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Research article

# Impact of egg yolk and cysteine on cryopreserved indigenous black rabbit sperm quality in Mekong Delta

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**Abstract**

**Importance of the work**: Cryopreservation of indigenous black rabbit sperm poses challenges due to induced damage that affects viability, motility and membrane integrity.
**Objectives**: To evaluate the impact of varying concentrations of cysteine and egg yolk on the cryopreservation medium.
**Materials & Methods**: In Experiment 1, egg yolks were used at concentrations of 0%, 5%, 10% or 15%. In Experiment 2, cysteine was used at concentrations of 0 mM, 1.25 mM, 2.5 mM or 5 mM. Samples were preserved in liquid nitrogen for 72 hours, before being thawed and evaluated.
**Results**: Based on the results in Experiment 1, 15% egg yolk was the optimal concentration for preserving rabbit sperm, with values for overall mobility, progressive motility, viability rate and membrane integrity of 52.31%, 38.74%, 59.32% and 44.79%, respectively. These differences were significant ($p < 0.05$) compared to the other concentrations. In Experiment 2, the tris citrate glucose (TCG) medium supplemented with 15% egg yolk and combined with 2.5 mM cysteine produced the best results and was significantly ($p < 0.05$) different from the other concentrations, with overall values for mobility, progressive motility, viability rate and membrane integrity of 63.85%, 45.26%, 74.50% and 51.56%, respectively.
**Main finding**: Using a TCG storage solution enhanced with 15% egg yolk and 2.5 mM cysteine improved rabbit sperm quality during cryopreservation, demonstrating the positive effects of egg yolk and cysteine in sperm cryopreservation.

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## Introduction

Rabbit husbandry has gained significant importance in the Mekong Delta of Vietnam, offering a promising avenue for sustainable income generation among local farmers (Chau and Thu, 2014). This is vital considering the challenges confronting the agricultural sector such as soil erosion, climate change and population growth (Silagadze, 2022). Rabbit meat holds a notable position in the market due to its low cholesterol content and widespread acceptance across various age groups (Thu and Dong, 2011). Additionally, the pharmaceutical industry benefits by using the rabbit-derived products to produce antibodies, surgical sutures and vaccinations (Ros et al., 2020). Notably, Vietnam leads Southeast Asian countries in rabbit production, with a total production value accounting for 3.24% of the country's gross domestic product (Lukefahr, 2007). The black rabbit is one of Vietnam's indigenous rabbit breeds, known for its outstanding characteristics, including disease resistance and the ability to adapt to low-feeding conditions and climatic variation throughout the country (Thu and Dong, 2011), making it a valuable genetic resource that must be preserved. However, the introduction of foreign rabbit breeds, such as English Spot, Chinchilla, New Zealand, and Californian rabbits, among others, has led to hybridization with indigenous black rabbits, causing notable changes in both their phenotype and genotype (Chau and Thu, 2014). Therefore, preserving and developing the population of Vietnamese indigenous black rabbits could be a major contributor to the growth of the rabbit farming industry in the Mekong Delta region.

A viable option for rapidly establishing disease-free, well-bred and productive local rabbit herds is to combine artificial insemination with cryopreservation techniques. However, spermatozoa suffer sub-lethal damage from the lowered temperature during cryopreservation, which jeopardizes sperm quality and the effectiveness of artificial breeding (Rizkallah et al., 2022). Although tris citrate glucose (TCG) medium with 5% glycerol is one of the fundamental media used for rabbit sperm cryopreservation (Tran et al., 2023) it cannot completely protect sperm during cryopreservation. Therefore, it is essential to add cryoprotective agents to the TCG medium. Egg yolks contain substances that provide energy for sperm, protein sources, lipoproteins that help maintain sperm membranes and lecithine that acts as a cryoprotectant for sperm (Bustani and Baiee, 2021). Egg yolks have been shown to provide optimal results in the cryopreservation of rabbit sperm (Iaffaldano et al., 2014). In semen cryopreservation media, egg yolks can regulate the transport of proteins, phospholipids and cholesterol, thereby protecting the plasma membrane against temperature-related damage (Forouzanfar et al., 2010). However, Peris et al. (2007) noted that cryopreservation could cause significant harm to sperm function due to lipid peroxidation (LPO) induced by reactive oxygen species (ROS). LPO occurs easily in tissues rich in highly oxidizing polyunsaturated fatty acids (PUFAs; Bansal and Bilaspuri, 2011). Sperm cells have major levels of PUFAs and hence are vulnerable to LPO (Collodel et al., 2022). Cysteine, a sulfur-containing amino acid, enhances glutathione production both *in vitro* and *in vivo* (Tuncer et al., 2010). Cysteine is an important endogenous antioxidant in animals, being one of the substances that helps to eliminate ROS present in sperm plasma and semen and thereby protecting the sperm from the harmful effects of oxidation (Abdullah et al., 2021).

Therefore, this study was conducted to evaluate the effects of egg yolk and cysteine on the quality of indigenous black rabbit sperm in cryopreservation in the Mekong Delta region.

## Materials and Methods

### *Animals*

Due to the concern regarding the declining population of indigenous black rabbits, limiting access to sufficient number of specimens, this study utilized male indigenous black rabbits sourced from various regions in the Mekong Delta, Vietnam. The rabbits were gathered at the Animal Experimental Farm of the Stem Cell Lab at Can Tho University, Vietnam. In total, six indigenous black male rabbits were included, aged 12–18 mth and weighing 2.5–3.5 kg. The animals were individually housed in flat-floor cages and provided with a sufficient supply of drinking water. The rations for each rabbit were formulated to meet the nutrient requirements of mature male rabbits according to guidelines of National Research Council (1977). All animals were fully vaccinated against hemolytic diseases and parasites.

### *Experimental design*

Experiment 1: Semen samples were collected from the six healthy male rabbits using a warmed artificial vagina lubricated with gel and stimulated by a doe (Naughton et al., 2013).

To ensure consistent, high-quality samples with over 60% motility, the samples were obtained twice per week at the same time in the early morning (3 ejaculates/male rabbit). The samples were diluted with TCG medium (250 mM tris-hydroxymethylaminomethane, 88 mM citric acid, 47 mM D-glucose and 80 mg/L gentamycin) supplemented with 5% glycerol. The diluted samples were further supplemented with egg yolk at concentrations of 0%, 5%, 10% or 15% (v/v) to achieve a concentration of $10 \times 10^7$ cells/mL. Then, the samples were loaded into 0.5 mL French straws and stabilized at 15°C for 30 min, followed by cooling to 5°C for 60 min. Subsequently, the straws were exposed to liquid nitrogen vapor for 15 min and finally immersed in liquid nitrogen for long-term storage. After 72 hr storage, the samples were thawed at 37°C for 60 s and the semen quality was evaluated. Each treatment group consisted of samples obtained from the six individual rabbits, resulting in a total sample size of 18 for each treatment group. The most optimal egg yolk concentration was used for Experiment 2.

Experiment 2: The collected semen samples were diluted with TCG medium supplemented with 5% glycerol and the appropriate egg yolk concentration identified in Experiment 1. The diluted samples were further supplemented with cysteine at concentrations of 0 mM, 1.25 mM, 2.5 mM or 5 mM, achieving a concentration of $10 \times 10^7$ cells/mL. The samples were refrigerated according to the procedure described in Experiment 1. Each treatment group consisted of samples obtained from the six individual rabbits, resulting in a total sample size of 18 for each treatment group (Fig. 1).

### *Assessment of sperm motility*

Each sample (10 µL drops of sperm suspension) was placed on a pre-warmed, clean glass slide covered with a clean coverslip. Each slide was viewed under an optical microscope with a bright field lens (Nikon, Japan) at a total magnification of 200×. Sperm motility was evaluated based on visual estimation and categorization into three types: progressive motility, non-progressive motility and immotility. A minimum of 200 spermatozoa across at least five fields were counted (Fumuso et al., 2018).

### *Assessment of sperm viability*

The eosin-nigrosin method was used to measure sperm viability (Agha-Rahimi et al., 2014). Approximately 100 spermatozoa/smear were counted using microscopy (magnification 40×) and the proportion of viable spermatozoa was calculated based on the total number of cells (Fig. 2).

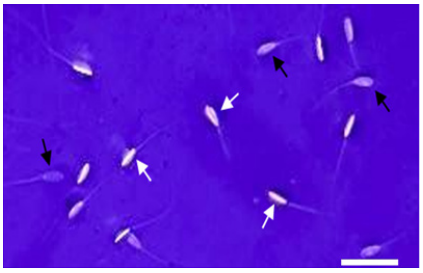

**Fig. 2** Assessment of sperm viability of indigenous black rabbit spermatozoa using eosin-nigrosine staining, where live sperm are unstained (white arrows), dead sperm are stained (black arrows) and scale bar = 50µm

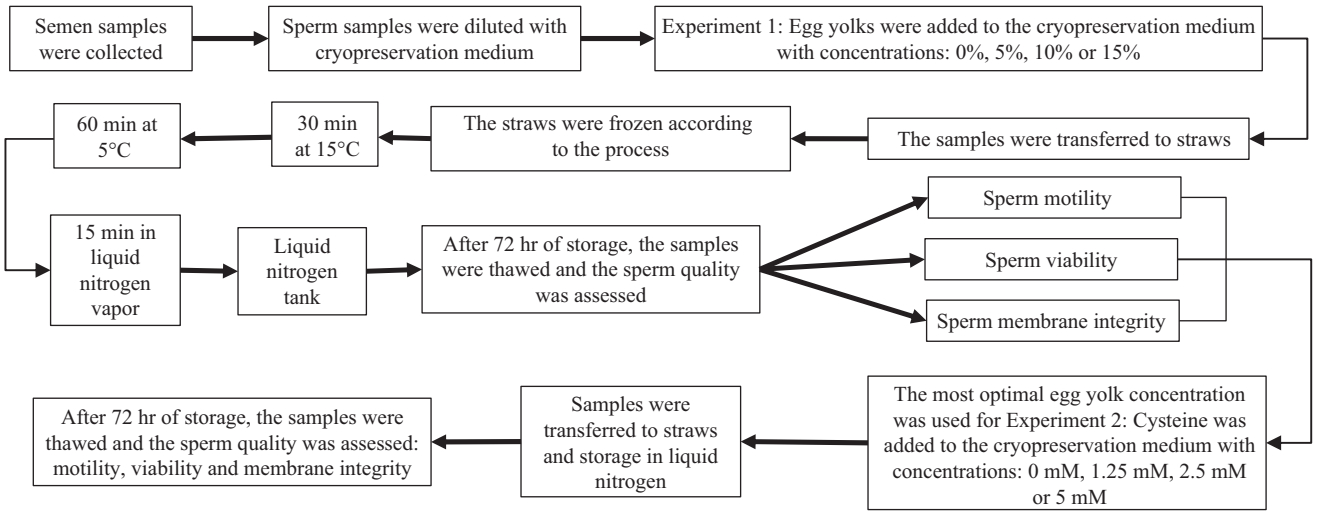


**Fig. 1** Experimental design

### *Assessment of sperm membrane integrity*

The hypo-osmotic swelling test (HOS) was applied to assess each sample (Ramu and Jeyendran, 2013). An Eppendorf tube containing 20 µL of semen sample and 80 µL of HOS solution was incubated at 37°C for 40 min. After incubation, a 10 µL portion of the mixture was placed on a glass slide for microscopic examination. Spermatozoa with intact membranes exhibited swelling in the tail region, whereas those with compromised membranes showed no swelling (Fig. 3).

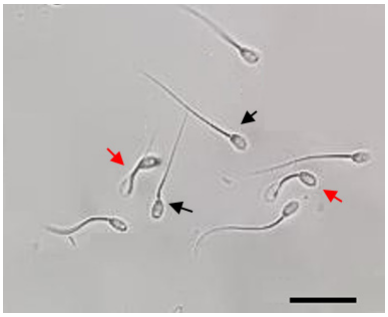

**Fig. 3** Assessment of sperm membrane integrity of indigenous black rabbit spermatozoa using hypo-osmotic swelling test, where sperm with intact membrane have swelling in tail (red arrows), sperm with compromised membrane have no swelling in the tail (black arrows) and scale bar = 50µm

### *Statistical analysis*

Statistical analysis was conducted to examine the impact of egg yolk concentration on the semen quality parameters in Experiment 1 and in Experiment 2, to examine the effect of cysteine concentration. A linear mixed model analysis of variance was applied to analyze the data after confirming normality and homogeneity of variance. Mean comparisons between treatments were conducted using the Tukey method in the R software version 4.3.1 (R core team, 2023). In Experiment 1, the fixed effect was egg yolk concentration and the random effects were rabbit and ejaculation. In Experiment 2, the fixed effect was cysteine concentration, while the random effects were rabbit and ejaculation. The results were presented as mean ± SD. Statistical significance was set at $p < 0.05$, indicating a high level of confidence in the obtained results.

### *Ethical review*

The study adhered to Ethical Guidelines for Animal Care, Housing and Semen Collection Procedures, as approved by the Animal Ethics Committee of Can Tho University (CTU-AEC24013).

## Results

### *Influence of egg yolk on sperm quality*

Figs. 4 and 5 present the results of Experiment 1, displaying the changes in sperm quality before and after cryopreservation using four different egg yolk concentrations: 0%, 5%, 10% or 15%. The parameters evaluated were: concentration, overall motility, progressive motility, viability and membrane integrity.

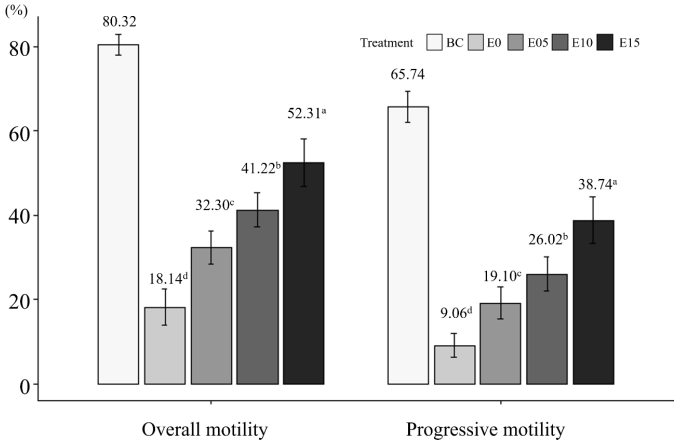


**Fig. 4** Sperm motility before and after cryopreservation in Experiment 1, where mean values with different lowercase superscripts are significantly ($p < 0.05$) different for each criterion, BC = before cryopreservation, E0 = egg yolk 0%, E05 = egg yolk 5%, E10 = egg yolk 10%, E15 = egg yolk 15% and error bars indicate ± SD

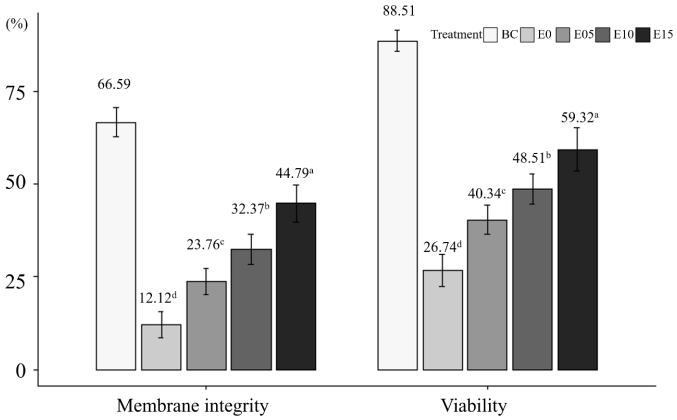


**Fig. 5** Sperm membrane integrity and viability before and after cryopreservation in Experiment 1, where mean values with different lowercase superscripts are significantly ($p < 0.05$) different for each criterion, BC = before cryopreservation, E0 = egg yolk 0%, E05 = egg yolk 5%, E10 = egg yolk 10%, E15 = egg yolk 15% and error bars indicate ± SD

Figs. 4 and 5 show that sperm quality decreased after cryopreservation. Notably, egg yolk concentration significantly impacted sperm quality, which progressively improved with increasing egg yolk concentration. The highest sperm quality was observed at a 15% egg yolk concentration, which was significantly different from other concentrations. Specifically, the overall motility rate was 52.31%, the progressive motility rate was 38.74%, the viability rate was 59.32% and the membrane integrity rate was 44.79%. Conversely, the significantly lowest sperm quality was observed in the TCG medium without egg yolk supplementation. Therefore, the TCG medium with 15% egg yolk (TCG-E15) was used for Experiment 2.

### *Influence of cysteine on sperm quality*

Figs. 6 and 7 present the results of Experiment 2, displaying the changes in sperm quality before and after cryopreservation with four different cysteine concentrations: 0 mM, 1.25 mM, 2.5 mM or 5 mM. The parameters evaluated were: concentration, overall motility, progressive motility, viability and membrane integrity.

Figs. 6 and 7 show that adding cysteine to the TCG-E15 medium helped to increase sperm quality ($p < 0.05$). The best sperm quality was recorded in the TCG-E15 medium supplemented with 2.5 mM cysteine for all assessment criteria ($p < 0.05$). Specifically, the overall motility, progressive motility, viability and membrane integrity were 63.58%, 45.26%, 74.50% and 51.56%, respectively. There was no significant difference in sperm quality between the medium supplemented with 0 mM cysteine and the medium supplemented with 5 mM cysteine.

## Discussion

In Experiment 1, the TCG medium supplemented with 15% egg yolk had the most optimal effect on rabbit sperm quality parameters after thawing, followed by the media supplemented with 10%, 5% and finally 0% egg yolk. The sperm overall motility in the TCG-15 egg yolk was 52.31%. Egg yolk concentration of 15% was also reported to be the optimal concentration for cryopreservation of ram sperm, with a motility rate of 54% (Kulaksız et al., 2010). Hall et al. (2017) showed that adding 17% egg yolk to the medium was the most effective method for cryopreservation of New Zealand white rabbit sperm in terms of post-thaw motility and viability. In Experiment 2, the medium treated with 2.5 mM cysteine produced the best sperm quality, followed by media supplemented with 1.25 mM, 5 mM and 0 mM cysteine. These findings were consistent with Sariözkan et al. (2009), who reported that cysteine helps enhance sperm motility. Based on the results from Experiment 2, increasing the cysteine concentration significantly improved sperm quality, while increasing the concentration to 5 mM reduced sperm quality, proving that a high concentration of cysteine affected the mobility, viability and membrane integrity of sperm after cryopreservation.

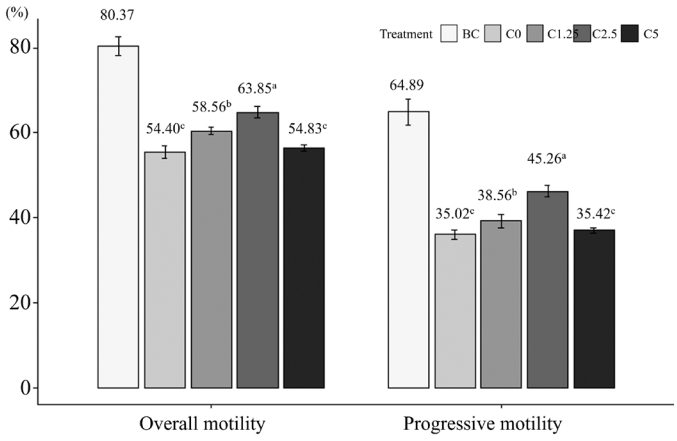


**Fig. 6** Sperm motility before and after cryopreservation in experiment 2, where mean values with different lowercase superscripts are significantly ($p < 0.05$) different for each criterion, BC = before cryopreservation, C0 = cysteine 0 mM, C1.25 = cysteine 1.25 mM, C2.5 = cysteine 2.5 mM, C5 = cysteine 5 mM and error bars indicate ± SD

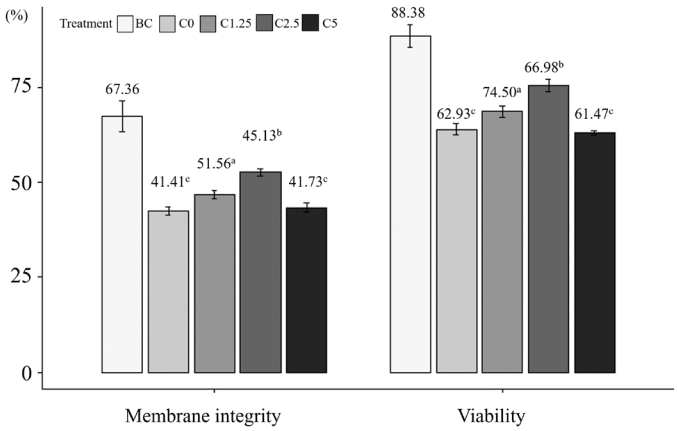


**Fig. 7** Sperm membrane integrity and viability before and after cryopreservation in Experiment 2, where mean values with different lowercase superscripts are significantly ($p < 0.05$) different for each criterion, BC = before cryopreservation, C0 = cysteine 0 mM, C1.25 = cysteine 1.25 mM, C2.5 = cysteine 2.5 mM, C5 = cysteine 5 mM and error bars indicate ± SD

Le et al. (2019) considered that the mitochondrial membrane damage generated by cryopreservation may be the cause of impaired sperm motility and that furthermore, sperm cells are vulnerable to increased LPO due to the creation of various oxidation processes that put strain on the cell membrane, thereby contributing to decreased motility. Sperm motility, the survival rate and sperm membrane integrity are all affected (Contreras et al., 2020). Egg yolk lecithin works as a cryoprotectant for sperm, with the egg yolk producing optimal results in the cryopreservation of rabbit sperm (Iaffaldano et al., 2014). In addition, egg yolks contain low-density lipoproteins (LDLs) that can resist cold shock and improve sperm motility after cryopreservation, since the LDLs adhere to the cell membrane during the freeze-thaw process, thereby protecting the sperm membrane (Iaffaldano et al., 2014).

The process of sperm cryopreservation, including cooling, freezing, and thawing, results in cellular damage to the cell membrane, cytoplasm and genomic structures (Nishijima et al., 2021). ROS are caused by osmotic stress, cold shock, intracellular ice crystal formation and overproduction of spermine (Amidi et al., 2016). ROS are mostly formed in sperm mitochondria, with higher ROS levels being reported in various studies in cows and sheep after cooling, freezing and thawing (Bansal and Bilaspuri, 2011; Santiani et al., 2014). These findings suggested that cysteine had preserved the rabbit spermatozoa during cryopreservation. Several studies have shown that adding cysteine to the cryopreservation medium promoted thawed-frozen sperm motility and membrane integrity in cats, buffalo, rams and boars (Kaeoket et al., 2010; Topraggaleh et al., 2014; Sharafi et al., 2015).

The current study on rabbit sperm cryopreservation identified both benefits and weaknesses. On the one hand, the study demonstrated the effects of egg yolk and cysteine using baseline measurements, establishing their functions in increasing the motility, viability and integrity of the rabbit sperm membrane. Additionally, cryopreservation of rabbit sperm with the TCG medium supplemented with egg yolk and cysteine could offer a good approach for the conservation of black rabbit breeds and other indigenous rabbit breeds in Vietnam. The results from the current study could help to identify an effective and inexpensive method of preserving rabbit sperm for rabbit breeding programs. However, Ahe limitation of this study was that other indications, such as acrosome activity status and DNA fragmentation, are required to provide a more thorough understanding of the effects of glycine. Furthermore, the experimental animal population must be expanded to better analyze the influence of egg yolk and cysteine on the sperm quality of different rabbit breeds when refrigerated for an extended period of time. Studies should be conducted on the effects of egg yolk and cysteine on frozen sperm quality in different rabbit breeds based on larger sample sizes to confirm the role of these various parameters in rabbit sperm cryopreservation. Furthermore, frozen semen samples need to be evaluated *in vivo* through artificial insemination techniques in female rabbits to test the fertilizing ability of the sperm. The current authors intend to conduct new research to find the best medium for rabbit sperm cryopreservation.

Notably, although in the current study, egg yolk and cysteine, when combined, had the ability to protect Vietnamese native black rabbit sperm, studies on other additives, such as trehalose or vitamins, also need to be conducted to find the most effective environment for the cryopreservation of Vietnamese black rabbit sperm.

Based on the results of the current study, the developed TCG storage solution, enhanced with egg yolk and cysteine, improved rabbit sperm quality during cryopreservation. Such findings are vital for the rapid development of local rabbit populations, as well as for the larger field of reproductive biotechnology and cryobiology. The results from this study could be a useful reference source for other studies on rabbit sperm cryopreservation. However, egg yolk and cysteine could have different effects on the sperm of different species. Therefore, careful consideration should be given before using egg yolk and cysteine concentrations in the cryopreservation of sperm from other species. As mentioned before, cryopreservation of rabbit sperm combined with artificial insemination not only helps to quickly develop rabbit herds but is also meaningful in protecting the environment. Specifically, semen samples collected from male rabbits are diluted and after cryopreservation, several sperm samples can be obtained. Furthermore, one sperm sample can be used to breed with a female rabbit using artificial insemination techniques, leading to a reduced requirement to maintain a larger number of male rabbits. This would lower farm investment costs and minimize waste generation and environmental pollution in the Mekong Delta region.

## Conclusion

The developed TCG storage solution enhanced with 15% egg yolk and 2.5 mM cysteine was the best combination of those tested for the cryopreservation of indigenous black rabbit sperm. Although these findings were promising, further validation is necessary based on larger sample sizes and different rabbit breeds to confirm the generalizability of the results.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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## References

Abdullah, F., Nor-Ashikin, M.N.K., Agarwal, R., et al. 2021. Glutathione (GSH) improves sperm quality and testicular morphology in streptozotocin-induced diabetic mice. Asian J. Androl. 23: 281–287. doi: 10.4103/aja.aja_81_20

Agha-Rahimi, A., Khalili, M., Nabi, A., Ashourzadeh, S. 2014. Vitrification is not superior to rapid freezing of normozoospermic spermatozoa: Effects on sperm parameters, DNA fragmentation and hyaluronan binding. Reprod. Biomed. Online 28: 352–358. doi.org/10.1016/j.rbmo.2013.11.015

Amidi, F., Pazhohan, A., Nashtaei, M.S., Khodarahmian, M., Nekoonam, S. 2016. The role of antioxidants in sperm freezing: A review. Cell Tissue Bank 17: 745–756. doi.org/10.1007/s10561-016-9566-5

Bansal, A.K., Bilaspuri, G.S. 2011. Impacts of oxidative stress and antioxidants on semen functions. Vet. Med. Int. 2011: 686137. doi: 10.4061/2011/686137

Bustani, G.S., Baiee, F.H. 2021. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. Vet World 14: 1220–1233. doi.org/10.14202/vetworld.2021.1220-1233

Collodel, G., Moretti, E., Noto, D., Corsaro, R., Signorini, C. 2022. Oxidation of polyunsaturated fatty acids as a promising area of research in infertility. Antioxidants 11: 1002. doi.org/10.3390/antiox11051002

Contreras, M.J., Treulen, F., Arias, M.E., Silva, M., Fuentes, F., Cabrera, P., Felmer, R. 2020. Cryopreservation of stallion semen: Effect of adding antioxidants to the freezing medium on sperm physiology. Reprod. Domest. Anim. 55: 229–239. doi.org/10.1111/rda.13611

Chau, N.T.V., Thu, N.V. 2014. Current status of rabbit raising in the Mekong Delta. Can Tho University Journal of Innovation and Sustainable Development 32: 1–8.

Forouzanfar, M., Sharafi, M., Hosseini, S.M., et al. 2010. *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology 73: 480–487. doi.org/10.1016/j.theriogenology.2009.10.005

Fumuso, F.G., Giulianob, S.M., Chavesa, M.G., Neilda, D.M., Miragayaa, M.H., Gambarottac, M.C., Carreteroa, M.I. 2018. Seminal plasma affects the survival rate and motility pattern of raw llama spermatozoa. Anim. Reprod. Sci. 192: 99–106. doi.org/10.1016/j.anireprosci.2018.02.019

Hall, S.E., Negus, C., Johinke, D., Bathgate, R. 2017. Adjusting cryodiluent composition for improved post-thaw quality of rabbit spermatozoa. PloS One 12: e0175965. doi.org/10.1371/journal.pone.0175965

Iaffaldano, N., Iorio, M.D., Rosato, M.P., Manchisi, A. 2014. Cryopreservation of rabbit semen using non-permeable cryoprotectants: Effectiveness of different concentrations of low-density lipoproteins (LDL) from egg yolk versus egg yolk or sucrose. Anim Reprod Sci. 151: 220–228. doi.org/10.1016/j.anireprosci.2014.10.020

Kaeoket, K., Chanapiwat, P., Tummaruk, P., Techakumphu, M. 2010. Supplemental effect of varying L-cysteine concentrations on the quality of cryopreserved boar semen. Asian J. Androl. 12: 760–765. doi: 10.1038/aja.2010.48

Kulaksız, R., Çebi, C., Akçay, E., Daşkin, A. 2010. The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen. Small Rumin. Res. 88: 12–15. doi.org/10.1016/j.smallrumres.2009.11.014

Le, M.T., Nguyen, T.T.T., Nguyen, T.T., Nguyen, V.T., Nguyen, T.T.A., Nguyen, V.Q.H., Cao, N.T. 2019. Cryopreservation of human spermatozoa by vitrification versus conventional rapid freezing: Effects on motility, viability, morphology and cellular defects. Eur. J. Obstet. Gynecol. Reprod. Biol. 234: 14–22. doi.org/10.1016/j.ejogrb.2019.01.001

Lukefahr, S. 2007. Strategies for the development of small- and extender-scale rabbit farming in South-East Asia. The International Conference on Rabbit Production. Bogor, Indonesia, pp. 24–25.

Naughton, C.K., Nelson, D.R., Thomas Jr, A.J. 2013. Development of an inexpensive artificial vagina for semen collection from rabbits. J. Androl. 24: 712–715. doi.org/10.1002/j.1939-4640.2003.tb02731.x

Nishijima, K., Kitajima, S., Matsuhisa, F., Niimi, M., Wang, C.C., Fan, J. 2021. Strategies for highly efficient rabbit sperm cryopreservation. Animals 11: 1220. doi.org/10.3390/ani11051220

National Research Council. 1977. Nutrient Requirements of Rabbits, 2nd ed. National Academy of Sciences. Washington DC, USA.

Peris, S.I., Bilodeau, J.F., Dufour, M., Bailey, J.L. 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol Reprod. Dev. 74: 878–892. doi.org/10.1002/mrd.20686

R Core Team. 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Ramu, S., Jeyendran, R.S. 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. Methods Mol. Biol. 927: 21–25. doi: 10.1007/978-1-62703-038-0_3

Rizkallah, N., Chambers, C.G., de Graaf, S.P., Rickard, J.P. 2022. Factors affecting the survival of ram spermatozoa during liquid storage and options for improvement. Animals 12: 244. doi.org/10.3390/ani12030244

Ros, F., Offner, S., Klostermann, S., et al. 2020. Rabbits transgenic for human IgG genes recapitulating rabbit B-cell biology to generate human antibodies of high specificity and affinity. Mabs 12: 1846900. doi.org/10.1080/19420862.2020.1846900

Santiani, A, Evangelista, S., Sepulveda, N., Risopatron, J., Villegas, J., Sanchez, R. 2014. Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. Theriogenology 82: 884–889. doi.org/10.1016/j.theriogenology.2014.07.002

Sariözkan, S., Bucak, M.N., Tuncer, P.B., Ulutaş, P.A., Bilgen, A. 2009. The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. Cryobiology 58: 134–138. doi.org/10.1016/j.cryobiol.2008.11.006

Sharafi, M., Zhandi, M., Sharif, A.A. 2015. Supplementation of soybean lecithin-based semen extender by antioxidants: Complementary flowcytometric study on post-thawed ram spermatozoa. Cell Tissue Bank 16: 261–269. doi.org/10.1007/s10561-014-9458-5

Silagadze, D. 2022. Major challenges of agriculture. Georgian Scientists 4: 145–153. doi.org/10.52340/gs.2022.04.02.11

Thu, N.V., Dong, N.T.K. 2011. Rabbit: Technology for Raising and Processing Products. Agriculture Press. Ho Chi Minh City, Vietnam.

Topraggaleh, T.R., Shahverdi, A., Rastegarnia, A., Ebrahimi, B., Shafiepour, V., Sharbatoghli, M., Esmaeili, V., Janzamin, E. 2014. Effect of cysteine and glutamine added to extender on post-thaw sperm functional parameters of buffalo bull. Andrologia 46: 777–783. doi.org/10.1111/and.12148

Tran, T.T.K., Chiem, T.N., Tran, V.B.N., Duong, N.D.T. 2023. Cryopreservation of local black rabbit spermatozoa in the Mekong delta region of Vietnam: Evaluating cryoprotectant effectiveness. Reprod. Domest. Anim. 58: 1139–1145. doi.org/10.1111/rda.14415

Tuncer, P.B., Bucak, M.N., Buyukleblebici, S., et al. 2010. The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. Cryobiology 61: 303–307. doi.org/10.1016/j.cryobiol.2010.09.009