EFFECTS OF STORAGE MEDIUM AND STORAGE TIME ON THE GOAT SPERMATOZOA QUALITY

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ABSTRACT

The storage medium for liquid semen and storage time play an important role in maintaining and stabilizing sperm quality during storage. The objective of the study was to determine the carbon source in the appropriate storage environment and optimal storage time to preserve the best sperm quality. The study evaluated the role of three different carbon sources in the storage medium: glucose, fructose and sucrose. This study included 6 treatments corresponding to 6 types of storage media, each solution was repeated 8 times. After collection, semen samples were diluted with storage medium at a ratio of 1:10, then the samples were stored at 15°C. The quality assessment according to specific time points were 0 hours, 6 hours, 12 hours, 24 hours, 48 hours and 72 hours. Research results showed that sperm quality decreases with storage time. The results of microscopic evaluation showed that, after 72 hours of storage, the best quality of goat sperm was recorded in Tris Citrate Sucrose-2 medium. Specifically, the overall motility rate was 71.61%, progressive motility rate was 64.91%, sperm viability rate was 74.50% and the membrane integrity was 57.59%. The results showed that sucrose improved sperm quality better than glucose and fructose, Tris Citrate Sucrose-2 medium helped keep the best sperm quality and the appropriate storage time was 72 hours.

ẢNH HƯỞNG CỦA MÔI TRƯỜNG BẢO QUẢN VÀ THỜI GIAN BẢO QUẢN ĐẾN CHẤT LƯỢNG TINH TRÙNG DÊ

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TỪ KHÓA

Dê
Môi trường bảo quản
Nguồn carbon
Tinh dịch
Tinh trùng

TÓM TẮT

Môi trường bảo quản dạng lỏng và thời gian bảo quản đóng vai trò quan trọng trong việc duy trì và ổn định chất lượng tinh trùng. Mục tiêu của nghiên cứu là xác định nguồn carbon thích hợp và thời gian tối ưu để bảo quản tinh trùng tốt nhất. Nghiên cứu đã đánh giá vai trò của ba nguồn carbon khác nhau trong môi trường bảo quản: glucose, fructose và sucrose. Thí nghiệm được thiết kế bao gồm 6 nghiệm thức tương ứng với 6 loại môi trường bảo quản, mỗi nghiệm thức lặp lại 8 lần. Mẫu tinh dịch sau khi thu nhận được pha loãng với môi trường bảo quản theo tỷ lệ 1:10, sau đó bảo quản mẫu ở nhiệt độ 15°C. Việc đánh giá chất lượng theo các mốc thời gian cụ thể là 0 giờ, 6 giờ, 12 giờ, 24 giờ, 48 giờ và 72 giờ. Kết quả nghiên cứu cho thấy chất lượng tinh trùng giảm dần theo thời gian bảo quản. Đánh giá tinh trùng dưới kính hiển vi cho thấy, sau 72 giờ bảo quản, tinh trùng dê có chất lượng tốt nhất được ghi nhận trên môi trường Tris Citrate Sucrose-2. Cụ thể, tỷ lệ di động tổng số là 71,61%, tỷ lệ di động tiến tới là 64,91%, tỷ lệ sống của tinh trùng là 74,50% và độ nguyên vẹn của màng tinh trùng là 57,59%. Từ nghiên cứu này cho thấy sucrose cải thiện chất lượng tinh trùng tốt hơn glucose và fructose, môi trường Tris Citrate Sucrose-2 giúp giữ chất lượng tinh trùng tốt nhất và thời gian bảo quản thích hợp là 72 giờ.

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1. Introdution

Goats represent a prominent livestock species due to their widespread cultivation, yielding substantial economic returns attributed to their ease of management, heightened productivity, and inherent disease resistance. The goat herd of the Mekong Delta accounts for a fairly large proportion, about 15% of the total herd of the country. Goat farming in the Mekong Delta is mainly on a household scale. Therefore, to be able to achieve productivity in livestock and livestock effectively, it is necessary to breed goats that are suitable for geographical conditions and consumer needs. One of the popular and highly effective methods that can help fast hybridization is artificial insemination (AI) [1]. Timing of artificial insemination relative to the onset of estrus is critical to the success of the procedure because both sperm and ovum have a short lifespan in goat reproduction. Insemination performed between 0-12 hours after the onset of estrus using preserved liquid semen is thought to yield higher conception rates [2]. The quality of sperm after liquid storage depends greatly on the storage medium [3]. The storage medium mainly consists of Tris hydroxymethyl aminomethane, citric acid and a carbon source (usually mono-saccharides or di-saccharides).

Spermatozoa are haploid cells that are highly specialized. The cellular processes of the spermatozoa, such as motility, hyperactivation, capacitation, and acrosome reaction, are necessary for fertilization to be successful [4]. The energy required for sperm to carry out these functions is provided by ATP [5]. The generation of ATP may be achieved either in the presence (aerobic) or in the absence (anaerobic) of oxygen [6]. Glycolysis and oxidative phosphorylation are two metabolic pathways known to generate ATP energy in sperm. The carbon source plays an important role as a substrate and is the main source of raw materials for Glycolysis and oxidative phosphorylation [7].

In studies of liquid sperm storage, the commonly used carbon sources are glucose [8] and fructose [9]. Di-saccharides such as sucrose are considered impermeable to cells. These sugars interact with plasma membrane phospholipids, increasing sperm viability after liquid storage [10]. However, no studies have investigated the effect of sucrose as a useful carbon source in liquid sperm storage medium. Therefore, this study wanted to compare the different effects of glucose, fructose and sucrose in storage medium on the storage quality of liquid goat semen. Moreover, in order to serve artificial insemination technique with high efficiency in livestock production, it is necessary to choose a storage environment with a suitable carbon source and storage time. This study was conducted to find out the best type of storage medium and storage time to keep the best sperm quality.

2. Materials and methods

2.1. Chemicals

The chemicals used in the study included Citric Acid (Sigma, USA, PHR1071), 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), Sucrose (Biotech, Vietnam) and Tris-hydroxylmethyl aminomethane (Biobasic, Canada, TB0196).

2.2. Animals

The study involved 2 male crossbred Boer (♂) x Bach Thao (♀) goats, aged approximately 2-3 years with an average weight of 42-45 kg. The rations of goats were formulated to meet nutrient requirement of matured male goats [11]. Goats were fed 3 times/day according to rations. Drinking water was fully prepared to prevent goats from getting thirsty. The barn facility was constructed to possess elevated dimensions, ensuring a cool environment, and was equipped with a roof, mosquito nets, and maintained cleanliness. Goats were fully vaccinated against diseases,

including Enterotoxemia, Anaplasmosis, Caseous Lymphadenitis, and Foot-and-Mouth Disease. The study received ethical approval for animal care, housing, and semen collection procedures under the Animal Welfare Assessment (BQ2022-03/VCNSHTP).

2.3. Experiment design

In this study, semen was collected from the goats twice a week for a duration of four months using an artificial vagina maintained at a temperature of 40-42°C. To ensure successful collection, the female goat was placed in the male goat's cage, and the semen collector positioned the artificial vagina between the male goat's hind legs. The samples were obtained at the same time in the early morning for 2 weeks (2 ejaculates/goat/week) to ensure consistent high-quality samples. The collected semen was meticulously evaluated for both macroscopic characteristics such as volume, color, and pH, as well as semen quality parameters including sperm concentration, motility, viability, and membrane integrity.

Samples were diluted with storage medium in a ratio of 1:10. This study used 6 different types of storage medium, the composition of which was shown in Table 1.

Table 1. *Composition of storage medium (100mL)*

Medium	Tris hydroxymethyl aminomethane (g)	Citric acid (g)	Carbon source (g)	Gentamycin (µL)	Sterile distilled water
TCG-1	3.0285	1.69	Glucose: 0.8469	200µL	Up to 100mL
TCG-2	3.0285	1.48	Glucose: 1.25	200µL	Up to 100mL
TCF-1	3.0285	1.69	Fructose: 0.8469	200µL	Up to 100mL
TCF-2	3.0285	1.48	Fructose: 1	200µL	Up to 100mL
TCS-1	3.0285	1.69	Sucrose: 0.8469	200µL	Up to 100mL
TCS-2	3.0285	1.48	Sucrose: 1	200µL	Up to 100mL

Semen samples were stored at 15°C and sperm parameters were examined at the following time points: 0 hours, 6 hours, 12 hours, 24 hours, 48 hours and 72 hours of storage.

2.4. Assessment of Sperm concentration

After loading 9µL of sample, the counting chamber was kept at room temperature for at least 4 min. A 40x microscope was used to count at least 200 intact spermatozoa (full head and tail) per counting chamber. Sperm located on the dividing line between the 2 squares are counted once and the sperm whose head is on the dividing line above and to the left of the square to be counted to prevent miscounting the number of sperm in the adjacent cells. The sperm count was determined according to the guidelines established by the World Health Organization [12].

2.5. Assessment of Sperm motility

For each sample, two wet mounts were prepared on the counting chamber, each with a depth of about 20 µm. There are 3 main types of sperm motility: progressive motility (PR), non-progressive motility (NP), and immobility (IM). A random counting area was selected and avoiding areas with only motile sperm for the purpose of ensuring an objective assessment. Preliminary examination is conducted in each field without waiting for sperm to swim into the evaluation area. A minimum of 200 spermatozoa were counted from at least 5 microfields in each wet binding. Counting was repeated twice on two different wet mounts and the results of the two mounts were compared. A mean was calculated for each motion classifier (PR, NP and IM) if the variation in sample rate was within an acceptable range [13].

2.6. Assessment of Sperm viability

The Eosin-Nigrosin method was used for assessment of sperm viability. First, a volume of 50 µL of Eosin-Nigrosin solution was mixed with 50 µL of the semen sample and allowed to incubate for 30 seconds. Then, the mixture was placed on a glass slide and air-dried. 100 spermatozoa were

examined and categorized under a microscope. Dead spermatozoa exhibited a reddish or dark pink coloration in the head region. In contrast, 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. The percentage of live spermatozoa was calculated [14].

2.7. Assessment of Sperm membrane integrity

The Hypo-Osmotic Swelling Test (HOS Test) was used for assessing the sample. An Eppendorf tube containing 80 µL of HOS solution and 20 µL of semen sample 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 [15].

2.8. Statistical analysis

Data analysis was performed using Excel (2016) and the R.4.3.1 program. 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 (SE). Statistical significance was set at $p < 0.05$, indicating a high level of confidence in the obtained results.

3. Results and Discussion

3.1. The quality of fresh semen

The quality of fresh semen after collection was shown in Table 2. The results showed that the average pH was 6.92. If semen has a pH lower or higher between 6.8 and 7.5, it is abnormal semen which is not good for sperm vitality and fertility. The sperm concentration was 2690 (*10^6 cell/ml) and the semen volume was 0.76 mL. In general, the quality evaluation parameters are quite high and meet the standards of macroscopic survey to perform the experiment.

Table 2. *The quality of fresh semen after collection (mean ± SEM, N = 8)*

Color	Milky white
pH	6.92± 0.07
Volume	0.76 ± 0.02 mL
Concentration	2690 ± 0.04 (*10^6 cell/ml)

3.2. The sperm motility during the storage

Storage proccess has a great influence on sperm motility. The results of sperm motility assessment during the storage were shown in Table 3 and Table 4.

Table 3. *Sperm overall motility (%) during time points (mean ± SEM, N = 8)*

Time point	TCG1	TCG2	TCF1	TCF2	TCS1	TCS2
0h	89.95^{bc}±0.23	82.33^{e}±0.22	93.38^{a}±0.35	87.41^{d}±0.33	89.65^{c}±0.28	91.06^{b}±0.30
6h	85.13^{ab}±0.30	79.21^{d}±0.31	86.00^{a}±0.28	81.88^{c}±0.30	84.24^{b}±0.30	85.14^{ab}±0.28
12h	81.14^{b}±0.27	76.72^{d}±0.29	82.90^{a}±0.29	79.03^{c}±0.30	80.98^{b}±0.32	82.00^{ab}±0.30
24h	76.89^{a}±0.27	71.04^{b}±0.24	77.02^{a}±0.25	76.76^{a}±0.25	76.99^{a}±0.23	77.48^{a}±0.29
48h	71.09^{b}±0.28	70.50^{b}±0.26	75.34^{a}±0.28	75.91^{a}±0.28	76.02^{a}±0.26	76.07^{a}±0.28
72h	60.49^{e}±0.29	60.08^{e}±0.27	67.21^{b}±0.30	63.47^{d}±0.27	65.94^{c}±0.31	71.61^{a}±0.29

Note: $^{a, b, c, d, e}$ Values within rows with different superscripts are different; P < 0.05

Nonetheless, following the storage time, a notable deterioration in sperm overall motility was observed (Table 3). Specifically, after 72 hours of storage, the highest overall motility was observed in TCS2 medium (71.61%), while the lowest overall motility was recorded in TCG2 medium (60.08%), the difference was statistically significant ($P < 0.05$).

The results from Table 4 showed that the progressive motility of sperm in all media decreased gradually with storage time. After 72 hours of storage, the progressive motility of sperm in TCS2

medium reached the highest with 64.91%, while TCG2 medium showed the lowest one (49.42%), the difference was statistically significant (P < 0.05).

Table 4. *Sperm progressive motility (%) during time points (mean ± SEM, N = 8)*

Time point	TCG1	TCG2	TCF1	TCF2	TCS1	TCS2
0h	71.04^{c}±0.22	75.08^{a}±0.28	69.70^{d}±0.25	67.79^{e}±0.32	72.73^{b}±0.29	74.52^{a}±0.27
6h	70.33^{a}±0.28	65.80^{c}±0.32	66.59^{cd}±0.29	64.92^{d}±0.28	69.34^{ab}±0.32	69.02^{b}±0.26
12h	65.39^{b}±0.28	63.47^{c}±0.30	65.18^{b}±0.29	63.14^{c}±0.28	66.19^{ab}±0.29	67.15^{a}±0.28
24h	63.25^{cd}±0.29	61.56^{e}±0.25	64.31^{bc}±0.24	62.39^{de}±0.23	64.55^{b}±0.25	66.06^{a}±0.25
48h	59.25^{c}±0.26	56.22^{d}±0.28	63.26^{b}±0.28	60.07^{c}±0.28	63.26^{b}±0.28	65.03^{a}±0.26
72h	50.33^{e}±0.27	49.42^{e}±0.29	61.26^{b}±0.27	55.26^{d}±0.30	57.99^{c}±0.29	64.91^{a}±0.31

Note: $^{a, b, c, d, e}$ Values within rows with different superscripts are different; P < 0.05

3.3. The sperm viability during the storage

The results of sperm viability assessment during the storage were shown in Table 5. The data in Table 5 showed that, sperm viability had dropped significantly following the storage time. Specifically, after 72 hours of storage, the highest viability was observed in TCS2 medium (73.73%), while the lowest one was recorded in TCG2 medium (64.11%), the difference was statistically significant (P < 0.05).

Table 5. *Sperm viability (%) during time points (mean ± SEM, N = 8)*

Time point	TCG1	TCG2	TCF1	TCF2	TCS1	TCS2
0h	92.81^{ab}±0.25	89.07^{d}±0.36	93.68^{a}±0.33	89.44^{d}±0.30	92.34^{bc}±0.36	91.45^{c}±0.27
6h	87.66^{b}±0.31	84.08^{c}±0.30	89.14^{a}±0.28	85.15^{c}±0.27	87.59^{b}±0.33	89.63^{a}±0.33
12h	84.42^{c}±0.29	79.17^{d}±0.33	85.44^{ab}±0.32	82.99^{a}±0.30	84.92^{b}±0.27	85.87^{a}±0.25
24h	76.12^{d}±0.24	72.92^{e}±0.21	80.86^{b}±0.27	79.47^{c}±0.24	78.98^{c}±0.28	82.05^{a}±0.25
48h	72.47^{b}±0.24	73.42^{b}±0.25	78.37^{a}±0.28	78.93^{a}±0.27	79.11^{a}±0.27	79.10^{a}±0.30
72h	63.77^{e}±0.26	64.11^{e}±0.30	71.17^{b}±0.24	66.39^{d}±0.29	69.02^{c}±0.22	73.73^{a}±0.27

Note: $^{a, b, c, d, e}$ Values within rows with different superscripts are different; P < 0.05

3.4. The sperm membrane integrity during the storage

The results of sperm membrane integrity assessment during the stotage were shown in Table 6. Nonetheless, following the storage time, a notable deterioration in sperm membrane integrity was observed. After 72 hours of storage, the percentage of sperm membrane integrity was highest in TCS2 medium (57.59%) and the lowest percentage of sperm membrane integrity was in TCG1 medium (44.67%), the difference was statistically significant (P < 0.05).

Table 6. *Sperm membrane integrity (%) during time points (mean ± SEM, N = 8)*

Time point	TCG1	TCG2	TCF1	TCF2	TCS1	TCS2
0h	70.05^{bc}±0.27	69.95^{c}±0.30	71.33^{b}±0.31	73.83^{a}±0.34	70.54^{c}±0.24	73.24^{a}±0.28
6h	66.99^{c}±0.27	66.73^{c}±0.27	69.15^{b}±0.27	71.09^{a}±0.29	68.81^{b}±0.29	69.65^{b}±0.25
12h	63.64^{d}±0.29	61.90^{e}±0.28	65.91^{c}±0.27	69.61^{a}±0.27	65.79^{c}±0.25	67.91^{b}±0.27
24h	59.41^{c}±0.28	55.66^{d}±0.27	63.28^{b}±0.21	66.49^{a}±0.28	63.41^{b}±0.24	66.36^{a}±0.28
48h	51.78^{c}±0.25	49.61^{d}±0.24	56.04^{b}±0.30	55.91^{b}±0.25	56.45^{b}±0.29	61.52^{a}±0.25
72h	44.67^{e}±0.30	44.91^{e}±0.26	51.26^{c}±0.29	52.99^{b}±0.24	46.37^{d}±0.27	57.59^{a}±0.22

Note: $^{a, b, c, d, e}$ Values within rows with different superscripts are different; P < 0.05

3.5. Discussion

The results showed that sperm parameters decreased gradually with storage time. The decrease in sperm motility, viability or membrane integrity is due to damage to the cell membrane and oxidative stress.

For medium using glucose as carbon source (TCG1 and TCG2), after 72 hours of storage, the differences of sperm quality in all parameters were not statistically significant (P > 0.05). Specifically, after 72 hours of storage, the overall motility of sperm in TCG1 medium was 60.49%,

this result was better than the results of Ngo [8] when studying the effect of dilution medium supplemented with egg yolk and caffeine on goat semen quality. The results showed that the percentage of overall motility of sperm in TCG medium after 72 hours of storage was 56.85%.

For medium using fructose as carbon source (TCF1 and TCF2), after 72 hours of storage, TCF1 medium gave better sperm quality results than TCF2 medium in all parameters, except sperm membrane integrity. Specifically, after 24 hours of storage, the sperm overall motility in TCF1 medium was 77.02%, this result was better than the results of Gororo [16] when studying the effects of different stretching devices and storage temperatures on the shelf life of East African small goat semen. The results showed that the percentage of sperm overall motility in TCF medium after 24 hours of storage was 54.3%. When comparing the effects of TCG and TCF medium on sperm overall motility, TCF medium gave better results than TCG medium, which was consistent with the results of Gororo [16] when showing that the percentage of sperm overall motility in the TCF medium was higher and significantly different from the sperm overall motility in the TCC medium ($P < 0.05$). This showed that fructose is more effective in preserving liquid sperm.

For medium using srucrose as carbon source (TCS1 and TCS2), after 72 hours of storage, TCS2 medium gave better sperm quality results than TCS1 medium in all parameters. Besides, TCS2 medium is the medium that gives the best sperm quality results among the 6 types of media surveyed, the difference was statistically significant ($P < 0.05$). This means that sucrose has a better positive effect than the other two sugars (glucose and fructose) in sperm storage.

Energy intake was responsible for the continued development and function of all living cells and gametes. Two metabolic pathways produce adenosine triphosphate (ATP), which fuels the primary functions of spermatozoa, oxidative phosphorylation and glycolysis. Glycolysis occurs in the cytoplasm of sperm cells and provides energy for sperm metabolism [5]. Fructose and glucose are considered the main sources of energy in sperm cells. There have been many significant advances in semen storage that have been made in the past few decades; however, the decline in sperm quality during this process remains unresolved. The excessive production of reactive oxygen species (ROS) induces oxidative stress (OS), which has been considered as the main factor that deteriorates sperm quality and functionality [17] during the freezing–thawing process and liquid storage [17], [18]. Excessive ROS damages cellular proteins and lipid structures of cell membranes, furthermore, excessive ROS causes gene mutations and accelerates cell death. Sperm motility and sperm cell membrane integrity are important for acrosome response and deeper penetration across the inner membrane of the ovum [19], [20]. Mitochondria play an important role in maintaining normal sperm function and energy homeostasis through oxidative phosphorylation and ATP synthase [21]. During liquid spermatogenesis, spermatozoa lose the ability to generate ATP through mitochondrial respiration due to mitochondrial senescence [22]. The reduction in mitochondrial activity and loss of ATP during liquid spermatogenesis are known to have adverse effects on sperm motility and thus on sperm function [23]. In this study, sperm quality decreased as semen storage time increased, and ROS accumulation resulted in loss of selectivity, permeability, and membrane changes, as well as decreased sperm motility and viability.

The research results can become the basis and foundation for further in-depth studies in the application of liquid goat sperm storage techniques for artificial insemination. The environment for preserving goat sperm is increasingly being improved. Based on the research results, sucrose can become one of the useful carbon sources to replace glucose and fructose in the storage of liquid sperm due to its positive effects. The study provides an overview of these effects, thereby helping to better see the role of carbon sources in the environment and storage time on mobility, viability and cell membrane integrity of goat sperm. Research has found a new discovery, a new application of sucrose in preserving liquid goat sperm.

The study used baseline assessments to check sperm quality. The advantages of these assessments are simplicity, uncomplicated operation and ease of application in not-so-modern farms. Short research time and low cost are also advantages of research. However, the disadvantage of this study is that other evaluation criteria such as acrosome activity status, ROS level and DNA fragmentation are needed to provide a more detailed view of the storage medium effects. Moreover, the study needs to expand the experimental animal population to better evaluate the effect of storage time and environment on the quality of goat sperm. In addition, the study needs to prolong the storage time and lower the storage temperature to further evaluate the influence of other factors on the storage of liquid goat spermatozoa.

In summary, after 72 hours of storage, TCS2 medium still kept the percentage of sperm progressive motility reaching over 60%. TCS2 medium also helped keep the best sperm quality after 72 hours with overall motility rate of 71.61%, viability rate of 74.5% and sperm membrane integrity rate of 57.59%.

4. Conclusion

Sucrose improves sperm quality better than glucose and fructose. TCS2 medium helps sperm keep the best quality during liquid sperm storage at 15°C. The best storage time is 72 hours.

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