



The Use of Ethylene Glycol Cryoprotectant with Varying Concentrations on the Integrity of the Plasma Membrane and DNA Damage in Gaga Chickens Semen

Muhammad Robbaani^{1,*}, Khaeruddin Khaeruddin², Achadiah Rachmawati¹,
Ita Wahyu Nursita¹, and Sri Wahjuningsih¹

¹Faculty of Animal Science, Universitas Brawijaya, Malang 65145, Indonesia

²Faculty of Agriculture, Universitas Muhammadiyah Sinjai, Sinjai 92615, Indonesia

*banihk2@gmail.com

Abstract. This study aimed to assess the effectiveness of cryoprotectant ethylene glycol with graded concentrations on cryopreserved Gaga chicken semen. The treatment repetitions in this study were ten times, included Dimethyl Sulfoxide (DMSO) 7% as control, Ethylene Glycol 5%, Ethylene glycol 7%, and Ethylene glycol 9%. The research procedures included semen collection, semen dilution, semen packaging, prefreezing and freezing. The variables analyzed were plasma membrane integrity and DNA damage before freezing and after thawing, as well as a decrease in plasma membrane integrity and an increase in DNA damage after thawing. Data were analyzed using analysis of variance (ANOVA). The results showed that there was no significant effect of all treatments on the variables evaluated. This study found that there was no significant effect on all variables with an average plasma membrane integrity after thawing getting the results of 48-55% and the average DNA damage was 6-8%.

Keywords: gaga chickens, ethylene glycol, plasma membrane integrity, DNA damage, sperm.

1 Introduction

Gaga Chicken is one of Indonesia's local chickens originating from South Sulawesi. Its distinctive feature is its crowing sound that resembles human laughter, as well as its good adaptability to tropical climates and resistance to disease. Although not as popular as other local chickens such as Kampung Chicken and Pelung Chicken, Gaga Chicken has economic value and potential as a local Indonesian poultry germplasm that needs to be preserved. Conservation of the genetic diversity of local chickens is important, one of which is through semen cryopreservation. However, research on the cryopreservation of local chicken semen, especially Gaga Chicken, is still very limited. In this study, ethylene glycol was used as a cryoprotectant because it has a low molecular weight and high permeability. Ethylene glycol possesses a molecular weight of 62.1 g mol⁻¹, which is comparatively lower than that of dimethyl sulfoxide (DMSO), recorded at 78.3 g mol⁻¹ [1].

© The Author(s) 2025

I. Subagiyo et al. (eds.), *Proceedings of the 11th International Conference of Innovation in Animal Science (ICIAS 2024)*, Advances in Biological Sciences Research 49,

https://doi.org/10.2991/978-94-6463-880-6_11

Ethylene glycol modifies the hydrogen bonding network upon mixing with water. When combined in a ratio of 40% water to 60% ethylene glycol, the solution experiences a significant decrease in freezing point and loses its ability to form crystalline structures. This characteristic makes ethylene glycol one of the most efficient substances for cryoprotectant [2]. Ethylene glycol has been utilized as a cryoprotectant for Muscovy duck semen, its effectiveness is reported to be lower compared to DMSO [3]. However, both DMSO and ethylene glycol have been found to be appropriate for the cryopreservation of semen in Venda chickens [4]. Some success parameters in semen cryopreservation are plasma membrane integrity and DNA damage before freezing and after thawing, as well as a decrease in plasma membrane integrity and an increase in DNA damage after thawing. This study aimed to find the most suitable level of ethylene glycol cryoprotectant used in the cryopreservation of Gaga Chicken seme.

2 Materials and Methods

2.1 Management of chicken rearing and semen collection

Semen of Gaga Chicken (*Gallus gallus domesticus*) was obtained from 3 Gaga Chickens aged approximately 10 months and kept using individual cages. The feed given was BR 1 Geet Feed, ration 100g/head/day with a composition of 20% crude protein, 5% crude fiber, 5% crude fat and 8% ash, and drinking water was given ad libitum. The semen collection method used was the massage method [5]. Milky white semen came out, immediately collected using a 1 ml syringe.

2.2 Preparation of Diluent

This study is an experimental study using the Completely Randomised Design method with 4 treatments and 10 replicates, aimed at evaluating the effect of cryoprotectant treatment on the preservation of Gaga Chicken semen, with the following treatment groups: P0 (Dimethyl Sulfoxide 7%) as control, P1 (Ethylene Glycol 5%), P2 (Ethylene Glycol 7%), and P3 (Ethylene Glycol 9%). The semen samples were diluted using Ringer Lactate Egg Yolk diluent, consisting of 90% lactated ringer (PT Widatra Bakti, Indonesia) and 10% egg yolk, then centrifuged for 30 minutes at 3000 rpm [6]. From the supernatant, 10 ml were taken and added with 1000iu of penicillin and 1mg/ml of streptomycin, then homogenised and tris(hydroxymethyl)aminomethane was gradually added until the solution reached pH 8.

2.3 Pre-Freezing, Freezing, and Thawing Process

After semen was collected, it was divided into four microtubes. Each microtube was diluted with egg yolk lactated Ringer's diluent (RLKT) with 7% dimethyl sulfoxide, 5%, 7%, and 9% ethylene glycol. The diluted semen was packed into a straw. Then the straw was equilibrated by putting it in a 5°C refrigerator for two hours [7], then the straw was put into sterfoam for pre-freezing which was evaporated by liquid nitrogen

for 10 minutes [8]. Semen is ready for freezing by placing it in liquid nitrogen at -196°C . After a day of storage in liquid nitrogen, the semen is thawed in warm water at 37°C for 30 seconds [9].

2.4 Plasma Membrane Integrity Observation Procedure

Semen was diluted with HOST solution, then incubated in a 37°C waterbath for 30 minutes [10]. After that, it was smeared on a glass plate and dried. After drying, the sample was observed for at least 200 sperm under a microscope at 400 magnification in 10 field of view. Sperm with intact (tail, head, or bent centre) and non-intact (without bending) membranes were counted [11].

2.5 DNA Damage Observation Procedure

Semen is dripped on the preparation glass and smeared on top using another glass, waiting for it to dry. The preparations were then fixed in ethanol-acetone solution, kept in the refrigerator for 30 minutes at 4°C , then dried for 30 minutes at room temperature. After drying, the preparations were hydrolysed in 0.1 N HCL solution in the refrigerator for 5 minutes at 4°C . Then rinsed three times with running water every 2 minutes. After rinsing the preparations were then dabbed with toluidine blue O and allowed to stand for 20 minutes [12]. After that, the preparations were rinsed and then dried and observed with a 1000 magnification microscope at 10 field of view for. Sperm with intact DNA are coloured light blue, while damaged ones are coloured dark blue [13].

2.6 Data Analysis

Data processing of this study used analysis of variance (ANOVA) using the SPSS version 25 application and continued with the Duncan test if there were significant differences or significantly different results.

3 Results and Discussion

3.1 Plasma Membrane Integrity

Plasma membrane integrity is an important indicator of spermatozoa viability during the cryopreservation process. This study aimed to evaluate the effect of different concentrations of ethylene glycol as a cryoprotectant on the integrity of the plasma membrane of Gaga chicken sperm before and after freezing. The results of this study, the use of ethylene glycol cryoprotectant in cryopreservation of Gaga Chicken semen on the integrity of the plasma membrane showed that the P value > 0.05 (Table 1). Thus, the factor of using cryoprotectant ethylene glycol 5%, 7%, and 9% with 7% dimethyl sulfoxide control on plasma membrane integrity before freezing is that there is no difference between the treatments used, besides the results after thawing also show the same thing, namely there is no difference between the treatments used (Figure 1).

Table 1. Table of plasma membrane integrity before freezing and post thawing.

Treatment	Before Freezing	Post-Thawing
P0	94.55±0.61	53.64±1.51
P1	93.98±0.72	52.50±0.67
P2	92.63±0.65	51.84±0.78
P3	93.09±0.65	51.71±0.74

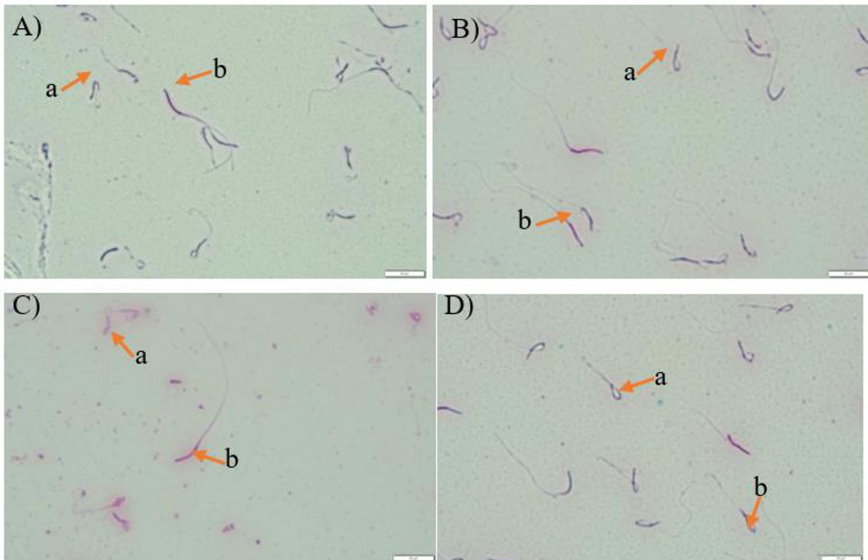


Fig. 1. Plasma Membrane Integrity. A=P0(Dimethyl Sulfoxide 7%), B=P1(Ethylene Glycol 5%), C=P2(Ethylene Glycol 7%), and D=P3(Ethylene Glycol 9%).

Notes: a=Sperm with intact membrane (tail, head or centre bent) and b=Non-intact (no bending).

3.2 DNA damage

DNA damage can affect the fertilizing potential of sperm, making it important to assess the effectiveness of cryoprotectants in maintaining DNA stability. This study aimed to compare the extent of DNA damage in Gaga chicken sperm using different concentrations of ethylene glycol as cryoprotectants before and after freezing. The results of this study, the use of ethylene glycol cryoprotectant in cryopreservation of Gaga Chicken semen against DNA damage showed that the P value > 0.05 (Table 2). Thus the factor of using cryoprotectant ethylene glycol 5%, 7%, and 9% with 7% dimethyl sulfoxide

control on DNA damage before freezing is that there is no difference between the treatments used, besides the results after thawing also show the same thing, namely there is no difference between the treatments used (Figure 2).

Table 2. Table of DNA damage before freezing and post thawing.

Treatment	Before Freezing	Post-Thawing
P0	2.42±0.25	5.47±0.51
P1	2.52±0.27	6.80±0.65
P2	2.78±0.32	8.59±1.20
P3	2.77±0.22	7.63±1.00

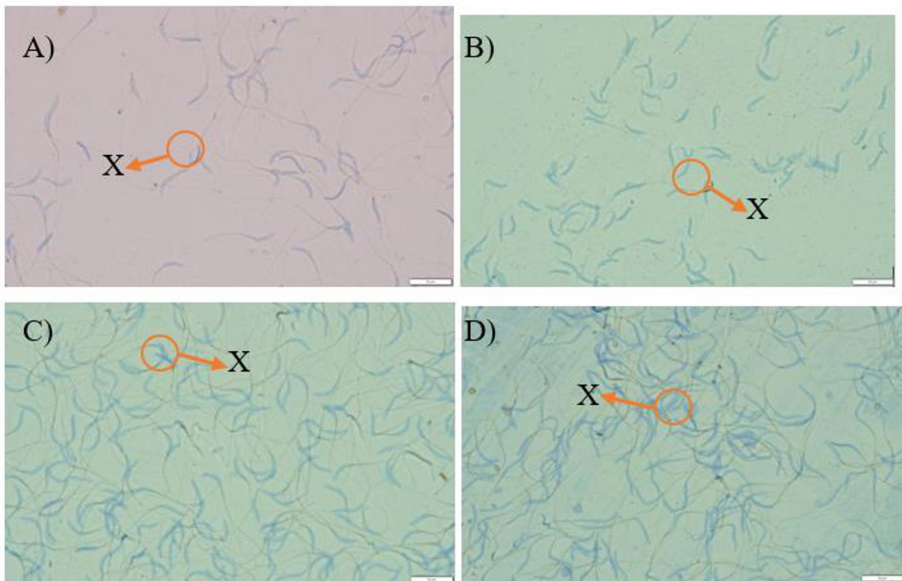


Fig. 2. DNA damage. A=P0(Dimethyl Sulfoxide 7%), B=P1(Ethylene Glycol 5%), C=P2(Ethylene Glycol 7%), and D=P3(Ethylene Glycol 9%).

Notes: Sperm with intact DNA are light blue, while those with damaged DNA are dark blue (X).

3.3 Decreased Plasma Membrane Integrity

The results of this study, the use of ethylene glycol cryoprotectants in the cryopreservation of Gaga Chicken semen obtained a P value > 0.05 (Figure 3). This the use of ethylene glycol cryoprotectants 5%, 7%, and 9% with 7% dimethyl sulfoxide control after thawing decreased plasma membrane integrity which was not significantly different in each treatment used.

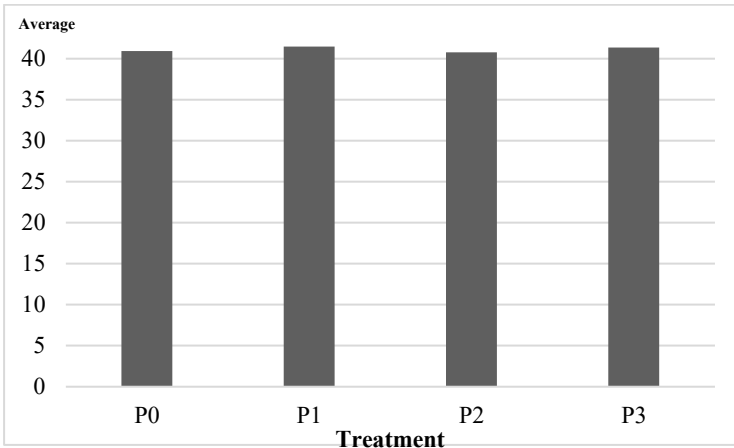


Fig. 3. Diagram of Plasma Membrane Integrity Decrease.

Notes: P0 (DMSO 7%), P1 (EG 5%), P2 (EG 7%), and P3(EG 9%)

3.4 Increased DNA damage

The results of this study, the use of ethylene glycol cryoprotectants in the cryopreservation of Gaga Chicken semen obtained a P value > 0.05 (Figure 4). Thus the use of ethylene glycol cryoprotectants 5%, 7%, and 9% with 7% dimethyl sulfoxide control after thawing increased DNA damage which was not significantly different in each treatment used.

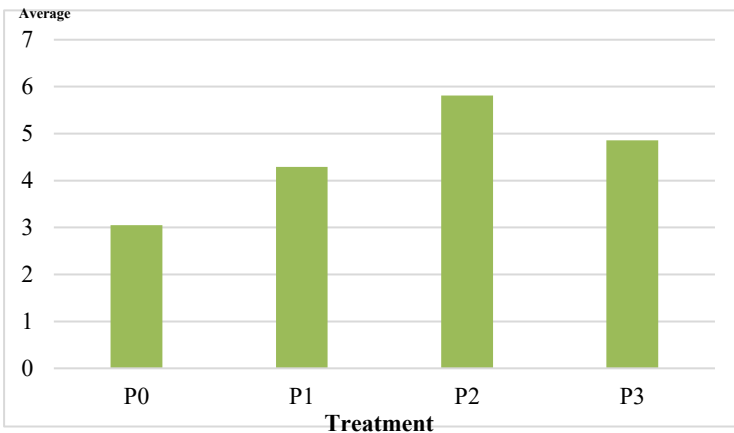


Fig. 4. Diagram of Increased DNA Damage

Notes: P0 (DMSO 7%), P1 (EG 5%), P2 (EG 7%), dan P3(EG 9%)

4 Discussion

The use of cryoprotectants in the cryopreservation process is to protect sperm from damage caused by the formation of ice crystals during the freezing and thawing process [14]. Without cryoprotectants, ice crystals can damage sperm cell structures, such as the plasma membrane and DNA leading to decreased plasma membrane integrity and increased DNA damage. This study is similar to the research of Choi who used Korean black bull semen, after passing the thawing process the results showed no significant difference in plasma membrane integrity between ethylene glycol treatments of 3%, 5%, and 7% [15]. Similarly, the research of Khaeruddin stated that there was no difference in the quality of chicken spermatozoa post thawing using ethylene glycol 3%, 5%, and 7% [16]. The sperm plasma membrane consists of a lipid layer that is very sensitive to temperature changes. Ethylene glycol, when interacting with the lipid membrane, can affect membrane fluidity, especially at low temperatures during the cryopreservation process. When fluidity decreases, the plasma membrane becomes rigid and more easily damaged, either due to freezing of ice crystals or temperature changes during thawing.

In this study, the observation of DNA damage was seen from the head of the spermatozoa, as reported by Esteves and Verza [17] DNA is at the front of the spermatozoa head. Partyka [18] and Hernandez [19] reported that increased DNA damage occurs during the freezing process. This is because freezing and thawing processes cause abrupt changes in osmotic pressure. Rota [20] reported that the use of ethylene glycol as a cryopreservation of dog semen obtained results above 20% of the integrity of the plasma membrane after thawing. Meanwhile, this study obtained an average value of 52.42% on the integrity of the plasma membrane after thawing. This suggests that the use of ethylene glycol for cryopreservation of chicken semen may be better than cryopreservation of dog semen. However, García [21] reported that sorubim cuspicaudus sperm showed that the use of ethylene glycol at a concentration of 6% to 10% actually caused increased mitochondrial damage, plasma membrane disruption, and DNA fragmentation, both before freezing and after thawing. This has an impact on decreasing fertilization capacity, indicating that ethylene glycol at certain concentrations can cause stress to cells, rather than protecting them.

5 Conclusion

The results of this study indicate that the use of ethylene glycol with graded concentrations of 5%, 7%, and 9% did not have a significant effect on the integrity of the plasma membrane or DNA damage in Gaga chicken semen, either before freezing or after thawing. This indicates that ethylene glycol in this concentration range is likely less effective as a cryoprotectant in maintaining the structure and genetic integrity of Gaga chicken spermatozoa. Therefore, further research is recommended to explore alternative cryoprotectants, optimize doses, or study combinations with antioxidants to increase the success of cryopreservation.

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

References

1. S. P. Leibo, and T. B. Pool, *Fertility and Sterility* 96, 269-276 (2011)
2. S. Bhattacharya, and B. G. Prajapati, *Asian J. of Pharmaceutics* 10, 154-159 (2016)
3. V. Gerzilov, *J. Agricultural Science and Technology* 2, 57-60 (2010)
4. M. L. Mphaphathi, M. M. Seshoka, D. Luseba, B. Sutherland, and T. L. Nedambale, *Asian Pacific J. of Reproduction* 5, 132-139 (2016)
5. Khaeruddin, *Gaga Chicken Semen Quality and Intracellular Calcium Intensity of Spermatozoa Using RLKT Diluent with Butylated Hydroxytoluene and Sorbitol Supplementation*, PhD Dissertation, Brawijaya University (2024)
6. Junaedi, C. Sumantri, dan A. Gunawan, *J. Veteriner* 17, 300-308 (2016)
7. Khaeruddin, S. Wahjuningsih, G. Ciptadi, dan M. Yusuf, *Kriopreservasi Semen Ayam* (UB Press, Malang, 2024)
8. X. F. Han, Z. Y. Niu, F. Z. Liu, and C. Z. Yang, *J. Poultry Science* 4, 197-201 (2005)
9. S. Wahjuningsih, A. A. Arif, Khaerudin, H. Pratiwi, and A. R. I. Putri, *Adv. in Animal and Veterinary Sciences* 12, 807-814 (2024)
10. A. Shahverdi, M. Sharafi, H. Gourabi, A. A. Yekta, V. Esmaili, M. Sharbatoghli, E. Janzamin, M. Hajnasrollahi, and F. Mostafayi, *Theriogenology* 83, 78-85 (2015)
11. S. J. Moreno, C. Castaño, M. A. Coloma, A. Gómez-Brunet, A. Toledano-Díaz, A. López-Sebastián, and J. L. Campo, *Poultry Science* 88, 2661-2669 (2009)
12. B. R. Rui, D. S. Angrimani, J. D. A. Losano, B. L. de Cássia, M. Nichi, and R. J. Pereira, *Animal Reproduction Science* 187, 133-140 (2017)
13. N. M. Esfahani, and M. Tavalae, *Sperm Chromatin Structure: Toluidine Blue Staining* (Cambridge University Press, England, 2021)
14. S. Iskandar, R. Mardalestari, R. Hernawati, E. Mardiah, and E. Wahyu, *Indonesian J. of Animal and Veterinary Sciences* 11, 34-38 (2006)
15. S. H. Choi, M. H. Ko, T. Y. Kang, S. R. Cho, Y. S. Park, and S. Oh, *Reproductive and Developmental Biology* 35, 377-383 (2011)
16. Khaeruddin., G. Ciptadi, M. Yusuf, W. Sawitri, C. Chotimah, and S. Wahjuningsih, *International J. of Agriculture and Biology* 32, 62-70 (2024)
17. S. C. Esteves, and J. S. Verza, *Reproductive Science Journal* 3, 72-84 (2011)
18. A. Partyka, W. Ni-zanski, and E. Lukaszewicz, *Theriogenology* 74, 1017-1027 (2010)
19. P. J. E. Hernandez, R. F. Fernandez, S. J. L. Rodriguez, M. Y. G. Soto, J. E. H. Verona, and R. A. D. Garcia, *Rev De Salud Animal* 34, 84-88 (2012)
20. A. Rota, C. Milani, G. Cabianca, and M. Martini, *Theriogenology* 65, 1848-1858 (2006)
21. V. A. García, D. P. Izquierdo, J. R. González, M. P. Guevara, S. P. Carrasco, and J. E. Araujo, *Animals* 13, 235-246 (2023)

Open Access This chapter is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits any noncommercial use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

