World Rabbit Sci. 2023, 31: 229-236
doi:10.4995/wrs.2023.19690
WRSA, UPV, 2003

IMPROVING INDIGENOUS VIETNAMESE BLACK RABBIT FROZEN SPERM QUALITY: THE ROLE OF GLYCINE AND SPERM SELECTION METHODS

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Abstract: Rabbit sperm are known to undergo damage during both cryopreservation and thawing, leading to decreased viability, motility and membrane integrity. Glycine can protect sperm and reduce damage during freezing. Swim-up is a simple semen processing method for selecting good motile sperm. The study evaluated the effect of the swim-up method and glycine with different concentrations supplemented to the frozen medium. Three indigenous black rabbits were selected for semen collection by artificial vagina. Next, semen was selected by swim-up method and diluted with glycine-added frozen medium. The samples were then transferred to 0.5 mL straws, cooled to 15°C and 5°C, placed in liquid nitrogen vapour, and finally placed directly into liquid nitrogen (-196°C). The samples were thawed and evaluated for sperm quality. The results showed that the medium supplemented with 10mM glycine in combination with swim-up method for 30 min gave the best results and was significantly different from the remaining concentrations (P<0.01), with viability rate, overall mobility and membrane integrity of 68.0%, 58.7% and 49.7%, respectively. In conclusion, 10 mM glycine concentration combined with swim-up for 30 min is the optimal choice for freezing local black rabbit semen. The study highlights the importance of optimising freezing protocols to improve the quality of frozen rabbit sperm, which can have important implications for animal breeding and conservation efforts.

Key Words: cryopreservation, glycine, indigenous black rabbit, rabbit sperm, swim-up.

INTRODUCTION

Rabbit farming has become increasingly important in the Mekong Delta of Vietnam due to its potential to provide a sustainable source of income for farmers in the region. This is especially important considering the challenges faced by the agriculture sector, such as climate change, soil degradation and population growth (Silagadze, 2022). The indigenous black rabbit is a domestic rabbit breed originating in Vietnam. In Vietnam, the rabbit farming industry has a total production value of 3.24‰ of the gross national product, ranking first among Southeast Asian countries (Lukefahr, 2007). The number of indigenous black rabbits in Vietnam is at risk of decreasing, leading to a decrease in biodiversity and an imbalance in the eco-system. Cryopreservation, such as vitrification, is an important method to preserve the genetic resources of rare animals and restore populations through genetic engineering or artificial insemination. However, the reduced temperature during cold storage inflicts sub-lethal damage on spermatozoa, compromising sperm quality and the success of artificial breeding

Correspondence: T.T.T. Khuong, *tttkhuong@ctu.edu.vn*. Received *May 2023* - Accepted *August 2023.*
*https://doi.org/10.4995/wrs.2023.*19690

Cite as: Khuong T.T.T., Duy N.L.K., Hang N.T., Ngoc P.K, Tuyen D.N. 2023. Improving indigenous Vietnamese Black Rabbit frozen sperm quality: The role of glycine and sperm selection methods. *World Rabbit Sci., 31: 229-236. https://doi.org/10.4995/wrs.2023.19690*

(Rizkallah *et al.*, 2022). Glycine is an amino acid that plays a crucial role in preventing spermatozoa cell damage during cryofreezing and has been reported in goats and rams (Wang *et al.*, 2013; Ugur *et al.*, 2020). Addition of glycine with glycerol in extender improved post-thaw spermatozoa quality. Sperm selection has been used to select the best quality sperm from poor quality semen samples (Podico *et al.*, 2020). A selection method such as swim-up improves the quality of stallion semen by selecting sperm with progressive motility and normal sperm morphology (Hoogewijs *et al.*, 2012). There have been many studies on the application of sperm swim-up technique in human, bull and buffalo (Jameel, 2008; Husna *et al.*, 2016; Magdanz *et al.*, 2019). Therefore, this study was carried out to find out the swim-up time and the optimal concentration of glycine for the best indigenous black rabbit sperm quality during cryopreservation, in order to establish a cryopreservation protocol in an endangered species.

MATERIALS AND METHODS

Animals

In light of the concerning decline in the population of local black rabbits, which has made it difficult to acquire a sufficient number of specimens, this study utilised local black rabbit bucks sourced from different regions of the Mekong Delta, Vietnam. The rabbits were gathered at the animal experimental farm of the Stem Cell Lab, Can Tho University. A total of 3 indigenous black male rabbits weighing between 2.5-3.4 kg were included in the study. The rabbits were fed with a recommended nutritional diet from the Department of Animal Sciences, College of Agriculture, Can Tho University. The animals were individually housed in flat-floor cages at the animal experimental farm and exposed to a cycle of 16 h of light and 8 h of darkness. They were provided with a standard diet and a sufficient supply of drinking water. All animals were fully vaccinated against haemolytic diseases and parasites, and the study was conducted with ethical approval for animal care, housing and semen collection procedures under the Animal Welfare Assessment (BQ2022-02/VCNSHTP).

Experimental design

Experiment 1

Semen samples were collected from three healthy male rabbits using an artificial vagina. The samples were taken twice per week at the same time in the early morning (3 ejaculates/male rabbit) to ensure consistent high-quality samples with over 60% motility. The swim-up method, based on Magdanz *et al.* (2019), was utilised. In a 15 mL falcon tube, 1 mL of Tris Citrate Glucose (TCG) medium (pH=7.0) was added, followed by gently injecting 0.5 mL of fresh semen along the tube wall to form two layers. The falcon tube was tilted at a 45° angle to maximise contact between the semen and TCG medium. The swim-up process was conducted at 37°C for three time points: 30 min, 45 min and 60 min. Subsequently, 0.5 mL of the supernatant containing healthy sperm was aspirated and microscopically examined for overall motility, viability and membrane integrity. Each treatment group consisted of samples collected from the three individual rabbits, resulting in a total sample size of nine for each treatment group.

Experiment 2

The sperm selected using the optimised swim-up method from Experiment 1 were diluted with TCG-EggYolk frozen medium supplemented with 5% glycerol and 8% egg yolk. The diluted samples were further supplemented with glycine at concentrations of 0, 5, 10 and 15 mM, achieving a concentration of 10×10^7 cells/mL. The samples were then loaded into 0.5 mL French straws and stabilised at 15°C for 30 min, followed by cooling to 5°C for 60 min. Subsequently, the straws were exposed to liquid nitrogen vapour for 15 min and finally immersed in liquid nitrogen for long-term storage. After 72 h of storage, the samples were thawed at 37°C for 60 s, and the semen quality, including motility, viability and membrane integrity, was evaluated. Each treatment group consisted of samples taken from the three individual rabbits, resulting in a total sample size of nine for each treatment group.

Sperm concentration

The Neubauer haemocytometer counting chamber was fixed at room temperature for 4 min after drawing up 9µL of the sample. Using a microscope with a magnification of 40×, at least 200 intact spermatozoa (with full head and tail) were counted per counting chamber. The WHO guidelines were followed to calculate the sperm count (Ros *et al.*, 2020).

Sperm motility

Sperm motility assessment involved the preparation of two wet mounts in a counting chamber, with a depth of approximately 20 µm, for each sample. The evaluation of sperm motility included the classification of all spermatozoa within a designated field into three categories: progressive motility (PR), non-progressive motility (NP) and immotility (IM). To ensure representative results, a random counting area was selected, avoiding regions where only motile spermatozoa were observed. A rapid evaluation of the field was conducted without waiting for spermatozoa to swim into the assessment area. A minimum of 200 spermatozoa across at least 5 fields were counted in each wet mount. The counting process was repeated on two separate wet mounts and the results were compared. If the variation in the percentage of motility categories fell within an acceptable range, the average values were calculated for each motility classification (PR, NP, and (IM).) (Fumuso *et al.*, 2018).

Sperm viability

The viability of sperm was measured by the Eosin-Nigrosin method (Agha-Rahimi *et al.*, 2014). About 100 spermatozoa in each smear were counted by microscopy (magnification 40×) and the proportion of viable spermatozoa was calculated on the total number of cells.

Sperm membrane integrity

The test was assessed using the Hypo-Osmotic Swelling Test (HOS Test) (Luong and Thu, 2005). A total of 20 µL of semen sample was mixed with 80 µL of HOS solution in an Eppendorf tube and placed in an incubator at 37°C. After incubation, 10 µL of the mixed sample was placed on a glass slide and observed under a microscope. Spermatozoa with intact membranes showed swelling in the tail, while those with damaged membranes did not show swelling.

Statistical analysis

Statistical analysis was conducted to examine the impact of the swim-up method at different time points on semen quality parameters in Experiment 1. Additionally, the effect of glycine concentration was investigated in Experiment 2. A Linear Mixed Model ANOVA was employed to analyse the data, after confirmation of normality and homogeneity of variance. In Experiment 1, the fixed effect was the swim-up time, while the random effects included rabbits and ejaculations. Similarly, in Experiment 2, the fixed effect was glycine concentration, and the random effects were rabbits and ejaculations. Mean comparison was carried out using the Tukey method in the Minitab program (2016). The results are presented as mean±standard error (SE). Statistical significance was set at $P<0.01$, indicating a high level of confidence in the obtained results.

RESULTS

Influence of swim-up on sperm quality

Table 1 presents the results of Experiment 1, displaying the changes in sperm quality (%) before and after the swim-up process at three different time points: 30, 45 and 60 min. The parameters evaluated include concentration, overall motility, progressive motility, viability and membrane integrity. Prior to the swim-up process, the average sperm concentration was 617.94×10^6 cells/mL. Following swim-up, the concentration decreased to 323.19×10^6 cells/mL at 30 min and slightly increased to 354.93×10^6 cells/mL at 45 min. In terms of overall motility, the swim-up process had a significant positive impact. The initial overall motility was 73.69%, which increased to 92.14% at 30 min and remained high at 90.31% at 45 min. However, a slight decline was observed at 60 min, with a value of 87.31%.

Table 1: Sperm quality (%) before and after swim-up (mean±standard error of mean, N=3).

		After swim- up		
	Before swim-up	30 min	45 min	60 min
C	617.94^{b}±1.32	323.19^{a}±11.36	354.93^{a}±14.32	364.38^{a}±15.48
OM (%)	73.69^{a}±0.26	92.14^{c}±0.42	90.31^{c}±0.74	87.31^{b}±0.56
PM (%)	65.80^{a}±0.14	86.48^{d}±0.61	83.56^{c}±0.68	79.39^{b}±0.78
V (%)	77.91^{a}±0.20	94.71^{d}±0.32	91.78^{c}±0.21	89.03^{b}±0.54
MI (%)	66.21^{a}±0.16	83.73^{d}±0.26	80.66^{c}±0.32	78.25^{b}±0.15

^{a,b,c,d}Values within rows with different superscripts are different; $P<0.01$. C: Concentration ($\times 10^6$ cells/mL); OM: Overall Motility; PM: Progressive Motility; V: Viability; MI: Membrane Integrity.

Progressive motility exhibited a similar pattern. The mean progressive motility prior to swim-up was 65.80%, which significantly improved to 86.48% at 30 min. However, it gradually decreased to 83.56% at 45 min and further declined to 79.39% at 60 min. Viability and membrane integrity also demonstrated improvement after the swim-up process. The initial viability was 77.91%, which increased to 94.71% at 30 min and remained relatively high at 91.78% at 45 min. However, viability decreased to 89.03% at 60 min. Similarly, membrane integrity increased from 66.21% before swim-up to 83.73% at 30 min. Subsequently, there was a gradual decline at 45 min (80.66%) and 60 min (78.25%). Overall, the results of Experiment 1 indicate that the swim-up method effectively enhanced sperm quality in terms of motility, viability and membrane integrity.

Effect of glycine concentrations (0, 5, 10, 15mM) on thawed black rabbit sperm quality

The results of Experiment 2 are shown in Table 2 and Table 3. Table 2 presents the results of the sperm quality analysis in freshly collected rabbit semen and after a 30-min swim-up process. The parameters assessed include concentration, overall motility, progressive motility, viability and membrane integrity. In the freshly collected semen, the mean concentration was 610.83×10^6 cells/mL. Following the 30-min swim-up process, the concentration significantly decreased to 337.36×10^6 cells/mL. A similar trend was observed for overall motility, which showed a significant increase from 73.06% in the freshly collected semen to 91.32% after swim-up. Progressive motility also exhibited a substantial improvement, rising from 62.27 to 86.60% after the swim-up process. Viability displayed a notable increase from 77.95% in the freshly collected semen to 93.61% following swim-up. Moreover, membrane integrity showed a significant enhancement, with an increase from 67.13 to 78.72% after swim-up. Statistical analysis confirmed the significant differences between the two groups ($P<0.01$). These findings indicate that the 30-min swim-up process positively influenced sperm quality, as evidenced by the improvements in motility, viability and membrane integrity. Based on these favourable results, the 30-min swim-up method was chosen for the subsequent experiments.

Table 3 provides the findings regarding sperm quality variables (%) in rabbit semen subsequent to cryopreservation utilising varying concentrations of glycine. The variables assessed encompass overall motility, progressive motility, viability and membrane integrity. Prior to cryopreservation, the sperm exhibited favourable outcomes in terms of overall motility (91.32%), progressive motility (86.60%), viability (93.61%) and membrane integrity (78.72%). Nonetheless, following the cryopreservation procedure, a notable deterioration in sperm quality was observed with increasing concentrations of glycine. Analysis of the impact of glycine concentration on sperm quality post-thawing

Table 2: Sperm quality (%) recorded in freshly collected rabbit semen and after 30-min swim-up (mean±standard error of mean, N=3).

	C	OM (%)	PM (%)	V (%)	MI (%)
Fresh semen	610.83^{b}±1.30	73.06^{a}±0.27	62.27^{a}±0.13	77.95^{a}±0.21	67.13^{a}±0.16
30 min Swim-up	337.36^{a}±2.50	91.32^{b}±0.18	86.60^{b}±0.24	93.61^{b}±0.21	78.72^{b}±0.47

^{a,b}Values within columns with different superscripts are different; $P<0.01$. C: Concentration ($\times 10^6$ cells /mL); OM: Overall Motility; PM: Progressive Motility; V: Viability; MI: Membrane Integrity.

Table 3: Sperm quality variables (%) recorded in rabbit semen after cryopreservation with 3 concentrations of glycine (mean±standard error of mean, N=3).

	Before Cryopreservation	After Cryopreservation			
		0mM	5mM	10mM	15mM
OM (%)	91.32^{d}±0.18	48.13^{a}±0.57	54.43^{b}±0.90	58.69^{c}±1.04	50.96^{a}±0.49
PM (%)	86.60^{d}±0.24	31.93^{a}±1.13	37.74^{b}±0.64	42.68^{c}±1.02	34.49^{ab}±0.85
V (%)	93.61^{d}±0.21	57.37^{a}±0.48	62.75^{b}±0.89	67.98^{c}±0.80	58.09^{a}±0.64
MI (%)	78.72^{d}±0.47	40.18^{a}±0.67	44.74^{b}±0.95	49.70^{c}±0.78	43.51^{b}±0.53

^{a,b,c,d}Values within rows for with different superscripts are different; $P<0.01$. OM: Overall Motility; PM: Progressive Motility; V: Viability; MI: Membrane Integrity.

revealed discernible disparities. Specifically, the highest overall motility was observed with 10 mM glycine (58.69%), followed by 5 mM glycine (54.43%) and 15 mM glycine (50.96%), while the lowest overall motility was recorded with 0 mM glycine (48.13%). Similar trends were discerned for progressive motility, viability and membrane integrity, with the optimum outcomes observed at 10 mM glycine and the least favourable outcomes observed at 0 mM glycine. Statistical analysis substantiated the significance of the variations observed between the groups ($P<0.01$). These findings emphasise the influence of glycine concentration during cryopreservation on sperm quality, with an optimal concentration of 10 mM yielding superior outcomes in terms of motility, viability and membrane integrity.

DISCUSSION

Compared with fresh semen, the sperm concentration obtained after swim-up was decreased significantly. The sperm concentration was 337.4×10^6 cells/mL, a drop of 55.23% compared to sperm before swim-up (610.8×10^6 cells/mL). Indeed, after swim-up, the sperm concentration was reduced by nearly half because only sperm with good motility could swim up. However, the motility and viability of sperm were very high, both above 90%. This result is consistent with the studies of Butt *et al.* (2016) and Khanam *et al.* (2018), when comparing the efficiency of sperm preparation methods, as the swim-up method gave an average motility of about 92.28%; morover, the sperm concentrations before and after swim-up method were 106.9×10^6 cells/mL and 54.2×10^6 cells/mL, respectively. The study found that rabbit sperm quality was highest with 30 min' swim-up and statistically significant differences compared with 45 and 60 min ($P<0.01$). These findings are consistent with previous research that observed improved sperm quality was higher at 30 min than at 45 and 60 min (Karabekir and Özgörgülü, 2019). The swim-up technique is easy to perform, cost-effective, and usually recovers a very clean fraction of highly motile spermatozoa (Henkel & Schill, 2003). This method accumulated the most motile sperm in the upper fraction and left sperm with low or no motility in the lower fraction. Sperm that swim up into the upper medium display increased motility, higher average velocity, higher percentage of normal morphology and generate improved fertilisation rates *in vitro* in mammals. Several studies have utilised the swim-up method for cryopreservation of semen in humans (Petyim *et al.*, 2014; Palomar *et al.*, 2018), buffalo (Husna *et al.*, 2016), bull (Chaveiro *et al.*, 2007) and dogs (Dorado *et al.*, 2016). Consequently, the swim-up method serves as an effective model for preparing sperm for cryopreservation, demonstrating its applicability and usefulness in preserving high-quality sperm for future reproductive purposes.

In this study, the frozen extender supplemented with glycine at a concentration of 10 mM gave the most optimal effect on the rabbit sperm quality indices after thawing. Specifically, with respect to motility, it can be seen that the percentage of total motility and the rate of progressive motility of sperm in the treatment with 10 mM glycine supplementation were higher and significantly different from the other concentrations. The progressive motility of sperm in the treatment supplemented 10 mM glycine reached 42.7% (accounting for 72.8% of overall motility). This was higher than the study of Nazif *et al.* (2022), where the progressive motility of sperm was 45.81% (56.77% of overall motility). Besides, according to another study by El-Sheshtawy *et al.* (2008), 5 mM glycine increased motility after thawing, while also improving the plasma membrane and acrosome integrity of spermatozoa. Cryopreservation causes physicochemical damage leading to reduced sperm viability and fertility (Gadea et al., 2011). Research by Khalili *et al.* (2010) demonstrated that the addition of glycine at a concentration of 5-15 mM to Tris frozen extender

improved sperm viability after thawing in Moghani rams. In this study, the viability rate of sperm samples supplemented with glycine was significantly higher than that of the control samples. For frozen extender supplemented with 10, 5 and 15 mM glycine, the viability rates of spermatozoa after thawing were 68.0, 62.8 and 58.1%, respectively, both higher than the viability rate of sperm in control samples control (57.4%). The results of this study are similar to those of Nazif *et al.* (2022), as post-thaw viability and sperm plasma membrane integrity were significantly ($P<0.01$) higher at 10 mM glycine concentrations, followed by 5, 15 and 20 mM glycine concentrations and finally the control group.

In general, thawed spermatozoa were lower in both viability, motility and membrane integrity than pre-frozen sperm. The degree of reduction of these parameters depends on the use of freezing, thawing or different types of cryoprotectants. The decrease in sperm motility could be due to freezing-induced damage to the mitochondrial membrane (Tam *et al*., 2019) and lower sperm viability may be related to changes in membrane structure during freezing and thawing. The cryodamage, which can occur when sperm is frozen, causes structural and molecular alterations in spermatozoa (Ozimic *et al*., 2023). This could be explained by the addition of cryoprotectant during freezing, plus thawing process can damage cell membranes and reduce sperm motility due to osmotic damage of glycerol, which occurs when glycerol enters cells before freezing and during thawing (Di Santo *et al*., 2012). In addition, the sperm cells' susceptibility to lipid peroxidation is increased due to various types of oxidative reactions, which exert pressure on the cell membrane. Furthermore, thawing causes the flow of water from the inside to the outside, increasing the solute concentration and osmotic pressure, leading to injury, dehydration and changes in cell volume.

Semen cryopreservation causes sperm to experience abnormalities and acrosomal damage, thereby reducing sperm quality parameters after thawing (Nazif *et al*., 2022). Sperm viability was reduced by 50% during cryopreservation due to several stressors, e.g. cold shock, oxidative stress and osmotic stress (Kumar *et al*., 2019). The study by Bansal and Bilaspuri (2010) showed that cryopreservation can cause cellular damage through stimulation of cells to produce reactive oxygen species (ROS). Spermatozoa contain large amounts of unsaturated fatty acids in the plasma membrane, making them highly susceptible to ROS stress (Aitken, 2020). ROS induces an oxidative attack on the sperm lipid membrane, leading to the initiation of lipid peroxidation (Bansal and Bilaspuri, 2010). This leads to loss of cell membrane integrity and impaired cellular function, along with impaired sperm motility and apoptosis of spermatozoa (Bucak *et al*., 2010). Oxidative stress in semen occurs due to temperature changes during cryopreservation, resulting in damage to sperm membranes and altered antioxidant profiles. These are some of the factors that lead to reduced sperm motility, lower viability and compromised sperm membrane integrity (Contreras *et al*., 2020). According to some studies by Alonge *et al.* (2019) and Alvarez *et al*. (1995), the plasma membrane of spermatozoa is composed of large unsaturated fatty acids that render it susceptible to oxidation, leading to breakdown of the sperm membrane during the freezing and thawing steps of cryopreservation. In order to improve overall motility after thawing of sperm and avoid damage when a semen extender is used at the appropriate level for cattle, the addition of antioxidants is essential (Zhong and Zhou, 2013). Carotenoids and flavonoids are antioxidants capable of protecting sperm from the damaging effects of cryopreservation and oxidation through co-enzymes and vitamins (Marri and Richner, 2014). Glycine is one of the three main amino acids in the structure of glutathione (GSH). GSH (a small peptide) is the most widely studied antioxidant in cooled semen, mainly in porcine and ovine (Zhang *et al*., 2016; Shi *et al*., 2020). Glycine provides this small peptide with the -SH group, a chemical functional group essential for the scavenging activities of free oxygen radicals in the cell (Silvestre *et al*., 2021). Thus, glycine has an indirect effect on the ability of sperm to resist oxidative stress. Studies of Wang *et al.* (2013) and Ugur *et al.* (2020) shown that glycine has a positive effect on preventing sperm damage during freezing and has been reported in rams and goats. The study by Buranaamnuay (2020) reported that glycine and other amino acids positively affect the quality of thawed semen in goats during cryopreservation.

The study on cryopreservation of rabbit sperm had both strengths and limitations. On one hand, the study showed the effects of glycine through baseline assessments, thereby confirming the role of glycine in improving the motility, viability and integrity of the rabbit sperm membrane. On the other hand, the limitation of this study is that other indicators such as acrosome activity status and DNA fragmentation are needed to provide a more detailed view of the effects of glycine. Besides, it is necessary to expand the experimental animal population to more closely evaluate the influence of glycine on the sperm quality of other rabbit breeds when refrigerated for a long time.

The study's results have significant implications for the preservation of the local black rabbit population and contribute to the broader field of reproductive biotechnology and cryobiology. Further research with larger sample sizes and long-term evaluation of the spermatozoa's viability and fertility is necessary to fully validate the effectiveness of the cryopreservation method developed.

CONCLUSION

In conclusion, it is clear that swim-up method in 30 min and using TCG-EY storage medium supplemented with 10 mM glycine showed a significant beneficial effect on improving sperm quality during cryopreservation.

Acknowledgements: This study is funded in part by the Can Tho University, Code: T2022-133

Authors contribution: Khuong T.T.T.: methodology, writing – original draft, project administration and funding acquisition. Duy N.L.K.: investigation. Hang N.T.: investigation. Ngoc P.K.: writing – review & editing. Tuyen D.N.D.: data curation.

Conflict of interest: The authors declare no conflicts of interest.

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