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Effect of dilution ratio, storage time and storage temperature on goat sperm quality

Tran Thi Thanh Khuong*, Nguyen Thi Bich Thuy

Stem Cell Laboratory, Institute of Food and Biotechnology, Can Tho University, Campus II, 3/2 Street, Ninh Kieu Ward, Can Tho City, Vietnam

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Abstract:

Artificial insemination is a technique that facilitates the rapid development of livestock herds, including goats, improves breeds, and produces healthy, high-yield livestock. The liquid storage of semen is an effective method for short-term preservation to support artificial insemination. The Mekong delta offers natural conditions favourable for goat farming. This study aimed to determine the optimal temperature, dilution ratio, and storage duration for preserving goat sperm in Tris-Citrate-Glucose (TCG) medium. The experiment comprised six treatments, each repeated eight times. After collection, semen samples were diluted in TCG medium at ratios of 1:25, 1:250, and 1:2500, and stored at two temperatures: 5°C and 15°C. Sperm quality was evaluated at six specific preservation intervals: 0, 6, 12, 48, and 72 hours. The results indicated that goat sperm quality was best after 72 hours of storage when diluted at a ratio of 1:25 and stored at 15°C. Specifically, after 72 hours of storage at 15°C, samples with a dilution ratio of 1:250 recorded overall motility, progressive motility, viability, and membrane integrity rates of 70.10, 44.79, 72.33, and 48.50%, respectively. In conclusion, the optimal conditions for preserving goat sperm were achieved with a dilution ratio of 1:250 at 15°C for up to 72 hours.

Keywords: **dilution ratio, goat, liquid storage, sperm, storage environment.**

Classification numbers: **3.1, 3.5**

1. Introduction

Goat farming plays a significant role in global agriculture [1], and it is currently thriving in Vietnam. Goats are regarded as reliable suppliers of various valuable products. Goat meat and milk provide essential nutrition, particularly for children, the elderly, and the cosmetics industry [2]. To meet consumer demand and enhance the genetic quality of goat herds in the Mekong delta, it is vital to breed and produce high-yield herds efficiently. Artificial insemination (AI) is a widely adopted and highly successful technique [3].

The development of effective semen storage protocols is critical for advancing artificial insemination techniques. Various diluents, including saline, sodium citrate-yolk, sodium citrate-fructose-yolk, and saccharose - EDTA, have been employed in laboratory and fertility testing [4]. A commonly used medium for liquid sperm storage is Tris-Citrate-Glucose (TCG), which provides adequate nourishment and protection to sperm during storage [5, 6]. Additionally, TCG medium has demonstrated efficacy in maintaining sperm motility and viability [7]. However, determining the optimal dilution ratio, storage temperature, and storage duration is essential to maximise outcomes.

K. Iusupova, et al. (2022) [8] showed that sperm quality of chilled Majorera buck samples corresponded to the donor's age and two distinct extenders: EY (tris-glucose, 12% egg yolk) and CEY (tris-glucose, 12% clarified egg yolk) at 4°C. Semen samples were able to be kept in EY/CEY media at 4°C without experiencing any changes in semen quality after a 96-hour preservation period in both age groups. Following 24 hours of preservation at 4°C, the fertility rate in goats following artificial insemination was approximately 70% and reduced drastically after 96 hours in preservation media. These results are consistent with those from earlier investigations that used 4°C chilled semen for inseminations [9, 10].

Rapid cooling and direct storage at 5°C have been found to reduce sperm motility and increase the proportion

*Corresponding author: Email: tttkhuong@ctu.edu.vn

of morphologically abnormal sperm. Additionally, storage duration affects sperm motility [11]. Artificial insemination performed within 0-12 hours after the onset of oestrus using liquid-stored semen has been shown to result in higher conception rates [10]. TCG medium supplemented with 10% egg yolk provides better semen quality and remains suitable for artificial insemination after 72 hours of storage at 5°C. Preserving goat sperm with dilution medium helps make subsequent experiments highly accurate.

Although semen cryopreservation has many advantages, the limited viability of sperm after freezing poses a significant challenge to the widespread use of frozen semen in AI studies. The storage medium, temperature, and duration provide the nutrients necessary for sperm metabolism, while maintaining pH and osmotic pressure, and thus play a crucial role in preserving goat semen [12, 13]. Based on these foundations, this study was conducted to determine the optimal dilution ratio, storage temperature, and duration for preserving goat sperm.

2. Materials and methods

2.1. Chemicals

Chemicals used in this study include citric acid (Sigma, USA), tris-hydroxyl methyl aminomethane (Biotech, Vietnam), glucose (Sigma, USA), $NaHCO_3$ (Thermo Fisher Scientific, USA), fructose (Sigma, USA), sodium citrate (Biotech, Vietnam), eosin (China), nigrosin (Himedia, USA) and cysteine (Biotech, Vietnam).

2.2. Animals

This study was conducted at the animal experimental farm of the Stem Cell Laboratory, Can Tho University. The study involved 2 male crossbred Boer (♂) x Bach Thao (♀) goats (ID code: 1002 and 1004), aged approximately 2-3 years, with an average weight of 42-45 kg. The goats' diets were formulated to meet the nutritional requirements of mature male goats [14]. Drinking water was provided ad libitum to prevent dehydration, allowing the goats free access to water. The barn facility featured elevated construction for a cooler environment, a roof, mosquito nets, and a clean and hygienic setup. The goats were reared entirely in confinement.

2.3. Experiment design

Semen was collected twice weekly for four months using an artificial vagina maintained at a temperature of 40-42°C. For successful semen collection, a female goat was introduced into the male goat's enclosure during each session. The semen collector positioned the artificial vagina between the male goat's hind legs during ejaculation, after which the female goat was returned to her enclosure. The semen collected was thoroughly evaluated for macroscopic characteristics, including volume, colour, and pH, alongside quality parameters such as sperm concentration, motility, viability, and membrane integrity.

Semen samples were diluted with TCG medium at ratios of 1:25, 1:250, and 1:2500, and stored at temperatures of 5°C and 15°C. Sperm parameters were assessed at six time points: 0, 6, 12, 24, 48, and 72 hours of storage.

The names of the treatments are defined as follows: E1 - dilution ratio 1:2500 and stored at 15°C, E2 - dilution ratio 1:250 and stored at 15°C, E3 - dilution ratio 1:25 and stored at 15°C, E4 - dilution ratio 1:2500 and stored at 5°C, E5 - dilution ratio 1:250 and stored at 5°C, E6 - dilution ratio 1:25 and stored at 5°C.

2.4. Assessment of sperm concentration

A 9 µl semen sample was loaded into the counting chamber, which was then maintained at room temperature for at least 4 minutes. A 40× microscope was used to count at least 200 intact spermatozoa (with full head and tail) per chamber. Sperm located on the dividing lines between two squares were counted once, with sperm whose heads lay on the upper and left dividing lines of a square included to avoid overestimation. The sperm count was determined in accordance with the guidelines established by the World Health Organization (2010) [15].

2.5. Assessment of sperm motility

For each sample, two wet mounts were prepared on the counting chamber, each with a depth of approximately 100 µm. Sperm motility was classified into three categories: progressive motility, non-progressive motility, and immobility. A random counting area was selected, avoiding regions containing only motile sperm, to ensure an objective evaluation. Preliminary examinations were conducted without waiting for sperm to swim into the assessment area. A minimum of 200 spermatozoa was counted from at least five microfields on each wet mount. Counts were repeated for two separate wet mounts, and the results were compared. If the variation between the two counts was within an acceptable range, the mean values were calculated

for each motility parameter, including overall motility and progressive motility [16].

2.6. Assessment of sperm viability

The Eosin-Nigrosin staining method was used to assess sperm viability. A 50 µl aliquot of Eosin-Nigrosin solution was mixed with 50 µl of semen sample and incubated for 30 seconds at 37°C. The mixture was then placed on a glass slide and air-dried. A total of 100 spermatozoa were examined under a microscope and categorised based on staining. Dead spermatozoa were identified by reddish or dark pink colouration in the head region, whereas live spermatozoa were identified by their white appearance or partial red/dark pink staining limited to the neck region, with the remaining head unstained. The percentage of live spermatozoa was calculated [17].

2.7. Assessment of sperm membrane integrity

The Hypo-Osmotic Swelling (HOS) Test was used to assess sperm membrane integrity. An Eppendorf tube containing 80 µl of HOS solution and 20 µl of semen sample was incubated at 37°C for 40 minutes. Following incubation, a 10 µl aliquot of the mixture was placed on a glass slide for microscopic evaluation. Spermatozoa with intact membranes exhibited tail swelling, whereas those with compromised membranes showed no swelling [18].

2.8. Statistical analysis

Data analysis was conducted using Microsoft Excel (2016) and R version 4.3.1. A Linear Mixed Model ANOVA was applied to analyse the data, followed by mean comparisons between treatments using the Tukey method in R.4.3.1. Results are presented as mean ± standard error (SE). Statistical significance was set at $p<0.05$, providing a high level of confidence in the findings. The independent variables in the study were the semen dilution rate and storage temperature. The dependent variables included sperm quality parameters such as motility, viability, and membrane integrity.

3. Results

The quality of fresh semen after collection is shown in Table 1. The results indicated an average pH of 7.0±0.2. Semen with a pH lower than 6.8 or higher than 7.5 is considered abnormal and unsuitable for maintaining sperm vitality and fertility. The sperm concentration was 2.685 ($\times 10^9$ cells/ml), and the semen volume was 0.74±0.03 mL. Similarly, sperm concentration plays a critical role in fertilisation capacity, and the measured values were within normal ranges, aligning with the experimental criteria. The data for pH, volume, and sperm concentration are consistent with the findings of T.T.T. Khuong, et al. (2024) [19].

Table 1. Quality of fresh semen after collection (mean ± SE, N=8).

pH	7.0±0.2
Volume	0.74±0.03 ml
Concentration	2.685±0.07 ($\times 10^9$ cells/ml)

The results of sperm viability assessment by Eosin-Nigrosin staining and sperm membrane integrity assessment by HOS test were showed in Fig. 1.

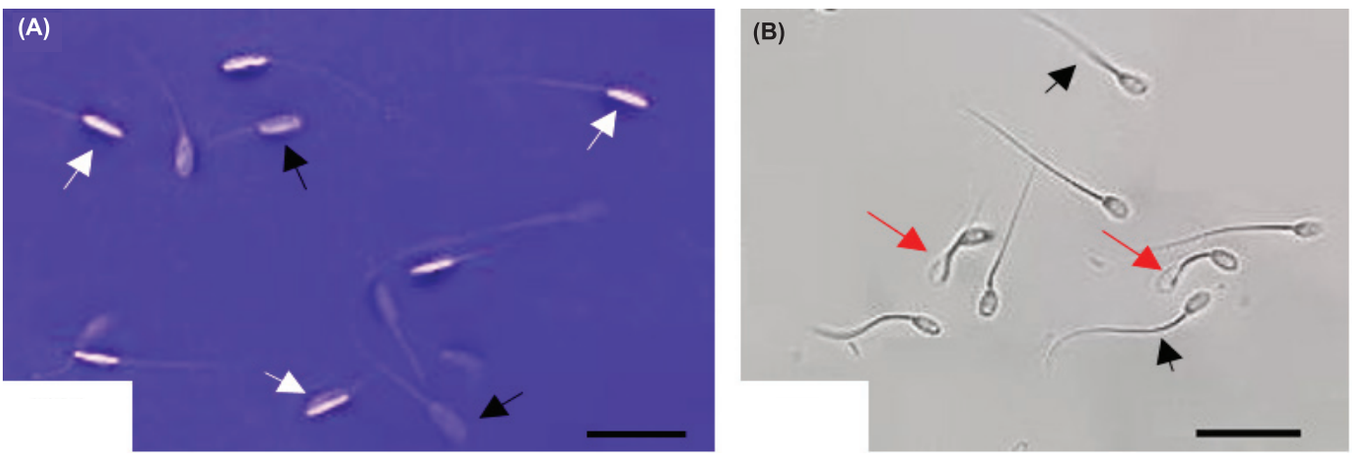

Fig. 1. Assessment of goat sperm quality. (A) Assessment of sperm viability using eosin-nigrosine staining. Live sperm (unstained, white arrow); dead sperm (stained, black arrow); **(B)** Assessment of sperm membrane integrity using the HOS Test. Sperm with an intact membrane (swelling in the tail, red arrow); Sperm has a compromised membrane (no swelling in the tail, black arrow). Scale bar =50 µm.

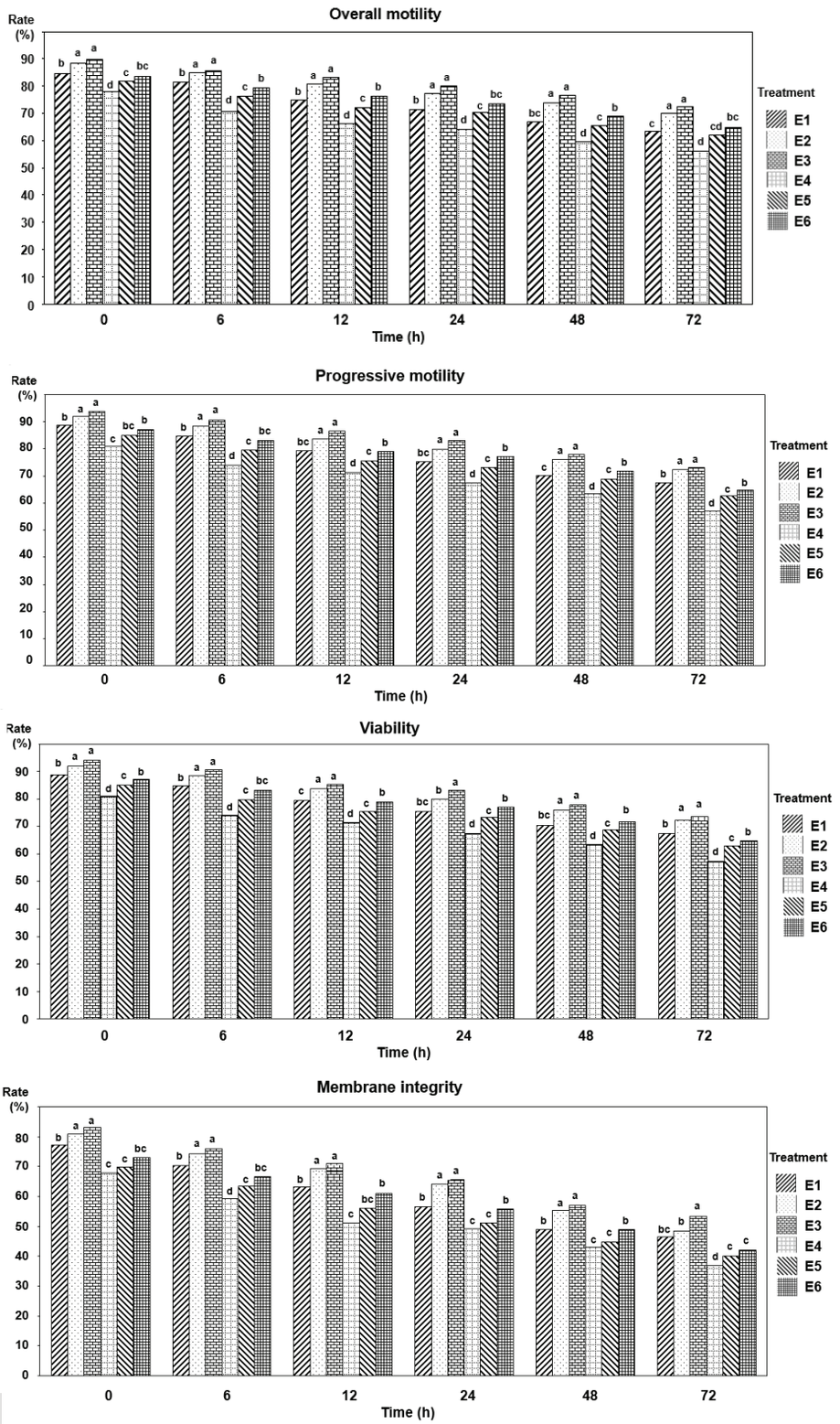

Fig. 2. Sperm quality during time points. [a, b, c, d]: values with the same superscript letters are not significantly different within one timepoint; E1 - dilution ratio 1:2500 and stored at 15°C; E2 - dilution ratio 1:250 and stored at 15°C; E3 - dilution ratio 1:25 and stored at 15°C; E4 - dilution ratio 1:2500 and stored at 5°C; E5 - dilution ratio 1:250 and stored at 5°C, E6 - dilution ratio 1:25 and stored at 5°C.

The assessment of sperm quality during storage, as illustrated in Fig. 2, showed a progressive decline in motility, viability, and membrane integrity over time. After 72 hours of storage, treatment E3 exhibited the highest overall motility (72.50%) and progressive motility (52.45%), whereas treatment E4 recorded the lowest values (56.10% and 29.07%, respectively). Notably, the difference in overall motility between treatments E2 and E3 at 72 hours was not statistically significant ($p>0.05$).

Sperm viability also decreased with increasing storage duration. At 72 hours, treatment E3 maintained the highest viability rate (75.28%), while treatment E4 displayed the lowest rate (57.13%). However, the difference in viability rates between treatments E2 and E3 at 72 hours was not statistically significant ($p>0.05$).

Similarly, membrane integrity declined during the storage period. After 72 hours, treatment E3 achieved the highest membrane integrity rate (53.22%), whereas treatment E4 exhibited the lowest rate (36.93%). As with motility and viability, the difference in membrane integrity between treatments E2 and E3 at 72 hours was not statistically significant ($p>0.05$).

4. Discussion

The results of this study revealed a gradual decline in sperm quality over the storage period, primarily due to damage to the cell membrane. J.H. Qiu, et al. (2016) [20] observed that goat sperm stored at 15°C exhibited higher motility than sperm stored at 5

or 25°C. In this study, for the diluted medium, the differences in sperm quality parameters after 72 hours of storage were not statistically significant ($p>0.05$). Similar findings were reported by I. Salvador, et al. (2006) [21], though their study utilised only PBS phosphate buffer without additional supplements to enhance sperm protection.

Progressive motility is strongly associated with successful fertilisation, making the maintenance of sperm motility a critical aspect of sperm preservation [22]. A comparison of dilution ratios of 1:25 and 1:250 at the same storage temperature of 15°C indicated no statistically significant differences in overall motility, progressive motility, viability, or membrane integrity after 72 hours of storage ($p>0.05$). These findings align with the results of R.J. Aitken (1995) [23]. As previously documented, sperm motility in goats declines as storage time increases [24].

Sperm storage induces molecular changes, including increased plasma membrane fluidity and permeability, overproduction of reactive oxygen species (ROS), reduced acrosome integrity, impaired mitochondrial membrane function, and decreased sperm motility. Minimising or preventing cold-induced damage requires a deeper understanding of the molecular mechanisms affected during storage. Since mature sperm exhibit minimal transcription and translation activity, proteomic studies are a preferred approach to investigate the regulation of sperm function. Additionally, cryopreservation's impact on sperm RNA transcripts warrants exploration, as these transcripts contribute to fertilisation, embryo development, motility, and metabolism. Oxidative damage during liquid storage has multifaceted effects. Beyond membrane damage, oxidative stress disrupts mitochondrial activity, increases intracellular enzyme flux, and weakens axonal proteins, leading to reduced motility [25]. Lipid peroxidation derivatives may diffuse into cellular compartments, forming adducts with protein nucleophiles, which can impair protein structure and function [26].

The findings of this study form the basis for further research on optimising liquid storage techniques for goat sperm in artificial insemination. As noted, treatments E2 and E3 showed no statistically significant differences across all evaluated semen quality parameters at the storage time points. However, using a dilution ratio of 1:250 (E2) allows for the production of more diluted samples, thereby increasing the number of semen doses available for artificial insemination. This makes E2 the most optimal and effective solution. Additionally, this study highlights the potential to adjust dilution ratios, storage temperatures, and durations to enhance goat sperm preservation. However, the research is limited by the small sample size of only two goats, which necessitates caution in generalising the results. Future studies should involve larger populations to better evaluate the effects of storage conditions on sperm quality. Additional evaluation criteria, such as acrosome activity, ROS levels, and DNA fragmentation, would further enhance our understanding of the impacts of different storage media.

Chilled semen has a limited shelf life, which restricts its suitability for prolonged storage or long-distance transport. Reducing storage temperature below body temperature slows cellular metabolism and extends shelf life but may also have adverse effects on cell integrity. Therefore, future research should expand the experimental population and focus on optimising storage conditions to mitigate these challenges. Exploring longer storage durations, lower temperatures, and additional factors affecting preservation could provide more comprehensive insights into goat sperm storage techniques.

5. Conclusions

The results of this study demonstrated that the optimal semen dilution ratio for goat sperm preservation was 1:250, with a storage temperature of 15°C and a maximum effective storage duration of 72 hours.

CRediT author statement

Tran Thi Thanh Khuong: Study concept and design, Data acquisition, Critical revision of the manuscript; Nguyen Thi Bich Thuy: Data analysis, Drafting of manuscript.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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