al.*, 2025).

It is important to note that viability is generally slightly higher than motility because some spermatozoa may remain alive but non-motile (Oyeleye *et al.*, 2025). The T1 group (0.005 mg extract) showed higher viability than T0 at 0 hours and 4 hours, demonstrating a protective effect at a low dose; however, its viability remained lower than T2, indicating that 0.005 mg was not optimal. Conversely, the T3 (0.015 mg) showed lower viability than T2, which may reflect disruption of redox balance due to altered ROS or excessive antioxidant levels, leading to negative effects on spermatozoa function (Salchi *et al.*, 2018).

Other factors influencing viability included storage duration and ambient temperature. Storage at room temperature (25 °C) allows continued spermatozoa metabolism, contributing to time-dependent declines in viability. Physical changes during diluent addition, such as friction between spermatozoa and lipid globules, can cause cellular damage and death (Ratchamak *et al.*, 2025). Dead spermatozoa may release toxic substances that harm remaining viable cells, and oxidation of diluent components during storage can increase free radicals and compromise plasma membrane integrity (Chaithongsri *et al.*, 2025). In conclusion, supplementation with green tea leaf extract at an optimal concentration (0.010 mg) improved and helped maintain the viability of Gaok spermatozoa during storage at room-temperature, although only to a certain extent; lower doses were insufficient, while higher doses risked perturbing redox balance and reducing viability.

### **Spermatozoa morphological abnormalities of Gaok roosters after treatment**

The predominant spermatozoa morphological defects observed in Gaok roosters were secondary abnormalities, including bent or coiled necks, kinked heads, tail coiling or breakage, detached heads or tails, and midpiece defects. Bustani and Baie (2021) explained that secondary abnormalities arise after spermatozoa

leave the seminiferous tubules during ejaculation and often result from environmental contamination. Primary abnormalities, such as bifurcated tails and protoplasmic-droplet heads, were also detected; these are generally associated with disturbances in spermatogenesis and may reflect pathological conditions, hereditary factors, suboptimal rearing environments, or osmotic stress during spermatogenesis (Juiputta *et al.*, 2025).

Green tea (*Camellia sinensis* L.) leaf extract supplementation did not significantly affect the proportion of morphological abnormalities. This lack of effect suggests that, within the dosage range tested, green tea extract did not substantially alter spermatozoa morphology. This finding is consistent with Raei *et al.* (2022), who reported that low antioxidant doses may be insufficient to influence morphology. Changes in spermatozoa morphology often require longer exposure periods compared to parameters such as motility or viability (Kim *et al.*, 2022). It is also plausible that spermatozoa morphology in Gaok roosters is relatively stable and less responsive to short-term interventions. Overall, all treatment groups (T0-T3) showed abnormality rates below commonly accepted thresholds for artificial insemination up to 6 h of storage. Poultry semen with more than 20% abnormal spermatozoa is generally considered unsuitable for artificial insemination (Yaman *et al.*, 2022).

### **Spermatozoa plasma membrane integrity of Gaok roosters after treatment**

The proportion of spermatozoa with intact plasma membranes (IPM) in Gaok roosters was only 60% at initial assessment, despite supplementation with green tea leaf extract. This finding suggests that, although green tea possesses antioxidant properties, the applied treatment did not prevent early post-ejaculatory membrane damage. The hypo-osmotic swelling test and aniline blue staining indicated that spermatozoa with a straight but thickened tail are classified as having intact plasma membranes, whereas spermatozoa with straight and long tails are classified as non-intact (Santiago-Moreno *et al.*, 2009).

Green tea is rich in catechins, particularly EGCG, which exhibit strong antioxidant activity. Previous studies by Hernawati *et al.* (2025) reported improvements in certain spermatozoa

quality parameters (e.g., motility and viability) following green tea extract supplementation; however, effects on plasma membrane integrity are not always consistent. In the present study, suboptimal dosage, exposure time, or treatment protocol may have limited the protective effect of the extract on membrane integrity.

The oxidative stress experienced by Gaok spermatozoa under the experimental conditions may have exceeded the protective capacity of the administered extract. Wang *et al.* (2025) explained that under severe oxidative stress, antioxidant defenses may be insufficient to prevent lipid damage to membranes; thus, the low IPM observed may reflect an inability of the extract to reverse preexisting oxidative damage. Insufficient dose or short exposure duration could also explain the lack of effect, as optimization of antioxidant concentration and exposure time is critical for protecting spermatozoa membrane (Berean *et al.*, 2024).

Intact plasma membrane declined during storage across all treatments. At 2 h, T2 differed significantly from T0, T1, and T3; at 4 h, T0 differed significantly from T1 and T3; and at 6 h, T0 and T2 differed significantly from T1 and T3. The decline in spermatozoa IPM during storage is associated with changes in diluent polarity; depolarization of atomic or molecular membrane constituents during storage can impair membrane physiological function (Xue *et al.*, 2025). The most pronounced decline in IPM occurred in T0, likely due to the absence of antioxidant supplementation and longer storage duration. Accumulation of toxic compounds from dead spermatozoa and oxidative degradation of diluent components during storage promotes lipid peroxidation of the spermatozoa plasma membrane. Lipid peroxidation produces malondialdehyde (MDA), a cytotoxic compound that damages membranes, thereby reducing membrane integrity and overall spermatozoa quality (Ratchamak *et al.*, 2025).

## CONCLUSIONS

Green tea leaf extract (*Camellia sinensis* L.) incorporated into a skim-milk-egg yolk-fructose diluent helped preserve the motility, viability and plasma membrane integrity of Gaok rooster spermatozoa during storage at 25 °C for up to 6 h, with the optimal effect observed in treatment

T2 (0.010 mg per 10 mL diluent). However, within the tested dosage range, the extract did not effectively preserve spermatozoa morphological abnormalities under the experimental conditions.

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## AUTHOR CONTRIBUTIONS

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RDJ, NH, SH: conceived the idea and designed the main framework of this manuscript, RDJ: data acquisition, analysis, interpretation, and manuscript drafting. TH, TDL and PS: critically reviewed and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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## ARTIFICIAL INTELLIGENCE (AI) DISCLOSURE

In the preparation of this work, the authors using Scopus AI for in depth analysis of discussion section. The narration generated from this application was then reviewed and manually edited, and relevant, valid and up-to-date references were added. Th authors are fully responsible for the content of the publication.

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