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Improving Rabbit Fertility: The Relationship Between Semen Dilution Rate and Artificial Insemination Effectiveness

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ABSTRACT

This study aimed to assess the impact of different dilution ratios of rabbit semen to Tris Citrate Glucose (TCG) extender on doe fertility through artificial insemination in the Mekong Delta region of Vietnam. The experiment included three dilution ratios (1:10, 1:15, and 1:20), with each treatment replicated 12 times. Semen quality variables were evaluated and does were artificially inseminated using a standardized technique. Our findings revealed that the 1:20 dilution exhibited the highest percentage of successful insemination (66.67%) and the shortest gestation period (31.6 days). Furthermore, the 1:20 dilution treatment resulted in an average litter size at birth of 5.25 kits, a litter weight at birth of 290.37 g, and a litter weight at weaning of 1963.04 g. These results suggest that the 1:20 dilution ratio holds promise for artificial insemination in rabbit breeding programs in the Mekong Delta of Vietnam.

Keywords: artificial insemination; dilution ratios; fertility; rabbit breeding; Vietnam

INTRODUCTION

Rabbit farming has become economically significant in the Mekong Delta of Vietnam since 2014, providing sustainable income to farmers in the region amidst challenges such as climate change, soil degradation, and population growth (Silagadze, 2022). With their abilities to digest green roughage efficiently and adapt to diverse climates, rabbits are well-suited for small-scale farming in the delta (Nguyen, 2019). The rabbit farming industry thrives due to low initial investment and a short breeding cycle, providing rapid returns (Lukefahr, 2017). Rabbit meat is highly desirable due to its low cholesterol content and appeal to consumers of all ages (El-Sabrout *et al.*, 2023). Rabbits also have potential for pharmaceutical applications as animal models for experimental drug research (Ros *et al.*, 2020). As the rabbit farming industry expands in the Mekong Delta, it becomes crucial to understand the factors that influence doe fertility in artificial insemination (AI).

Although AI is widely used in large livestock in Vietnam, such as cows (Thanh *et al.*, 2023), pigs (Son *et al.*, 2020), and buffalo (Cai *et al.*, 2011), its use in smaller livestock, particularly rabbits in the Mekong Delta, has received limited research attention (Khuong *et al.*, 2022). Ovulation does not occur spontaneously in does but must be induced by a neuro-hormonal reflex stimulated during mating (Soliman & El-Sabrout, 2020). AI in rabbits brings great benefits, such as reducing the number of males needed, improving the quality of the rabbit herd, and avoiding diseases transmitted through the reproductive tract by direct mating. Compared to natural mating, AI has proven to be more suitable for both small and large commercial rabbit farms (Shuji, 2009). Due to intensive cyclic production and genetic selection for improved prolificacy, the use of AI has increased the output of rabbit farms over the past 20 years (Castellini *et al.*, 2010). The success of AI relies on understanding the mating and reproductive processes of rabbits, as the dilution ratio of semen is a critical factor influencing breeding efficiency and cost. Previous studies have investigated AI in rams, pigs, and cows (Di Iorio *et al.*, 2014; EL-Azzazi *et al.*, 2017; Singh *et al.*, 2021; Hamid *et al.*, 2021). The application of semen dilution in AI has remained largely unexplored in the context of rabbit farming in the Mekong Delta.

The Tris citrate glucose (TCG) extender is a commonly used extender in preserving rabbit semen for AI. The TCG extender is a Tris-buffered extender containing citric acid and glucose (Tran *et al.*, 2023). It is known for providing adequate protection and nourishment to sperm during the dilution process and storage period (Kubovicova *et al.*, 2022; Khuong *et al.*, 2023). The TCG extender has a balanced pH level of 7.0, which ensures an environment conducive to sperm survival and functionality (Roca *et al.*, 2000). The TCG extender has also been shown to effectively maintain sperm motility and viability (Viudes-de-Castro *et al.*, 2021). Several studies have evaluated the effectiveness

of the TCG extender in preserving rabbit semen and compared it to other extenders (Eo *et al.*, 2019; Di Iorio *et al.*, 2020). The results have shown that TCG extender is effective in preserving rabbit semen and maintaining sperm motility and viability. There is no published research on semen dilution rates using the TCG extender in AI on rabbits in the Mekong Delta. Therefore, this study was conducted.

This study addresses a significant research gap by investigating semen dilution rates with the TCG extender in AI of rabbits in the Mekong Delta—an area lacking prior investigation. Our research systematically assesses AI in this context, emphasizing the impact of semen dilution ratios on doe fertility. With limited exploration in current literature, this study enhances understanding and offers insights to improve local rabbit breeding practices. By unraveling the intricacies of insemination, our research contributes to scientific knowledge and fosters sustainable development in the Mekong Delta's rabbit farming. The objective is to evaluate the effects of various semen dilution ratios on doe fertility, to expand knowledge, and to set the stage for advancements in rabbit breeding programs in Vietnam.

MATERIALS AND METHODS

Animals

The study was conducted on 6 local crossbred bucks (New Zealand x Black) and 36 local crossbred does (New Zealand x Black) weighing 2.8 kg, aged 18 months at Cam Nhung rabbit farm, Can Tho City, Vietnam. The animals were individually housed in flat-floor cages and provided with a sufficient supply of drinking water. The rabbit rations were formulated to meet the nutritional requirements of matured male rabbits (NRC, 1977). All animals were fully vaccinated against hemolytic diseases and parasites. The study was conducted with ethical approval for the animal care, housing, semen collection, and AI procedures under the Animal Welfare Assessments (BQ2022-01/VCNSHTP).

Chemicals

The chemicals used in the study include 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), and Tris-hydroxymethyl aminomethane (Biobasic, Canada, TB0196).

Experimental Design

Six healthy bucks were selected for semen collection, which took place twice a week same period using a warmed artificial vagina lubricated with gel and stimulated by a doe (Cathy *et al.*, 2013). One ejaculation from one buck was one repetition. The collected semen samples were then diluted with TCG (TCG: 250 mM tris-hydroxymethyl aminomethane, 88 mM citric acid, 47 mM D-glucose, and 80 mg/L gentamycin) extender to create three treatment groups with dilution ratios of 1:10, 1:15, and 1:20. After incubation for 10 minutes at 37 °C, the diluted semen samples were evaluated to assess quality parameters, including sperm concentration, motility, viability, and membrane integrity. Furthermore, 36 local crossbreeding does were randomly assigned to each treatment group and artificially inseminated with the corresponding diluted semen. Throughout the study, reproductive parameters such as insemination rate, pregnancy duration, and number of newborns per litter were diligently recorded for each treatment group. Additionally, the quality of the offspring was assessed by considering factors such as birth weight, number of animals weaned per litter, and weaning weight per litter. The experiment was designed using a completely randomized approach to ensure unbiased results.

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 (Nikon Eclipse e200, Japan) with 40x magnification, a minimum of 200 intact spermatozoa (with complete heads and tails) were counted per hemocytometer chamber (Neubauer improved, Germany). To avoid double counting, spermatozoa located on the dividing line between two squares were counted once, while 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, 2021).

Assessment of Sperm Motility

For each sample, two wet mounts were prepared on a hemocytometer chamber (Neubauer improved, Germany), each with a depth of approximately 20 μm. The evaluation of spermatozoa motility involved categorizing them into three types: progressive motility (PR), non-progressive motility (NP), and immotility (IM). A random counting area was selected to ensure an unbiased assessment, avoiding areas where only motile spermatozoa were present. A preliminary examination was conducted in each field without waiting for spermatozoa to swim into the evaluation area. A minimum of 200 spermatozoa were counted from at least 5 fields in each wet mount. The count was repeated twice on two different wet mounts, and the results of the two mounts were compared. Then, the average was calculated for each motility classification (PR, NP, and IM) (Fumuso *et al.*, 2018).

Assessment of Sperm Viability

Sperm viability was measured by the Eosin-Nigrosine method (5% Eosin and 5% Nigrosine). Approximately 100 spermatozoa in each smear were counted by microscopy (40× magnification) and the

proportion of viable spermatozoa was calculated based on the total number of cells (Agha-Rahimi *et al.*, 2014).

Assessment of Sperm Membrane Integrity

Sperm membrane integrity was assessed using the hypo-osmotic swelling (HOS) test (Ramu & Jeyendran, 2013). A total of 20 µL of semen sample was mixed with 80 µL of HOS solution (13.5 mM sodium citrate and 35 mM D-fructose) in an Eppendorf tube and placed in an incubator at 37 °C for 30 minutes. After incubation, 10 µL of the mixed sample was applied to a glass slide and observed under a microscope. Spermatozoa with intact membranes showed swelling in the tail, while those with damaged membranes did not show swelling.

Artificial Insemination

In preparation for AI, the doe's cage was positioned adjacent to the buck's enclosure. The doe's receptivity is typically indicated by a red vulva. At this stage, the doe is deemed ready for AI. The procedure for AI in doe rabbits, as outlined by Soliman & El-Sabrout (2020), involves several steps. First, the semen was diluted and stored in a sterile 15 mL Falcon tube, followed by incubation at 37 °C for 10 minutes to allow the sperm to adjust to the temperature of the doe's reproductive organs. Subsequently, the diluted semen was loaded into an insemination straw, which was then inserted into an insemination sheath. The sheath, in turn, was placed in an insemination gun. To initiate the insemination process, a doe was carefully restrained on a table and the base of its tail was grasped firmly. The forearm was aligned along the doe's spine, to facilitate gradual elevation of the hindquarters to achieve the lordosis posture, while ensuring minimal movement from the doe. The insemination gun was then cautiously introduced into the orifice at a depth of 12 cm from the vulva while being angled toward the spine to avoid inadvertent bladder insertion. The sperm was then injected by exerting gentle pressure on the gun piston. After the injection, the gun was slowly withdrawn, and at least one 0.2 mL intramuscular injection of buserelin (Porceptal, Intervet, Germany) was administered to support ovulation.

Statistical Analysis

Data were analyzed using Excel (2016) and the R 4.3.1 program. A linear mixed model ANOVA was used to analyze the data, followed by mean comparisons between treatments using the Tukey 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 confidence level in the obtained results.

RESULTS

The Rabbit Semen Quality

Table 1 provides an overview of the rabbit semen quality at different dilutions in the semen extender. The data showed that the 1:10 dilution exhibited the highest sperm quality while the 1:20 dilution exhibited the lowest, and this difference was statistically significant ($p<0.05$). Specifically, the concentration, viability, overall motility, progressive motility, and membrane integrity of sperm in the 1:10 dilution were 2285.25 (10^6/mL), 98.71%, 83.71%, 66.58%, and 52.08%, respectively. In the 1:20 dilution, the concentration, viability, overall motility, progressive motility, and membrane integrity of sperm were 1104.41 (10^6/mL), 72.02%, 57.02%, 43.60%, and 40.08%.

The Results of Insemination Parameters and Fertility Outcomes

Table 2 presents the results of insemination parameters and fertility outcomes at different dilution ratios in rabbit breeding. Regarding the insemination success rate, the percentages were 41.67%, 50.00%, and 66.67% for the 1:10, 1:15, and 1:20 dilution ratios, respectively. The gestation period ranged from 31.60 ± 0.24 to 32.00 ± 0.19 days across the dilution ratios, with no significant variation observed. The average litter size at birth was 6.60 ± 0.40, 5.50 ± 0.76, and 5.25 ± 0.65 for the 1:10, 1:15, and 1:20 dilution ratios, respectively, with no statistically significant differences. These results indicate that the dilution ratios did not significantly affect the number of inseminations, insemination success rate, gestation period, or litter size at birth in this study.

Table 3 shows the results of reproductive performance and offspring quality in this study, with different dilution ratios used in AI. The data shows that in terms of birth weight, the 1:10 dilution group had the highest litter weight at birth (393.60 ± 22.63 g) compared to the other two groups. However, this difference was not

Table 1. Rabbit semen quality at different dilution rates (mean ± SEM, N=6)

Variables	Dilution rates		
	1:10	1:15	1:20
C (*10^6 sperm/mL)	2288.25±31.3^a	1525.55±30.5^b	1104.41±29.6^c
OM (%)	83.71±0.9^a	68.49±1.0^b	57.02±0.8^b
PM (%)	66.58±1.8^a	55.16±1.9^b	43.60±1.8^c
V (%)	98.71±1.3^a	83.49±1.1^b	72.02±1.2^b
MI (%)	52.08±0.7^a	45.21±0.9^{ab}	40.08±0.8^b

Note: ^{a,b,c}Means in the same row with different superscripts differ significantly ($p<0.05$). C= Concentration; OM= Overall motility; PM= Progressive motility; V= Viability; MI= Membrane integrity.

Table 2. Insemination parameters and fertility outcomes at different dilution rates in rabbit breeding (mean ± SEM, N=12)

Variables	Dilution rates			p
	1:10	1:15	1:20	
ISR (%)	41.67	50.00	66.67	
GP (days)	31.60±0.24	31.67±0.33	32.00±0.19	0.249
LSB (kits)	6.60±0.40	5.50±0.76	5.25±0.65	0.180

Note: ISR= Insemination success rate; GP= Gestation period; LSB= Litter size at birth.

Table 3. Reproductive performance and offspring characteristics in does (mean ± SEM, N=12) at different dilution ratios of semen

Variables	Dilution rates			p
	1:10	1:15	1:20	
LSB (kits)	6.60± 0.40	5.50± 0.76	5.25± 0.65	0.180
LWB (grams)	393.60±22.63	306.01± 20.83	290.37± 36.34	0.104
LSW (kits)	6.60± 0.40	5.33± 0.66	5.13± 0.69	0.146
LWW (grams)	2184.68±52.40	2096.32±142.93	1963.04±127.91	0.210

Note: LSB= Litter size at birth; LWB= Litter weight at birth; LSW= Litter size at weaning; LWW= Litter weight at weaning.

statistically significant. Similarly, there was no significant difference in the litter size at weaning and weaning weight per litter among the three dilution rates. Overall, the data suggests that the different dilution rates used in AI do not significantly affect the reproductive performance and offspring quality in rabbits. However, it is important to note that these results are specific to the experimental conditions and may not be generalizable to other contexts.

DISCUSSION

The study examined the effects of different dilution ratios (1:10, 1:15, and 1:20) on rabbit semen quality in AI. The results showed significant variations in sperm concentration, viability rate, motility rate, and membrane integrity among the dilution ratios. Although the 1:20 dilution resulted in lower sperm quality than the 1:10 dilution, all dilution ratios exceeded the World Health Organization (WHO, 2021) standards for normal semen parameters. In this study, the overall motility of rabbit sperm in TCG extender at 1:10 dilution is over 80%, a result similar to that of Roca *et al.* (2000). These findings highlight the importance of selecting the appropriate dilution ratio to optimize sperm quality and improve fertility outcomes in rabbit breeding.

The observed decrease in sperm quality with higher dilution ratios is consistent with previous research, suggesting that higher dilution can compromise sperm functionality and viability. It is important to note that the WHO (2021) guidelines are designed for assessing human fertility and may not directly apply to rabbits. However, using these standards as a reference provides a useful benchmark for evaluating rabbit semen quality. Overall, the study emphasizes the significant influence of the dilution ratio on rabbit semen quality and underscores the need for careful consideration of the dilution ratio in AI practices to maximize reproductive success.

The results of our study provide insight into the relationship between sperm quality, sperm dilution ratio, and successful insemination in rabbit breeding. The highest quality sperm, including those with the highest concentration, vitality, motility, and membrane integrity, are produced by a 1:10 semen dilution ratio, according to earlier studies (Castellini *et al.,* 2010). This emphasizes how crucial it is to keep sperm quality at its highest level for effective AI. However, a fascinating new finding involving insemination success rates was made from our study. The 1:20 dilution group had the highest insemination success rate even though the 1:10 dilution had better sperm quality. This unexpected finding challenges the widespread assumption that better reproductive outcomes are invariably associated with higher-quality sperm. This unexpected outcome could be attributed to several things. First off, despite being linked to worse sperm quality, the lower sperm concentration in the 1:20 dilution may have produced a less viscous semen sample, facilitated smoother and more effective insemination techniques, and possibly increased the likelihood of successful fertilization (Nosi *et al.,* 2019). The success of AI may have also been influenced by intricate physiological and biochemical processes within the female reproductive system (Soliman & El-Sabrout, 2020). The complex nature of rabbit fertility is highlighted by the complex interactions that can partially mitigate the effects of lower-quality sperm. As a result, while our work highlights the importance of semen dilution ratios in maintaining sperm quality, it also emphasizes the need for a thorough understanding of the variables that affect AI outcomes. The unexpectedly higher insemination success of the 1:20 dilution group challenges long-held beliefs and emphasizes the complex interplay of numerous elements in the reproductive process. To improve the overall effectiveness of this crucial agricultural technique, more research is needed to understand the underlying principles and to optimize AI procedures in rabbit breeding programs.

The success rate of AI in rabbits is influenced by the dilution ratio and choice of extender. This study revealed that although there was no statistically significant difference between treatments, the dilution ratio affected the success rate of insemination. The highest successful insemination rate of 50.69% was achieved with a dilution ratio of 1:20 using a basic TCG extender. The choice of extender can also affect the optimal dilution ratio. For example, Motedayen *et al.* (2007) reported a successful insemination rate of 60% in Dutch rabbits using Galap commercial preservatives at a 1:10 dilution ratio. Variations in the composition and components of storage media and dilution ratios can affect the success rate of insemination. Furthermore, the temperature at which semen is stored plays a crucial role in maintaining its quality and viability. Research conducted by Rosato & Iaffaldano (2011) indicated that storing rabbit semen at 5 °C is preferable to 15 °C for long-term storage in TCG and Cunigel extenders. Therefore, careful consideration should be given to the extender, dilution ratio, and storage temperature when performing AI in rabbits. It is also important to note that the optimal dilution ratio may vary between species.

For instance, AI in sheep commonly uses dilution ratios ranging from 1:1 to 1:4, with ratios higher than 1:8 being uncommon (Kukovics *et al*., 2011). In the case of common carp brooders, Betsy *et al*. (2019) found that a dilution ratio of 1:40 with FWFS allowed for the highest post-thaw motility duration, fertilization rate, and hatching rate when cryopreserving milt.

The implementation of a 1:20 dilution ratio in rabbit breeding shows promise for practical integration. Our study demonstrates notable efficiency, where only 0.75 mL of semen diluted at 1:20 can inseminate 30 does, while a 1:10 dilution can inseminate 15, highlighting the economic advantages. Supporting the TCG extender at a 1:20 dilution for subsequent studies also offers a reliable method to improve sperm quality and fertility results. This combination is proposed as a standard protocol for AI in rabbits. These results set the stage for further investigation and establish this study as a pioneer in AI of rabbits in the Mekong Delta. The success of the 1:20 dilution can potentially improve breeding practices by reducing costs, minimizing male requirements, and maximizing valuable genetics. This study provides scientific and practical insights for refining rabbit breeding practices in the Mekong Delta.

The study on AI practices for rabbit breeding had both strengths and limitations. On the one hand, the study used a large sample size and measured multiple parameters to evaluate semen quality and fertility rates. On the other hand, the study did not investigate the effects of different extenders on semen quality and fertility rates, potentially affecting the results. To overcome these limitations, future research should evaluate the physiological and biochemical parameters of mother and baby rabbits, expand the experimental animal population, and study different types of extenders while improving the TCG extender to achieve better results.

This study evaluated the fertility of does via AI using a TCG extender by testing different semen dilution ratios. The results suggest that the 1:20 dilution ratio is a viable option for AI in rabbit breeding programs in the Mekong Delta of Vietnam. However, the differences in insemination parameters, fertility outcomes, reproductive performance, and offspring characteristics between the treatments were not statistically significant. The 1:10 dilution treatment had the highest average number of newborn rabbits and weaning rate, while the 1:15 dilution treatment had the highest rabbits weight at weaning. The study's limitations may limit the generalizability of the findings, and further research is required to investigate the effects of different dilution ratios and extenders on fertility rates in diverse rabbit breeds.

CONCLUSION

In conclusion, this study highlights the importance of dilution ratio in AI practices in rabbit breeding. The dilution ratio of 1:20, with the lowest sperm concentration, was found to enhance fertility rates and reproductive performance in does. This study is the first AI investigation on rabbits in the Mekong Delta region of Vietnam, highlighting the need for further research to optimize breeding strategies and improve rabbit production.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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