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The role of sodium bicarbonate in improving cold storage preservation of indigenous black rabbit spermatozoa

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ABSTRACT

The population of indigenous black rabbits in Viet Nam has been steadily declining, primarily due to the increasing preference for commercial meat rabbit varieties. Consequently, the conservation and systematic breeding of indigenous black rabbits in the Mekong Delta are critical for preserving genetic diversity and sustaining local biodiversity. Sperm conservation is essential for the artificial reproduction of this species. This study aimed to find the optimal concentration of sodium bicarbonate ($NaHCO_3$) buffer added to Tris Citrate Glucose (TCG) storage medium. Samples after collection were diluted with a storage medium containing sodium bicarbonate at concentrations of 0 mM, 15 mM, 25 mM and 35 mM, then refrigerated at 15°C. The results showed that rabbit sperm achieved the best quality when supplemented with 25 mM $NaHCO_3$ after 72 hours of storage. Specifically, the sperms had 59.25% overall motility, 22.34% progressive motility, 62.19% viability and 46.77% membrane integrity, the differences were statistically significant when compared with the remaining treatments ($p<0.05$). The study results indicate that supplementing the TCG preservation medium with 25 mM $NaHCO_3$ significantly improves the quality of sperm during cold storage. This study has contributed to the development of the sperm conservation medium for indigenous black rabbits in the Mekong Delta.

1. INTRODUCTION

Viet Nam, an agriculturally advanced nation, places significant economic emphasis on animal husbandry. The favorable climatic conditions of the Mekong Delta have fostered an environment conducive to rabbit growth and development. The black rabbit is one of the indigenous breeds of rabbit. However, the introduction of foreign rabbit breeds, such as English Spot, Chinchilla, New Zealand, and Californian rabbits, among others, has caused the hybridization of the indigenous black rabbits, resulting in noticeable alterations to both their physical characteristics and behavioral qualities.

Artificial insemination is an essential tool in rabbit husbandry in an effort to restore and stabilize the indigenous black rabbit population. The concept of rabbit artificial insemination became more popular on European farms in the late 1980s (Theau-Clement, 2007). Notably, the pregnancy rates produced by artificial insemination utilizing fresh sperm are on par with or even better than those obtained from natural mating. Beyond the advantages for reproduction, artificial insemination

also avoids the necessity for close animal contact, reducing the likelihood of disease transmission.

Effective semen preservation techniques are essential given the increasing need for semen, a crucial resource for artificial insemination programs in both livestock (Zhao et al., 2009) and rabbits (Di Iorio et al., 2014). Semen quality and fertility rates are compromised by storage times longer than 24 to 48 hours (Rosato & Iaffaldano, 2011; Di Iorio et al., 2014). The choice of an appropriate storage medium, which serves as a barrier against the toxins produced during sperm metabolism as well as against the chilling and osmotic shocks experienced during freezing and transportation, determines the effectiveness of semen preservation.

Due to its possible impact on sperm motility, sodium bicarbonate stands out as an important element. Its effects are dual: CO_2 (Johnson et al., 1983) inhibits sperm motility, whereas HCO_3^- activation promotes it (Okamura et al., 1985; Morisawa & Morisawa, 1988; Ohta et al., 1997). The current study aims to determine the ideal sodium bicarbonate content in the storage medium to obtain higher sperm quality as well as the ideal time for sperm preservation at 15°C. By examining these elements, this research advances our knowledge of improving sperm preservation methods, aiding in the maintenance of the wild population of black rabbits.

2. MATERIALS AND METHOD

2.1. Chemicals

The study utilized chemicals from various suppliers. These included Citric acid, D-glucose, and Fructose from Sigma (USA), Eosin Y and Nigrosine from Himedia (India), $NaHCO_3$ and NaOH from Sigma (USA), sodium citrate and Sucrose from Biotech (Viet Nam), and Tris-hydroxylmethyl aminomethane from Biobasic (Canada).

2.2. Animals

Three rabbits, weighing 2.5-3.4 kg each, were housed individually at the Stem Cell Lab's animal experimental farm, Can Tho University. The rabbits received a standard diet (NRC, 1977), free access to water, and were vaccinated against infectious diseases. 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-AEC24036).

2.3. Experimental design

Semen samples were collected from three healthy rabbits using an artificial vagina, twice weekly in the early morning (3 ejaculates/rabbit), ensuring high-quality samples with over 60% motility. The samples were diluted with TCG medium supplemented with sodium bicarbonate at concentrations of 0 mM, 15 mM, 25 mM, and 35 mM (dilution ratio 1:10) and stored at 15°C. Sperm quality was assessed at 0, 6, 12, 24, 48, and 72 hours in terms of motility, viability, and membrane integrity.

2.4. Assessment of sperm motility

Sperm motility was assessed using two wet mounts prepared on a counting chamber (depth ≈ 20 μm) for each sample. A random counting area was chosen to ensure representative results, with at least 200 spermatozoa counted across 5 fields per wet mount. Results were averaged if the variation fell within an acceptable range (Fumuso et al., 2018).

2.5. Assessment of sperm viability

Sperm viability was determined using the Eosin-Nigrosin method (Agha-Rahimi et al., 2014). Approximately 100 spermatozoa per smear were counted under a microscope (40× magnification), and the proportion of viable spermatozoa was calculated based on the total count

2.6. Assessment of sperm membrane integrity

The Hypo-Osmotic Swelling Test (HOS Test) was conducted by mixing 20 μL of semen sample with 80 μL of HOS solution in an Eppendorf tube, followed by incubation at 37°C for 40 minutes. A 10 μL portion of the mixture was then examined microscopically on a glass slide. Intact sperm membranes were indicated by tail swelling, while compromised membranes showed no swelling. (Ramu & Jeyendran, 2013).

2.7. Statistical analysis

Data were analyzed using a Linear mixed model ANOVA after confirming normality and homogeneity of variance. Mean comparisons between treatments were conducted using the Tukey method in R software (version 4.3.1; R Development Core Team; New Zealand). Sodium bicarbonate concentrations served as fixed effects, with rabbits and ejaculations as random effects. Results are presented as mean ± standard error (SE), with statistical significance set at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Sperm motility

The data in Table 1 showed that prior to storage, the sperm exhibited favorable outcomes in terms of overall motility and progressive motility. Nonetheless, following the storage time, a notable deterioration in sperm motility was observed. Specifically, after 72 hours of storage, the highest overall motility was observed with 25 mM $NaHCO_3$, followed by 15 mM $NaHCO_3$ and 35 mM $NaHCO_3$, while the lowest overall motility was recorded with 0 mM $NaHCO_3$. After 72 hours of storage, the highest progressive motility was observed with 25 mM $NaHCO_3$, followed by 15 mM $NaHCO_3$ and 35 mM $NaHCO_3$, while the lowest overall motility was recorded at 0 mM $NaHCO_3$. After 72 hours, the addition of 25 mM sodium bicarbonate storage medium showed the best overall and progressive motility; the difference was significant from the remaining concentrations ($p<0.05$). According to the standards of World Health Organization (WHO, 2010), the overall motility of sperm when treated with 25 mM sodium bicarbonate supplement for 72 hours still ensures the standard quality.

Table 1. Sperm motility over time of storage at 15°C (mean ± SE)

Survey criteria	Time of storage (hour)	Sodium bicarbonate concentration			
		0 mM	15 mM	25 mM	35 mM
Overall motility (%)	0	84.04 ± 0.51^{c}	87.29 ± 0.51^{b}	90.20 ± 0.54^{a}	85.60 ± 0.59^{bc}
	6	80.32 ± 0.42^{d}	83.58 ± 0.40^{b}	86.60 ± 0.43^{a}	81.49 ± 0.38^{c}
	12	77.37 ± 0.31^{c}	80.34 ± 0.29^{b}	83.13 ± 0.44^{a}	78.60 ± 0.30^{bc}
	24	71.75 ± 0.33^{c}	73.85 ± 0.28^{b}	79.49 ± 0.22^{a}	72.97 ± 0.24^{bc}
	48	52.16 ± 0.29^{b}	54.87 ± 0.32^{b}	69.57 ± 0.21^{a}	53.50 ± 0.23^{b}
	72	40.95 ± 0.28^{c}	43.28 ± 0.31^{b}	59.25 ± 0.33^{a}	42.65 ± 0.39^{bc}
Progressive motility (%)	0	72.98 ± 0.47^{c}	74.94 ± 0.54^{b}	78.40 ± 0.52^{a}	75.41 ± 0.61^{b}
	6	67.46 ± 0.32^{c}	70.93 ± 0.53^{b}	73.24 ± 0.43^{a}	67.95 ± 0.42^{c}
	12	63.62 ± 0.31^{c}	67.91 ± 0.32^{b}	70.37 ± 0.38^{a}	64.96 ± 0.33^{c}
	24	47.90 ± 0.33^{c}	53.48 ± 0.19^{b}	59.91 ± 0.23^{a}	53.28 ± 0.19^{b}
	48	26.53 ± 0.39^{c}	32.33 ± 0.31^{b}	40.92 ± 0.20^{a}	31.77 ± 0.21^{b}
	72	12.55 ± 0.33^{c}	17.01 ± 0.28^{b}	22.34 ± 0.30^{a}	16.29 ± 0.40^{b}

$^{a, b, c, d}$ *Values in the same row with different superscripts are significant difference ($p<0.05$)*

3.2. Sperm viability

The data in Table 2 shows that sperm viability dropped significantly with storage time. Specifically, after 72 hours of storage, the highest viability was observed with 25 mM $NaHCO_3$ (62.19%), followed by 15 mM $NaHCO_3$ (45.78%) and 35 mM $NaHCO_3$ (44.75%), while the lowest viability was recorded with 0mM $NaHCO_3$ (42.95%). After 72 *hours*, the addition of 25 mM sodium bicarbonate storage medium showed the best viability; the difference was significant from the remaining concentrations ($p<0.05$). According to the standards of WHO (2010), the viability of sperm when treated with 25 mM sodium bicarbonate for 72 hours still ensures the standard quality.

Table 2. Sperm viability over time of storage at 15°C (mean ± SE)

Time of storage (hour)	Sodium bicarbonate concentration			
	0 mM	15 mM	25 mM	35 mM
0	86.03 ± 0.51^{c}	89.79 ± 0.52^{b}	92.96 ± 0.49^{a}	87.72 ± 0.62^{c}
6	82.38 ± 0.42^{c}	86.08 ± 0.43^{b}	89.53 ± 0.42^{a}	83.59 ± 0.38^{c}
12	79.37 ± 0.34^{c}	82.84 ± 0.32^{b}	86.07 ± 0.40^{a}	80.70 ± 0.32^{c}
24	73.75 ± 0.29^{b}	76.35 ± 0.22^{b}	82.43 ± 0.21^{a}	58.52 ± 0.18^{b}
48	54.16 ± 0.30^{c}	57.37 ± 0.31^{b}	72.51 ± 0.19^{a}	48.95 ± 0.21^{b}
72	42.95 ± 0.33^{c}	45.78 ± 0.28^{b}	62.19 ± 0.32^{a}	44.75 ± 0.43^{bc}

$^{a, b, c}$ *Values in the same row with different superscripts are significant difference ($p<0.05$)*

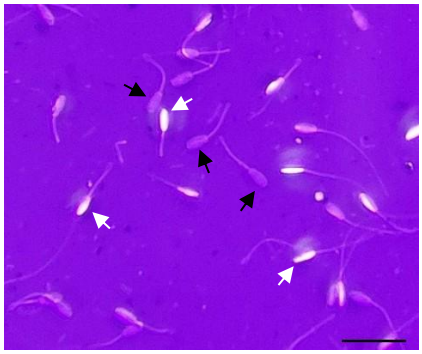

Figure 1. Assessment of sperm viability of indigenous black rabbit spermamotoza using eosin-nigrosine staining. Live sperm (unstained, white arrow); Dead sperm (stained, black arrow). Scale bar = 50µm

3.3. Sperm membrane integrity

The data presented in Table 3 demonstrates a significant reduction in sperm membrane integrity with increasing storage time. Specifically, after 72 hours of storage, the highest membrane integrity was observed with 25mM $NaHCO_3$ (46.77%), followed by 15mM $NaHCO_3$ (40.49%) and 35mM $NaHCO_3$ (39.2%). In contrast, the lowest membrane integrity was recorded with 0 mM $NaHCO_3$ (36.67%). After 72 hours, the addition of 25 mM sodium bicarbonate to the storage medium exhibited the most favorable membrane integrity, displaying a statistically significant difference from the other concentrations ($p<0.05$).

In general, the quality of rabbit sperm exhibited a gradual decline over the storage period. This study investigated the impact of sodium bicarbonate at varying concentrations on the quality of indigenous black rabbit sperm during storage at 15°C. The results indicate that the inclusion of sodium bicarbonate in the storage medium led to an enhancement in sperm quality following storage. Specifically, the addition of 25 mM Sodium bicarbonate after 48 hours of storage resulted in a total sperm motility of 69.57% and a motility rate of 40.92%. Notably, increasing the concentration of sodium bicarbonate to 35 mM led to a significant reduction in sperm motility, indicating the negative effect of excessively high sodium bicarbonate concentrations on rabbit sperm motility.

Table 3. Sperm membrane integrity over time of storage at 15°C (mean ± SE)

Time of storage (hour)	Sodium bicarbonate concentration			
	0 mM	15 mM	25 mM	35 mM
0	73.09 ± 0.67^{b}	75.62 ± 0.71^{b}	80.58 ± 0.45^{a}	74.22 ± 0.72^{b}
6	69.26 ± 0.71^{c}	72.20 ± 0.69^{b}	77.28 ± 0.33^{a}	70.88 ± 0.81^{c}
12	65.02 ± 1.11^{bc}	69.46 ± 0.72^{b}	74.41 ± 0.29^{a}	68.10 ± 0.89^{bc}
24	55.57 ± 0.92^{c}	60.07 ± 0.66^{b}	65.27 ± 0.31^{a}	58.52 ± 0.88^{bc}
48	45.66 ± 1.02^{c}	50.25 ± 0.63^{b}	56.17 ± 0.50^{a}	48.95 ± 1.03^{bc}
72	36.67 ± 0.83^{c}	40.49 ± 0.65^{b}	46.77 ± 0.68^{a}	39.20 ± 0.92^{b}

$^{a, b, c}$ *Values in the same row with different superscripts are significantly different ($p<0.05$)*

The findings of this study are consistent with previous research, such as the study of Hinrichs and Loux (2012), who reported improved sperm quality with the addition of 25 mM sodium bicarbonate. Moreover, further storage with 25 mM sodium bicarbonate exhibited the most positive impact on cryopreservation, demonstrating both sperm viability and cell membrane integrity. Specifically, after 72 hours of storage, the viability of sperm supplemented with 25 mM sodium bicarbonate decreased by only around 30%, and membrane integrity reduced by 33%. In contrast, other preservation methods showed reductions of 42 – 44% in sperm viability and 35 – 37% in membrane integrity after the same storage duration (Di Iorio et al., 2014).

This study is pioneering in its demonstration that rabbit spermatozoa can be effectively preserved using a medium containing a bicarbonate buffer system (HCO3-), which helps maintain a physiological pH of approximately 7.2– 7.4. The bicarbonate buffer system is advantageous due to its presence in the gametophyte or embryo culture fluid within the mammalian body. The study establishes that appropriate concentrations of sodium bicarbonate added to the storage medium significantly enhance sperm motility, viability, and membrane integrity. This protective effect is attributed to the maintenance of the culture medium's pH (pHe).

The discussion underscores the connection between progressive sperm motility and the likelihood of

successful pregnancy (Larse et al., 2000; Zinaman et al., 2000), emphasizing the pivotal role of controlling sperm motility in preservation. Additionally, Sodium bicarbonate's influence on intracellular pH (pHi) is discussed, recognizing pH as an intrinsic factor in spermatogenesis initiation (Lee et al., 1983). Sodium bicarbonate ($NaHCO_3$) dissociation into $CO_2 + H_2CO_3$ (free CO_2), CO_3^- and HCO_3^- in an aqueous solution is noted, with their ratio dependent on pH and sodium bicarbonate concentration in the storage medium. The study emphasizes the role of an appropriate concentration of sodium bicarbonate buffer system in stabilizing pHe, balancing cell membrane osmotic pressure, and regulating sperm pH and activation in vitro.

While the study holds strengths, including its establishment of the sodium bicarbonate buffer system's effect through baseline assessment, confirming its role in enhancing rabbit sperm mobility, viability, and integrity, there are limitations. Notably, the study lacks indications, such as acrosome activity status and DNA fragmentation, for a more thorough examination of sodium bicarbonate effects. Moreover, expanding the experimental animal population would enhance the assessment of the sodium bicarbonate buffer system's impact on sperm quality in different rabbit breeds during prolonged cryopreservation.

4. CONCLUSION

In conclusion, the study findings demonstrate that TCG storage medium supplemented with 25 mM sodium bicarbonate significantly enhances sperm quality during storage. The optimal storage duration identified is 72 hours.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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