

Research Article

IMPROVING POST-THAWED SPERM QUALITY IN BOER GOATS: THE ROLE OF THAWING DURATION AND VITAMIN E

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ABSTRACT

Cryopreservation is a widely used technique for the long-term storage of animal genetic material, but freezing and thawing can cause significant damage to sperm cells. This study aimed to optimize thawing protocols and post-thaw conditions to improve the quality of cryopreserved Boer goat sperm. Two thawing durations (30 and 60 seconds) and four concentrations of vitamin E (0 mM, 1 mM, 2 mM, and 3 mM) were tested to determine their effects on sperm motility, viability, and membrane integrity. Semen samples from two Boer bucks were cryopreserved and later thawed at 37°C for either 30 or 60 seconds. Post-thaw, sperm were incubated in TCG medium with vitamin E supplementation. The results showed that sperm thawed for 30 seconds with 2 mM vitamin E achieved the highest motility (63.66%), viability (72.16%), and membrane integrity (46.68%), significantly outperforming other treatments ($p < 0.05$). Although thawing time had no significant effect on membrane integrity, the addition of 2 mM vitamin E consistently improved overall sperm quality. These findings suggest that a 30-second thawing time combined with 2 mM vitamin E supplementation is the optimal protocol for maintaining the quality of cryopreserved Boer goat sperm.

Keywords: Boer goat sperm, cryopreservation, post-thawed sperm, thawing duration, vitamin E.

INTRODUCTION

Cryopreservation plays a crucial role in the long-term preservation of genetic resources, particularly in livestock species, where maintaining genetic diversity is essential for breeding programs, disease resistance, and overall population health (Engdawork *et al.*, 2024). This technology is especially vital for

conserving the genetic material of endangered species, allowing future restoration efforts and preventing the loss of valuable traits (Bolton *et al.*, 2022). Additionally, sperm cryopreservation is widely applied in biotechnological research and artificial reproductive technologies (ART), contributing to advancements in genetic improvement, livestock production,

and reproductive efficiency (Menchaca, 2023). Cryopreservation not only preserves genetic material but also facilitates its efficient transport, supporting sustainable animal genetic resource management.

Cryopreservation poses significant biological challenges to sperm cells, primarily due to the extreme temperature changes during freezing and thawing. These processes can result in mechanical and biochemical damage, reducing sperm motility, viability, and membrane integrity (Morris *et al.*, 2012). Freezing induces ice crystal formation, dehydration, and oxidative stress, which compromise sperm functionality (Hai *et al.*, 2024). Thawing, while necessary for sperm recovery, may worsen damage if performed improperly. Current thawing protocols are limited in their ability to fully restore sperm quality, and there remains a critical need for improvement to ensure higher post-thaw viability.

Thawing time is a key factor in minimizing damage. Warming the cells quickly is necessary to prevent ice recrystallization (Whaley *et al.*, 2021). Additionally, the use of cryoprotectants and antioxidants, such as vitamin E, has been shown to play a protective role during cryopreservation (Penitente-Filho *et al.*, 2014). Vitamin E at a concentration of 2 mM has been shown to reduce oxidative stress by neutralizing free radicals, thereby protecting membrane integrity and enhancing overall post-thawed ram sperm quality (Kurmi *et al.*, 2018). Therefore, optimizing thawing protocols, including thawing duration and the use of cryoprotectants, is essential for improving the efficiency of cryopreservation and ensuring the recovery of functional sperm cells.

The objective of this study is to investigate the impact of thawing duration and vitamin E supplementation on the quality of post-thaw Boer goat sperm. We aim to determine the optimal thawing time and vitamin E concentration that maximizes sperm motility, viability, and membrane integrity. The hypothesis is that shorter thawing times, combined with appropriate levels of vitamin E supplementation, will significantly improve the post-thaw quality of cryopreserved Boer goat sperm compared to longer thawing durations or the absence of antioxidants.

MATERIALS AND METHODS

Chemicals

The study utilized several chemicals, including vitamin E (Merk, Germany), citric acid (Sigma, USA), tris-hydroxymethyl-aminomethane (Biotech, Vietnam), glucose (Sigma, USA), NaOH (Thermo Fisher Scientific, USA), NaHCO₃ (Thermo Fisher Scientific, USA), fructose (Sigma, USA), sodium citrate (Sigma, USA), Eosin Y (China), and Nigrosin (Himedia, USA).

Animals

The subjects of this study were two Boer bucks, each over 12 months of age, sourced from the experimental animal facility of the Stem Cell Laboratory at Can Tho University. Their diet was specifically designed to fulfill the nutritional requirements of adult male goats (NRC, 2007). All bucks were vaccinated against common diseases and underwent regular health checks. Ethical approval was obtained for the animal care, housing, and semen collection procedures, following the guidelines of the Regulation

on Ethics in Animal Experimentation of Can Tho University (CTU-AEC24013).

Experimental design

Semen was collected from the two adult male goats using an artificial vagina technique. Following collection, the semen samples underwent initial examination and evaluation. They were then diluted in a preservation medium and stored in 0.5 mL straws in liquid nitrogen at -196°C . Thawing occurred at 37°C for two time intervals: 30 seconds and 60 seconds. Post-thawing, the sperm samples were incubated for 15 minutes at 37°C in a Tris-Citrate-Glucose (TCG) basal medium supplemented with vitamin E at concentrations of 0 mM, 1 mM, 2 mM, and 3 mM. Sperm quality is assessed within 15 minutes of thawing.

Assessment of sperm concentration

Highly concentrated semen samples are diluted for easier and more accurate evaluation. To determine sperm concentration, 9 μL of the semen sample was loaded into a counting chamber, which was allowed to equilibrate to room temperature for four minutes. To prevent double counting, spermatozoa located precisely on the dividing line between two squares were counted only once. Sperm with heads positioned on the upper and left edges of a square were included in the count. A minimum of 200 intact spermatozoa were evaluated under a microscope at $40\times$ magnification. The sperm count was calculated following the criteria established by the World Health Organization (WHO, 2021).

Assessment of sperm motility

Motility assessments involved creating two wet mounts for each semen sample. Sperm

motility was classified into three categories: immotile (sperm do not move), non-progressive (sperm move but do not swim, only stay in a fixed position), and progressive motility (sperm swim and move everywhere). Each of the two wet mounts was counted separately, and the average results were compiled for analysis (Nguyen *et al.*, 2024).

Assessment of sperm viability

Sperm viability was determined using the Eosin-Nigrosin staining method. Viable spermatozoa appeared white or exhibited partial red or dark pink staining in the neck region, while the head remained unstained. In contrast, dead spermatozoa displayed a reddish or dark pink coloration in the head. The viability percentage was calculated based on the counts observed (Tran *et al.*, 2024).

Assessment of sperm membrane integrity

The Hypo-Osmotic Swelling Test (HOS Test) was conducted to evaluate sperm membrane integrity. A sample comprising 20 μL of semen and 80 μL of HOS solution was placed in an Eppendorf tube and incubated at 37°C for 40 minutes. Following incubation, a 10 μL aliquot of the mixture was placed on a glass slide for microscopic analysis. Spermatozoa with intact membranes demonstrated swelling in the tail region, while those with compromised membranes showed no swelling (Tran *et al.*, 2025).

Statistical analysis

Data analysis was performed using R version 4.3.1 and Excel. The primary factors of interest were the concentrations of vitamin E and thawing times. A Linear

Mixed Model ANOVA was employed for data analysis, with subsequent mean comparisons conducted using the Tukey method in R. Results are presented as mean \pm standard deviation (SD). Statistical significance was established at $p < 0.05$, indicating a high level of confidence in the data collected. Graphs were generated using R 4.3.1.

RESULTS

Fresh semen quality

The samples were assessed for volume, pH, sperm concentration, overall motility, progressive motility, viability, and membrane integrity (Table 1).

Table 1. Fresh sperm quality variables (%). Data are expressed as mean values \pm SD (n = 8).

pH	Volume (mL)	Concentration (*10 ⁹ cells/mL)	Overall motility (%)	Progressive motility (%)	Viability (%)	Membrane integrity (%)
6.99 \pm 0.16	0.79 \pm 0.05	2.70 \pm 0.08	84.28 \pm 0.64	74.40 \pm 0.92	92.53 \pm 0.77	78.50 \pm 0.88

The average pH of the samples was recorded at 6.99 ± 0.16 , which falls within the normal physiological range for goat semen, suggesting suitable conditions for sperm viability. The progressive motility of the sperm samples was quantified at $74.40 \pm 0.92\%$, indicating a high percentage of sperm demonstrating forward movement. The concentration of sperm was measured at $2.70 \pm 0.08 \times 10^9$ sperm/mL. The overall motility, which encompasses both progressive and non-progressive motile sperm, was assessed at $84.28 \pm 0.64\%$, highlighting the overall activity of the sperm population. Viability, an essential indicator

of sperm health, was found to be $92.53 \pm 0.77\%$, suggesting that the majority of the sperm cells were alive and capable of functional activity. The HOS test used to evaluate the integrity of the sperm plasma membrane, yielded a result of $78.50 \pm 0.88\%$, demonstrating that a substantial proportion of sperm maintained their membrane integrity, which is crucial for fertilization potential. All criterias are within the normal value and consistent with the study of Nguyen *et al.* (2024). Figure 1 depicted goat sperm evaluated for viability and membrane integrity.

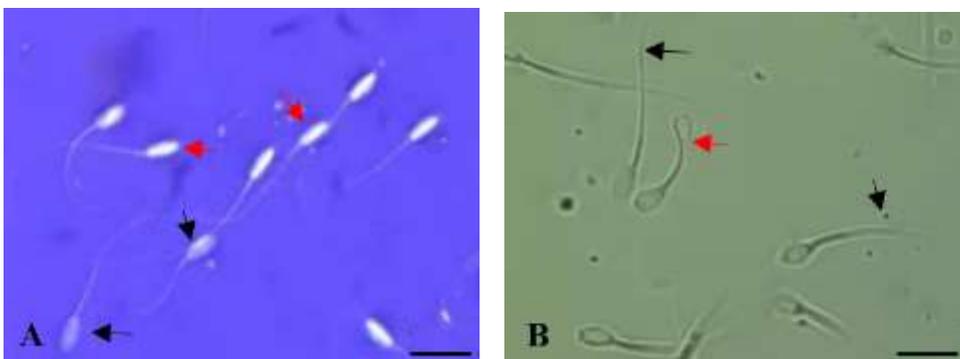


Figure 1. Sperm quality test results. (A) Sperm stained with Eosin-Nigrosin. Live sperm do not take up the dye (red arrow), dead sperm take up the dye (black arrow). (B) Sperm tested for HOS. Sperm with intact cell membranes show a tail curling reaction (red arrow), sperm with damaged cell membranes do not have a reaction (black arrow). Scale bar = 50 μm .

Effect of vitamin E in thawing medium and thawing times on sperm quality during thawing

The results depicted in Figures 2, 3, 4, and 5 showed that vitamin E and thawing time affected sperm quality.

The sperm overall motility showed substantial variation depending on the concentration of vitamin E and thawing time (Figure 2). The highest overall motility was 63.66% in samples treated with 2 mM vitamin E and thawed for 30 seconds,

indicating that vitamin E at this concentration protects sperm from the stresses of thawing. In contrast, the control group (0 mM vitamin E) displayed the lowest motility at 44.43% after 60 seconds of thawing. This drop suggests that the absence of vitamin E leads to greater cellular damage during prolonged thawing. The statistical analysis confirmed that these differences in overall motility across treatments were significant ($p < 0.05$), underscoring the protective role of vitamin E in maintaining motility.

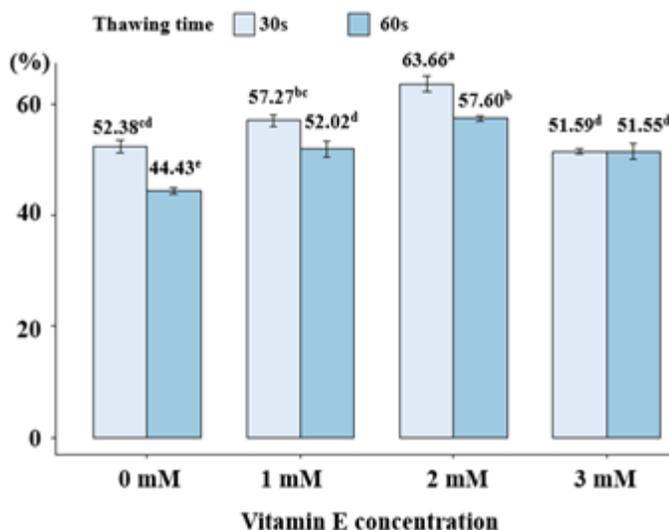


Figure 2. Sperm overall motility after thawing in medium supplemented with vitamin E at 2 thawing times. a, b, c, d, e values for each data with different superscripts are statistically significant differences; $p < 0.05$.

Progressive motility, which measures sperm's capacity for forward movement, followed a similar trend (Figure 3). The highest progressive motility was observed at 50.05% in the 2 mM vitamin E group after 30 seconds of thawing. This demonstrates that vitamin E not only improves overall motility but also enhances the directional movement of sperm. On the other hand, the control group had the lowest progressive motility at 29.53% after 60 seconds of thawing. The statistical analysis showed

highly significant differences between treatments ($p < 0.05$), confirming the beneficial effect of vitamin E on progressive motility during thawing. This study showed that at 3 mM concentration of vitamin E, sperm progressive motility after 60 seconds of thawing was lower than that observed at 30 seconds. This finding suggests that, in addition to antioxidant supplementation, thawing conditions play a critical role in determining post-thaw sperm quality.

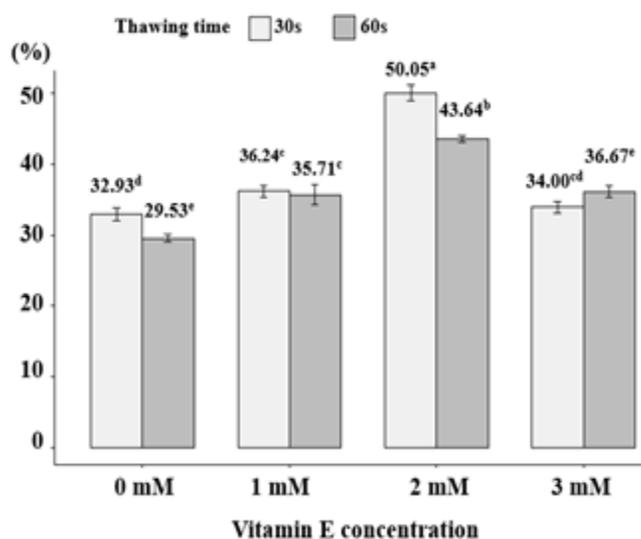


Figure 3. Sperm progressive motility after thawing in medium supplemented with vitamin E at 2 thawing times. ^{a, b, c, d, e} values for each data with different superscripts are statistically significant differences; $p < 0.05$

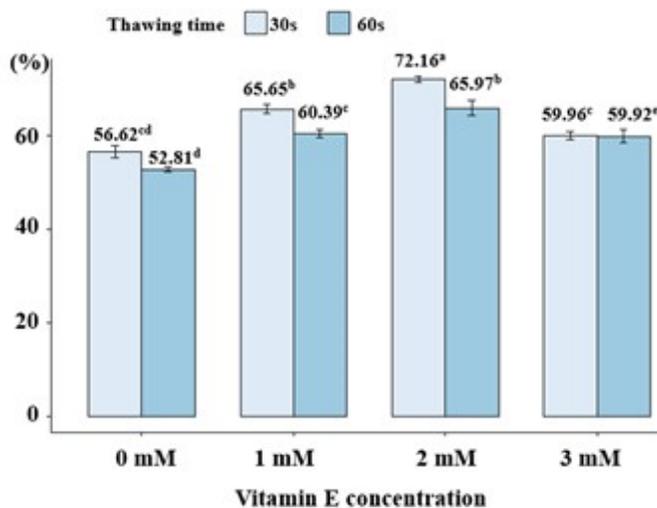


Figure 4. Sperm viability after thawing in medium supplemented with vitamin E at 2 thawing times. ^{a, b, c, d, e} values for each data with different superscripts are statistically significant differences; $p < 0.05$.

Sperm viability was markedly enhanced by vitamin E supplementation (Figure 4). The highest viability, 72.16%, was recorded in the 2 mM vitamin E group after 30 seconds of thawing, suggesting a strong protective effect of vitamin E in maintaining sperm cell function during thawing. By contrast, the

control group showed a much lower viability of 52.81% after 60 seconds of thawing, indicating the negative impact of vitamin E absence on sperm survival. Statistical analysis confirmed the significance of these differences ($p < 0.05$), highlighting the role of vitamin E in preserving sperm viability.

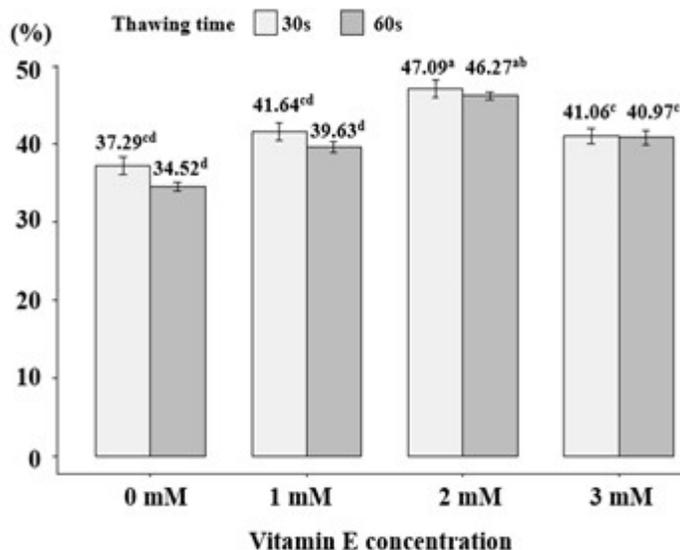


Figure 5. Sperm membrane integrity after thawing in medium supplemented with vitamin E at 2 thawing times. a, b, c, d, e Values for each data with different superscripts are statistically significant differences; $p < 0.05$.

The HOST results also reflected the positive effect of vitamin E on membrane integrity (Figure 5). The 2 mM vitamin E group exhibited the highest membrane integrity, with 47.09% of sperm showing intact membranes after 30 seconds of thawing. This suggests that vitamin E helps maintain the structural integrity of the sperm membrane during thawing. Conversely, the control group, with no vitamin E supplementation, had the lowest membrane integrity at 34.52% after 60 seconds of thawing. However, statistical analysis for membrane integrity did not show significant differences between treatments ($p > 0.05$), indicating that while vitamin E does contribute to membrane preservation, its effect on this parameter was less pronounced compared to motility and viability.

DISCUSSION

In this study, we investigated the effects of different concentrations of vitamin E (0 mM, 1 mM, 2 mM, and 3 mM) and thawing times

(30 and 60 seconds) on the quality of frozen-thawed goat sperm. Our results demonstrate that the addition of vitamin E significantly improves post-thaw sperm quality, particularly in terms of motility, viability, and membrane integrity. The most notable improvements were observed with 2 mM vitamin E, yielding 63.66% overall motility, 72.16% viability, and 47.09% intact membranes, outperforming the control group. These findings suggest that vitamin E at this optimal concentration mitigates cryodamage, primarily by reducing oxidative stress, thereby preserving sperm function.

The present study demonstrated that vitamin E supplementation improved post-thaw sperm quality, with 2 mM identified as the optimal concentration for enhancing progressive motility. This finding contrasts with the results of Penitente-Filho *et al.* (2014), who reported no significant effect of vitamin E on goat semen cryopreservation. The discrepancy may be attributed to

differences in experimental conditions, particularly the range of concentrations evaluated and cryopreservation protocols, as the effectiveness of antioxidants is highly dose-dependent.

The positive effects of vitamin E observed in this study align with the well-documented role of antioxidants in protecting cells from oxidative stress during cryopreservation. Bozkurt *et al.* (2021) and Kalthur *et al.* (2011) both emphasized the ability of vitamin E to enhance sperm motility and viability post-thaw by reducing oxidative damage. This is consistent with our findings, where vitamin E significantly improved sperm function by neutralizing reactive oxygen species (ROS), a critical factor in reducing lipid peroxidation and maintaining membrane integrity during freezing and thawing (Pan *et al.*, 1997; Sanders *et al.*, 2001). The enhancement of these parameters underscores the potential for incorporating vitamin E into cryopreservation protocols to optimize post-thaw sperm performance across species.

However, our results diverge slightly from the findings of Ball *et al.* (2001), who reported that concentrations of vitamin E higher than 2 mM may have adverse effects on sperm quality due to potential pro-oxidant activity. While Ball *et al.* (2001) found that higher concentrations of vitamin E could lead to diminished sperm quality, our results indicate that 2 mM is an optimal concentration for improving sperm function without inducing oxidative stress. This variation could be attributed to differences in species-specific responses, cryopreservation methods, or the antioxidant systems of different sperm samples. Thus, the precise concentration of vitamin E required for optimal sperm preservation appears to be species- and protocol-dependent.

Thawing time also plays a crucial role in determining sperm quality post-cryopreservation. Our data showed that thawing for 30 seconds produced significantly better sperm motility (56.23%) than thawing for 60 seconds (51.40%), consistent with the results of Carlos *et al.* (2020), who reported that shorter thawing durations improve sperm quality in equine and bovine samples. These findings highlight the importance of optimizing both antioxidant concentration and thawing duration for improving cryopreservation outcomes. Furthermore, the interaction between 2 mM vitamin E and a 30-second thawing time produced the highest motility (63.66%), supporting the notion that carefully calibrated protocols are essential for maximizing post-thaw sperm function (Goswami *et al.*, 2021).

The practical implications of these findings are significant. In the context of animal breeding programs, the improved motility and viability achieved through the use of 2 mM vitamin E could lead to higher success rates in artificial insemination and improved reproductive outcomes. This is particularly important for species with high economic or conservation value, where optimizing sperm cryopreservation protocols is critical for genetic diversity and species preservation (Dewry *et al.*, 2020). Moreover, in assisted reproductive technologies, the incorporation of vitamin E into cryopreservation media could enhance fertilization rates by preserving sperm quality and reducing oxidative damage. This has potential applications in both human and animal fertility treatments, as well as in the long-term genetic preservation of endangered species.

One limitation of this study is the relatively small sample size, which may affect the

generalizability of the results. Moreover, the sperm antioxidant capacity was not assessed through ROS assay for evaluating the ability of vitamin E to reduce oxidative damage during thawing. Additionally, this study focused exclusively on Boer goats, meaning that the effects of vitamin E on sperm quality may vary across other species, including humans. Furthermore, only a limited range of vitamin E concentrations (0 mM to 3 mM) was tested, leaving open the possibility that other concentrations could yield different or even more optimal results. Another limitation of this study is the variability in thawing protocols and the impact of semen collection and handling practices. Future studies should address these limitations by increasing sample sizes, testing a broader range of species, and investigating additional concentrations of vitamin E.

Further research is also needed to explore the potential benefits of combining vitamin E with other antioxidants, such as vitamin C or selenium, to enhance cryopreservation outcomes. Additionally, studies should investigate long-term fertility outcomes, including in vivo fertility rates, offspring health, and genetic integrity, to fully understand the practical implications of vitamin E supplementation in cryopreservation protocols. Extending this research to different species and optimizing thawing times, storage conditions, and cryoprotectant formulations could further enhance the effectiveness of antioxidant-based cryopreservation strategies across diverse contexts.

CONCLUSION

In conclusion, our study demonstrates that supplementation with 2 mM vitamin E significantly improves the post-thaw quality of frozen Boer goat sperm, particularly in

terms of motility, viability, and membrane integrity. The optimal combination of 2 mM vitamin E and a 30-second thawing duration effectively mitigates oxidative stress, highlighting its potential as a key additive in cryopreservation protocols. These findings have practical applications in enhancing reproductive success in animal breeding, fertility treatments, and genetic preservation efforts. Future research should investigate the synergistic effects of vitamin E with other antioxidants and adapt protocols for species-specific requirements to expand the applicability of these results across diverse biological and ecological contexts.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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