Research Article

The Quality of KUB Rooster Sperm During Cryopreservation in Extender with Genistein and Glutathione Supplementation

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Abstract | The increase in lipid peroxidation during rooster semen cryopreservation reduces sperm functioning and creates reproductive issues. Antioxidants are crucial in semen cryopreservation to inhibit lipid peroxidase, thereby preserving sperm quality. This study aimed to investigate the effects of genistein and glutathione supplementation on the quality of cryopreserved sperm. Semen was collected from six KUB roosters and then diluted with the LREY extender using five different treatments: LREY without antioxidant as control (LREY0), LREY supplemented with genistein at 5 µM (LREY5), genistein at 10 µM (LREY10), glutathione at 0.2 mM (LREY2), and glutathione at 0.4 mM (LREY4). The semen was evaluated after dilution, equilibration, and thawing. The evaluation criteria included sperm motility, recovery rate, viability, membrane integrity, and DNA fragmentation. In the post-dilution and post-equilibration phases, there was no significant effect on motility, viability, and membrane integrity ($P > 0.05$). However, supplementing genistein and glutathione improved the post-thawing results significantly ($P < 0.05$). The addition of 10 µM genistein and 0.02 mM glutathione to KUB rooster semen post-thawing improved motility (43.00±1.41% and 41.75±1.70%), viability (54.75±1.70% and 53.75±1.50%), membrane integrity (57.50±2.64% and 57.25±2.21%), recovery rate (51.19±1.68% and 49.69±2.03%), and DNA fragmentation (3.25±0.50% and 3.25±0.95%). This study concluded that genistein at 10 µM and glutathione at 0.2 mM may preserve the quality of KUB rooster semen.

Keywords | Cryopreservation, Genistein, Glutathione, KUB rooster, Semen quality

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INTRODUCTION

Ayam Kampung Unggul Badan Penelitian dan Pengembangan Pertanian (KUB Chicken) is one of the Indonesian selected-native chickens with high egg productivity and high-quality meat (Sartika, 2016; Udjianto, 2016). The high productivity of KUB chickens facilitates the effective fulfillment of public demands. Cryopreserved semen and artificial insemination represent reproductive biotechnological methods with the potential to contribute to the expansion of the KUB chicken population. However, the cold shock and oxidative stress elicited by reactive oxygen

species (ROS) may inhibit semen cryopreservation (Hendiyani et al., 2018; Len et al., 2019).

The excessive formation of ROS is associated with elevated lipid peroxidation, resulting in compromised sperm functioning and fertility issues (Silvestre et al., 2015; Hendiyani et al., 2018; Masoudi et al., 2019a). Rooster sperm, characterized by high concentration of polyunsaturated fatty acids (PUFA), is susceptible to lipid peroxidation. Prolonged lipid peroxidation can lead to structural damage of lipids, causing the cell membranes unstable, disrupting the membrane functions, and diminishing the membrane fluidity (Prihantoko et al., 2022a). According to Shah et al. (2016), cryopreservation-induced damage to sperm cells predominantly impacts cellular membranes, including the plasma and mitochondrial membrane, resulting in a particularly detrimental impact on DNA. The effects extend to membrane-related attributes, such as motility, viability, metabolic activity, functionality, and sperm morphology, which is ultimately influencing the fertilization process.

Antioxidants are crucial to maintain the sperm quality during cryopreservation and in suppressing lipid peroxidation, as the endogenous antioxidant levels are insufficient against excessive ROS (Masoudi et al., 2019b). The heightened concentration of polyunsaturated fatty acids within the plasma membrane, following with a moderate level of antioxidants in the sperm cytoplasm, renders the sperm cells vulnerable to oxidative stress and peroxidative harm (Prihantoko et al., 2022b). Certain antioxidants, such as genistein and glutathione, can preserve sperm and semen quality. Glutathione (GSH), functions as an antioxidant, contributing to the establishment of a defense mechanism against oxidative stress (Ugur et al., 2019). Research across several species, including lamb, porcine, mice, equine, human, and bovine has demonstrated the beneficial impact of GSH supplementation on in-vitro semen quality (Masoudi et al., 2019b). The addition of GSH to semen dilution promotes various aspects of sperm function, including motility, membrane functionality, mitochondrial activity, acrosomal integrity, viability, and fertility. Furthermore, it effectively mitigates lipid peroxidation and DNA fragmentation following freezing at -196°C (Masoudi et al., 2019a).

Genistein, as an antioxidant against oxidative stress, enhances the sperm motility and viability (Garcia et al., 2015) and protects sperm DNA integrity (Silvestre et al., 2015; Elsayed et al., 2019). Supplementation of genistein to ram semen has demonstrated improvements in semen characteristics (motility, viability, acrosomal integrity, and membrane integrity), accompanied by a reduction in DNA fragmentation (Elsayed et al., 2019). Previous studies have investigated that the presence of genistein in the equine semen dilution does not affect the viability, motility, or acrosomal status of sperm (Garcia et al., 2015). Our study aimed to investigate the effect of genistein and glutathione supplementation on the quality of KUB rooster sperm during cryopreservation.

MATERIALS AND METHODS

Animal trial and ethics

The Research Ethics Committee Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, approved the procedures in the current study under code number 0027/EC-FKH/Int/2020. Six healthy KUB roosters, aged 48 weeks, were selected based on their semen quality. They were then housed in a 60 x 60 x 80 cm cage and provided with a standard daily diet of 150 g, along with *ad libitum* water.

Extender preparation

The lactate ringer and 20% egg yolk (LREY) were homogenized using a stirrer and centrifuged at 2000 rpm for 15 minutes. The supernatant was suspended as a main extender, then added with 1000 IU/ml penicillin and 1 mg/ml streptomycin as antibiotics. Antibiotic buffers placed into five treatments: genistein 5 µM, genistein 10 µM, glutathione 0.2 mM, glutathione 0.4 mM, and a control group (without antioxidants). The extenders were then distributed into two compositions (compositions A and B) (Table 1). The 10% Dimethylsulfoxide (DMSO) concentrations were added to extender composition B as a cryoprotectant (Junaedi et al., 2016; Telnoni et al., 2017).

Semen collection and evaluation

Semen was collected twice a week, every morning, utilizing the abdominal massage method (Ardhani et al., 2018), with four repetitions. Semen evaluation was conducted following the method described by Telnoni et al. (2017) with slight modifications. Macroscopic evaluations examined the volume by using a pipette, pH with acidity indicator paper, and colors visually. Microscopic evaluations examined the mass and individual motility, viability, abnormality, and sperm concentration.

Semen cryopreservation

In this study, the semen exhibited sperm motility >70%, abnormality <20%, and sperm concentration >3x10^9 sperm cells/ml. Semen collected from six roosters was pooled and then allocated to five different treatments, diluted with extender composition A; $LREY_0$, $LREY_5$, $LREY_{10}$, $LREY_2$, and $LREY_4$ and equilibrated at 5 °C for three hours. Following equilibration, extender composition B was added, and the mixture was loaded into straws. Subsequently, the semen was frozen for 10 minutes above liquid nitrogen fumes and stored in liquid nitrogen at -196 °C (Telnoni et al., 2017; Masoudi et al., 2019a).

Table 1: Composition of extenders A and B

Composition	Treatments				
	$LREY_0$	$LREY_5$	$LREY_{10}$	$LREY_2$	$LREY_4$
Composition of Extender A					
Antibiotics buffer (ml)	5	4.75	4.5	4.975	4.950
GN (stock solution 100µM) (ml)	-	0.25	0.5	-	-
GSH (stock solution 40mM) (ml)	-	-	-	0.025	0.05
Composition of Extender B					
Antibiotics buffer (ml)	4	3.75	3.5	3.975	3.950
GN (stock solution 100µM) (ml)	-	0.25	0.5	-	-
GSH (stock solution 40mM) (ml)	-	-	-	0.025	0.05
DMSO 10% (ml)	1	1	1	1	1
Total extenders (ml)	10	10	10	10	10

GN: Genistein; GSH: Glutathione; $LREY_0$: LREY without antioxidant; $LREY_5$: LREY supplemented genistein 5µM; $LREY_{10}$: LREY supplemented genistein 10µM; $LREY_2$: LREY supplemented glutathione 0.2 mM; $LREY_4$: LREY supplemented glutathione 0.4 mM.

Spermatozoa quality evaluation

The evaluation was performed post-dilution, post-equilibration (three hours after equilibration), and post-thawing (two hours after freezing). Straws were thawed at 37 °C for 30 seconds. The examination measures motility (%), viability (%), recovery rate (%), membrane integrity (%), and DNA sperm fragmentation (%).

Sperm motilities: Sperm motility evaluation was subjectively assessed under a microscope. A drop of semen and four drops of saline solution were placed on a microscope slide and thoroughly mixed. The mixture was then covered with a glass cover. Sperm motility was evaluated at 40x magnification across five fields of view on the glass object.

Sperm viabilities: Viability was assessed by eosin-nigrosin staining method. A mixture of 10 µl semen and 50 µl eosin-nigrosin staining solution (1:5) was placed on an object glass, then slide and heated with a bunsen burner. Morphometric measurements were performed on 200 sperm cells in each eosin-nigrosin-stained sample under microscope at 40x magnification. Living sperm appeared as white heads, while dead sperm exhibited a red color (Telnoni et al., 2017).

Recovery rate: The sperm recovery rate was calculated by dividing post-thawing sperm motility by fresh sperm motility and multiplying by 100%. (Telnoni et al., 2017).

Membrane integrity: The hypo-osmotic swelling test (HOST), as described by Gangwar et al. (2018), was used to determine membrane integrity with slight modifications. For the HOST method, 10 µl of semen and 100 µl of HOST solution (a mixture of 0.9 fructose, 0.49 g of citrate sodium, and distillate water to a final volume of 100 ml) were diluted and briefly incubated at 37 °C for 30 minutes. Solutions were then smeared onto a slide, dried, and fixated. A total of 200 spermatozoa were counted under a microscope at magnification 40x. Normal membrane plasm of spermatozoa appeared with swollen or curled tail, while those with damaged membrane displayed a straight tail (Bebas and Gorda, 2016).

DNA fragmentation: This study used TMR Red version 12th tunnel assay method with In Situ Cell Death Detection Kit (Sigma-Aldrich, USA) to detect DNA fragmentation. Post-thawing sperm was then smeared onto a slide, dried, and fixated at 15-25 °C for an hour, followed by rinsing with Phosphate Buffer Saline (PBS). For sample permeability, the sample was treated with 0.1% Triton X-100 in 0.1% sodium citrate for two minutes at 2-8 °C before being gently washed two times with PBS. Addition of 50 µl Label solution served as a negative control. For the positive control, 3000 U/ml - 3 U/ml of incubated DNAse recombinant in 50 mM Tris-HCL, pH 7.5, 1 mg/ml BS was used for 10 minutes at 15-25 °C to induce DNA separation. After drying, the sample and control were combined with 50 µl of TUNEL reaction mixture for 60 minutes at 37 °C, followed by three gentle rinses with PBS. A laser-scanning confocal microscope, with 517 nm wavelengths, was used to analyze the results. DNA fragmentation of sperm was indicated by a green fluorescent signal.

Data analysis

SPSS was used to analyze the data using the one-way ANOVA method. Statistical differences among distinct groups were determined using Duncan's multiple-range test. Results were presented as Mean±SD. The P values < 0.05 were considered statistically significant.

Table 2: Data (mean ± SD) of motility, viability, and membrane integrity (%) in LREY extender supplemented with glutathione and genistein.

Semen Evaluation	Treatments				
	$LREY_0$	$LREY_5$	$LREY_{10}$	$LREY_2$	$LREY_4$
Post dilution					
Motility	78.50±2.38	79.50±2.08	80.50±2.38	80.00±2.44	78.75±2.21
Viability	86.25±1.70	87.75±2.06	88.50±2.51	88.75±1.25	87.00±1.41
Membrane	88.25±0.95	89.50±1.29	90.25±1.25	90.00±1.41	88.75±1.50
Post equilibration					
Motility	71.25±1.50	74.50±2.38	75.25±2.63	74.25±2.21	71.75±1.70
Viability	82.50±2.51	84.75±2.75	85.25±3.20	84.75±3.30	83.25±2.63
Membrane	85.25±1.89	87.00±1.41	87.75±1.89	87.75±1.25	86.50±1.73
Post thawing					
Motility	$39.25±1.25^{c}$	$41.50±1.29^{abc}$	$43.00±1.41^{a}$	$41.75±1.70^{ab}$	$40.25±1.50^{bc}$
Viability	$48.50±1.91^{c}$	$52.50±1.91^{ab}$	$54.75±1.70^{a}$	$53.75±1.50^{a}$	$50.00±2.44^{bc}$
Membrane	$51.50±2.64^{c}$	$55.50±1.91^{ab}$	$57.50±2.64^{a}$	$57.25±2.21^{ab}$	$53.50±2.38^{bc}$

[a,b,c] Different superscripts within the same row indicate significant differences between treatments ($P < 0.05$).
$LREY_0$: LREY without antioxidant; $LREY_5$: LREY supplemented with 5µM genistein; $LREY_{10}$: LREY supplemented with 10µM genistein; $LREY_2$: LREY supplemented with 0.2 mM glutathione; $LREY_4$: LREY supplemented with 0.4 mM glutathione.

Table 3: Data (mean±SD) of recovery rate (%) in LREY extender supplemented with glutathione and genistein.

Treatments	Sperm motility (%)		Recovery rate
	Fresh Semen	Post thawing	
$LREY_0$	84.00±0.81	39.25±1.25	$46.17±1.49^{c}$
$LREY_5$	84.00±0.81	41.50±1.29	$49.40±1.54^{abc}$
$LREY_{10}$	84.00±0.81	43.00±1.41	$51.19±1.68^{a}$
$LREY_2$	84.00±0.81	41.75±1.70	$49.69±2.03^{ab}$
$LREY_4$	84.00±0.81	40.25±1.50	$47.91±2.19^{bc}$

[a,b,c] Different superscripts within the same column indicate significant differences between treatments ($P < 0.05$).
$LREY_0$: LREY without antioxidant; $LREY_5$: LREY supplemented with 5µM genistein; $LREY_{10}$: LREY supplemented with 10µM genistein; $LREY_2$: LREY supplemented with 0.2 mM glutathione; $LREY_4$: LREY supplemented with 0.4 mM glutathione.

RESULTS

The evaluation of fresh semen from KUB rooster indicated normal semen quality based on macroscopic and microscopic results, which revealed volume (0.23±0.02ml), pH (7.25±0.50), color (white), mass movement (+++), motility (84.00±0.81%), viability (93.25±0.95%), abnormality (6.50±1.91%), and sperm concentration (4.11±0.74 x10^9/ml). The percentages of motility, viability, membrane integrity (Table 2), and recovery rate (Table 3) during the post-dilution and post-equilibration phases indicated no significant differences, but the addition of genistein and glutathione during the post-thawing phase showed significant differences ($P < 0.05$). The current study revealed that the quality of post-thawed semen was highest with genistein at 10 µM, followed by glutathione at 0.2 mM, genistein at 5 µM, glutathione at 0.4 mM, and control without antioxidants. Genistein 10 µM and glutathione 0.2 mM could maintain the post-thawing sperm quality with motility rates of (43.00±1.41% and 41.75±1.70%), viability (54.75±1.70% and 53.75±1.50%), membrane integ-

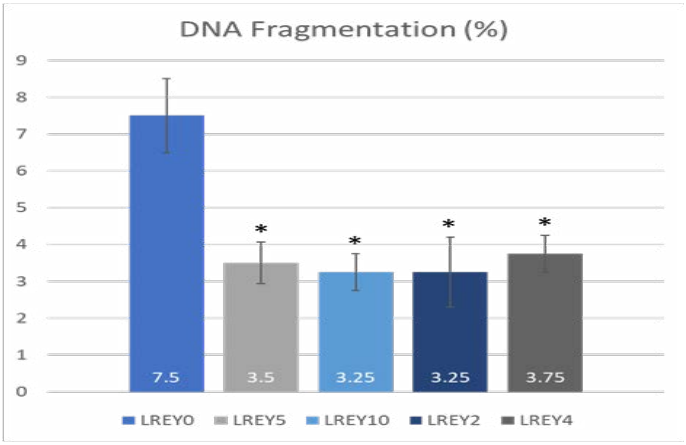

Figure 1: DNA fragmentation (mean ± SD) in KUB rooster frozen sperm.
Asterisks indicate significant differences between treatments (*$P < 0.01$ vs LREY0/control).

rity (57.50±2.64% and 57.25±2.21%), and recovery rate (51.19±1.68% and 49.69±2.03%). Post-thawing DNA fragmentation in $LREY_5$ (3.50±0.57%), $LREY_{10}$ (3.25±0.50%), $LREY_2$ (3.25±0.95%), and $LREY_4$ (3.75±0.50%) were significantly lower compared to $LREY_0$ (7.50±1.00%) ($P < 0.01$) (Figure 1).

DISCUSSION

In cryopreservation, both cold shock and oxidative failure may reduce sperm quality (Hendiyani et al., 2018; Len et al., 2019). Sperm cold shock is associated with oxidative stress induced by ROS (Gadea et al., 2005). The composition of the sperm plasma membrane in roosters contains a large amount of polyunsaturated fatty acids (PUFA), which makes the membrane vulnerable to lipid peroxidation in the presence of ROS. A defective membrane led to sperm cells death, highlighting the crucial role of membrane stability in facilitating successful oocyte and sperm conjugation. Elevated level of ROS may lead to DNA damage within sperm (Ciftci and Aygun, 2018). Thus, the recent study showed that cryopreservation of KUB rooster semen was affected by membrane damage, resulting in decreased motility, viability, and membrane integrity, along with increased DNA fragmentation in sperm.

The conditions to maintain sperm viability and fertility require the balanced production of ROS and antioxidants (Neuman et al., 2002; Gibb et al., 2020). The endogenous antioxidant capacities may not be sufficient to prevent lipid peroxidation during cryopreservation, leading to decreased motility and viability. In this study, genistein and glutathione were used as antioxidants in the extenders. Genistein effectively eliminates hydrogen peroxide and inhibits tyrosine-kinase (Garcia et al., 2015; Jalili et al., 2015). The antioxidants agent in genistein may significantly reduce the ROS level (Jalili et al., 2015), protect DNA integrity (Soto et al., 2010; Elsayed et al., 2019), and increase the motility and viability of sperm. Glutathione, derived from the thiol group (-SH), can directly react with ROS and act as a cofactor for glutathione peroxidase (GPx). It is recognized as a catalyst for reducing the toxicity of H_2O_2 and hydroperoxide (Masoudi et al., 2019b).

The result showed that the post-thawing motility rate of $LREY_{10}$ was the highest (43.00±1.41%), as correlated with the study of lamb cryopreservation by Elsayed et al. (2019). Tyrosine phosphorylase is known to contribute to spermatozoa motility. Moreover, the low concentration of genistein (10 μM) may inhibit tyrosine kinase production (Jiao et al., 2022). This study showed that 10μM of genistein was better at maintaining sperm quality than 5μM of genistein (Elsayed et al., 2019; Soto et al., 2010). The addition of glutathione showed increased sperm motility at concentrations of 0.2 mM which was greater than 0.4 mM. Shamiah et al. (2017) demonstrated that a 0.2 mM glutathione concentration might increase sperm motility (44.50%) at 5 °C. This is correlated to the thiol group, which affects the protection of sperm motility (Gadea et al., 2005).

The control group, without antioxidants, exhibited the lowest viability rate (48.50±1.91%), while the addition of genistein 10 μM in the extender exhibited the highest viability rate (54.75±1.70%) (Elsayed et al., 2019). Genistein significantly decreased the ROS due to its antioxidant properties (Jalili et al., 2015), leading to increased motility and viability rates (Garcia et al., 2015). Viability showed a positive correlation with a higher rate of membrane integrity (Patti et al., 2021). Studies by Masoudi et al. (2019b) and Elsayed et al. (2019) investigated the effects of glutathione and genistein on maintaining the integrity of frozen semen membranes, which align with the findings of the current study. It was indicated that a certain concentration of antioxidants might increase sperm fertility, as assessed by the hypo-osmotic swelling test (HOST) method used as sperm integrity functions indicator (Ramu and Jayendran, 2013). Sperm capacitation was correlated with sperm functional integrity or acrosomal reaction resulting from the sperm binding to the ovum (Ramu and Jayendran, 2013).

The recovery rate was influenced by the sperm's ability to recover after freezing (Mosca et al., 2020). The current study showed the average recovery rate was highest for genistein at 10 μM (51.19±1.68), followed by glutathione at 0.2 mM (49.69±2.03), genistein at 5μM (49.40±1.54), glutathione at 0.4 mM (47.91±2.19), and control (46.17±1.49). The KUB Rooster recovery rate was lower than that of ducks (73.12%) (Han et al., 2005). Junaedi et al. (2016) stated that the elliptical shapes of the sperm morphology could be easily damaged, potentially affecting the recovery rate, thus emphasizing the importance of proper cryopreservation and extenders techniques. The combinations of LREY and Genistein at 10 μM demonstrated protective effects on membrane integrity and sperm motility.

Glutathione and genistein proved its ability to reduce DNA fragmentation in cryopreserved semen of KUB roosters. The study by Elsayed et al. (2019) reported that genistein concentrations at 5 μM and 10 μM reduced DNA fragmentation to 0.42% and 4.12%, respectively, in frozen lamb semen. Glutathione in extenders was found to reduce DNA fragmentation by approximately 0.9%. The increase in oxidative stress in cryopreservation is associated with DNA fragmentation, as well as apoptosis and caspase activation (Gualtieri et al., 2021). Genistein and glutathione act as antioxidants against oxidative stress to protect DNA during cryopreservation (Soto et al., 2010;

Elsayed et al., 2019; Masoudi et al., 2019a).

In this study, genistein at 5 µM and 10 µM successfully maintained the sperm quality after freezing, consistent with the findings from previous studies (Soto et al., 2010; Elsayed et al., 2019). While glutathione at 0.2 mM and 0.4 mM was utilized similarly to a study by Shamiah et al. (2017). A slight disparity concentration was observed compared to previous studies utilizing glutathione (GSH) to preserve semen quality. In Buras breed chicken semen used GSH at concentration 0.05 mM (Iswati et al., 2017), 1 mM in duck semen (Sarangi et al., 2017), and 0.5 - 8 mM in Ross breed chicken semen (Masoudi et al., 2019a). The variation of GSH concentration in semen dilution was influenced by the different levels of enzymatic antioxidants among chicken breeds. A review by Partyka et al. (2021) and a study from Partyka et al. (2012) reported varying values for the main enzymatic antioxidant system (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT)) in poultry semen (chickens, turkeys, ducks, swans, guinea fowls). Additionally, a study from Mavi et al. (2020) found that there were different values of SOD, GPx, and CAT levels in different chicken breeds (RIR, Punjab Red, RIR cross, Aseel, and Kadaknath).

CONCLUSION

The addition of genistein and glutathione during the post-thawing phase exhibited significant effects on sperm quality. The supplementation of the extender with genistein at 10 µM and glutathione at 0.2 mM enhanced motility, viability, membrane integrity, recovery rate, and reduced DNA fragmentation in KUB rooster sperm after the freeze-thaw process. Supplementation with genistein and glutathione could be an effective strategy for transporting KUB rooster frozen semen to different locations while maintaining sperm quality standards. Further studies are needed to quantify the total capacity of antioxidants, and in-vivo sperm fertility levels which have not been covered in this study.

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CONFLICT OF INTERESTS

The authors declare that no conflict of interest is associated with publishing this work.

NOVELTY STATEMENT

Our study represents the first investigation on the quality of frozen semen from KUB roosters using genistein and glutathione. Our research findings highlight the efficacy of genistein at 10 µM and glutathione at 0.2 mM in preserving the quality of KUB rooster sperm.

AUTHORS CONTRIBUTION

All authors participate from the beginning of this research until the publications are released.

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