



Ultrastructure Analysis of Gaga Chicken Spermatozoa after Freezing with Coenzyme Q10 Supplementation in the Diluent

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Abstract. The purpose of this study was to analyze the ultrastructure of Gaga chicken spermatozoa after freezing-thawing with Coenzyme Q10 (CoQ10) supplementation in egg yolk lactate ringer diluent. The 3 Gaga chickens used were 10 months old, semen was collected using massage technique, diluted with egg yolk lactate ringer (EOL) with 2 treatments, namely EOL without CoQ10 and EOL with 300 μ M CoQ10 supplementation. The diluted semen was packed in straws, equilibrated for 2 hours, pre-freezing for 10 minutes, freezing for 24 hours and thawing. Fresh semen and post-thawing semen were prepared, coated and observed using Field Emission Scanning Electron Microscope (FESEM). The results of the study were observed descriptively. The results of the study showed that fresh semen had intact spermatozoa ultrastructure conditions in the head, middle and tail sections, while post-thawing semen experienced damage to the head, middle and tail sections with control treatment and no damage to spermatozoa occurred with CoQ10 treatment. The conclusion of this study is that Coenzyme Q10 supplementation in egg yolk lactate ringer diluent is able to maintain intact ultrastructure in Gaga chicken spermatozoa.

Keywords: Coenzyme Q10, Freezing, Gaga chicken, Sperm, Ultrastructure

1 Introduction

Gaga chicken is one of the local chicken breeds originating from South Sulawesi, Indonesia. This chicken is famous for its unique crowing sound, which is similar to the sound of a person laughing, so this chicken is highly valued in chicken singing contests, where the quality of the laughing chicken's crowing is assessed based on its length, rhythm, and variation [1]. According to the Decree of the Minister of Agriculture No. 2920 / Kpts / OT.140 / 6/2011, Gaga chicken is declared as one of the rich genetic resources of Indonesian livestock that needs to be protected and preserved [2].

Cryopreservation of chicken sperm has a very important role in the world of animal husbandry and biotechnology, especially in poultry breeding programs and the preservation of genetic resources. Through cryopreservation, chicken sperm can be stored for a very long time at ultra-low temperatures, making long-term storage easier. The

process of freezing and re-thawing sperm often causes increased production of reactive oxygen species (ROS), which can damage sperm structures, including membrane lipids, proteins, and reduce their fertilization ability [3][4][5][6].

Chicken spermatozoa have limitations in the preservation process when compared to mammalian sperm, namely having high polyunsaturated fatty acids (PUFA) in their cell membranes, this makes them more susceptible to damage due to lipid peroxidation due to free radicals [7], for this reason it is necessary to add antioxidants in the diluent. The use of antioxidants in semen preservation aims to protect sperm from oxidative stress that occurs during the cooling or freezing process. However, the effectiveness of antioxidants is highly dependent on the dose, animal species, and storage method. Coenzyme Q10 (CoQ10) can act as an antioxidant by scavenging reactive oxygen species and free radicals [8]. CoQ10 helps protect male sperm from structural and functional damage due to cold storage [9], so it has the potential to be used to maintain semen quality and chicken sperm fertility [10]. The use of CoQ10 has also been shown to be able to maintain the quality of chicken sperm after the freeze-thaw process [11].

Observation of sperm ultrastructure using an electron microscope is very important because it provides in-depth and detailed information about the sperm structure that cannot be seen with an ordinary light microscope. Electron microscopy allows the identification of systematic and non-systematic spermatozoa damage and organelle changes in the head and tail of spermatozoa [12]. Based on this, the aim of this study was to analyze the ultrastructure of Gaga chicken spermatozoa after freezing-thawing with Coenzyme Q10 (CoQ10) supplementation in egg yolk lactate ringer diluent.

2 Materials and Methods

2.1 Ethical Clearance

This study has been approved by the Research Ethics Commission of Brawijaya University No. 96-KEP-UB-2023.

2.2 Diluent Preparation

The basic diluent used was RLKT (90% Ringer lactate and 10% egg yolk) which was centrifuged at 3000 rpm for 15 minutes. The supernatant was added with 1000 IU/ml penicillin, 1 mg/ml streptomycin and 7% DMSO [13]. The basic diluent was divided into 2 tubes, each consisting of a control treatment (without CoQ10) and a treatment with the addition of 300 µL CoQ10.

2.3 Semen Collection, Dilution and Packaging of Semen

Semen was collected from 3 10-month-old Gaga chickens using the massage method [14]. Semen was taken to the laboratory to be diluted with a ratio of 1:10, then packaged in straws.

2.4 Equilibration, Freezing dan Thawing

The packaged semen was then equilibrated at 5 °C for 2 hours followed by pre-freezing for 10 minutes by placing the straw in liquid nitrogen vapor at a height of 3 cm for 10 minutes then the straw was frozen in nitrogen for 24 hours [15]. Thawing of semen was carried out in a water bath for 30 seconds at 37 °C [16].

2.5 Evaluation of Sperm Ultrastructure

Fresh semen (1 sample) and post-thawing semen (2 samples) were prepared by washing with 0.9% NaCl, the fixation process using 2.5% glutaraldehyde for 3-4 hours, then washed again with PBS 3 times for 5 minutes followed by a dehydration process with graded alcohol. The preparation was attached to a stub and coated with gold, observations were made on Field Emission-Scanning Electron Microscopy (FE-SEM) with a magnification of 20000x [17].

3 Results

The results of ultrastructural observations of the spermatozoa head showed that the spermatozoa head in fresh semen (**Figure 1a**) looked intact, as did the spermatozoa head after freezing with CoQ10 supplementation treatment (**Figure 1b**), but head damage (**Figure 1c**) occurred in spermatozoa after freezing without CoQ10 supplementation.

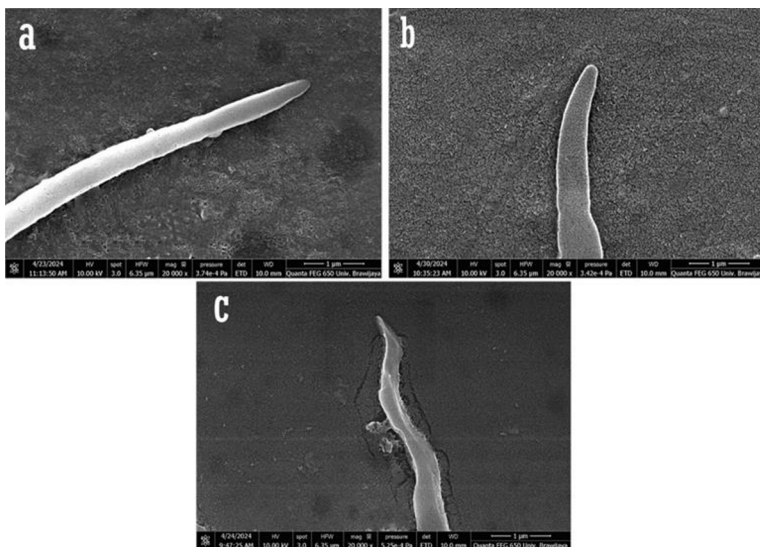


Fig. 1. Results of ultrastructure observations of spermatozoa heads using FE-SEM at 20000x magnification. a. Fresh semen, b. Post-thawing semen with CoQ10 treatment and c. Post-thawing semen without CoQ10.

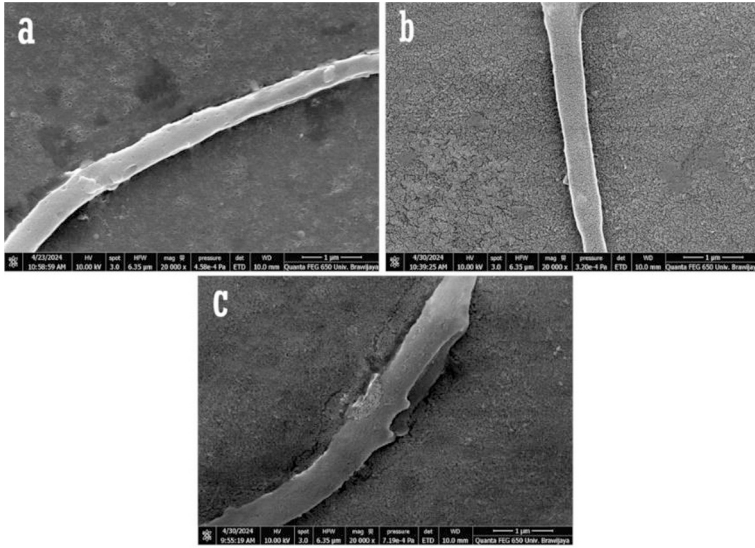


Fig. 2. Results of ultrastructure observations of the midpiece of spermatozoa using FE-SEM at 20000x magnification. a. Fresh semen, b. Post-thawing semen with CoQ10 treatment and c. Post-thawing semen without CoQ10.

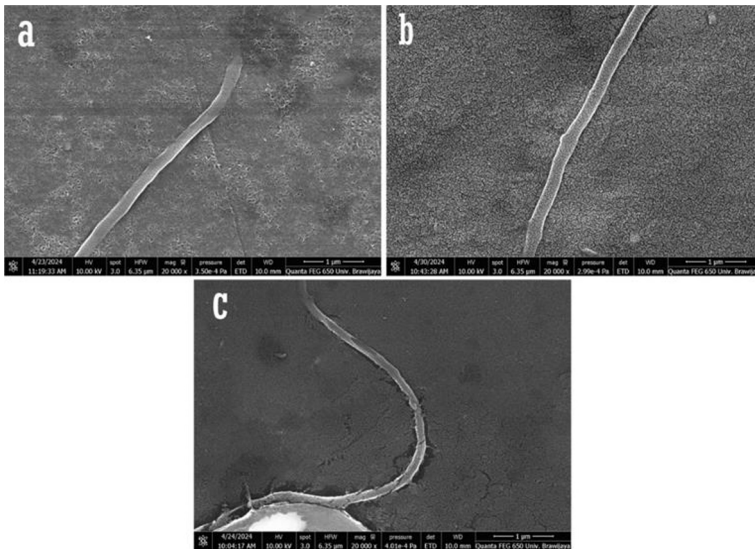


Fig. 3. Results of ultrastructure observations of spermatozoa tails using FE-SEM at 20000x magnification. a. Fresh semen, b. Post-thawing semen with CoQ10 treatment and c. Post-thawing semen without CoQ10.

The results of ultrastructural observations of the midpiece of spermatozoa showed that the midpiece of spermatozoa in fresh semen (**Figure 2a**) looked intact, as did the mid-piece of spermatozoa after freezing with CoQ10 supplementation treatment (**Figure**

2b), but damage to the midpiece (**Figure 2c**) occurred in spermatozoa after freezing without CoQ10 supplementation.

The results of ultrastructural observations of spermatozoa tails showed that the spermatozoa tails in fresh semen (**Figure 3a**) looked intact, as did the spermatozoa tails after freezing with CoQ10 supplementation treatment (**Figure 3b**), but tail damage (**Figure 3c**) occurred in spermatozoa after freezing without CoQ10 supplementation.

4 Discussion

Scanning electron microscopy (SEM) has been used to determine the surface topology of sperm [18]. Overall, SEM ultrastructural observation of sperm provides important insights into functional, morphological, and biological aspects of sperm that are not accessible by conventional observation methods. SEM allows high-resolution visualization of sperm down to the ultrastructural level, such as the head, neck, tail, and acrosome. This provides a detailed picture of the shape and structure of sperm that is important for understanding its functionality. Sperm ultrastructure and the relationship between sperm structure and function are major determinants of fertility [19].

Cryopreservation causes changes in fluidity, permeability, and the internal environment of the membrane, in addition to oxidative stress that causes damage to the plasma membrane and a decrease in mitochondrial membrane potential [20]. There are striking differences between the ultrastructure of mammalian and avian sperm, where avian sperm has poor tolerance to cryopreservation related to its subcellular structure. The shape of poultry sperm is different from the shape of mammalian sperm such as cows and pigs, where poultry sperm has a long cylindrical head that is not conducive to cryoprotectants, besides it also has a longer tail that is susceptible to breaking during the cryopreservation process [21]. Various classes of plasma membrane damage in cryopreserved chicken spermatozoa can be identified using electron microscopy [22].

Good quality rooster sperm has an intact acrosome membrane, nucleus, mitochondria, axoneme, centriole, and perforator [23], this can be seen in fresh semen in this study. The sperm head contains a nucleus as a carrier of genetic material and an acrosome at the tip of the head which plays a role in the fertilization process. Previous studies have shown that cryopreservation and thawing processes have an impact on the ultrastructure of poultry spermatozoa, especially mitochondria, the middle part, and perforator [24]. In this study, the freezing to thawing process caused damage to several parts of the spermatozoa (without CoQ10 supplementation). In the head, damage was seen to the plasma membrane and acrosome, in line with the report of Chen et al. [20] that chicken spermatozoa after freezing caused the plasma membrane in the head to be damaged and several parts of the acrosome were lost. While Heng et al. [21] reported that freezing chicken sperm causes structural damage including fractures and broken heads, swelling or loss of the head plasma membrane, cytosol overflow, and damage to the plasma membrane. The freezing to thawing process causes swelling of the plasma membrane and damage around the perforatorium [23].

In the midpiece of the sperm there are mitochondria that play a role in the metabolic process to produce energy [25], this energy is then used for tail movement so that sperm can travel in the female reproductive tract to the egg. In this study, damage after freezing also occurred in the middle and tail of the spermatozoa (without CoQ10 supplementation), where the plasma membrane in this section was damaged. A similar thing was reported by Chen et al. [20] that freezing chicken sperm causes the mitochondrial sheath to appear blurry and elliptical, unevenly distributed, and some mitochondria swell. Likewise, the report of Heng et al. [21] that there is damage to the mitochondrial sheath in chicken sperm after freezing.

CoQ10 as an antioxidant in this study was able to protect chicken sperm from ultra-structural damage after freezing. Coenzyme Q10 acts as a fat-soluble antioxidant and stops chain reactions, and plays a role in the regeneration of natural antioxidants such as superoxide dismutase, which helps reduce oxidative stress [26], thus, because CoQ10 is a fat-soluble antioxidant, it is able to diffuse directly into the polyunsaturated lipid chains found in the plasma membrane and protect it [10]. The presence of CoQ10 has also been shown to be able to maintain the integrity of the acrosome, mitochondrial activity and functionality of the chicken sperm plasma membrane after freezing [11].

The most important function of CoQ10 is as a carrier in the mitochondrial electron transport chain [27]. Due to this electron transport property, its function as an antioxidant is very relevant and has been shown to protect cell membranes from lipoperoxidation caused by free radicals [28]. CoQ10 also has an important function in the provision of cellular energy by transferring protons to the mitochondrial intermembrane space, which supports the formation of a proton gradient capable of producing energy to produce adenosine triphosphate (ATP) [29][30].

5 Conclusion

The conclusion of this study is that supplementation of Coenzyme Q10 in egg yolk lactate ringer diluent is able to maintain intact ultrastructure in Gaga chicken spermatozoa. The use of Coenzyme Q10 has the potential to be used as a chicken semen diluent for artificial insemination and opens up opportunities for research towards molecular interactions.

Disclosure of Interests. The authors have no competing interests to declare that are relevant to the content of this article.

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