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# Cryopreservation of Gaga' chicken semen from South Sulawesi, Indonesia with the addition of L-carnitine, hyaluronic acid, sucrose and their combination in diluent

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**Abstract.** *Khaeruddin, Wahjuningsih S, Ciptadi G, Yusuf M, Hermawansyah, Sahiruddin. 2022. Cryopreservation of Gaga' chicken semen from South Sulawesi, Indonesia with the addition of L-carnitine, hyaluronic acid, sucrose and their combination in diluent. Biodiversitas 23: 3297-3302.* Gaga' chicken (*Gallus gallus domesticus*) is Indonesian germplasm that needs to be preserved with cryopreservation technology. Therefore, this study aims to determine the effect of adding L-Carnitine, hyaluronic acid, and sucrose as well as their combination in a diluent on the quality of Gaga' chicken spermatozoa after freeze-thawing. A completely randomized design was used along with 6 treatments, namely P0, P1, P2, P3, P4, and P5, containing Ringer acetate-egg yolk (RAEY) diluent (P0), RAEY + 0.063 mM hyaluronic acid (P1), RAEY + 1 mM L-carnitine (P2), RAEY + 1 mM sucrose (P3), RAEY + 0.063 mM hyaluronic acid + 1 mM L-carnitine (P4), and RAEY + 0.063 mM hyaluronic acid + 1 mM L-carnitine + 1mM sucrose (P5). Liquid semen was packed in a 0.25 mL straw, equilibrated at 5°C for 2 hours, and placed 3 cm above the surface of liquid nitrogen for 10 minutes. It was then immersed in nitrogen for 24 hours and thawed at 60°C for 5 seconds. The results showed that there was a significant difference ($P<0.05$) in the motility, viability, and kinematic variables of spermatozoa after freeze-thawing. P1, P2, P3, and P4 increased progressive motility, P1 and P4 increased curvilinear velocity, while viability increased with P4 treatment. Furthermore, the addition of Hyaluronic acid, L-carnitine, or their combination in the RAEY diluent can improve the sperm quality of Gaga' chickens.

**Keywords**: Chicken sperm kinematic, freeze-thawing, hyaluronic acid, L-carnitine, sucrose

## INTRODUCTION

Indonesia has a relatively high biodiversity of flora and fauna, but attention must be paid to some animal species, such as birds to prevent their extinction. Chicken (*Gallus gallus domesticus*) is a bird species that is widely spread in various islands and clumps. Indonesia is one of the centers of chicken domestication in the world, which has various types of local or native chickens that are raised for the purpose of producing meat, eggs, ornamental or fighting chickens (Sulandari et al. 2008; Ulfah et al. 2015). According to Ulfah et al. (2015) conservation of chicken genetic diversity in Indonesia should focus on unique local populations and exotic breeds. Gaga' chicken is a native Indonesian chicken, which originates from South Sulawesi, and is known to have a unique sound (Bugiwati and Ashari 2013). In addition to having a different sound from other ornamental chickens in Indonesia, these chickens also have different physical forms and genetic compositions, so support is needed for their protection and conservation (Abinawanto and Effendi 2017). The Minister of Agriculture stated that the Gaga' chicken is one of the wealth of genetic resources for livestock that must be protected and preserved with the Decree No. 2920/Kpts/OT.140/6/2011.

Over the years, several efforts have been made to conserve germplasm by preserving chicken sperm using cryopreservation technology. Thélie et al. (2019) revealed that based on the amount of time spent in storage, chicken semen stored for 18 years in liquid nitrogen did not affect sperm fertility after freeze-thawing. It is effective in recovering lost genetic resources but causes low post-thawing quality. Partyka et al. (2012) reported that it led to a decrease in plasma membrane integrity, acrosome integrity, and mitochondrial activity after freeze-thawing. Several studies also stated the cryopreservation of chicken sperm causes an increase in lipid peroxidation. This is because the cell membrane contains a large amount of polyunsaturated fatty acids (Cerolini et al. 2006; Mussa et al. 2021), which can lead to a loss of fluidity and integrity of the membrane structure (Colagar et al. 2013).

Consequently, it is necessary to add antioxidants, such as L-carnitine and hyaluronic acid to the diluent to prevent lipid peroxidation in sperm. L-Carnitine (beta-hydroxy-gamma-N-trimethylaminobutyric acid) is a water-soluble vitamin-like amino acid that plays a role in the preservation of membrane integrity, mitochondrial function, as well as the inhibition of apoptosis (Surai 2015a). Several studies also reported that it is an effective antioxidant for the cryopreservation of cat (*Felis catus*) (Manee-in et al. 2014), sheep (*Ovis aries*) (de Souza et al. 2019), broiler (*Gallus gallus domesticus*) (Fattah et al. 2016) and Green-legged Partridge (*Gallus gallus domesticus*) sperms (Partyka et al. 2017). Hyaluronic acid is a nonsulfated glycosaminoglycan that is the main constituent of the extracellular matrix (Williams et al. 2019). Ke et al. (2011) revealed that its low molecular weight variant is an antioxidant with free radical scavenging activity. Previous studies also reported that it is effective for inhibiting lipid peroxidation and has the potential as a cryoprotectant in pig sperm (Qian et al. 2016). Hyaluronic acid supplementation in diluent can improve broiler sperm quality after freeze-thawing (Lotfi et al. 2017).

Antioxidants and cryoprotectants are needed to protect spermatozoa from the negative effects of freezing. Several studies revealed that sucrose could function as a nonpermeable cryoprotectant (Chen et al. 2019). Its addition in a concentration of 1 mM improved membrane and acrosome integrity, membrane potential as well as sperm fertility of Thai native breed chickens (Thananurak et al. 2018). Therefore, this study aims to determine the effect of adding L-Carnitine, hyaluronic acid, and sucrose as well as their combination in a diluent on the quality of Gaga' chicken spermatozoa after freeze-thawing.

## MATERIALS AND METHODS

### Extender preparation

The basic diluent used was ringer acetate (Asering®, PT. Otsuka, Indonesia) containing 1.9 g sodium acetate, 3 g sodium chloride, 0.15 potassium chloride, and 0.1 g calcium chloride in 500 mL sterile water, with an osmolarity of 273 mOsm L- 1. Ringer acetate 90% added chicken egg yolk 10% and centrifuged 2000 rpm for 20 minutes. The supernatant was taken and added with 2% glucose (Merck, KgaA, Darmstadt Germany), 1000 IU penicillin (PT Meiji, Indonesia), streptomycin 1 mg ml -1 (PT Meiji, Indonesia), and 7% dimethyl sulfoxide (Merck, KgaA, Darmstadt, Germany). The diluent was divided into six tubes and each was given a treatment. The first tube (P0) was controlled group, the second tube (P1) was filled with 0.063 mM low molecular hyaluronic acid (DNA Code®), the third tube (P2) was filled with 1 mM L-carnitine (Now Food®, US), the fourth tube (P3) was filled with 1 mM sucrose (Himedia®, India), the fifth tube (P4) was filled with hyaluronic acid 0.063 mM + L-carnitine 1 mM, and the sixth tube (P5) was filled with 0.063 mM + L-carnitine 1 mM + sucrose 1 mM.

### Collection and dilution of the semen

This study used 1 selected Gaga' chicken with a semen volume of more than 0.35 mL ejaculate$^{-1}$ and sperm concentration is more than 950 million ejaculate$^{-1}$. It was kept individual cages of 40 x 50 x 70 cm$^3$, given a daily feed was 150 grams and drinking water ad libitum. Subsequently, semen was collected in the morning at 07.30 by massaging the soft sides of their abdomen between the gizzard and the pelvic bones, as proposed by Burrows and Quinn (1937). The samples obtained were then divided into 6 tubes containing the diluent and treatments. Each tube contains a minimum of 100 million sperm cells.

### Cryopreservation procedures and evaluation of the sperm

A 0.25 mL straw (IMV, France) was filled with diluted semen and stored at 5°C for 2 hours (Santiago-Moreno et al. 2011). It was placed 3 cm above the surface of liquid nitrogen (Madeddu et al. 2016) for 10 minutes (Mosca et al. 2016) and then immersed for 24 hours. Subsequently, an evaluation of semen was carried out after thawing at 60°C for 5 seconds (Salih et al. 2021). The sperm viability was assessed by staining with eosin-nigrosine (Merck, KgaA, Darmstadt, Germany) and observing the color-absorbing sample on a light microscope (Boeco, Germany) at 40x magnification spermatozoa that absorb the dye is considered dead and those that do not absorb the dye are considered viable. Observation of motility (total motility, progressive motility, local motility and immotile) and kinematic variable [distance curve-line (DCL), distance straight-line (DSL) and distance average path (DAP), velocity curvilinear (VCL), velocity average pathway (VAP), velocity straight line (VSL), linearity (LIN), straightness (STR), wobble (WOB), beat cross frequency (BCF), amplitude lateral head (ALH) and average orientation and change of head (AOC)] was performed with a computer-assisted sperm analysis system (CASA, Sperm Vision, Minitube, Tiefenbach, Germany), which was connected to a trinocular light microscope (Zeiss AXIO Scope A1, US).

### Statistical analysis

This study used a completely randomized design with six treatment levels. The Shapiro-Wilks test was used to detect the normality of sperm quality data after freeze-thawing. Normally distributed data were analyzed using ANOVA and continued with the Duncan multiple range tests when the F-value was significant (P<0.05). Statistical analysis was carried out using the SPSS 16 software on a Windows computer.

## RESULTS AND DISCUSSION

### Sperm motility parameters

The result showed that there were significant differences (P<0.05) in all motion rate variables where the progressive motility increased with the use of treatments P1, P2, P3, and P4. Furthermore, the local motility recorded in other groups was lower than that of P0, P2 and P3. The percentage of immotile spermatozoa obtained after supplementation with P2, P3, and P4 was low (Table 1).

### Sperm viability

The viability of the spermatozoa was significantly different (P<0.05) between the treatments, where the highest value was obtained from samples in P4, P1, and P2 while P5 had the lowest (Table 2).

**Table 1.** Motility of Gaga' chicken sperm after freeze-thawing with addition of hyaluronic acid, L-Carnitine and sucrose in RAEY diluent

| Treat-ment | Variable of motility (%) (Mean ± SEM) | | | |
|---|---|---|---|---|
| | Total motility | Progressive motility | Local motility | Immotile |
| P0 | 42.12±2.75[b] | 20.28±3.43[a] | 21.84±2.46[c] | 57.88±2.75[b] |
| P1 | 50.92±6.11[bc] | 37.18±5.19[b] | 13.74±1.18[ab] | 49.08±6.11[ab] |
| P2 | 57.22±6.15[c] | 37.68±5.54[b] | 19.54±2.18[c] | 42.78±6.15[a] |
| P3 | 57.84±2.80[c] | 35.92±2.87[b] | 21.92±1.39[c] | 42.16±2.80[a] |
| P4 | 59.80±1.56[c] | 41.82±1.28[b] | 17.98±0.70[bc] | 40.20±1.56[a] |
| P5 | 27.74±3.62[a] | 17.40±3.62[a] | 10.27±0.72[a] | 72.26±3.62[c] |

Note: Different superscripts in the same column showed a significant effect (P <0.05)

**Table 2.** Viability of Gaga's chicken sperm after freeze-thawing with addition of hyaluronic acid, L-Carnitine and sucrose in RAEY diluent

| Treatment | Viability (%) |
|---|---|
| P0 | 36.82±3.67[b] |
| P1 | 42.83±4.11[bc] |
| P2 | 41.17±3.79[bc] |
| P3 | 31.91±4.03[ab] |
| P4 | 51.19±1.53[c] |
| P5 | 22.50±3.82[a] |

Note: Different superscripts in the same column showed a significant effect (P <0.05)

### Sperm kinematics parameters

The results showed there was a significant difference (P<0.05) in DSL, DCL, DAP, VCL, VSL, VAP, STR, and LIN, and ALH. Samples in group P1 and P4 experienced an increase in VCL. The administration of treatment P1, P2, P3 and P4 increased the DCL, while P5 decreased the DAP, VCL, VAP, VSL, and ALH but increased STR (Table 3).

## Discussion

Cryopreservation of semen in poultry can be carried out with the aim of increasing production, conserving or preserving the genetic diversity of endangered poultry species or breeds (Woelders 2021). Genetic conservation of chickens in Indonesia is important because it has many types of native or local chickens that have not received serious attention to be developed. There are still very few studies on the cryopreservation of native Indonesian chicken spermatozoa, some of which are Sentul chicken (Junaedi et al. 2016), Kampung chicken (Khaeruddin et al. 2020) and Kampung Sentul Kedu crossed chicken (Telnoni et al. 2021). In this study, it is the first time to report the quality of Gaga' chicken spermatozoa after cryopreservation. In addition, this study was also the first to examine the sperm kinematic parameters of Indonesian native chickens.

In the present study, hyaluronic acid, L-carnitine, sucrose and their combination were tested to improve Gaga' chicken sperm survival and motion ability after cryopreservation in semen packaged in straws. Hyaluronic acid can increase progressive motility and its combination with L-Carnitine increases the viability of Gaga chickens' sperm after freeze-thawing. This is in line with Lotfi et al. (2017) that hyaluronic acid can decrease lipid peroxidation as well as increase viability, membrane functionality, and acrosome integrity in broiler's sperm. The total sperm motility obtained using 1 mM hyaluronic acid was similar to that of Lotfi et al. (2017), namely 50%. Furthermore, progressive motility of 37.18% was recorded in this study, which was higher than that of the previous study (Lotfi et al. 2017).

**Tabel 3.** Kinematic parameters of Gaga' chicken sperm after freeze-thawing with addition of hyaluronic acid, L-Carnitine and sucrose in RAEY diluent

| Kinematic parameters | P0 | P1 | P2 | P3 | P4 | P5 |
|---|---|---|---|---|---|---|
| DCL (µm) | 29.35±2.42[a] | 37.99±1.36[b] | 34.46±1.45[b] | 34.85±1.39[b] | 37.33±1.44[b] | 24.69±1.39[a] |
| DAP (µm) | 16.68±1.13[b] | 18.67±0.73[b] | 18.45±0.57[b] | 17.75±0.63[b] | 18.66±0.51[b] | 12.91±0.61[a] |
| DSL (µm) | 11.65±0.79[ab] | 12.42±0.39[b] | 13.22±0.44[b] | 12.13±0.53[b] | 12.69±0.30[b] | 10.14±0.66[a] |
| VCL (µm $s^{-1}$) | 76.31±7.86[b] | 93.18±2.93[c] | 85.88±3.93[bc] | 85.85±3.58[bc] | 91.98±3.83[c] | 61.82±4.19[a] |
| VAP (µm $s^{-1}$) | 43.86±3.93[b] | 46.41±1.65[b] | 46.14±1.64[b] | 43.84±1.32[b] | 46.34±1.43[b] | 32.34±1.29[a] |
| VSL (µm $s^{-1}$) | 30.71±2.41[b] | 31.04±1.08[b] | 33.34±1.30[b] | 30.14±1.21[b] | 31.59±0.71[b] | 25.57±1.29[a] |
| LIN (%) | 40.92±2.55 | 33.40±1.77 | 39.00±0.98 | 36.00±2.57 | 34.40±1.58 | 41.80±2.87 |
| STR (%) | 70.47±2.86[a] | 67.00±1.36[a] | 72.25±1.05[a] | 68.80±2.16[a] | 68.28±1.08[a] | 79.20±2.54[b] |
| WOB (%) | 57.80±1.77 | 50.00±1.95 | 54.20±1.46 | 52.00±2.59 | 50.60±1.96 | 52.80±2.52 |
| BCF (Hz) | 21.59±1.52 | 21.21±0.66 | 20.72±0.37 | 20.43±1.44 | 20.64±0.39 | 21.06±1.38 |
| ALH (µm) | 5.37±0.39[b] | 5.52±0.16[b] | 5.37±0.14[b] | 5.50±0.16[b] | 5.64±0.16[b] | 4.53±0.26[a] |
| AOC | 14.20±2.34 | 13.72±0.82 | 16.14±0.29 | 15.59±0.67 | 17.88±1.05 | 13.64±1.17 |

Note: Different uppercase superscripts on the same line showed significant differences (P < 0.05). DCL: distance curve-line, DSL: distance straight-line, DAP: distance average path, VCL: velocity curvilinear, VAP: velocity average pathway, VSL: velocity straight line, LIN: linearity, STR: straightness, WOB: wobble, BCF: beat cross frequency, ALH: amplitude lateral head, AOC: average orientation and change of head.

Cryopreservation causes a decrease in plasma membrane integrity, acrosome integrity and mitochondrial activity of chicken spermatozoa (Partyka et al. 2012). But, one of the roles of hyaluronic acid that is related to membrane functionality and acrosome integrity is its ability to form a glassy protective layer on the outer part of the sperm membrane when frozen damaged, thereby reducing the physical injury caused by the cryopreservation process (Singh et al. 2015; Qian et al. 2016). Cryopreservation of chicken spermatozoa has also been reported to increase the production of reactive oxygen species (ROS) and lipid peroxidation (Nguyen et al. 2015). It has long been recognized that high levels of reactive oxygen species (ROS) can inflict direct damage on lipids (Ayala et al. 2014). ROS production and lower antioxidant enzyme activity in spermatozoa induce apoptotic pathways that can lead to a reduction in sperm viability (Di Santo et al. 2012). But, hyaluronic acid, also known as an antioxidant, inhibits lipid peroxidation (Braga et al. 2015; Qian et al. 2016). Low molecular hyaluronic acid showed strong inhibition of lipid peroxidation and scavenging activities of hydroxyl radical (Ke et al. 2011). One method of protection hyaluronic acid is known to perform is neutralizing free radicals, all reactions between ROS and hyaluronan result in the fragmentation of the hyaluronic acid chain (Dovedytis et al. 2020).

On the kinematic parameters, the addition of hyaluronic acid, as well as its combination with L-Carnitine, increased the velocity of curvilinear (VCL) and distance of curvilinear (DCL). VCL is the average velocity measured over the actual point-to-point track traveled by the cell (Sloter et al. 2006). In this study, a range of 61.82-93.18 $ms^{-1}$ was obtained, along with a VAP of 32.34-46.41 $ms^{-1}$. Compared to the standard of quail sperm, the velocity was classified as a medium because it was above 45 $ms^{-1}$ and below 160 $ms^{-1}$ (Farooq et al. 2017). This is in line with Sayed et al. (2017) that the velocity of a chicken sperm cell is in the middle category when the VAP is above 20 $ms^{-1}$ and below 50 $m\ s^{-1}$. However, the VCL, VAP, VSL, ALH, LIN, and STR in this study were lower than that of Lotfi et al. (2017).

The addition of L-Carnitine in the RAEY diluent can increase total and progressive motility as well as DCL. This finding is consistent with Fattah et al. (2016) on broiler sperm using Beltsville diluent that there was an increase in the motility variable. Meanwhile, several studies reported that L-Carnitine could maintain membrane integrity and mitochondrial function as well as inhibit apoptosis in chicken sperm (Partyka et al. 2017). Its addition in a concentration of 1-2 mM to cryopreservation media increased motility, membrane functionality and viability of broiler sperm cells after freeze-thawing (Fattah et al. 2016). L-Carnitine plays an important role in metabolism by providing energy via β-oxidation, which positively affects the motion rate (Lisboa et al. 2012). Carnitine is involved in several metabolic pathways, such as oxidative phosphorylation, transport of long-chain fatty acids into the mitochondria for energy production, as well as buffering of the intra-mitochondrial ratio of free and acetylated co-enzyme A (Mongioi et al. 2016). L-carnitine is an antioxidant because it can scavenge free radicals, destroy hydrogen peroxide and chelate metals (Banihani et al. 2014). It also protects sperm membranes against toxic reactive oxygen species (ROS) that are associated with the transfer of β-oxidation products to the mitochondria for oxidation into $CO_2$ and $H_2O$ in the Krebs cycle (Surai 2015b).

The total motility in this study was lower compared to Fattah et al. (2016), which reported 68.2% after the addition of 1 mM L-carnitine in Beltsville diluent. However, the value was higher than that of Partyka et al. (2017), which recorded 49.06% in indigenous chicken spermatozoa using EK diluent and 1 mM L-carnitine. The progressive motility was higher compared to previous studies, namely 28.4% (Fattah et al. 2016), 16.48% (Partyka et al. 2017), and 24% using Sasaki diluent with 1mM L-carnitine (Kumar et al. 2019). The sperm viability obtained after the addition of L-carnitine was higher than that of Kumar et al. (2019), namely 27.55%. The VCL, VSL, VAP and LIN were also higher than the values recorded by Fattah et al. (2016) but lower compared to Partyka et al. (2017). Furthermore, the STR was similar to the results reported by Partyka et al. (2017), namely 70.75%.

The results showed that the addition of sucrose in the RAEY diluent increased the total and progressive motility as well as DCL of Gaga' chicken sperm. This is in line with Thananurak et al. (2018) that its supplementation in a concentration of 1 mM can improve membrane integrity and mitochondrial function. Mitochondria is a cell organelle that supplies energy for the movement of sperm flagellum (Zhang et al. 2018). This finding is consistent with Sangani et al. (2017) that it is associated with movement in chicken sperm. In addition, sucrose is an example of a disaccharide that has also been reported to act as a cryoprotectant (Chen et al. 2019). Disaccharides work by forming a coating that may include strong covalent interactions or electrical van der Waals interactions with the sperm membrane components (Patist and Zoerb 2005). The progressive motility obtained in this study was higher than that of Thananurak et al. (2018), which used BHSV diluent with 1 mM of sucrose, but the total motility, VAP and VSL were lower. In the present study, the viability and almost all kinematic parameters of spermatozoa did not differ from that without sucrose, in line with the report of Mosca et al. (2016) that there is no difference in viability, VSL, VAP, ALH, BCF of Lohman chicken sperm after freeze-thawing with the addition of sucrose. The combination of sucrose, L-carnitine and hyaluronic acid was not beneficial because it reduced the overall quality of the samples.

In conclusion, the addition of Hyaluronic acid, L-carnitine or their combination in ringer acetate-egg yolk (RAEY) diluent can improve the sperm quality of Gaga' chickens after freeze-thawing.

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