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EFFECTS OF CYSTEINE ON GOAT SPERM QUALITY IN CRYOPRESERVATION

Tran Thi Thanh Khuong✉, Nguyen Lam Khanh Duy, Le Thi Yen Nhi

Stem Cell Laboratory, Institute of Food and Biotechnology, Can Tho University, Can Tho City, Vietnam

✉To whom correspondence should be addressed. E-mail: tttkhuong@ctu.edu.vn

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ABSTRACT

Cryopreservation is an effective method for long-term storage of sperm. It is essential to investigate the optimal conditions for preserving semen for the purpose of artificial insemination. Cysteine has been proven to reduce the extent of sperm damage caused by oxidative stress during cryopreservation. The aim of this study was to determine the concentration of cysteine required for cryopreservation of goat semen. The experiment was carried out on 2 bucks with 4 treatments, each treatment repeated 8 times. Goat semen was diluted with cryopreservation medium supplemented with cysteine at 4 concentrations: 0 mM, 2 mM, 5 mM, and 10 mM. The sample was transferred to a straw at 0.5 mL, then stored in a liquid nitrogen container. The samples were then thawed, and the sperm quality was evaluated after 72 hours of storage. The results showed that the treatment with 5 mM cysteine showed the best sperm quality results, and the difference was statistically significant with the rest of the treatments ($P < 0.05$). Specifically, the overall motility, viability, and membrane integrity of spermatozoa in the most optimal treatment were: 82.21%, 90.71%, and 76.09%, respectively. In summary, the study showed that adding a 5 mM concentration of cysteine to the cryopreservation medium significantly enhanced sperm quality.

Keywords: cysteine, cryopreservation, goat, oxidative stress, sperm.

INTRODUCTION

Goat farming has been developing in Vietnam, particularly in the Mekong Delta. There is an increasing demand to improve goat breeds in order to produce more productive, disease-resistant, and environmentally-adapted breeds. Artificial insemination and cryopreservation of sperm are particularly important techniques for selecting and improving goat breed quality (Dalen *et al.*, 2021; Anel-Lopez *et al.*, 2017). However, cryopreservation significantly reduced sperm functions due to lipid peroxidation (LPO) caused by reactive oxygen species (ROS). Lipid peroxidation occurs readily in tissues rich in highly oxidizing polyunsaturated fatty acids (PUFAs) (Chatterjee *et al.*, 2001). Sperm cells contain high concentrations of PUFA, which makes them easily oxidized. In addition, every stage of cryopreservation

(cooling, freezing, and thawing) exerts biochemical stress on the membrane and plasma structure of the spermatozoa, resulting in loss of motility, integrity of the spermatozoa membrane, fertilization capacity, and metabolic changes of the sperm (Wang *et al.*, 1997). One strategy to improve goat sperm quality after thawing is to reduce oxidative stress by adding cysteine to the cryopreservation medium. (Kampon *et al.*, 2010). Cysteine is a sulfur-containing amino acid naturally found in seminal plasma and sperm nucleic acids, which helps maintain DNA integrity. Cysteine also acts as an antioxidant directly and/or indirectly, helping to protect spermatozoa from ROS-mediated harmful effects. As a precursor to intracellular glutathione biosynthesis, cysteine increases glutathione levels. It should be mentioned that a significant decrease in endogenous antioxidant levels during freezing-thawing of bovine semen has been reported (Beheshti *et al.*, 2011). However, each animal species requires its own cryopreservation procedure and optimal preservative concentration. In Vietnam, studies on the effect of cysteine on cryopreservation of goat semen are limited. Therefore, this study was carried out to determine the appropriate cysteine concentration for the cryopreservation of goat spermatozoa.

MATERIALS AND METHODS

Chemicals

The chemicals used in the study included Citric Acid (Sigma, USA, PHR1071), Cysteine (Biotech, Vietnam), D-glucose (Thermo Fisher Scientific, USA, A16828.36), Eosin Y (Himedia, India, GRM938), Fructose (Sigma, USA, PHR1002), $NaHCO_3$ (Sigma, 89 USA, S6014), NaOH (Sigma, USA, S5881), Nigrosine (Himedia, India, GRM247), Sodium Citrate 90 (Biotech, Vietnam), and Tris-hydroxylmethyl aminomethane (Biobasic, Canada, TB0196).

Animals

The study involved 2 crossbred bucks Boer (♂) x Bach Thao (♀) goats, aged approximately 2–3 years, with an average weight of 42–45 kg. The rations for bucks were formulated to meet the nutrient requirements of mature male goats (NRC, 2007). Bucks were fed three times per day according to rations. Drinking water was fully prepared to prevent goats from getting thirsty. The barn area is built to be tall, and cool, with a roof, mosquito nets, and cleanliness. Bucks were fully vaccinated against diseases and regularly monitored for health. Ethical approval was obtained for the animal care, housing, and semen collection procedures, following the guidelines of the Animal Welfare Assessment (BQ2022-03/VCNSHTP).

Experimental design

In this study, semen was collected from the bucks twice a week for four months using an artificial vagina maintained at a temperature of 40–42 °C. To ensure successful collection, the does were placed in the buck’s cage, and the semen collector positioned the artificial vagina between the male goat's hind legs. The collected semen was meticulously evaluated for macroscopic characteristics such as volume, color, and pH, as well as semen quality parameters including sperm concentration, motility, viability, and membrane integrity.

For the experimental phase, the sperm samples were diluted with TCG-E medium

(250 mM tris-hydroxymethylaminomethane, 88 mM citric acid, 47 mM D-glucose, 80 mg/L gentamycin, and 10% egg yolk) supplemented with different concentrations of cysteine (0 mM, 5 mM, 10 mM) to achieve a targeted concentration of $10*10^9$ cells/mL. The samples were loaded into 0.5 mL French straws and stabilized through a series of steps: 15°C for 30 minutes, 5 °C for 60 minutes, placed on nitrogen vapor for 15 minutes, and finally plunged into liquid nitrogen. After 72 hours of freezing period, the samples were thawed at 37 °C for 60 seconds and evaluated for semen quality. Subsequent assessments were set to focus on evaluating sperm motility, viability, and membrane integrity, which provided valuable insights into the influence of cysteine concentration on sperm quality during the cryopreservation process.

Assessment of sperm concentration

After loading 9 µL of the sample, the counting chamber was allowed to equilibrate at room temperature for 4 minutes. Using a microscope with 40× magnification, a minimum of 200 intact spermatozoa (with complete heads and tails) were counted per counting chamber. To avoid double-counting, spermatozoa located on the dividing line between two squares were counted once. In contrast, those with heads positioned on the dividing line above and to the left of a square were included in the count. The sperm count was determined according to the guidelines established by the World Health Organization (WHO, 2010).

Assessment of sperm motility

For each sample, two wet mounts were prepared on a counting chamber, each with a depth of approximately 20 µm. The evaluation of spermatozoa motility involved categorizing them into three types: progressive motility, non-progressive motility, and immotility. To ensure objectivity, a random counting area is selected. A preliminary examination was conducted in each field without waiting for spermatozoa to swim into the evaluation area. A minimum of 200 spermatozoa from at least five fields in each wet mount were counted. The count was repeated twice on two different wet mounts, and the results of the two mounts were compared. If the variation in the percentage of samples fell within an acceptable range, the average was calculated for each motility classification (Fumuso *et al.*, 2018).

Assessment of sperm viability

Sperm viability was assessed using the eosin-nigrosin method. A volume of 50 µL of the semen sample was mixed with 50 µL of eosin-nigrosin solution and allowed to incubate for 30 seconds. Subsequently, the mixture was placed on a glass slide and air-dried. Under a microscope, 100 spermatozoa were examined and categorized. Live spermatozoa were identified by their white appearance or partial red or dark pink staining in the neck region, while the remaining head portion remained unstained. In contrast, dead spermatozoa exhibited a reddish or dark pink coloration in the head region. The percentage of live spermatozoa was calculated based on the observed counts (Agha-Rahimi *et al.*, 2014).

Assessment of sperm membrane integrity

The Hypo-Osmotic Swelling Test (HOS Test) was employed to assess the sample. An Eppendorf tube containing 20 µL of semen

sample and 80 µL of HOS solution was placed in a 37 °C incubator. After 40 minutes of 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 did not display any swelling (Ramu, Jeyendran, 2013).

Statistical analysis

Data analysis was performed using Excel and the R 4.3.1 program. The main factor examined was the effect of cysteine concentration. A Linear Mixed Model ANOVA was employed to analyze the data, followed by mean comparisons between treatments using the Turkey method in the R 4.3.1 program. The results are presented as mean ± standard error of mean (SEM). Statistical significance was set at $p < 0.05$, indicating a high level of confidence in the obtained results. The graphs were constructed using the R 4.3.1 program.

RESULTS

Fresh semen quality

The average volume, overall motility, progressive motility, viability, and membrane integrity of goat ejaculated spermatozoa are shown in Table 1. The results showed that the average pH was 6.96. Semen with a pH below 6.8 or above 7.5 is considered abnormal. This can negatively impact the vitality and fertility of sperm.. The mean overall motility of sperm was 85.18%, with progressive motility accounting for over 70% of the overall motile sperm. The average viability was 93.51%, indicating that the majority of sperm were survived. The mean membrane integrity was 77.3%, indicating that a significant proportion of the sperm cells had intact membranes. In general, the quality evaluation parameters are sufficiently rigorousand meet the standards of macroscopic surveys to perform the experiment.

Table 1. Sperm quality variables (%) recorded in freshly collected goat semen

Color	pH	Volume	Concentration (*10^9 cells/ mL)	Overall motility (%)	Progressive motility (%)	Viability (%)	Membrane integrity (%)
White	6.96 ±0.04	0.78 ± 0.04	2.69±0.03	85.18 ± 0.67	74.35±0.33	93.51± 0.73	77.3 ± 1.5

Note: Data are expressed as mean values ± SEM (n = 8).

Effect of different cysteine concentrations on frozen-thawed semen qualitative characteristics

The results regarding sperm motility at different cysteine concentrations are shown in Figure 1.

In particular, sperm motility increased significantly with 5 mM cysteine, compared to the other tested cysteine concentrations ($p < 0.05$). The highest overall motility value was observed in the medium supplemented with 5 mM cysteine (79.98%), while the lowest value was recorded in the medium without cysteine addition (67.40%). Similarly, the highest mean progressive motility was detected in the 5 mM cysteine (55.44%) while the lowest value was seen in

the 0 mM cysteine (35.71%). The difference in sperm motility between the three media supplemented with 0 mM, 2 mM, and 10 mM cysteine was not statistically significant.

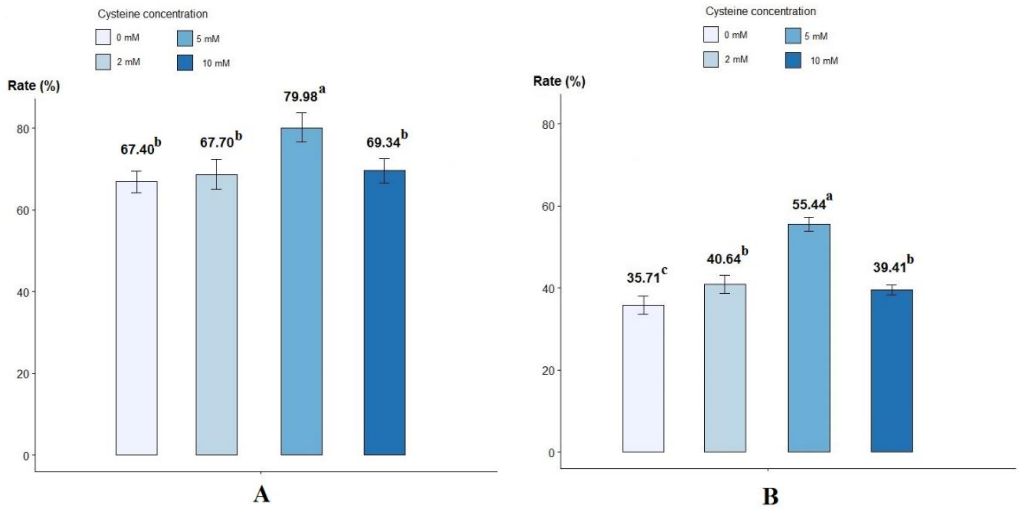

Figure 1. The sperm motility in different cysteine concentrations (n = 8). A. Overall motility; B. Progressive motility. [a, b, c] Values for each data with different superscripts are different; $p < 0.05$.

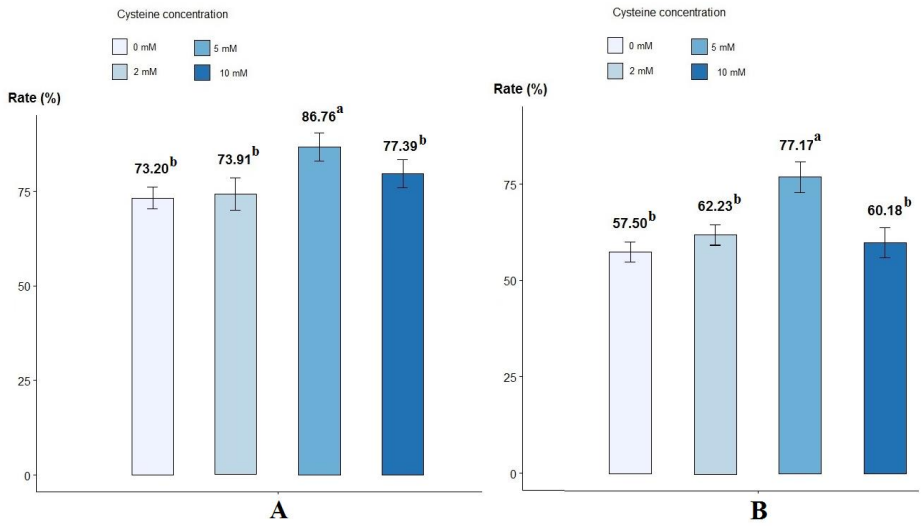

Figure 2. The sperm viability and sperm membrane integrity at different cysteine concentrations (n = 8). A. Viability; B. Membrane integrity. [a,b] Values for each data with different superscripts are different; $p < 0.05$.

The results regarding sperm viability and sperm membrane integrity at different cysteine concentrations are shown in Figure 2.

The highest viability value was observed in the medium supplemented with 5 mM cysteine (86.76%), while the lowest value was recorded in the medium without cysteine addition (73.20%). The difference

in sperm viability between the three media supplemented with 0 mM, 2 mM, and 10 mM cysteine was not statistically significant ($p > 0.05$).

The medium supplemented with 5 mM cysteine significantly increased membrane integrity compared to the control group (0 mM) ($p < 0.05$). The highest membrane integrity value was observed in the medium supplemented with 5 mM cysteine (77.17%), while the lowest value was recorded in the medium without cysteine addition and 90 minutes of cooling time (57.50%).

DISCUSSION

The cryopreserved sperm generally showed a lower quality than of fresh sperm. The study results showed that the addition of 5 mM cysteine in cryopreservation medium resulted in the highest progressive mobility (55.44%). This rate of forward mobility was higher than that of goat spermatozoa in the study of Atessahin *et al.* (2007). In their study, the forward mobility rate of goat spermatozoa was 50% when 5 mM cysteine was used. One of the explanations is that the plasma membrane of mammalian sperm contains high concentrations of polyunsaturated lipids, which makes them extremely susceptible to attack by free radicals, leading to lipid peroxidation (LPO) (Sharma, Agarwal, 1996). LPO reduces membrane integrity and increases permeable fluidity, which can lead to impaired sperm motility and viability (Ohyashiki *et al.*, 1988).

ROS is produced mainly in sperm mitochondria, and in several studies, elevated ROS levels have been reported during the cooling, freezing, and thawing processes in humans, cows, and sheep sperm (Wang *et al.*, 1997; Chatterjee, Gagnon, 2001; Santiani *et al.*, 2014). During cryopreservation, peroxides (a type of ROS) are the most dangerous metabolic free radicals formed, with H_2O_2 being formed in the highest quantities. H_2O_2 can move easily through different compartments and attack molecules inside the cell. To inhibit the formation of ROS and improve sperm quality after cryopreservation, the addition of antioxidants is an essential need (Naijian *et al.*, 2013).

Motility, viability, and membrane integrity are important parameters for assessing sperm quality and function. The results of this study showed that goat sperm exposed to cysteine improved the above three parameters of frozen-thawed sperm. These data indicate that cysteine protects goat spermatozoa from damage during cryopreservation. Many studies showed that the addition of cysteine to the cryopreservation medium increased the motility and membrane integrity of thawed frozen sperm from cats, buffalos, rams, and boars (Thuwanut *et al.*, 2008; Topraggaleh *et al.*, 2014; Sharafi *et al.*, 2015; Kaeoket *et al.*, 2010). The positive effect of cysteine on sperm motility, viability, and membrane integrity after thawing may be due to the fact that cysteine is an amino acid and a precursor of intracellular glutathione which can inactivate ROS and catalyze the detoxification of hydrogen or other superoxides (Atessahin *et al.*, 2007). In addition, cysteine acts as a glutathione peroxidase cofactor that destroys H_2O_2 (Anghel *et al.*, 2010). Furthermore, the increased sperm motility may be due to the cryoprotective effect of cysteine on the functional integrity of mitochondria and axons after thawing (Memon *et al.*, 2012).

Research on cryopreservation of goat sperm has both advantages and limitations. On the one hand, the study shows the effect of

cysteine through baseline assessments, thereby confirming the role of cysteine in improving the motility, viability, and membrane integrity of the goat sperm. However, this study's limitation lies in the need for additional indicators like acrosome activity status and DNA fragmentation to offer a clear view of cysteine's effects. Moreover, it is necessary to expand the experimental animal population in order to more closely evaluate the influence of cysteine on the sperm quality of other rabbit breeds when refrigerated for a long time.

The study showed that when supplementing with cysteine at a concentration of 5 mM, sperm quality was significantly improved in motility, viability, and cell membrane integrity compared with other experiments ($p < 0.05$).

CONCLUSION

The cryopreservation medium supplemented with 5 mM cysteine improved the quality of goat sperm after cryopreservation. Further research should be addressed the addition of other antioxidant compounds to goat sperm cryopreservation medium to develop the best cryopreservation method.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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