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EFFECTS OF COENZYME Q10 AND VITAMIN E IN LACTATED RINGER'S EGG YOLK EXTENDER ON PRE-FREEZING SEMEN QUALITY OF GAGA CHICKEN

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Abstract

This study aimed to evaluate the effect of Coenzyme Q10 (CoQ10) and vitamin E supplementation in lactated Ringer's egg yolk (LREY) extender on the pre-freezing semen quality of Gaga chicken. Semen was collected from six mature Gaga roosters using the dorso-abdominal massage method, pooled, and diluted into four treatment extenders arranged in a completely randomized design with ten replications: T0 (LREY extender as control), T1 (LREY + CoQ10 200 µM), T2 (LREY + vitamin E 5 µg/mL), and T3 (LREY + vitamin E 5 µg/mL + CoQ10 200 µM). The variables evaluated were sperm motility, viability, abnormality, plasma membrane integrity, and DNA damage before freezing. Fresh Gaga chicken semen showed favorable baseline quality, with motility of 88.67 ± 0.22%, viability of 94.54 ± 0.27%, and plasma membrane integrity of 94.53 ± 0.35%. Before freezing, sperm viability differed significantly among treatments ($P < 0.05$), with T1, T2, and T3 showing higher values than T0. Meanwhile, sperm motility, abnormality, plasma membrane integrity, and DNA damage were not significantly different among treatments ($P > 0.05$). The combined treatment showed a favorable numerical pattern, particularly for motility, abnormality, and DNA damage. These results indicate that CoQ10 and vitamin E supplementation can help preserve sperm viability during dilution and the pre-freezing stage.

Keywords: *Antioxidant, Coenzyme Q10, Gaga Chicken, Pre-Freezing, Vitamin E*

INTRODUCTION

Cryopreservation is an important strategy for preserving indigenous poultry genetic resources, including local chicken breeds with specific genetic and cultural value. In poultry semen preservation, the success of cryopreservation is commonly evaluated based on post-thawing sperm quality, because this parameter is closely related to fertilization potential in artificial insemination programs (Khaeruddin et al., 2020). However, the pre-freezing stage also plays an important role in determining the final quality of frozen semen. During dilution and the pre-freezing stage, spermatozoa are exposed to extender components, osmotic adjustment, cooling temperature, and antioxidant supplementation before the actual freezing process occurs (Partyka & Niżański, 2021). Therefore, evaluating sperm quality before freezing is necessary to understand whether an extender formulation can protect spermatozoa at the early stage of cryopreservation.

This issue is particularly relevant for Gaga chicken, an Indonesian native chicken breed from South Sulawesi. Gaga chicken is known for its distinctive crowing sound and is considered one of Indonesia's valuable local poultry genetic resources. The development of semen preservation technology for this breed is important to support artificial insemination and genetic conservation programs. Previous studies on Gaga chicken semen have mainly focused on post-thawing sperm quality, while information regarding sperm condition during the dilution and pre-freezing stage remains limited (Wahjuningsih et al., 2024). In fact, damage that occurs before freezing may reduce the ability of spermatozoa to survive the subsequent freezing and thawing process. Therefore, this study aimed to evaluate the effect of CoQ10 and vitamin E supplementation in LREY extender on the pre-freezing semen quality of Gaga chicken. The evaluated parameters included sperm motility, viability, abnormality, plasma membrane

integrity, and DNA damage. This study is expected to provide basic information on the protective effect of antioxidant supplementation during the dilution and pre-freezing stage prior to semen cryopreservation.

LITERATURE REVIEW

Semen extenders perform several functions during the pre-freezing stage, including nutrient supply, pH buffering, and osmotic stabilization. In the LREY extender, the lactated Ringer's fraction maintains electrolyte balance. The egg yolk fraction contains low-density lipoproteins and phospholipids that coat the sperm plasma membrane and limit the loss of its native lipids during cooling (Bergeron & Manjunath, 2006). This protection is structural and does not neutralize reactive oxygen species (ROS). ROS are produced as a normal by-product of sperm metabolism, and their accumulation increases once spermatozoa are removed from seminal plasma, which normally provides its own enzymatic antioxidant defenses (Masoudi et al., 2018). Avian spermatozoa are particularly vulnerable to this oxidative burden because their plasma membranes contain high levels of polyunsaturated fatty acids (PUFA). This composition gives the membrane the fluidity needed for fertilization, but it also makes the membrane a preferred target for lipid peroxidation (Partyka & Niżański, 2021).

Two classes of exogenous antioxidants have been proposed to offset this deficit. Vitamin E is a lipophilic, chain-breaking antioxidant that embeds within the phospholipid bilayer and intercepts peroxyl radicals before they can propagate further membrane damage Supplementation with vitamin E at 5 µg/mL has been reported to preserve motility, viability, and membrane integrity during liquid storage of KUB chicken semen (Farid et al., 2021) and after freeze-thawing of rooster semen (Moghbeli et al., 2016). Coenzyme Q10 acts through a different mechanism. It is a component of the mitochondrial electron transport chain, and its supplementation at 200 µM has been associated with improved post-thaw DNA integrity in Gaga chicken spermatozoa diluted in an LREY extender (Wahjuningsih et al., 2024). This effect is consistent with its role in supporting ATP-dependent flagellar motility and scavenging ROS generated during oxidative phosphorylation. However, the effects of these two antioxidants are not always consistent in the literature. A meta-analysis of rooster studies published between 2010 and 2022 found that the effects of vitamin E on sperm kinematic parameters were discordant across studies, and on several variables vitamin E performed worse than catalase (Díaz Ruiz et al., 2024). The optimum concentration of vitamin E is also not fixed. Moghbeli et al. (2016) reported that the optimum level shifts with sperm concentration in the extender, so a concentration that is protective in one protocol may not work in another.

CoQ10 shows a similar pattern of inconsistency. Supplementation with CoQ10 has improved post-thaw semen quality in several rooster studies (Masoudi et al., 2018). However, a similar concentration range, 100 to 200 µM, was reported to be harmful to post-thaw motility when tested in rabbit sperm (Gardela et al., 2023). This suggests that the effect of CoQ10 is not the same across species and may depend on species-specific membrane composition or metabolic rate. A related inconsistency has also been reported in Gaga chicken. In the closest antecedent study on this breed, Wahjuningsih et al. (2024) added CoQ10 at 100, 200, and 300 µM to an LREY extender and found a significant effect only on post-thaw DNA damage. Motility, viability, abnormality, and plasma membrane integrity did not differ significantly among the CoQ10 concentrations tested. This finding does not fully match the expectation that CoQ10, by supporting mitochondrial ATP production, should also improve motility. It instead suggests that the protective effect of CoQ10 in Gaga chicken spermatozoa may be more specific to DNA protection than to overall energy metabolism. These findings indicate that neither the 5 µg/mL vitamin E nor the 200 µM CoQ10 concentration used in this study can be assumed to behave the same way in Gaga chicken semen based on prior poultry research alone. Vitamin E acts mainly at the membrane level, while CoQ10 acts mainly at the mitochondrial level. CoQ10 can also regenerate the oxidized form of vitamin E, known as the tocopheroxyl radical. For these reasons, the combination of the two antioxidants is expected to provide broader and more consistent protection than either compound alone. However, this combined effect has not yet been tested in Gaga chicken semen at the pre-freezing stage.

MATERIALS AND METHODS

Animal Management and Semen Collection

Semen was collected from six mature Gaga roosters aged approximately 11 months with an average body weight of 2.3 kg. Each rooster was housed individually in a 100 × 80 × 80 cm cage and fed a complete ration (17% crude protein, 3% crude fat, 7% crude fibre, 14% ash) at 150 g/bird/day, with water provided ad libitum. Semen was collected twice weekly using the dorso-abdominal massage method and pooled prior to dilution. Milky white semen came out, immediately collected using a 1 ml syringe.

Preparation of Diluent

The semen samples were diluted using a Ringer Lactate Egg Yolk extender prepared following Hidayat et al. (2016). The base extender consisted of 90% lactated Ringer's solution (Wida RL Infus®, PT Widatra Bakti, Pandaan, Indonesia) and 10% fresh egg yolk. This mixture was centrifuged at 3,000 rpm for 30 minutes, and 10 mL of the resulting supernatant was supplemented with 1,000 IU penicillin (Crystalline Procaine Penicillin-G®, Bangil, Indonesia) and 1 mg/mL streptomycin (Streptomycin Sulfate Meiji®, Bangil, Indonesia). Tris hydroxymethyl aminomethane (Merck KGaA, Darmstadt, Germany) was then added gradually until the extender reached pH 8, after which it was divided into four aliquots corresponding to each treatment.

Experimental Design and Semen Dilution

The experiment used a completely randomized design (CRD) with four treatments and ten replications: T0 (LREY extender, control), T1 (LREY + CoQ10 200 µM/mL), T2 (LREY + vitamin E 5 µg/mL), and T3 (LREY + vitamin E 5 µg/mL + CoQ10 200 µM/mL). Fresh semen was diluted in a ratio of 1:10 using the extender according to treatment, together with 7% dimethyl sulfoxide (DMSO) as an intracellular cryoprotectant. Diluted semen was packaged into straws and equilibrated at 5°C for 2 hours before proceeding to the freezing stage. Only pre-freezing (equilibrated) samples are reported in this study.

Evaluation of Semen Parameters

Semen samples were collected and analyzed for standard quality parameters. Sperm concentration was determined using a haemocytometer at 100× magnification (Robbaani et al., 2026).

1. Motility was assessed microscopically as the proportion of progressively motile spermatozoa (Khaeruddin et al., 2025).
2. Viability was evaluated using eosin-nigrosin staining at 400× magnification with 200 spermatozoa counted per sample (Figure 1).
3. Morphological abnormalities were identified from the same preparation used for viability, based on head, midpiece, and tail defects (Figure 2).
4. Plasma membrane integrity was examined using the hypo-osmotic swelling test (HOST), in which semen samples were incubated in hypo-osmotic solution at 37°C for 30 minutes prior to observation at 400× magnification (Figure 3) (Fisabilillah et al., 2025).
5. DNA integrity was assessed using toluidine blue staining at 1000× magnification in 200 spermatozoa (Figure 4) (Wahjuningsih et al., 2026).

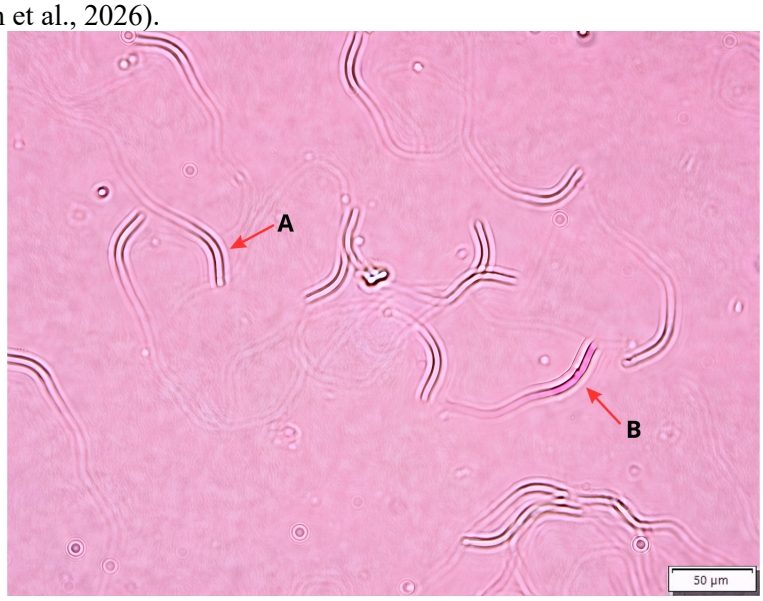

Figure 1. Sperm viability assessed by eosin-nigrosin staining. (a) live sperm (unstained head); (b) dead sperm (stained head).

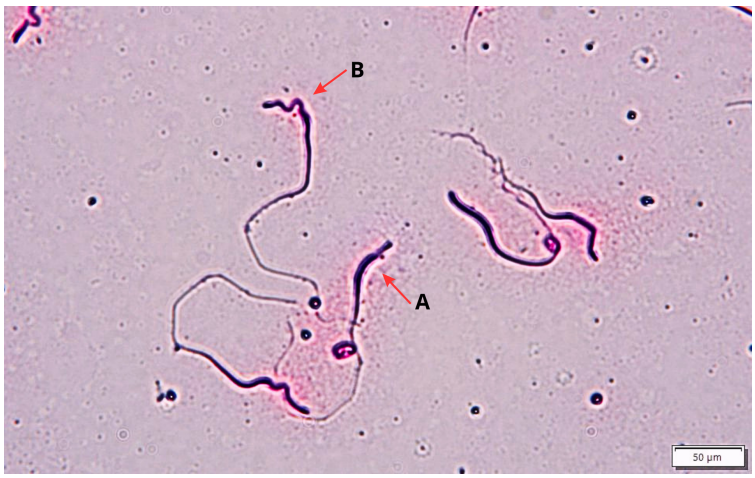

Figure 2. Sperm abnormality assessed by eosin-nigrosin staining.
(a) normal sperm; (b) abnormal sperm.

Figure 3. Sperm plasma membrane integrity assessed by the hypo-osmotic swelling test (HOST).
(a) non-intact membrane (straight tail); (b) intact membrane (coiled tail).

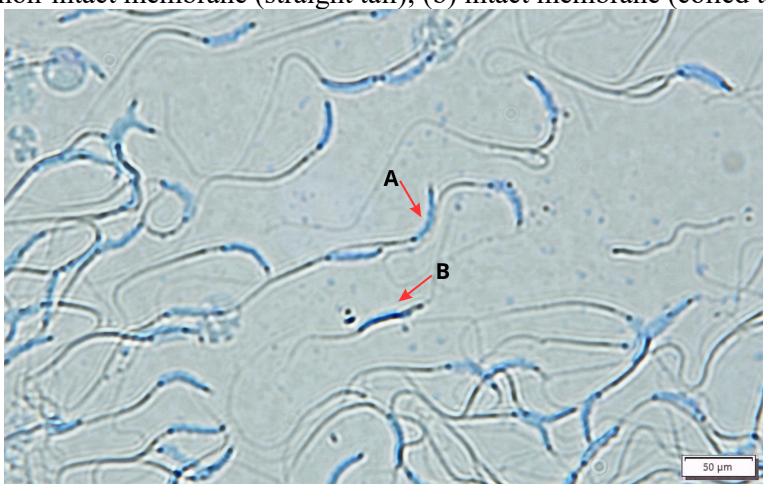

Figure 4. Sperm DNA integrity assessed by toluidine blue staining.
(a) intact DNA (light-stained head); (b) damaged DNA (dark-stained head).

Data Analysis

Data were analyzed by one-way analysis of variance (ANOVA) according to the CRD model, using IBM SPSS Statistics version 31 (IBM Corp., USA). Where treatment effects were significant ($P<0.05$), means were compared using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Fresh Semen Characteristics

The characteristics of pooled fresh Gaga chicken semen prior to dilution are summarized in Table 1.

Table 1. Characteristics of fresh Gaga chicken semen

Parameter	Mean ± SE
Volume (mL)	0.31 ± 0.01
Colour	Milky white
Consistency	Thick
pH	7.92 ± 0.02
Sperm concentration (×10⁹ cells/mL)	6.13 ± 0.37
Mass movement	+++
Motility (%)	88.67 ± 0.22
Viability (%)	94.54 ± 0.27
Abnormality (%)	4.05 ± 0.20
Plasma membrane integrity (%)	94.53 ± 0.35
DNA damage (%)	2.72 ± 0.70

Fresh semen showed a milky-white colour and thick consistency, indicating no visible contamination by faeces, urine, or blood. The overall baseline quality was favourable and was comparable to, or higher than, values reported for other Indonesian local chicken breeds (Udrayana et al., 2023; Khaeruddin et al., 2020). This confirms that the roosters used in this study were reproductively healthy and suitable for the dilution trial.

Sperm Quality Before Freezing

Motility, viability, abnormality, plasma membrane integrity, and DNA damage of diluted Gaga chicken spermatozoa before freezing are presented in Table 2.

Table 2. Quality of Gaga chicken spermatozoa before freezing (mean ± SE, %)

Parameter	T0	T1	T2	T3
Motility (%)	79.50 ± 1.17	80.00 ± 1.05	80.50 ± 0.90	83.00 ± 0.82
Viability (%)	90.71 ± 0.60^{b}	92.83 ± 0.59^{a}	92.48 ± 0.49^{a}	92.64 ± 0.49^{a}
Abnormality (%)	6.27 ± 0.19	6.86 ± 0.43	7.17 ± 0.31	6.10 ± 0.32
Plasma membrane integrity (%)	91.72 ± 0.95	92.85 ± 0.43	92.07 ± 1.32	92.26 ± 0.73
DNA damage (%)	3.84 ± 0.45	4.23 ± 0.59	3.95 ± 0.73	3.49 ± 0.56

Note: Different superscripts within a row indicate significant differences (Duncan's test, $P<0.05$) T0 = LREY extender (control), T1 = LREY + CoQ10 200 µM/mL, T2 = LREY + vitamin E 5 µg/mL, T3 = LREY + vitamin E 5 µg/mL + CoQ10 200 µM/mL

Before freezing, motility ranged from 79.50±1.17% in T0 to 83.00±0.82% in T3. No significant differences were found among treatments ($P>0.05$), although T3 showed the numerically highest value. Viability differed significantly among treatments ($P<0.05$). T1, T2, and T3 were all higher than T0, and T1 (CoQ10 alone) showed the highest numerical value. This result is consistent with the antioxidant mechanisms of vitamin E and CoQ10. Vitamin E interrupts lipid peroxidation chain reactions in the plasma membrane (Amini et al., 2015), while CoQ10 scavenges ROS and supports mitochondrial ATP production (Masoudi et al., 2018). Both mechanisms may already protect cell survival during the initial handling stage, before the additional stress of freezing and thawing occurs. The vitamin E concentration used in this study, 5 µg/mL, falls within the range previously reported to support rooster and KUB chicken sperm quality after freeze-thawing (Moghbeli et al., 2016; Farid et al., 2021). Since those studies evaluated post-thaw semen, a direct numerical comparison with the pre-freezing results of the present study is not appropriate. The present findings instead indicate that this concentration already confers a measurable protective effect at the dilution and pre-freezing stage, before the additional stress of freezing is introduced. Whether this early advantage translates into comparable post-thaw benefits in Gaga chicken semen remains to be established in future work.

Abnormality (6.10-7.17%) and DNA damage (3.49-4.23%) remained low in all treatments, with no significant differences ($P>0.05$). Plasma membrane integrity also showed no significant differences among treatments (91.72-92.85%), although all antioxidant-supplemented treatments were numerically higher than the control. This absence of significant treatment effects before freezing suggests that, under the dilution and short pre-freezing period used in this study, oxidative stress had not yet reached a level that produced clear differences between the antioxidant-supplemented and unsupplemented extenders. This is in line with previous reports that some antioxidant effects on

avian spermatozoa only become apparent after the additional stress of the freeze-thaw cycle (Wahjuningsih et al., 2024). Even so, the values in T3 were consistently higher across all five parameters, although not always statistically significant. This suggests that the combination of vitamin E and CoQ10 already provides a numerical advantage before freezing. This early advantage may lead to a stronger protective effect once the sample undergoes the more severe stress of cryopreservation, including cold shock, osmotic stress, and the formation of intracellular ice crystals (Partyka & Niżański, 2021).

Only viability differed significantly among treatments, while motility, abnormality, membrane integrity, and DNA damage did not. This can be explained by the fact that each test measures a different level of sperm damage. Eosin-nigrosin staining identifies a cell as dead once its plasma membrane allows the dye to enter, so it detects an early and relatively mild form of membrane damage. The HOST assay, on the other hand, measures whether the membrane can still control water movement under osmotic stress, which requires a more advanced degree of membrane damage before a difference can be seen. Sicherle et al. (2020) reported that membrane permeability changes were among the first signs of cell damage to appear during cryopreservation, occurring earlier than mitochondrial or DNA damage. The two-hour pre-freezing period used in the present study was therefore probably sufficient to reveal weaker spermatozoa through the viability test, but not long enough to change the average membrane resistance, motility, or DNA integrity of the whole sperm population.

The slightly higher numerical viability in T1 (CoQ10 alone) compared with T2 and T3 can also be explained by the different mechanism of action of the two antioxidants. Vitamin E is located within the lipid bilayer and only neutralizes peroxyl radicals after a peroxidation chain has already started, so it interrupts only the propagation stage of lipid peroxidation. CoQ10 is distributed in the mitochondria, plasma membrane, and other cell compartments at concentrations several times higher than vitamin E. This allows CoQ10 to intercept free radicals at both the initiation and propagation stages of lipid peroxidation, in addition to donating electrons back to oxidized vitamin E. In rooster semen extenders, this broader antioxidant action has been associated with higher viability and membrane functionality than membrane protection alone would explain (Sharideh et al., 2019). Under the relatively mild oxidative conditions at the pre-freezing stage in this study, this wider mode of action may explain why T1 gave a slightly higher mean viability than T3, even though the difference among T1, T2, and T3 was not statistically significant.

Abnormality was the only parameter that did not improve numerically with antioxidant supplementation, with T2 (7.17%) even slightly higher than the control. This finding is consistent with the biological origin of sperm morphological defects, which are formed during spermatogenesis and epididymal maturation rather than during handling after ejaculation. Henning et al. (2021) reported that the proportion of boar spermatozoa with retained cytoplasmic droplets, the most common morphological abnormality in that species, did not decrease during liquid storage regardless of the extender composition used. In the same way, an antioxidant added to the dilution medium after semen collection cannot be expected to correct a structural defect that was already present in the cell before ejaculation. The small differences in abnormality among treatments observed in this study are therefore more likely due to normal biological variation than to a genuine treatment effect.

Even so, T3 gave the most favourable, or close to the most favourable, mean value in four of the five parameters measured. CoQ10 is able to regenerate the oxidized form of vitamin E, known as the tocopheroxyl radical, back to its active form. Because of this, vitamin E and CoQ10 do not simply add their effects together, since each antioxidant also extends how long the other remains active (Sharideh et al., 2019). At the pre-freezing stage, this regenerating effect may not yet be strong enough to produce a statistically significant advantage over CoQ10 alone, since the level of oxidative stress at this stage is still relatively low. The benefit of combining the two antioxidants would be expected to become clearer once the semen is exposed to the greater and more prolonged oxidative and osmotic stress of the freezing and thawing process, consistent with the treatment-specific and concentration-dependent antioxidant responses previously reported for this breed (Wahjuningsih et al., 2024).

CONCLUSION

This study shows that antioxidant protection can already be detected before Gaga chicken semen is frozen. Viability was significantly higher in all CoQ10- and vitamin E-supplemented groups than in the unsupplemented control. Motility, abnormality, plasma membrane integrity, and DNA damage were statistically comparable among treatments at this stage. T3 gave the most favorable result for motility, abnormality, and DNA damage, while T1 gave the highest viability and plasma membrane integrity. This suggests that vitamin E and CoQ10 protect sperm quality through different mechanisms rather than one treatment being superior for every parameter. These pre-freezing

findings complement the post-thawing evaluations reported elsewhere and support the use of combined vitamin E and CoQ10 supplementation in extender formulations for the cryopreservation of Gaga chicken semen.

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