The Impact of Sexual Rest and Scrotal Circumference on the Quality of Fresh and Chilled Semen in Local Ram

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ABSTRAK

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Kualitas semen dipengaruhi oleh berbagai faktor, salah satunya istirahat kawin. Tujuan penelitian ini adalah mengevaluasi kualitas semen cair domba lokal Indonesia yang diistirahatkan kawin. Semen dikoleksi dari empat ekor domba menggunakan vagina buatan. Semen segar dievaluasi secara makroskopis (volume, warna, konsistensi, pH, dan gerakan massa) serta mikroskopis (konsentrasi, motilitas progresif, viabilitas, dan abnormalitas spermatozoa). Semen kemudian diencerkan menggunakan pengencer Tris-fruktosa-kuning telur (Tris) atau Na sitrat-fruktosa-kuning telur (Na-sitrat) hingga konsentrasi 50x106 sel/0,2 mL. Semen cair dikemas dalam tabung sentrifugasi tertutup dan disimpan dalam refrigerator bersuhu 4−5◦C. Motilitas progresif dan viabilitas spermatozoa semen cair dievaluasi setiap 24 jam selama 4 hari. Pengaruh lingkar skrotum (LS) terhadap semen segar maupun semen cair juga dievaluasi. Hasil penelitian menunjukkan bahwa semen segar memiliki volume 1,08±0,12 mL, warna putih susu, konsistensi encer hingga kental, pH 6,42±0,13, gerakan massa 2,94±0,13, konsentrasi 4941±926,84x106/mL, motilitas progresif 79,38±2,17%, viabilitas 87,74±3,48%, dan abnormalitas 7,99±3,14%. Istirahat kawin meningkatkan konsentrasi dan abnormalitas spermatozoa; sedangkan LS memengaruhi motilitas progresif, viabilitas, konsentrasi, dan abnormalitas spermatozoa semen segar. Istirahat kawin tidak memengaruhi kualitas semen cair. Media Tris mempertahankan motilitas dan viabilitas spermatozoa semen cair lebih baik (P< 0,05) dibandingkan dengan Na-sitrat. Motilitas dan viabilitas spermatozoa semen cair dipengaruhi oleh LS. Istirahat kawin meningkatkan beberapa parameter kualitas semen segar, namun perlakuan ini tidak berdampak pada kualitas semen cair domba lokal. Peningkatan abnormalitas spermatozoa setelah istirahat kawin dapat berdampak negatif pada efisiensi reproduksi, sehingga kualitas semen perlu dioptimalkan kembali sebelum digunakan dalam inseminasi buatan.

Kata Kunci: Domba, Istirahat Kawin, Lingkar Skrotum, Pengencer Semen, Semen Cair

ABSTRACT

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Semen quality is influenced by various factors, including sexual rest. This study aimed to evaluate the quality of chilled semen from Indonesian local rams subjected to sexual rest. Semen was collected from four rams using an artificial vagina and evaluated macroscopically (volume, colour, consistency, pH, and mass movement) and microscopically (sperm concentration, progressive motility, viability, and abnormality). Semen was then diluted using either Tris-fructose-egg yolk (Tris) or sodium citrate-fructose-egg yolk (Na-citrate) extenders to a concentration of 50×10^6 cells/0.2 ml. The diluted semen was stored in sealed centrifuge tubes at 4–5°C. Progressive motility and viability of spermatozoa in chilled semen were assessed every 24 hours for four days. The influence of scrotal circumference (SC) on both fresh and chilled semen quality was also evaluated. Results showed that fresh semen had a volume of 1.08±0.12 mL, milky white colour, thin to thick consistency, pH of 6.42±0.13, mass movement score of 2.94±0.13, concentration of $4941\pm926.84\times10^6$/mL, progressive motility of 79.38±2.17%, viability of 87.74±3.48%, and abnormality of 7.99±3.14%. Sexual rest increased sperm concentration and abnormality, while SC significantly influenced progressive motility, viability, concentration, and abnormality in fresh semen. However, sexual rest had no significant effect on chilled semen quality. The Tris extender could maintain sperm motility and viability significantly better ($P < 0.05$) than the Na-citrate extender. Additionally, chilled semen quality was influenced by SC. In conclusion, sexual rest improved certain parameters of fresh semen quality but did not significantly affect the quality of chilled semen in local Indonesian rams. An increase in spermatozoa abnormalities after a period of sexual rest can negatively affect reproductive efficiency; therefore, semen quality needs to be re-optimized before being used in artificial insemination.

Key Words: Chilled Semen, Ram, Scrotal Circumference, Semen Extenders, Sexual Rest

INTRODUCTION

Sheep (*Ovis aries*) are among the domesticated livestock species that have been widely cultivated in Indonesia for centuries. These animals are recognized for their high fertility rates, numerous benefits, and ease of management. They serve as an essential source of protein and animal fat through their meat, a valuable alternative to cow's milk through their milk production, and as suppliers of wool and hides for the textile industry. Sheep farming is particularly popular in Indonesia due to its cost-efficiency and minimal land requirements. Additionally, sheep are also one of the national food sources, ranking fifth in meat production, with a population reaching 17,902,991 in 2021 (BPS 2021). Thus, sheep farming holds significant potential for economic development.

One strategy to enhance sheep productivity is through the application of artificial insemination (AI) technology. Gibbons et al. (2019) define AI as a key biotechnology used to enhance the population and genetics of livestock. AI can utilize fresh, chilled, or frozen semen. Approximately 95% of AI procedures in livestock employ preserved semen (Bustani & Baiee 2021). The use of chilled semen in AI offers several advantages, including practicality, simplicity, cost-effectiveness, and reliability (Gibbons et al. 2019). Although unsuitable for extended storage or long-distance transport, chilled semen mitigates the physiological stress associated with cryopreservation processes, such as freezing and thawing, which can induce substantial damage to sperm (Richardson et al. 2017, Schoelerman et al. 2026). Reports indicate that sperm motility in chilled semen is superior to that observed in frozen semen after storage (Acharya et al. 2020). Furthermore, chilled semen exhibits lower rates of morphological damage, reduced fertilization failure, and decreased embryonic loss compared to frozen semen.

The quality of semen is a critical determinant of fertility and AI success. High-quality chilled semen relies on the use of superior fresh semen, extenders capable of maintaining sperm viability outside the male reproductive tract, and appropriate preservation techniques. Ejaculation frequency is a key factor influencing fresh semen quality, with prolonged sexual rest shown to significantly affect sperm physiology. Research on evaluating the chilled semen of local ram rested from breeding programs has been limited, thus prompting this research. The primary objective is to evaluate the quality of chilled semen from local rams following a rest period from breeding activities. Furthermore, the study investigates the effects of scrotal circumference and the use of two yolk-based extenders on the quality of chilled semen from ram undergoing breeding rest, providing a foundation for this research.

MATERIALS AND METHODS

Determination of age and scrotal circumference

The ram used in this study was thin-tailed. Each ram was manually restrained, ensuring proper immobilization by securely gripping the ram's body. The age of each ram was determined by assessing dental eruption and wear patterns, following standard age estimation protocols. Scrotal circumference (SC) was measured using a flexible measuring tape, following the procedures outlined by Tibary et al. (2018). The neck of the scrotum was gently grasped to ensure the testes were fully descended to the base of the scrotum. The measuring tape was then carefully wrapped around the widest circumference of the scrotum, and the measurement was recorded.

Fresh semen collection and evaluation

Fresh semen was collected biweekly using an artificial vagina (AV) designed for sheep. Adult ewes were used as teasers to stimulate ejaculation. The semen was collected into a tube and immediately protected from light exposure by placing it in a plastic cup wrapped with opaque paper to prevent light-induced sperm damage. The semen samples were then transported to the laboratory for evaluation. Both macroscopic and microscopic assessments were performed to analyse the quality of the semen.

Macroscopic evaluation

Macroscopic evaluation of semen was conducted according to the protocols described by Susilawati (2011). The evaluation included assessments of volume, colour, consistency, and pH. The volume of semen was measured directly using the collection tube. The colour of the semen was observed directly after collection in the collection tube. Consistency was determined by gently tilting the collection tube and then returning it to its original position, observing the rate at which the semen flowed back to the bottom of the tube. The pH of the semen was measured by applying a small drop of the sample onto narrow-range pH indicator paper (6.4–8.0). The pH was determined by comparing the colour change of the pH indicator strip to a standardized colour chart.

Microscopic evaluation

Microscopic evaluation of semen was conducted according to the protocols described by Susilawati (2011), which involved assessing several key parameters, including sperm mass movement,

concentration, progressive motility, viability, and total morphological abnormalities. Mass movement was evaluated by placing a drop of semen on a pre-warmed microscope slide and observing it under a microscope at 100x magnification (10x10).

Sperm concentration was measured using a Neubauer chamber. The semen sample was diluted 500 times (2 µL semen + 998 µL buffered formol saline), homogenized, and loaded into the Neubauer chamber, which was then covered with a special cover slip. The sperm were counted under a microscope at 400x magnification (40x10) across five large squares, measured diagonally.

Sperm progressive motility was evaluated by mixing fresh semen with 0.95% physiological saline at a ratio of 1:10, which was then placed on a pre-warmed microscope slide, homogenized with a cover glass, and a portion was transferred onto another slide and covered with a cover glass. The sample was examined under a microscope at 400x magnification (40x10) for a minimum of five fields of view. The percentage of progressive motility was assessed subjectively by comparing the total number of progressively moving sperm to the total number of sperm observed.

Sperm viability was assessed using differential eosin-nigrosine staining. To evaluate viability, semen was mixed with eosin-nigrosine stain at a ratio of 1:10 on a microscope slide, and a portion of the mixture was spread onto another slide to create a smear. The smear was air-dried on a heated stage for fixation. The sample was then observed under a microscope at 400x magnification (40x10). Viable sperm appeared with a clear or white head, while dead sperm had a purple head. At least 200 sperm or five fields of view were examined, and viability was calculated by dividing the number of live sperm by the total number of sperm observed, multiplied by 100%.

Total sperm morphological abnormalities were evaluated by observing the head, neck, and tail of the sperm. Abnormality assessment was also performed using eosin-nigrosine differential staining, with at least 200 sperm or five fields of view observed.

The preparation of chilled semen

The extenders used were Tris-fructose-egg yolk (Tris) and sodium citrate-fructose-egg yolk (Na-citrate), as referenced by Rather et al. (2017). The Tris buffer consisted of 3.028 g tris(hydroxymethyl)aminomethane, 1.70 g citric acid monohydrate, 1.25 g fructose, and distilled water to a final volume of 100 ml. The Na citrate buffer consisted of 2.9 g sodium citrate dihydrate, 1.25 g fructose, and distilled water to a final volume of 100 ml. Each extender was prepared by mixing the buffer with egg yolk (EY) in a 4:1 ratio. The extender was then supplemented with antibiotics, penicillin, and streptomycin, at concentrations of 1000 IU and 1000 µg per mL of solution. Both extenders had a pH of 6.4.

Fresh semen, once evaluated, was diluted by mixing with either the Tris or Na-citrate extender. The dilution of semen followed the protocol for artificial insemination (AI) in sheep using chilled semen, at a concentration of 50×10^6 sperm/0.2 mL. The diluted semen was then packaged into sealed centrifuge tubes and stored in a refrigerator (4–5°C) using a water-jacketed container. The refrigerator used for semen storage was designated exclusively for this purpose and was only opened during semen evaluation.

Chilled semen evaluation

The evaluation of chilled semen included the assessment of sperm progressive motility and viability, conducted every 24 hours at time points 0, 24, 48, 72, and 96 hours. The evaluation of progressive motility was performed by placing approximately 50 µL (one drop) of chilled semen on a pre-warmed microscope slide, which was then covered with a cover glass. The sample was observed under a microscope at 400x magnification (40x10) for a minimum of five fields of view. The motility was recorded as a percentage from 0 to 100%.

For sperm viability evaluation, 50 µL or one drop of chilled semen was placed on a microscope slide, and a differential eosin-nigrosine stain was added in a 1:1 ratio. A smear was prepared on another microscope slide and air-dried on a heated stage for fixation. The sample was examined under a microscope at 400x magnification (40x10). A minimum of 200 sperm or five fields of view were observed.

Data analysis

This study used 16 samples from 4 local rams with 4 repetitions. Data were analysed to assess fresh semen characteristics based on SC, and the motility and viability of chilled semen using Tris and Na-citrate extenders. Effects of SC, extender type, and storage time on semen quality were evaluated. Means and standard deviations were tabulated in Excel 2013 and analysed with SPSS 16.0. Independent t-tests assessed differences in fresh semen based on SC, as well as motility and viability of chilled semen in the two extenders. One-way ANOVA tested SC effects on chilled semen. The Independent t-test was used because SC was only compared between two groups, whereas the one-way ANOVA was applied to the other parameters, as they involved four groups, making it more appropriate for comparing more than two groups simultaneously.

RESULTS AND DISCUSSION

Age and scrotal circumference

The four rams were approximately 4 years old. Rams 1 and 2 had a scrotal circumference (SC) of 29 cm,

while rams 3 and 4 had a SC of 23 cm. Age is a key factor influencing semen quality, as it is closely linked to the development of the male reproductive system.

Variation in SC size may be influenced by individual factors. Pugh et al. (2020), in their evaluation of breeding soundness exams (BSE), categorized rams aged 8–14 months with a SC greater than 28 cm as satisfactory, while those with a SC smaller than 28 cm were considered unsatisfactory. The Indonesian National Standardization Agency (BSN 2015) mandates a minimum SC of 25 cm for Garut ram aged 18–24 months. Due to the differences of SC, SC was selected as one of the variables for this study.

Characteristics of fresh semen

Based on both macroscopic and microscopic evaluations, the fresh semen used in this study was of good quality (Table 1). The results of the fresh semen evaluation are necessary to determine the suitability of the semen for further processing. The analysis of fresh semen in this study showed characteristics that were similar to those previously reported. The volume of fresh semen in adult ram has been reported as 1.20 mL (Solihati et al. 2018), 0.5–2 mL (Hafez & Hafez, 2013), and 0.7–1.0 mL (Nubatonis et al. 2022). Normal ram semen is typically cream-colored (Solihati et al. 2018) or milky white (Hafez & Hafez 2013). The consistency of local ram semen has been described as slightly watery (Solihati et al. 2018) or ranging from thick to very thick (Hafez & Hafez 2013). The normal pH range of ram semen is 5.9–7.3 (Hafez & Hafez 2013).

The semen volume obtained in this study was considered normal, despite the extended sexual rest period (no semen collection for one year). Normal ram semen ejaculates typically have a low volume but high concentration (Hafez & Hafez 2013). Semen volume can be influenced by the plasma content of the semen and the collection method. Ram semen collected using an artificial vagina has been reported to have a volume range of 0.5–1.5 mL (Abott 2018).

The microscopic evaluation of sperm mass movement in this study was similar to the results of Solihati et al. (2018), which was 2–3. The average sperm concentration was higher than that reported by Solihati et al. (2018) and Nubatonis et al. (2022), which were 3487.1x106/mL and 2940x106/mL, respectively. The sperm motility obtained in this study was lower than that of Solihati et al. (2018), which was 87.72%, but still within the normal range (70–90%) according to Hafez & Hafez (2013). The sperm viability was higher compared to Nubatonis et al. (2022), who reported 81.5±4.54%. The total sperm abnormalities in this study were higher than those reported by Solihati et al. (2018) at 1%, but still within the normal range, which is <15% (Hafez & Hafez 2013). Differences in the quality of ram semen can be influenced by breed, age, environmental temperature, season, and frequency of collection (Benmoula et al. 2018).

The high sperm concentration in this study was likely due to the long sexual rest period. Sperm are continually produced by the testes, regardless of the frequency of ejaculation. Sperm that are not ejaculated for a long time will accumulate in the cauda epididymis, vas deferens, and ampulla vas deferens, leading to a higher sperm concentration when ejaculated (Abott 2018). A long sexual rest period has also been reported to be associated with the secretion of the epididymis in providing a conducive environment for sperm. Sperm undergo various biochemical and physiological modifications in the epididymis to acquire fertilizing

Table 1. The quality of fresh ram semen

Characteristics	Mean±SD	References
Macroscopic		
Volume (mL)	1.08±0.12	1.20[(a)], 0.5–2[(b)]
Colour	Milky-white	Cream[(a)], Milky-white[(b)]
Consistency	Thin to thick	Think[(b)]
pH	6.42±0.04	5.9–7.3[(b)]
Microscopic		
Mass movement	2.94±0.13	2.00–3.00[(a)]
Sperm concentration (10^6/mL)	4941±926.84	3487.1[(a)], 2940±0.50[(c)]
Sperm motility (%)	79.38±2.17	87.72[(a)], 70–90[(b)]
Sperm viability (%)	87.74±3.48	81.5±4.54[(c)]
Total abnormality (%)	7.99±3.14	1[(a)], <15[(b)]

[(a)]= Solihati et al. (2018); [(b)]= Hafez & Hafez (2013); [(c)]= Nubatonis et al. (2022)

ability. These modifications may include chromatin condensation, changes in plasma membrane composition, and the ability of sperm to withstand hypoosmotic stress (Koziol & Armstrong 2022).

The epididymis is also responsible for processing degenerative sperm. Dead sperm release enzymes that may harm nearby live sperm. Cauda epididymal cells are believed to secrete fibrinogen-related proteins that coat damaged sperm. A long sexual rest period reduces the epididymis capacity to provide a conducive environment for sperm (Dcunha et al. 2022). In this study, the slightly lower sperm progressive motility and higher total sperm abnormalities may be related to the decreased capacity of the epididymis.

Sperm stored in the cauda epididymis after a long sexual rest period may undergo degeneration or be passively transported to the vas deferens. Hafez & Hafez (2013) reinforced the idea that sperm have a limited lifespan and, if not ejaculated due to a long sexual rest period, will gradually be eliminated via urine or undergo aging and disintegration. Besides decreased motility, prolonged storage of sperm in the cauda epididymis leads to the loss of fertilizing ability. Other factors affecting sperm motility include breed, body weight (BW), environment, physical activity, diet, and heat stress (Hafez & Hafez 2013).

Sperm viability did not seem to be affected by the long sexual rest period in this study. Sperm viability is more dependent on an intact plasma membrane, which is influenced by pH, osmolarity, and sperm metabolism (Prastika et al. 2018). Sperm abnormalities have been reported to be related to epididymal dysfunction caused by long sexual rest periods (Bhosrekar 1990). In this study, the most common abnormality found was secondary abnormalities in the tail, while primary abnormalities such as pear-shaped (pyriform head) and macrocephalus heads were also found in small numbers. Other factors causing sperm abnormalities include nutrition, body weight, accessory gland activity, season, and staining treatment (Koziol & Armstrong 2022). However, the total abnormality of 7.99% observed in this study wass still within the Indonesian national standard, which sets a maximum limit of 20%.

The results of this study indicate that, although the total sperm abnormalities were slightly higher and progressive motility was somewhat lower, the semen quality could still be considered good. This suggests that local ram have their own mechanisms to adapt and produce high-quality semen after a prolonged sexual rest period. Koziol & Armstrong (2022) also reported that ram that have had prolonged sexual rest periods adapt to produce high-quality sperm.

Characteristics of fresh semen based on SC

Based on scrotal circumference (LS), the local ram with an LS of 29 cm had better semen characteristics compared to the local ram with an LS of 23 cm, both macroscopically and microscopically (Table 2).

The results of both macroscopic and microscopic evaluations of fresh semen from local ram with scrotal circumference (LS) of 29 cm and 23 cm can be considered good. However, the sperm concentration, progressive motility, and viability of semen from ram with an LS of 29 cm were significantly higher ($P<0.05$) compared to those with an LS of 23 cm. The total sperm abnormalities in fresh semen from ram with LS 29 cm

Table 2. The quality of fresh ram semen based on scrotal circumference

Characteristics	Scrotal circumference (cm)	
	29	23
Macroscopic		
Volume (mL)	1.14±0.26[a]	1.02±0.36[a]
Colour	Milky white	Milky white
Consistency	Thick	Thin to thick
pH	6.40±0.00[a]	6.44±0.11[a]
Microscopic		
Mass movement	3.00±0.00[a]	2.88±0.35[a]
Sperm concentration (106/mL)	5480.63±737.64[a]	4399.38±792.85[b]
Sperm motility (%)	81.25±3.54[a]	77.50±2.67[b]
Sperm viability (%)	90.51±2.16[a]	84.96±2.96[b]
Total abnormality (%)	5.37±0.89[a]	10.61±1.90[b]

*Numbers followed by different lowercase letters (a, b, c, d) in the same row indicate significant differences at the significance level of $\alpha=0.05$.

were significantly lower ($P<0.05$) compared to those with LS 23 cm. LS is an indirect measurement of testicular size (Chaudari et al. 2018). Testicular size has been used as a standard for fertility and reproduction in ram. LS can estimate the number of healthy testicular parenchymal cells, which are related to sperm production (Tibary et al. 2018). This underscores the importance of measuring LS in the selection of males for breeding programs.

The testes consist of seminiferous tubules (ST), which are the only site for sperm production. ST is made up of peritubular myoid cells (PTM), Sertoli cells, and germ cells, and there are Leydig cells between the tubules (interstitial) that influence spermatogenesis (Choudhary & Choudhary 2022). Significant correlations between LS and semen quality have been reported for sperm motility (Susilawati et al. 2020; Rashid et al. 2015).

Normal testicular function depends on endocrine, autocrine, and paracrine control (Koziol & Armstrong 2022). The coordinated interaction of hormones from the hypothalamus, anterior pituitary, and gonads regulates spermatogenesis (Campbell et al. 2020). The hypothalamus secretes hormones that stimulate the release of gonadotropins (gonadotropin-releasing hormone/GnRH), which in turn stimulate the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the anterior pituitary. FSH promotes Sertoli cell activity, while LH stimulates Leydig cells.

Testosterone is the main androgen in the testes that regulates spermatogenesis. Testosterone is converted into a more active form, dihydrotestosterone, by 5α reductase and oestradiol by aromatase (Davey and Grossmann, 2016). Testosterone's actions are mediated by androgen receptors (AR), which are transcription factors activated by ligands (ligand-dependent nuclear transcription factors).

Testosterone in the testes interacts with AR in Leydig cells, PTM cells, arteriolar smooth muscle cells, and endothelial cells. Testosterone works synergistically with FSH to regulate Sertoli cells (Koziol & Armstrong 2022). Sertoli cells secrete androgen-binding protein (ABP), which binds testosterone and is responsible for maintaining high levels of testosterone in the testes and carrying it to the epididymis. Furthermore, testosterone regulates the levels of GnRH, FSH, and LH through feedback inhibition on the hypothalamus and anterior pituitary (Campbell et al. 2020).

Testosterone concentration has been reported to have a positive correlation with LS in bulls (Dasrul et al. 2020), buffaloes (Qadarsina et al. 2019), and ram (Maksimović et al. 2016). Testosterone stimulates the growth and development of spermatogenic tissue, influencing testicular size. Testosterone concentration has also been shown to have a positive correlation with sperm concentration, motility, and viability in cattle (Dasrul et al. 2020). Moreover, Dasrul et al. (2020) found a negative correlation between testosterone concentration and sperm abnormalities in cattle. Low testosterone levels may lead to an increase in sperm abnormalities due to impaired epididymal function (Azizunnesa et al. 2013). In this study, the total sperm abnormalities in rams with LS 23 cm were twice as high as in those with LS 29 cm, which may be related to testosterone levels.

Characteristics of chilled semen

Quality of chilled semen in Tris and sodium citrate

The study results indicated that the progressive motility and viability of local ram sperm in Tris were higher than in Na citrate. Progressive motility was significantly higher ($P<0.05$) in Tris compared to Na citrate at 72 and 96 hours of storage (Table 3). The viability of sperm was also significantly higher ($P<0.05$) in Tris compared to Na citrate at 0, 24, and 48 hours of storage (Table 4).

The type of extender is a crucial factor determining the success of sperm during chilled storage. Tris was able to maintain the motility and viability of ram sperm better than Na citrate during chilled storage at 4–5°C. Tris is a type of extender that has been proven to be superior to other extenders due to its ability to stabilize biomolecular structures and its low toxicity. Tris acts as a buffer against pH changes, electrolyte balance, and tonicity (osmotic pressure) during chilled storage (Soltanpour & Moghaddam 2013). Progressive motility (72 and 96 hours) and sperm viability (0, 24, and 48 hours) in Tris were significantly higher compared to Na citrate due to Tris's superior ability to maintain sperm plasma membrane integrity.

Tris is believed to form two hydrogen bonds that help stabilize protein bases (Rasad et al. 2019). These hydrogen bonds interact with the polypeptide chains in egg yolk, positively affecting the maintenance of the sperm plasma membrane. Sperm are considered viable if they have an intact plasma membrane (Glazar & McCue 2021). Sperm that can maintain their membrane integrity are better able to protect mitochondrial function. Intact mitochondrial function facilitates ATP production, which is essential for sperm motility and metabolic activity.

Both progressive motility and sperm viability in Tris and Na citrate extenders decreased gradually every 24 hours of chilled storage. The total decrease in progressive motility over 96 hours in Tris was 34.07% (from 79.38% to 45.31%), while in Na citrate it was 40.94% (from 79.38% to 38.44%). The total decrease in sperm viability over 96 hours in Tris was 15.51% (from 87.74% to 72.23%), while in Na citrate it was 18.71% (from 87.74% to 69.03%).

Table 3. Sperm progressive motility of chilled semen preserved with Tris and sodium citrate extenders

Storage (h)	Extenders	
	Tris	Sodium citrate
0	77.81±4.46^{aE}	75.63±4.03^{aE}
24	71.56±5.07^{aD}	68.75±4.65^{aD}
48	63.75±5.32^{aC}	60.00±5.16^{aC}
72	55.00±5.48^{bB}	50.00±5.16^{aB}
96	45.31±4.99^{bA}	38.44±5.07^{aA}

*Numbers followed by different lowercase letters (a, b, c, d) in the same row and different uppercase letters (A, B, C, D) in the same column indicate significant differences at the significance level of α=0.05

Table 4. Sperm viability of chilled semen preserved with Tris and sodium citrate extenders

Storage (h)	Extenders	
	Tris	Sodium citrate
0	83.91±3.42^{bD}	81.14±3.81^{aD}
24	81.09±3.67^{bCD}	77.80±4.13^{aC}
48	78.56±3.98^{bC}	75.20±4.58^{aBC}
72	75.66±4.28^{aB}	72.64±4.57^{aB}
96	72.23±4.6^{aA}	69.03±4.56^{aA}

*Numbers followed by different lowercase letters (a, b, c, d) in the same row and different uppercase letters (A, B, C, D) in the same column indicate significant differences at the significance level of α=0.05

The decline in sperm quality caused by storage of semen at 4–5°C does not completely halt the metabolic activity of sperm. Oxygen remains in the centrifuge tubes (the storage container for chilled semen), allowing for aerobic metabolism to occur. The accumulation of free radicals, the enzyme aromatic amino acid oxidase (AAAO), and lactic acid over prolonged storage becomes toxic and disrupts the intracellular balance of sperm, leading to a decline in their function (Khalifa & Khalil 2016).

The accumulation of free radical results in oxidative stress (Aitken & Drevet 2020). The enzyme AAAO, originating from dead sperm, has been shown to increase the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), leading to oxidative stress (Aitken et al. 2015). Oxidative stress induces lipid peroxidation in sperm. The ram sperm membrane is particularly susceptible to lipid peroxidation due to its high content of polyunsaturated fatty acids (PUFAs). Lipid peroxidation inhibits respiration and fructo-lysis, making it toxic to sperm. Similarly, excess lactic acid lowers pH, disrupting sperm motility.

Sperm viability should be higher compared to progressive motility, as not all viable sperm exhibit progressive movement. The decline in viability occurs gradually due to the presence of KT in the extender, which protects the sperm membrane from cold shock. KT has been reported to prevent oxidation of PUFAs and degenerative changes in the acrosome (Quan et al. 2016).

A prolonged sexual rest did not affect sperm during chilled storage in this study. Although the progressive sperm motility was slightly lower in fresh semen, the chilled semen exhibited good initial progressive motility (79.38±2.17%), providing a relatively high starting point for the subsequent decline during storage. This is in line with the initial decline in sperm viability, which started from a high percentage (87.74±3.48%).

Quality of chilled semen based on SC

The observations based on scrotal circumference (SC) indicated that the progressive motility and viability of sperm from chilled semen with a scrotal circumference of 29 cm were significantly higher ($P<0.05$) than those from a scrotal circumference of 23 cm. Within the same SC group, the progressive motility and viability of sperm in Tris were significantly higher ($P<0.05$) compared to those in sodium citrate (Na citrate) (Tables 5 and 6). The progressive motility of sperm in both Tris and Na citrate at an SC of 29 cm began to show significant differences ($P<0.05$) from 0 to 96 hours of storage, while at an SC of 23 cm, differences became significant ($P<0.05$) from 24 to 96 hours of storage

(Table 5). The viability of sperm from both SC 29 cm and 23 cm showed significant differences ($P<0.05$) from 0 to 96 hours of storage (Table 6). The highest progressive motility and viability of sperm during storage were found in Tris-SC 29 cm, followed by Na citrate-SC 29 cm, Tris-SC 23 cm, and Na citrate-SC 23 cm.

Sperm from ram with an SC of 29 cm were more resistant to detrimental effects during chilled storage in both Tris and Na citrate compared to sperm from ram with an SC of 23 cm in this study. In addition to predicting sperm production, testicular size can be used to predict sperm resistance during storage. Testicular size is a good indicator for estimating the quality of fresh semen, chilled semen, and frozen semen (Saurabh et al. 2018).

The estimation of a higher number of seminiferous tubules (ST) in larger scrotal circumferences (SC) suggests that the quantity and quality of the fresh semen produced are better. Rams produce approximately 21–25 $\times 10^6$ sperm per gram of testicular parenchyma per day (Montes-Garrido et al. 2023). Normal mitosis and meiosis processes, supported by components in the ST and testicular interstitial cells, determine the production of fertile sperm (Gordon 2017). Good quality fresh semen leads to semen that can better maintain its quality during chilled storage.

Sperm resistance during chilled storage is also influenced by the composition of seminal plasma. SC in ram and cattle has been positively correlated with testosterone, which affects the activity of the seminal plasma-producing organs (Maksimović et al. 2016; Dasrul et al. 2020). Rams with an SC of 29 cm have higher semen volume compared to those with an SC of 23 cm, suggesting that the semen from the SC of 29 cm contains more seminal plasma. Semen consists of one-third sperm and two-thirds seminal plasma (Kaushish 2019). Rams with an SC of 29 cm are better able to maintain sperm quality, both fresh and after dilution, compared to ram with an SC of 23 cm due to the higher content of seminal plasma and its biological components.

Lipids, cholesterol, and proteins in seminal plasma are reported to be related to the function, structure of membranes, and motility of ram sperm (Benmoula et al. 2017; Swelum et al. 2018). The main inorganic components in seminal plasma act as buffers to maintain an environment conducive to sperm metabolism, and the content of fructose has been shown to correlate with sperm fertility (Swelum et al. 2018).

Table 5. Sperm progressive motility in Tris and sodium citrate extenders based on SC

Storage (h)	Scrotal circumference (cm)			
	29		23	
	Tris	Sodium citrate	Tris	Sodium citrate
0	80.00 ± 3.78^{bE}	77.5 ± 3.78^{abE}	75.63 ± 4.17^{aE}	73.75 ± 3.54^{aE}
24	74.38 ± 4.17^{cD}	71.25 ± 4.43^{bcD}	68.75 ± 4.43^{abD}	66.25 ± 3.54^{aD}
48	66.88 ± 4.58^{cC}	63.13 ± 3.72^{bcC}	60.63 ± 4.17^{abC}	56.88 ± 4.58^{aC}
72	58.13 ± 4.58^{cA}	53.13 ± 4.58^{bA}	51.88 ± 4.58^{bA}	46.88 ± 3.72^{aA}
96	48.13 ± 5.30^{cB}	41.25 ± 3.54^{bB}	42.50 ± 2.67^{bB}	35.63 ± 4.96^{aB}

*Numbers followed by different lowercase letters (a, b, c, d) in the same row and different uppercase letters (A, B, C, D) in the same column indicate significant differences at the significance level of $\alpha=0.05$

Table 6. Sperm viability in Tris and sodium citrate extenders based on SC

Storage (h)	Scrotal circumference (cm)			
	29		23	
	Tris	Sodium citrate	Tris	Sodium citrate
0	86.23 ± 1.97^{cE}	82.81 ± 3.05^{bE}	81.60 ± 3.00^{abE}	79.48 ± 3.94^{aE}
24	83.00 ± 3.07^{cD}	79.83 ± 3.54^{bcD}	79.19 ± 3.34^{abD}	75.78 ± 3.81^{aD}
48	80.61 ± 3.41^{cC}	77.71 ± 3.64^{bcC}	76.51 ± 3.56^{bC}	72.69 ± 4.16^{aC}
72	77.90 ± 3.54^{cA}	75.35 ± 3.85^{bcA}	73.43 ± 3.90^{abA}	69.93 ± 3.62^{aA}
96	74.78 ± 4.03^{cB}	71.99 ± 4.11^{bcB}	69.69 ± 3.81^{abB}	66.08 ± 2.77^{aB}

*Numbers followed by different lowercase letters (a, b, c, d) in the same row and different uppercase letters (A, B, C, D) in the same column indicate significant differences at the significance level of $\alpha=0.05$

The data on sperm motility from the 29 cm SC group showed a significant difference (P<0.05) 24 hours earlier between Tris and Na citrate, compared to sperm from the 23 cm SC group. This difference may be due to the influence of seminal plasma composition on the extenders (Table 5). Seminal plasma has been reported to mitigate the effects of high dilution and even to improve sperm resistance to oxidative stress and cold shock (Swelum et al. 2018). However, prolonged exposure to seminal plasma is ineffective for maintaining sperm quality during dilution (Rajabi-Toustani et al. 2020). Seminal plasma is a complex biological fluid that is assumed to interact with the type of extender.

CONCLUSION

The quality of local ram semen was affected by the sexual rest period. A sufficiently long sexual rest period increased the concentration and abnormality of sperm in fresh semen, but did not affect chilled semen. Scrotal circumference significantly influenced the motility, viability, concentration, and abnormality of sperm in fresh semen, as well as the motility and viability of sperm in chilled semen. The Tris extender was more effective at maintaining the motility and viability of local ram sperm compared to Na-citrate when stored at 4–5°C for 96 hours. It is suggested that semen be collected approximately twice per week with an interval of around 3 days to support optimal sperm quality and sufficient replenishment of sperm reserves.

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