Research Article

Impact of Pre-Freezing Duration on Buck Semen Quality in Eppendorf Tubes

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Abstract | This study aims to evaluate a manual protocol for freezing semen in Eppendorf tubes. The research utilized semen from a healthy and fertile Kacang buck weighing 45 kg and aged 3 years. The semen extender employed was Tris-egg yolk. The study was conducted using a 3 x 5 Completely Randomized Design (CRD). The first factor examined was the pre-freezing duration, which included 10, 15, and 20 minutes (T1, T2, and T3, respectively). The second factor assessed was the post-thawing storage time of 0, 30, 60, 90, and 120 minutes. After equilibration, Eppendorf tubes containing liquid semen were placed four centimeters above liquid nitrogen (LN2) in a Styrofoam container and then frozen by immersion in LN2. Total motility, progressive motility, plasma membrane integrity, and viability were measured at 0 and every 30 minutes up to 120 minutes post-thawing. Data were analyzed using one-way ANOVA and Duncan's test. At 120 minutes post-thawing, total motility at T1 decreased from 51.02 ± 2.57 to 24.31 ± 4.25, which was significantly ($P < 0.05$) lower than T2 and T3, which showed motility values of 61.40 ± 2.31 to 29.11 ± 3.26 and 62.19 ± 3.15 to 41.19 ± 1.12, respectively. A similar trend was observed for the percentage of progressive motility, which was significantly ($P < 0.05$) higher in T3 (39.13 ± 1.02) compared to T1 (12.03 ± 3.16) and T2 (21.21 ± 1.26). Additionally, the PMI 120 minutes after thawing for F1, F2, and F3 were 29.07 ± 2.17, 33.04 ± 2.17, and 45.04 ± 2.17, respectively. The viability of sperm for T1, T2, and T3 were recorded as 31.02 ± 2.15, 30.08 ± 1.17, and 43.03 ± 1.06, respectively. In conclusion, the manual, cost-effective semen freezing method is suitable for small laboratories. Future research should examine how different extenders, equilibration, and pre-freezing durations affect post-thaw semen quality, and validate fertilization rates against modern methods.

Keywords | Eppendarf, Sperm quality, Pre-freezing, AI, Simple, Cheap

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INTRODUCTON

Semen preservation research has been conducted for an extended period, and its benefits are currently being validated, particularly in enhancing reproductive efficiency and livestock productivity. Chunrong *et al*. (2019) noted that semen preservation has become an integral component of the modern livestock industry. This technique is well-known in animal breeding, including for goat. Crossbreeding semen from superior exotic goat breeds with local goats

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enhances genetic quality and productivity. (Myagmarsuren *et al*., 2016). Agarwal *et al*. (2004), Tash *et al*. (2003) and Yimer *et al*. (2015) stated that semen preservation facilitates the distribution of superior breeds, which is essential for rapid genetic improvement and increased production of local livestock. Ultimately, this contributes to meeting the ever-growing human demand for livestock products. Chunrong *et al*. (2019) further explained that semen preservation not only directly supports artificial insemination (AI) programs but also significantly reduces the costs associated with maintaining breeding studs. Consequently, semen preservation remains a compelling area of research that continues to attract the attention of many researchers (Chunrong *et al*., 2019; Walters *et al*., 2009).

Semen cryopreservation, particularly in bucks and cattle, has recently benefited from the adoption of modern techniques and equipment, which necessitate significant financial investment (Chunrong *et al*., 2019). The entire process is facilitated by automated tools and machinery, enhancing efficiency and ensuring that the quality of the frozen semen produced is optimal (Arman, 2014). Nevertheless, post-thaw sperm motility remains approximately 50%, primarily due to damage sustained by sperm to the plasma membrane, nucleus, mitochondria, acrosome, and overall morphology during the freezing process (Walter *et al*., 2009). In contrast, the utilization of artificial insemination (AI) technology has been steadily increasing; however, not all institutions, particularly those involved in goat farming, possess the modern facilities required for the production of frozen semen.

Sperm cryopreservation has experienced significant advancements regarding the techniques utilized for freezing, the speeds at which samples are frozen and thawed, and the preservation media employed to maintain sperm functionality and DNA integrity (Hungerford *et al*., 2022). References concerning the freezing of buck semen are less abunandt compared to those available for bovine semen (Morrell *et al*., 2022). Frozen semen can be stored in a variety of packaging formats, including ampoules, pellets, mini-tubes, and fine straws. Of these options, fine straw packaging is currently the most widely utilized (Ramirez-Reveco *et al*., 2016).

Unlike contemporary freezing methods, this study was conducted in a small laboratory with very limited resources, focusing on a cost-effective, manual and applicable approach that can be implemented in small laboratories worldwide. Consistent with the findings of Morrell *et al*. (2022), the buck semen freezing method should be tailored to the specific conditions under which the work is conducted. The objective of this study was to investigate the effects of Eppendorf packaging and the duration of pre-freezing with a manual protocol on the quality of post-thaw Kacang buck semen. The goal is to make semen preservation more accessible to farmers by proposing simplified techniques that can be implemented in small, remote laboratories.

MATERIALS AND METHODS

Kacang Buck

The subject of this study is a local Indonesian buck, specifically the Kacang breed, three years old, and weighing 45 kg. This buck has been confirmed to be fertile, healthy, and free from both internal and external parasites. The buck is housed in a stage cage measuring 2×3 m^2. Its diet consists of high-quality fresh leaves from the legume tree (*Sesbania glandiflora*), provided in the morning and afternoon at a quantity equivalent to 15% of its body weight. Access to drinking water is freely available in a plastic bucket container.

Treatment Extenders

The most commonly used extender for semen cryopreservation in Indonesia is Tris-egg yolk (Nisfimawardah *et al*., 2023). The buffered experimental extender was prepared by dissolving 3.634 g of Tris (hydroxymethyl) aminomethane (Sigma, USA), 2.17 g of citric acid monohydrate (Merck, Germany), 0.50 g of fructose (Merck, Germany), 0.06 g of sodium penicillin G (Wonder, Japan), and 0.1 g of streptomycin (Wonder, Japan) in 100 ml of distilled water to achieve a pH of 7.0, following the protocol by Evans and Maxwell (1987). The prepared extender was stored in a refrigerator until needed. Just before semen collection, the extender was mixed with 2.5% egg yolk (v/v) at room temperature (Evans and Maxwell, 1987). The extender was then divided into three test tubes, each containing 10 ml, and kept at room temperature until use. Additionally, a tank of liquid nitrogen (LN2) was prepared for the preservation process.

Semen Collection, Extended, Freezed and Evaluation

Ten ejaculates were collected from the buck every four days using an artificial vagina (AV) and transported to the laboratory for assessment. Criteria for suitability included a volume of at least 0.8 ml, a wave motion rating of +++, total motility of at least 70%, and a concentration of at least 2.5 x 10^9 sperm per milliliter (Evans and Maxwell, 1987; Menchaca *et al*., 2005).The concentration of sperm was determined using a hemocytometer. The concentration of sperm per unit volume (ml) was calculated using Thoma haemocytometer (Evans and Maxwell, 1987).

The method to prepare a 3% NaCl solution (Susilawati, 2013). Briefly, by dissolving 1.5 grams of NaCl in 50 mL of distilled water and homogenizing it. Use a micropipette to take 10 μl of fresh semen and drop the semen into a

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test tube. Mix the fresh semen with 1990 µl of 3% NaCl solution, which means that the semen has been diluted 200×; homogenize the semen and 3% NaCl solution by shaking the tube slowly several times. Take 2 µl of semen sample using a micropipette and drop it on the side of the haemocytometer cover glass; then the sperm are observed using a light microscope with 400× magnification. Count the sperm in 5 large boxes, each box consisting of 16 small boxes, so that the total number of boxes counted is 80 small boxes. The concentration of sperm per ml is the number of sperm counted multiplied by 10^7.

Fresh semen meeting all criteria was promptly processed by mixing it with an extender at a 1:50 ratio (Menchacha *et al*., 2005). The liquid semen was then divided into three tubes, each containing 10 ml of diluted semen. These tubes were placed in a pre-equilibrated water jacket to gradually decrease the temperature of the semen by 0.25ºC per minute, reaching 5 ºC within five hours (Arman, 2014; Menchacha *et al*., 2005).

The addition of 7% (v/v) glycerol was carried out in a refrigerated environment three hours after semen stored in refrigerator (Arman, 2014) and then homogenized by gently shaking the tube. One hour before pre-freezing, each tube containing 10 ml of diluted semen was aliquoted into five Eppendorf tubes, each with a capacity of 1 ml, based on the observation frequency for each treatment, resulting in a total of 15 Eppendorf tubes. This protocol was repeated for the second through tenth semen collections.

The freezing process involves several steps. First, prepare a styrofoam box measuring 24 cm x 22 cm x 20 cm and pour liquid nitrogen into it. Next, use a second styrofoam box measuring 8 cm x 15 cm x 13 cm, placing a fine wire mesh 4 cm from the bottom to allow it to float on the LN2 surface, enabling vapor entry. Then, position an Eppendorf tube containing liquid semen in the second box. Pre-freezing treatments T1, T2 and T3 are conducted for 10, 15, and 20 minutes, respectively, before immersion in liquid nitrogen. Finally, label all treatment tubes and store them in a goblet.

Post-Thaw Evaluation

Thawing was conducted by immersing the Eppendorf tube in water at 50°C for 73 seconds, similar to thawing semen in ampoules (Ramirez-Reveco *et al*., 2016). The study evaluates sperm quality post-thawing, including motility, viability, and membrane integrity, with observations recorded immediately and every 30 minutes for 120 minutes. The evaluation of total motility, progressive motility, and viability followed the methodology outlined by Maxwell and Salamon (1993). A drop of fresh semen was mixed with 1 ml of extender, placed on a glass slide, and covered with a cover slip. Total motility was subjectively assessed at 200× magnification by estimating the proportion of motile sperm. Progressive motility, defined as sperm exhibiting straight-line movement, was evaluated at 400× magnification with 200 sperm analyzed (Swarna *et al*., 2022). The total total and progressive motility were quantified across multiple fields of view and expressed as percentages. The percentage of total and progressive motility was calculated by dividing the number of motile sperm by 200 and multiplying by 100% (Swarna *et al*., 2022; Zaenuri *et al*., 2023).

The integrity of the plasma membrane was evaluated using a hypo-osmotic solution (HOS) (Alcay *et al*., 2015) made with fructose and sodium citrate at concentrations of 8.72 g/L and 4.74 g/L, a pH of 8.05, respectively (Susilawati, 2013; Alcay *et al*., 2015). A 10 µL aliquot of semen was mixed with 200 µL of the HOS solution and incubated at 37°C for 40 minutes (Jeyendran *et al*., 1984; Susilawati, 2013; Suwimonteerabutr *et al*., 2020). After incubation, a drop of the semen mixture was placed on a glass slide, covered with a cover slip, and examined under a light microscope (Nikon, Japan) at 400× magnification. Microscope fields were randomly selected for analysis. Two hundred sperm were counted per slide, and the percentage of swollen-tailed sperm was calculated. The percentage of sperm with intact membranes was determined by dividing the number of sperm with circular tails by 200 (Jeyendran *et al*., 1984; Ahmad *et al*., 2003; Susilowati *et al*., 2021; Suwimonteerabutr *et al*., 2020).

Sperm viability was assessed by preparing smears from each semen treatment. To create a smear, a drop of post-thaw semen was mixed with Eosin-Nigrosin solution (Wonder, Japan). The smear was examined under a light microscope (Nikon, Japan) at 400× magnification. Sperm were classified as viable if they did not absorb the dye, and those that absorbed the dye were considered non-viable.

Data Analysis

Data obtained from the postthawing evaluation results were analyzed using a Completely Randomized Design (CRD) analysis of variance, a 3 x 2 factorial pattern with ten replications (Steel and Torrie, 1993). Data were analyzed using One Way ANOVA for completely randomized design using Co-Stat for windows statistical software (version 6.03). If there were differences between treatments, the analysis was continued with the Duncan test (α = 0.05).

RESULTS AND DISCUSSION

Fresh Semen

This study investigated the free-freezing duration of buck semen in Eppendorf tubes, using semen from one Kacang buck for homogeneity. Semen collection using an artificial vagina (AV) proved to be an effective method for obtaining high-quality semen in this study. The bucks displayed high libido and quickly adapted to ejaculating into the AV

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when presented with anoestrous does, even in the presence of other males. The average volume and pH of the semen, as well as the concentration, total motility, and viability of the buck sperm from ten collections using the AV, were recorded as 0.89 mL, 6.8, 2.85 x 10^9/mL, 90.2%, and 95%, respectively. This indicates that the semen from the buck meets the criteria for processing into frozen semen (Evans and Maxwell, 1987; Menchacha *et al*., 2005). Cryopreservation is essential for long-term storage of semen at -196°C while maintaining fertilization rates post-thawing (Lemma, 2011). However, it is important to note that negative effects on total motility, progressive motility, viability, and the integrity of the sperm plasma membrane may occur during the freezing and thawing processes (Watson, 2000). However, it could be noted that the main factor to evaluate quality characteristics of sperm such as motility, plasma membrane integrity, and viability (Amini *et al*., 2019).

Post-Thaw Semen Quality

Total sperm motility: The total motility of Kacang buck sperm after thawing (Table 1) shows a pattern where longer pre-freezing durations lead to higher total sperm motility. On the other hand, storing the sperm at room temperature for an extended period results in a decrease in total motility. Eppendorf tube packaging for freezing goat semen does not harm spermatozoa quality. A negative impact may stem from other factors. Ugur *et al*. (2019) propose that damage to sperm could be caused by factors such as hyperosmolarity, ionic imbalance, protein denaturation, generation of reactive oxygen species (ROS), and inadequate energy supply during cooling and freezing processes.

Table 1: The impact of pre-freezing duration and room temperature storage time on the total motility of post-thaw buck sperm packed in eppendorf tubes.

Post thawing (minute)	Treatments		
	T1	T2	T3
0	51.02 ±2.57[a]	61.40±2.31[b]	62.19±3.15[b]
30	48.10±6.91[a]	55.70±1.95[b]	57.20±1.44[b]
60	37.12±5.18[a]	44.18±3.26[b]	49.16±2.11[b]
90	29.25±4.21[a]	36.01±3.11[b]	48.23±3.21[c]
120	24.31±4.25[a]	29.11±3.26[a]	41.19±1.12[c]

Note: different superscripts in the same column indicate significant differences ($P < 0.05$).

The study findings indicate that total motility assessed at 0 minutes post-thawing is relatively high, with all three treatment groups showing motility rates exceeding 50%. However, there was a significant decline in total motility shortly after thawing from 51.02 ± 2.57 to 24.31 ± 4.25 at 120 minutes in T1. This reduction in T1 was statistically significant ($P < 0.05$) compared to T2 and T3, which had motility rates of 61.40 ± 2.31 to 29.11 ± 3.26 and 62.19 ± 3.15 to 41.19 ± 1.12, respectively. Table 1 shows that frozen semen at T1, T2, and T3 can be maintained respectively for 30, 60, and 120 minutes for AI purposes, as total motility remains above 40% (Standard Nasional Indonesia, 2014). Additionally, the percentage of total motility for Kacang buck sperm post-thawing in this study was slightly higher than the findings reported by Morrell *et al.* (2022), who recorded a total motility rate of 59 ± 3%.

This study positioned semen in an Eppendorf tube 4 cm above liquid nitrogen. The results align with Kabir *et al.* (2024), who found that the optimal distance for the highest total motile buffalo sperm count (62.67±1.12%) is 1.6 inches, compared to 1.5 inches (47.13±3.37%) and 2 inches (38.10±1.97%). Ramirez-Reveco *et al.* (2016) found no significant effect of storage duration at -196°C on the percentage of total motile sperm after thawing, with values ranging from 60 ± 2.4% to 66 ± 3.2%. In contrast, Zamfirescu and Nadolu (2013) reported an approximately 50% reduction in total motility of alpine buck sperm, decreasing from 86.81% in fresh semen to 46.35% in frozen-thawed semen. The total post-thaw motility results in this study were higher than those reported for sheep, which showed a motility of 40.3 ± 1.7% (Marco-Jiménez *et al*., 2005). The total movement of sperm is the key for successful reproduction, and sperm with low motility is the primary cause of infertility in most cases (Aitken, 1995).

Packaging, temperature, and thawing duration significantly influence total sperm motility. In this study, semen was packaged in Eppendorf tubes and thawed at 50°C for 37 seconds, resulting in total sperm motility similar to some previous research. Chaiprasat *et al.* (2006) observed that higher thawing temperatures led to increased bovine sperm motility, with percentages of 51.04%, 40.41%, and 39.79% at thawing temperatures of 37°C, 30°C, and 25°C for 30 seconds, respectively. Samsudewa and Suryawijaya (2008) reported that thawing with ice water for 30 and 60 minutes resulted in 37.50±1.8% and 25.00±1.82% motility in Simmental cattle, and 40.00±1.64% and 20.00±1.14% in Limousin cattle. Straw water jacket packaging preserved sperm motility at a rate of 52% for up to 96 hours when stored in the refrigerator (Roca *et al.* 2004). Total sperm motility of 39±2.0 in the Tris-egg yolk extender, significantly higher than the 34±2.0, 33±4.0, and 33±4.0 observed in Tris-albumin extenders with 4%, and 6% (v/v) fig fruit filtrate, respectively, after four days of refrigeration (Triyani *et al*., 2024).

Progressive motility of sperm: Progressively motile sperm is a crucial parameter for assessing sperm quality after thawing (Abdelnour *et al*., 2020). The decrease in the percentage of progressive motility corresponds to the reduction in total motility (Table 2). Significantly higher ($P < 0.05$) percentages of progressive motility were observed

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immediately post-thawing and at 120 minutes post-thawing at T3 (39.13±1.02) compared to T1 (12.03±3.16) and T2 (21.21±1.26). This difference may be due to the use of Eppendorf tubes for frozen semen packaging, which are thicker than straw packaging, requiring a longer pre-freezing duration to achieve optimal quality of frozen sperm. Kabir *et al.* (2024) found that manually pre-freezing buffalo semen in minitube packages at 1.5 inches and 1.6 inches above the LN2 surface yielded sperm progressive motility of 24.50 ± 1.29% and 38.97 ± 1.10%, respectively. The progressive motility percentage of frozen semen post-thawing at T1 indicates its suitability for immediate consecutive insemination. For T2 and T3 (as shown in Table 2), the semen remains suitable for AI for up to 60 and 90 minutes post-thawing, respectively, according to the Standard Nasional Indonesia (2014).

Table 2: The impact of pre-freezing duration and room temperature storage time on the progressive motility of post-thaw kacang buck sperm packed in eppendorf tubes.

Post thawing (minute)	Treatments		
	T1	T2	T3
0	49.31±1.21[a]	54.21±6.37[b]	56.19±2.05[b]
30	39.50±2.73[a]	47.23±1.05[b]	51.12±1.04[c]
60	26.12±1.03[a]	42.05±3.26[b]	46.11±1.11[c]
90	19.14±2.36[a]	31.18±1.11[b]	42.12±1.21[c]
120	12.03±3.16[a]	21.21±1.26[b]	39.13±1.02[c]

Note: Different superscripts in the same column indicate significant differences (P < 0.05).

Arifiantini and Yusuf (2006) found no significant differences in the percentages of progressively motile and live sperm when frozen in 0.3 ml minitub (52.16% and 69.4%) or 0.25 ml Cassou straw (49.59% and 70.44%). The post-thaw progressive motility percentages at T2 (54.21 ± 6.37) and T3 (56.19 ± 2.05) are consistent with Sitomorang's (2002) findings of 56.0% and 51.5% for Tris-citrate extenders enriched with 1 mg/ml and 0.5 mg/ml cholesterol in straw packaging. White (1993) highlighted the low cholesterol levels in seminal plasma and sperm, making sperm vulnerable to cold shock. Therefore, the inclusion of 2.5% egg yolk (Evans and Maxwell, 1987) containing lipoproteins can help mitigate the effects of cold shock. Zaenuri and Rodiah, (2025) reported, after six hours of preservation at 27°C, spermatozoa in Tris-albumin extender with 8% (v/v) Fig fruit filtrate showed significantly higher progressive motility (66±2.53; P < 0.05) compared to the 4%, and 6% (59±4.1, 60±2.74, and 62±2.24, respectively).

Plasma membrane integrity: The integrity of the plasma membrane in sperm is a critical factor influencing their viability. An intact membrane correlates with prolonged viability, which in turn enhances both total motility and progressive motility of sperm. The integrity of the plasma membrane directly affects the quality of sperm (Pawar and Kaul, 2011). Physiologically, sperm membrane integrity plays a role in various vital processes that occur in sperm, including protecting and maintaining sperm motility in the female reproductive tract, capacitation and fertilization (Mocé and Graham, 2008). The proportion of high-quality sperm is influenced by the presence of sperm with normal membrane integrity (Makarevich *et al*., 2011).

Table 3: The impact of pre-freezing duration and room temperature storage time of eppendorf tube-packed semen on plasma membrane integrity parameters of post-thaw buck sperm.

Post thawing (minute)	Treatments		
	T1	T2	T3
0	59.04±2.57[a]	64.04±1.07[b]	66.03±1.17[b]
30	41.09±1.27[a]	61.22±1.37[b]	63.09±2.16[b]
60	38.11±1.91[a]	46.17±2.05[b]	55.21±1.14[c]
90	31.14±1.08[a]	37.13±1.21[b]	47.17±3.06[c]
120	29.07±2.17[a]	33.04±2.17[a]	45.04±2.17[c]

Note: Different superscripts in the same column indicate significant differences (P < 0.05).

The percentage of sperm with intact plasma membrane (Table 3) at time point T1 was significantly lower (P < 0.05) compared to T2 and T3. Conversely, T3 showed a significantly higher percentage (P < 0.05) than T2 from 60 to 120 minutes post-thawing. Ramirez-Reveco *et al.* (2016) found that long-term storage at -196°C did not affect the plasma membrane integrity of bovine sperm, consistent with other studies. Morrell *et al.* (2022) reported 29.9 ± 14.7% membrane integrity in post-thawed buck sperm, while Barbas *et al.* (2024) observed a decline from 65.5% to 42.9% in buck sperm plasma membrane integrity pre- and post-thawing. Zaenuri *et al.* (2017) reported that the integrity of the plasma membrane of Kacang buck semen, when preserved in a Tris-egg yolk-based extender supplemented with 0.06 g (v/v) of free-drying fig fruit extract, was measured at 42.1 ± 5.1% after 92 h in refrigerated. Makarevich *et al.* (2011) found that plasma membrane integrity is crucial for sperm metabolism and function, making it necessary to recover a high percentage of the sperm with integrity of membrane after storage and processing.

The decrease in sperm integrity after thawing and room temperature storage may result from insufficient cholesterol in the extender. Arman (2014) noted that cholesterol is crucial for membrane integrity and sperm viability. Low cholesterol levels in semen and sperm increase cold shock susceptibility (White, 1993; Arman, 2014). Thus, more sperm with normal membranes leads to a higher percentage of high-quality sperm (Makarevich *et al*., 2011).

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Table 4: The impact of pre-freezing duration and room temperature storage time on the viability of post-thaw buck sperm packaged in eppendorf tubes.

Post thawing (minutes)	Treatments		
	T1	T2	T3
0	57.02±1.57[a]	65.01±0.07[b]	66.01±0.17[b]
30	53.02±1.17[a]	63.12±1.17[b]	65.02±1.12[b]
60	39.01±1.11[a]	47.07±1.05[b]	59.11±1.04[c]
90	34.11±2.08[a]	38.23±1.21[a]	54.12±1.05[b]
120	31.02±2.15[a]	30.08±1.17[a]	43.03±1.06[c]

Note: Different superscripts in the same column indicate significant differences ($P < 0.05$).

Viability of sperm: Sperm viability is also a critical factor in assessing sperm quality. The percentage of viable sperm at time point T1 was consistently significantly lower ($P < 0.05$) from the immediate post-thawing period up to 120 minutes thereafter, compared to time points T2 and T3 (Table 4). According to the sperm viability indicator, Kacang buck semen stored in Eppendorf tubes under T1 remained suitable for artificial insemination (AI) for up to 30 minutes post-thawing, while treatments T2 and T3 were suitable for 60 and 120 minutes post-thawing, respectively. Roca *et al*. (2004) found that sperm viability decreased along with preservation time. The sperm viability observed in this study was higher than the findings reported by Barbas *et al*. (2024), which indicated a decrease in viability from 75.0% before freezing to 42.8% post-thawing. The viability of post-thawed bull sperm, as reported by Putra and Ducha (2019), is 43.5 ± 1.5%.

The post-thaw viability percentage of buck sperm is reported to be 68±2.1, shows no significant decrease compared to samples stored for over ten years, with a viability of 60±1.8 (Ramirez-Reveco *et al*., 2016). In contrast, Malik *et al*. (2015) found that the viability and motility of bovine sperm are affected by the storage duration. Specifically, semen preserved at -196°C for six years showed lower viability and motility (64.50±0.39 and 42.08±0.13, respectively) than samples stored for one year (80.63±0.41 and 48.33±0.31, respectively). The equilibration rate of this study was 0.25 °C/min, in line with Januskauskas *et al*. (1999) that no different effects of different cooling rates during equilibration of semen from room temperature to 4°C, at 4.2°C/min or at 0.1°C/min on in vitro sperm viability, motility, membrane integrity, capacitation and acrosomal status post-thawing

It seems that enriched extender with antioxidant, energy sources or others effected to the sperm quality at cold or freezed. Putra and Ducha (2019) found that treating Boer buck semen samples with fructose alone or in combination with raffinose in a basic soya tris diluent resulted in the high post-thaw viability percentages, with values of 40.42 ± 0.44 and 41.77 ± 0.32, respectively. Arman (2014) noted that buck sperm can remain viable for extended periods, and fertility rates improve when cholesterol is added to the extender.

The utilization of Eppendorf tubes offers several advantages, including their accessibility, cost-effectiveness, and the capability of a single tube to facilitate the insemination of one to four female goats. However, it is important to emphasize that the fertilization potential of semen is not confirmed until the sample is used for insemination, regardless of the extender or packaging used.

CONCLUSIONS AND RECOMMENDATIONS

The manual, cost-effective semen freezing method in Eppendorf tube is suitable for small laboratories with limited facilities. Future research should explore how different extenders, equilibration, and pre-freezing periods affect post-thaw semen quality. Validation of fertilization rates compared to modern methods is essential.

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NOVELTY STATEMENTS

This study explored a manual, cost-effective semen freezing method using Eppendorf tubes and its effects on post-thaw Kacang buck semen quality, a previously underexplored area.
This method is ideal for small laboratories with limited resources.

AUTHOR'S CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest

The authors declare that they have no conflict of interests.

REFERENCES

Abdelnour SA, Abd El-Hack ME, Alagawany M, Taha AE, Elnesr SS, Abd Elmonem OM, Swelum AA (2020). Useful impacts of royal jelly on reproductive sides, fertility rate

and sperm traits of animals. J. Anim. Physiol. Anim. Nutr., 104:1798–1808. https://doi.org/10.1111/jpn.13303

Agarwal A, Nallella KP, Allamaneni SSR, Said TM (2004). Role of antioxiandts in treatment of male infertility: An overview of the literature. Reprod. Biomed. Online, 8(6): 616-627. https://doi.org/10.1016/S1472-6483(10)61641-0

Ahmad Z, Anzar M, Shahab M, Ahmad N, Andrabi SMH (2003). Sephadex and Sephadex ion exchange filtration Improves the quality and free ability of low-grade buffalo semen ejaculates. Theriogenology, 59: 1189-202. https://doi.org/10.1016/S0093-691X(02)01159-7

Aitken RJ (1995). Free radicals, lipid peroxidation and sperm function. Reproduction, Fertil. Dev., 7(4): 659–668. https://doi.org/10.1071/RD9950659

Alcay S, Toker MB, Gokce E, Ustuner B, Onder NT, Sagirkaya H, Nur Z, Soylu MK (2015). Successful ram semen cryopreservation with lyophilized egg yolk-based extender. Cryobiology, 71(2):329–333. https://doi.org/10.1016/j.cryobiol.2015.08.008

Amini S, Masoumi R, Rostami B, Shahir MH, Taghilou P, Arslan HO (2019). Effects of supplementation of Tris-egg yolk extender with royal jelly on chilled and frozen-thawed ram semen characteristics. Cryobiology, 88: 75–80. https://doi.org/10.1016/j.cryobiol.2019.03.008

Arifiantini RI, Yusuf TL (2006). Keberhasilan Penggunaan Tiga Pengencer Dalam Dua Jenis Kemasan Pada Proses Pembekuan Semen Sapi Frisien Holstein. Majalah Ilmiah Peternakan., 9(3): 164

Arman C (2014). Reproduksi Ternak. Graha Ilmu. Sleman. Yogyakatra. ISBN978-602-262-270-3

Barbas JP, Baptista MC, Carolino N, Simões J, Margatho G, Pimenta J, Claudino F, Ferreira FC, Grilo F, Pereira RMLN (2024). Effect of Breed and Season in Buck Semen Cryopreservation: The Portuguese Animal Germplasm Bank. Vet. Sci., 11: 326. https://doi.org/10.3390/vetsci11070326

Chaiprasat S, Benjakul W, Chartchue A, Joemplang P, Punyapornwithaya V (2006). Effect of Bull Semen Thawing Methods on Sperm Progressive Motility. Chiang Mai Vet. J., 4 (1) : 25 – 29.

Chunrong LV, Wu G, Hong Q, Quan G (2019). Sperm cryopreservation: State of Art and Future in Small Ruminants. Biopreserv. Biobank., 17(2): 171-182. https://doi.org/10.1089/bio.2018.0113

Evans G, Maxwell WMC (1987). Salamon's Artificial insemination of Sheep and Goats. Butterworths, Sydney, NSW, Australia: University Press. 22-141.

Hungerford A, Bakos HW, Aitken RJ (2022). Sperm cryopreservation: current status and future developments. Reproduction, Fertil. Dev., 35(3): 265–281. https://doi.org/10.1071/RD22219

Januskauskas A, Gil J, Söderquist L, Håård MG, Håård MC, Johannisson A, Rodriguez-Martinez H (1999). Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in Sweden. Theriogenology., 52(4): 641-58. https://doi.org/10.1016/S0093-691X(99)00159-4

Jeyendran RS, Van Der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil., 70(1): 219-228. https://doi.org/10.1530/jrf.0.0700219

Kabir Md A, Hossain SMJ, Deb GK, Shejuty SF, Das D, Noman AA (2024). Assessing the efficacy of different semen extenders on post-thaw quality of Bangladeshi buffalo sperm under a manual cryopreservation protocol. J. Adv. Biotechnol. Exp. Ther., 7(1): 83-94. https://doi.org/10.5455/jabet.2024.d08

Lemma A (2011). Effect of cryopreservation on sperm quality and fertility. In: M Manafi (ed), Artificial Insemination in Farm Animals. Published online by In Tech, 191-216. https://doi.org/10.5772/16563

Makarevich AV, Spalekova E, Olexikova L, Lukac N, Kubovicova E, Hegedusova Z (2011). Functional characteristics of ram cooling-stored sperm under the influence of epidermal growth factor. Gen. Physiol. Biophys., 30: 36–43. https://doi.org/10.4149/gpb_2011_SI1_36

Malik A, Laily M, Zakir MI (2015). Effects of long-term storage of semen in liquid nitrogen on the viability, motility and abnormality of frozen thawed Frisian Holstein bull sperm. Asian Pac. J. Reprod., 4 (1): 22-25. https://doi.org/10.1016/S2305-0500(14)60052-X

Marco-Jime´nez F, Puchades S, Gadea J, Vicente JS, Viudes-de-Castro MP (2005). Effect of semen collection method on pre- and post-thaw Guirra ram sperm. Theriogenology 64: 1756–1765. https://doi.org/10.1016/j.theriogenology.2005.04.006

Maxwell WMC, Salamon S (1993). Liquid storage of ram semen: a review. Reprod Fertil Dev., 5(6):613-38. https://doi.org/10.1071/RD9930613

Menchaca A, Pinczak A, Queirolo D (2005). Strorage of ram semen at 5 ºC: effects of preservation period and timed artificial insemination on pregnancy rate in ewes. Anim. Reprod., 2 (3):195-198.

Mocé E, Graham JK (2008). In vitro evaluation of sperm quality. Anim. Reprod Sci., 105: 104-118. https://doi.org/10.1016/j.anireprosci.2007.11.016

Morrell JM, Malaluang P, Ntallaris T, Johannisson A (2022). Practical Method for Freezing Buck Semen. Animals, 12: 352. https://doi.org/10.3390/ani12030352

Myagmarsuren P, Baatar A, Batsukh T (2016). Cryopreservation of Alpine Bucks Semen. J. Exp. Biol. Agric. Sci., 4 (5): 554-556. https://doi.org/10.18006/2016.4(5).554.556

Nisfimawardah L, Firmawati A, Ihsan MN, Susilawati T, Wahjuningsih S (2023). Semen Cryopreservation Quality and Sperm Kinematics of Saanen Bucks using Different Diluents. World Vet. J., 13 (2): 300-309. https://doi.org/10.54203/scil.2023.wvj32

Pawar K, Kaul G (2011). Assessment of buffalo (Bubalus bubalis) sperm DNA fragmentation using a sperm chromatin dispersion (SCD) test. Reproduction in Domestic Animals. https://doi.org/10.1111/j.1439-0531.2011.01766.x

Putra LM, Ducha N (2019).Viabilitas Sperm Kambing Boer Pasca Pembekuan dalam Pengencer Tris Dasar Soya dengan Kombinasi Gula yang Berbeda. LenteraBio, 8 (1): 31–35

Ramirez-Reveco A, Hernandez JL, Aros P (2016). Long Term of frozen semen at -196oC doses not effect the post-thawed sperm quality of bull semen. Book Chapter. https://doi.org/10.5772/64948

Roca J, Carrizosa JA, Canpos I, Lafuente A, Varquez JM, Martines E (2004). Viability and fertility of unwashed Muriciano-Granadina buck sperm diluted in Tris egg yolk extender and strored at 5ºC. Small Rumin. Res., 25: 147-153. https://doi.org/10.1016/S0921-4488(96)00978-9

Samsudewa D, Suryawijaya A (2008). Pengaruh Berbagai Methode Thawing terhadap Kualitas Semen Beku Sapi. Seminar Nasional Tekhnologi Peternakan dan Veteriner. Fakultas Peternakan Universitas Diponegoro. Semarang.

Swarna M, Saha NG, Biswas S, Paul AK (2022). Collection and evaluation of indigenous buck semen at the coastal region of Bangladesh. Insights Vet Sci., 6: 001-004. https://doi.org/10.29328/journal.ivs.1001034

Sitomorang P (2002). Pengaruh Kolesterol Terhadap Daya Hidup dan Fertilitas Sperm Sapi. JITV., 7(4): 251-258

Standar Nasional Indonesia (SNI) 4869.3. (2014). Semen beku–Bagian 3: Kambing dan domba. Badan Standardisasi Nasional Indonesia. Retrieved from: https://www.scribd.com/document/392812442/SNI-4869-3-2014-SemenBeku-Ba gian-3-Kambing-Dan-Domba-01

Steel RGD, Torrie JH (1993). Prinsip dan prosedur Statistika, Suatu Pendekatan Biometrik. Terjemahan Sumantri B. Jakarta: Gramedia Pustaka Utama.

Susilawati T (2013). Spermatologi. Universitas Brawijaya Press. Malang, Indonesia.

Susilowati S, Mustofa I, Wurlina W, Hernawati T, Oktanella Y (2021). Maintaining the quality of Kacang buck semen in chilled storage with the addition of green tea extract in extender. Trop. Anim. Sci. J., 44(4): 408-414. https://doi.org/10.5398/tasj.2021.44.4.408

Suwimonteerabutr J, Chumsri S, Tummaruk P, Nuntapaitoon M (2020). Butaphosphan and Cyanocobalamin Supplementation in Semen Extender on Chilled Boar Sperm Quality and Life Span. Front. Vet. Sci., 7:592162. https://doi.org/10.3389/fvets.2020.592162

Tash JA, Applegarth LD, Kerr SM, Fins JJ, Rosenwaks Z, Schlegel PN (2003). Postmortem Sperm Retrieval: The Effect of Instituting Guidelines. J. Urol., https://doi. org/10.1097/01.ju.0000092832.37190.94

Triyani RS, Zaenuri LA, Sumadiasa IWL, Lukman HY (2024). Jurnal Biologi Tropis, 24 (1b): 380 – 389. https://doi.org/10.29303/jbt.v24i1b.7675

Ugur MR, Abdelrahman AS, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, Purwantara B, Kaya A, Memili E (2019). Advances incryopreservation of bull sperm. Review. Front Vet Sci., 6: 268. https://doi.org/10.3389/fvets.2019.00268

Walters EM, Benson JD, Woods EJ, Critser JK (2009). The history of sperm Cryopreservation. Cambridge University Press and Assessment 978-0-521-61128-2 — Sperm Banking, Theory and Practice. Edited by Allan A. Pacey, Mathew J. Tomlinson., 1-17. https://doi.org/10.1017/CBO9781139193771.002

Watson PF (2000). The cause of reduced fertility with cryopreserved semen. Anim. Reprod. Sci., 60-61: 481-492. https://doi.org/10.1016/S0378-4320(00)00099-3

White IG (1993). Lipids and calcium uptake of sperm in relation to cold and preservation: a review. J. Reprod. Fertil. Dev., 5:639-658. https://doi.org/10.1071/RD9930639

Yimer N, Muhammad N, Sarsaifi K, Rosnina Y, Haron AW, Khumran AM, Kaka A (2015). Effect of honey supplementation into Tris Extender on Cryopreservation of Bull Sperm. Mal. J. Anim. Sci., 18(2): 47-54

Zaenuri LA, Lukman HY, Yanuarianto O, Sumadiasa IWL, Rodiah R (2017). Additional freezed drying fig fruit (*Ficus carica L*) filtrate into tris egg yolk extender and its effect on sperm membrane integity and acrosome of Kacang buck. Anim. Prod., 19(3): 161-166. https://doi.org/10.20884/1.jap.2017.19.3.647

Zaenuri LA, Rodiah R, Lukman HY, Dradjat AS, Yuliani E (2023). Sperm quality of liquid boer buck semen in tris egg yolk extender enriched with non-enzymatic antioxiandts. J. Kedokteran Hewan, 17(2): 55-61. https://doi.org/10.21157/j.ked.hewan.v17i2.26913

Zaenuri LA, Rodiah R (2025). Impact of fig-enriched tris-albumin extender on the quality of boer buck spermatozoa. Adv. Anim. Vet. Sci., 13(1): 26-35. https://doi.org/10.17582/journal.aavs/2025/13.1.26.35

Zamfirescu S, Nadolu D (2013). Results concerning the freezing pretablility of buck semen and fecundity after artificial insemination of local bucks, Universitatea de Ştiințe Agricole şi Medicină Veterinară