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Two-Step Cryopreservation of Goat (*Capra hircus*) Semen Using Fresh Coconut Water as Egg Yolk Substitute in a Tris–Citric Acid–Glycerol-Based Extender

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Abstract

This study evaluated the effectiveness of fresh coconut water (*Cocos nucifera L.)* as an alternative cryoprotective component in a Tris–citric acid–glycerol extender for goat (*Capra hircus*) semen using a two-step cryopreservation protocol. A completely randomized design was employed with four treatments, including a conventional egg yolk-based control and three coconut water concentrations (25%, 50%, and 75%), with four replicates per treatment across three collection sessions. Semen collected from three healthy bucks was pooled, processed, cryopreserved, and evaluated after 24 hours using Computer-Assisted Sperm Analysis for progressive and total motility. Results showed no statistically significant differences among treatments; however, the 50% coconut water formulation consistently produced the highest numerical values for both progressive and total motility, slightly exceeding the control. Lower and higher concentrations resulted in reduced motility, indicating a concentration-dependent response. The two-step protocol effectively minimized osmotic stress during cryopreservation. Despite the absence of statistical significance, findings suggest that 50% coconut water has potential as a viable alternative to egg yolk-based extenders. The study recommends increasing sample size, expanding sperm quality assessments, standardizing coconut water preparation, and conducting in vivo fertility trials. This research aligns with SDG 2 (Zero Hunger) and SDG 12 (Responsible Consumption and Production) by supporting improved livestock productivity and promoting sustainable, plant-based alternatives in agricultural biotechnology. The findings contribute to socio-economic and technological sustainability by offering a locally accessible, cost-effective solution for improving reproductive efficiency in goat production systems.

Keywords: Artificial Insemination, Coconut Water, Cryopreservation, Goat Semen, Two-Step Protocol

1. Introduction

Goat (*Capra hircus*) production remains a vital component of agricultural systems in the Philippines and other tropical regions, supporting food security and rural livelihoods. Advances in artificial insemination (AI) and semen cryopreservation offer opportunities for genetic improvement and efficient breeding. However, cryopreservation-induced stress significantly reduces sperm viability. Conventional egg yolk-based extenders present biosafety risks, prompting the search for safer alternatives such as fresh coconut water (FCW). This study evaluates FCW as a cryoprotective component in a Tris–citric acid–glycerol extender using a two-step cryopreservation protocol and assesses its effects on post-thaw sperm motility.

1.1 Background of the Study on Fresh Coconut Water as a Cryoprotective Component in Goat Semen Cryopreservation

Goat production plays a significant role in the Philippine livestock sector, particularly among smallholder farmers. The adoption of AI facilitates genetic improvement and broader dissemination of superior traits. However, cryopreservation exposes spermatozoa to mechanical, osmotic, and oxidative stress, reducing post-thaw motility and viability. Egg yolk is widely used in semen extenders due to its protective phospholipids and low-density lipoproteins, but it poses risks of microbial contamination. Fresh coconut water (FCW) has emerged as a natural alternative due to its composition of amino acids, phytohormones, electrolytes, and antioxidants that may enhance sperm survival during freezing. Despite promising findings, the optimal level of FCW substitution in Tris–citric acid–glycerol extenders using a two-step protocol remains insufficiently explored.

1.2 Themes and Insights from Related Literature on Coconut Water-Based Extenders and Two-Step Cryopreservation

Literature highlights key challenges in goat semen cryopreservation, particularly the interaction between seminal plasma components and conventional extenders. The bulbourethral gland secretion fraction BUIII exhibits lipase activity that degrades egg yolk lipids, producing toxic byproducts that impair sperm function (Leboeuf et al., 2000; Purdy, 2006). Additionally, goat sperm is highly susceptible to oxidative stress, necessitating antioxidant-rich alternatives. Coconut water contains sugars, amino acids, vitamins, minerals, and cytokinins that support sperm metabolism and membrane stability

(Prades et al., 2012). Evidence shows its positive effect on sperm motility, particularly in cooled semen (Oliveira et al., 2023), and its applicability across species including goats, cattle, swine, and canines (Cardoso et al., 2003; Cardoso et al., 2005; El-Sheshtawy et al., 2017; Castro et al., 2020; Silva et al., 2012; de Macêdo et al., 2022; Brito et al., 2022). The two-step cryopreservation protocol reduces osmotic stress by gradually introducing glycerol, improving sperm survival (Cardoso et al., 2005). Its effectiveness in goat semen has been supported by studies emphasizing the importance of stepwise dilution (Kundu et al., 2000). Meanwhile, Computer-Assisted Sperm Analysis (CASA) provides objective evaluation of motility parameters, enhancing accuracy and reproducibility.

1.3 Local, National, and Global Context of Goat Production and Semen Preservation Technologies

In the Philippines, goat production continues to expand, with a national inventory of 3.64 million heads in 2025 (PSA, 2025). Government initiatives promote AI to enhance productivity and genetic quality. However, inconsistent conception rates from frozen semen highlight the need for improved preservation techniques (Chowdhury et al., 2021). Globally, research has focused on improving semen extenders and cryopreservation protocols to address species-specific challenges. The development of plant-based, biosecure alternatives such as coconut water reflects broader efforts toward safer and more sustainable reproductive technologies.

1.4 Theoretical or Conceptual Basis of Coconut Water-Based Cryoprotection and Stepwise Freezing

The study is grounded in cryobiology principles, emphasizing membrane stability, osmotic balance, and oxidative stress mitigation during freezing. Coconut water functions as a biologically active medium providing energy substrates, osmotic regulation, and antioxidant protection (Prades et al., 2012). The two-step cryopreservation protocol is based on controlled osmotic adaptation, where gradual glycerol incorporation minimizes cellular damage (Cardoso et al., 2005). The integration of these principles supports the hypothesis that FCW-based extenders can improve post-thaw sperm quality compared to conventional methods.

1.5 Research Gaps and Rationale for Evaluating Fresh Coconut Water Concentrations in Goat Semen Extenders

Despite evidence supporting coconut water as a semen extender component, limited studies have examined its optimal concentration in Tris–citric acid–glycerol extenders under standardized two-step freezing conditions. Existing research often focuses on cooling rather than cryopreservation or lacks direct comparison across multiple concentration levels. This study addresses these gaps by systematically evaluating varying FCW concentrations and their effects on post-thaw sperm motility using CASA, providing a controlled and comparative assessment aligned with established cryopreservation protocols.

1.6 Alignment with Relevant Sustainable Development Goals (SDGs)

This study aligns with several Sustainable Development Goals (SDGs), particularly SDG 2 – Zero Hunger, by enhancing goat production efficiency and supporting food security through improved reproductive technologies. It also contributes to SDG 8 – Decent Work and Economic Growth, as more effective semen cryopreservation can increase productivity and income opportunities for smallholder farmers. Furthermore, the use of fresh coconut water as a plant-based alternative supports SDG 12 – Responsible Consumption and Production by promoting safer and more sustainable materials in livestock biotechnology, reducing reliance on animal-derived components such as egg yolk. Additionally, by minimizing biosafety risks associated with microbial contamination in semen extenders, the study supports SDG 3 – Good Health and Well-Being, ensuring safer practices in animal reproduction and agricultural systems.

1.7 Importance of the Study to Multidisciplinary Sustainability in Livestock Biotechnology

This study contributes to socio-economic sustainability by enhancing reproductive efficiency and income potential for smallholder farmers. It supports technological sustainability through the development of improved cryopreservation protocols and objective evaluation methods such as CASA. Additionally, the use of coconut water promotes environmental and biosecurity sustainability by reducing reliance on animal-derived extender components that carry contamination risks. The research also advances agricultural and institutional sustainability by supporting innovation in livestock biotechnology and strengthening evidence-based breeding programs.

2. Material and methods

2.1 Research Design

A completely randomized design (CRD) was utilized, consisting of four treatment groups with four replicates per treatment. Each experimental unit comprised a batch of semen straws derived from a single pooled ejaculate collected on the same day. The experiment was conducted across three collection sessions, each involving semen from at least three bucks to ensure replication and minimize variability.

2.2 Locale of the Study

The study was conducted at the Cagayan Valley Livestock Biotechnology Research Center, DA Southern Cagayan Research Center, located in Maguirig, Solana, Cagayan, Philippines, in October 2025. The facility provided a controlled laboratory environment equipped with cryogenic storage systems and a Computer-Assisted Sperm Analysis (CASA) unit necessary for semen processing and evaluation.

2.3 Respondents of the Study

The study utilized semen collected from a minimum of three healthy, sexually mature bucks (Capra hircus) of similar genetic background. Prior to collection, all animals underwent health screening, including evaluation of body condition, reproductive tract integrity, and libido. Semen collection was performed between 07:00 and 08:30 h to minimize the effects of heat stress. Ejaculates were pooled immediately after collection to reduce individual variation. Only samples with at least 70% progressive motility and a sperm concentration of 1×10^9 sperm/mL were included for processing.

2.4 Sample and Sampling Procedure

Semen samples were obtained using the artificial vagina method by trained personnel under supervised conditions. Pooling of ejaculates from multiple bucks per session served as the sampling strategy to ensure homogeneity and reduce biological variability. Each pooled sample meeting quality standards was randomly assigned to one of the four treatment groups. The study was replicated across three collection periods to strengthen experimental validity.

2.5 Research Instrument

The study utilized laboratory-based instruments and standardized protocols for semen processing and evaluation. Semen extenders were formulated using Tris–citric acid buffer combined with either egg yolk or varying concentrations of fresh coconut water. As summarized in Table 1, the control treatment consisted of Tris–citric acid buffer with 20% egg yolk and 7% glycerol, while experimental treatments replaced egg yolk with 25%, 50%, and 75% fresh coconut water, respectively. All formulations maintained a constant glycerol concentration of 7% in the second extender fraction and were filtered through 0.45 µm membranes to ensure sterility. Extenders were prepared fresh on each collection day. The buffer solution was heat-sterilized and cooled before incorporating either centrifuged egg yolk or filtered coconut water. Each extender was divided into a glycerol-free fraction and a glycerol-containing fraction to support the two-step cryopreservation process. A Computer-Assisted Sperm Analysis (CASA) system was used to measure sperm motility parameters, specifically progressive motility and total motility, ensuring objective and reproducible assessment of semen quality.

Table 1: *Composition of extender formulations used in the study*

Component	T1 (Control)	T2 (25% CW)	T3 (50% CW)	T4 (75% CW)
Tris-citric acid buffer	Balance	Balance	Balance	Balance
Egg yolk	20%	–	–	–
Fresh coconut water	–	25%	50%	75%
Glycerol (Extender B only)	7%	7%	7%	7%
Membrane filter (µm)	0.45	0.45	0.45	0.45

2.6 Data Gathering Procedure

The cryopreservation process followed a standardized two-step protocol. Initially, pooled semen was diluted with the glycerol-free extender at 37°C to achieve a concentration of 400×10^6 sperm/mL. Samples were then gradually cooled to 5°C over 30 minutes to prevent thermal shock. At 5°C, the glycerol-containing extender was added dropwise over five minutes, reducing osmotic stress while achieving a final concentration of 200×10^6 sperm/mL and 7% glycerol. The samples were equilibrated at 5°C for two hours before being loaded into 0.50 mL French straws using an automated filling and sealing system. Straws were exposed to liquid nitrogen vapor at approximately −80 to −100°C for 12 minutes before immersion in liquid nitrogen at −196°C for storage. After 24 hours, samples were thawed at 37°C for 30 seconds. Post-thaw motility was immediately evaluated using a Makler chamber placed on a heated CASA stage, with measurements conducted in triplicate for accuracy.

2.7 Data Analysis Techniques

Data were expressed as mean ± standard deviation. The effects of the different extender treatments on post-thaw progressive and total sperm motility were analyzed using one-way Analysis of Variance (ANOVA) through the Statistical Tool for Agricultural Research (STAR) software. Statistical significance was determined at $p < 0.05$. In cases where no significant differences were observed, treatment means were compared descriptively without post-hoc testing.

2.8 Ethical Considerations

All procedures adhered to ethical standards for animal research, following the principles of Replacement, Reduction, and Refinement. Animals were maintained under proper husbandry conditions with adequate nutrition and water. Semen collection was performed using a non-invasive artificial vagina method by trained personnel, ensuring that no procedures caused distress or harm to the animals.

3. Results and Discussion

3.1 Post-Thaw Sperm Motility Response to Coconut Water-Based Extenders

3.1.1 Pre-Freeze Semen Quality Standardization

All semen samples met the inclusion criteria prior to cryopreservation, with progressive motility consistently at or above 70% and sperm concentration at a minimum of 1×10^9 sperm/mL across all collection sessions. Ejaculates from three bucks were pooled in each session, ensuring uniform starting material and reducing variability among treatment groups. The consistent pre-freeze quality indicates that any observed differences in post-thaw motility can be attributed primarily to treatment effects rather than initial semen variability. Pooling of ejaculates is a recognized approach to minimize inter-individual differences, thereby improving the reliability of comparative evaluation across treatments.

3.1.2 Post-Thaw Progressive Motility Across Treatments

Post-thaw progressive motility showed no statistically significant differences among treatments ($F = 1.85$, $p = 0.1914$). However, numerical differences were evident, with the 50% coconut water treatment yielding the highest mean progressive motility at 30.67%, followed by the control at 27.02%. Lower values were observed in the 25% and 75% treatments at 16.20% and 17.70%, respectively. The superior performance of the 50% coconut water treatment suggests an optimal concentration that balances nutrient supply and osmotic stability. At this level, coconut water likely provides sufficient antioxidants and energy substrates to support sperm metabolism without compromising buffer integrity. Prades et al. (2012) attributed the protective effects of coconut water to its cytokinin content, which contributes to antioxidant defense and cellular energy processes. In contrast, lower concentrations may provide insufficient protective components, while higher concentrations may induce osmotic imbalance, as noted by Oliveira et al. (2023), who emphasized the importance of optimizing coconut water levels for sperm preservation. The observed motility values, although within reported ranges, remain below the 36.4% benchmark for AI programs and lower than values reported by Apu et al. (2012), possibly due to differences in breed, sample size, and formulation optimization**.**

3.1.3 Post-Thaw Total Motility Across Treatments

Total motility also showed no statistically significant differences among treatments ($F = 2.60$, $p = 0.1002$), although the p-value indicated a stronger numerical trend. The 50% coconut water treatment recorded the highest total motility at 77.88%, slightly exceeding the control at 75.00%, while the 25% and 75% treatments showed lower values of 58.62% and 51.33%, respectively. The consistent ranking of treatments across both progressive and total motility supports the reliability of the observed concentration-response pattern. The slightly stronger trend in total motility may reflect its inclusion of a broader sperm population, including non-progressive cells. The ability of the 50% coconut water treatment to match or exceed the control suggests that its antioxidant and nutritive components can compensate for the absence of lipid-based protection. This is particularly relevant in goats, where egg yolk lipids are susceptible to degradation by BUIII lipase, negatively affecting sperm viability (Leboeuf et al., 2000). The antioxidant and mineral composition of coconut water has been associated with improved membrane stability and oxidative stress reduction (El-Sheshtawy et al., 2017; de Macêdo et al., 2022), although excessive concentrations may disrupt osmotic balance, explaining the reduced performance at 75%.

3.2 Cryopreservation Protocol and Mechanistic Insights

3.2.1 Role of the Two-Step Cryopreservation Protocol

The two-step cryopreservation protocol enabled controlled glycerol incorporation, reducing osmotic shock during freezing. Gradual cooling and stepwise glycerolization allowed sperm cells to equilibrate before exposure to full cryoprotectant concentration, resulting in stable post-thaw motility outcomes across treatments. This approach aligns with findings by Cardoso et al. (2005), who demonstrated improved sperm survival with stepwise dilution methods. The protocol is particularly beneficial for coconut water-based extenders, where multiple solutes require gradual integration to prevent osmotic stress. Kundu et al. (2000) similarly emphasized the importance of multi-step dilution in enhancing post-thaw quality in goat semen, supporting the methodological robustness of the present study.

3.3 Practical Implications and Study Limitations

3.3.1 Implications for Goat Artificial Insemination Programs

The results indicate that a 50% coconut water extender can numerically match or slightly exceed the performance of conventional egg yolk-based extenders in both progressive and total motility. This suggests that coconut water is a viable, biosecure, and accessible alternative for semen preservation, particularly in regions where coconuts are abundant. The use of coconut water offers practical advantages, including natural sterility, ease of preparation, and reduced risk of pathogen transmission compared to egg yolk. Additionally, its lipid-free composition avoids BUIII-mediated degradation, which is a known limitation of conventional extenders (Purdy, 2006; Leboeuf et al., 2000). These attributes make coconut water a promising candidate for sustainable and locally adaptable AI technologies.

3.3.2 Limitations and Sources of Variability

The study did not detect statistically significant differences among treatments, primarily due to the limited sample size of three bucks per session, which reduced statistical power. Variability in semen quality across collections and the use of fresh coconut water may also have contributed to inconsistent results. Fresh coconut water introduces variability in composition depending on fruit maturity and environmental factors, which may affect extender consistency. Future studies should consider standardized formulations and larger sample sizes to improve statistical power. Additionally, the evaluation was limited to motility parameters, whereas comprehensive assessment including viability, membrane integrity, and DNA quality would provide a more complete understanding of cryopreservation outcomes.

4. Conclusion and Recommendations

The study demonstrated that the use of fresh coconut water in a two-step cryopreservation protocol produced comparable post-thaw sperm motility to a conventional egg yolk-based extender, with the 50% coconut water formulation yielding the highest numerical values for both progressive and total motility. Although no statistically significant differences were observed among treatments, the results revealed a concentration-dependent effect, where both lower and higher coconut water levels resulted in reduced motility compared to the intermediate concentration. These findings indicate that 50% coconut water has potential as a practical alternative cryoprotective component for goat semen preservation under the conditions tested.

Future studies should increase the number of experimental animals to improve statistical power and expand sperm quality evaluation beyond motility to include viability, membrane integrity, and other functional parameters. Standardization of coconut water preparation and characterization of extender physicochemical properties should also be conducted to improve consistency and interpretation of results. Further validation through in vivo fertility trials and economic assessment is necessary to determine the practical applicability and cost-effectiveness of coconut water-based extenders in goat artificial insemination programs.

The study was limited by the small sample size, which reduced the ability to detect statistically significant differences among treatments despite observable numerical trends. Variability in semen quality across collection sessions and the use of fresh coconut water, which may differ in composition depending on source and maturity, also contributed to potential inconsistencies. Additionally, the assessment of post-thaw quality was restricted to motility parameters, limiting the evaluation of other critical indicators of sperm functionality and fertility.

Compliance with ethical standards

The authors declare no conflict of interest, and this study received no external funding. The research was conducted at the DA–Southern Cagayan Research Center, and the researchers acknowledge the institution and its personnel for their support; participation of animal subjects was managed by trained personnel under standard husbandry conditions, with procedures performed using non-invasive methods. All processes adhered to ethical standards, ensuring humane treatment, and no identifying information was recorded or disclosed, thereby maintaining confidentiality.

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