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Semen Cryopreservation Quality and Sperm Kinematics of Saanen Goats Using Different Diluents

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ABSTRACT

The success of artificial insemination (AI) in small ruminants, especially goats, depends on the quality of frozen semen. Therefore, the current study aimed to determine the quality of various diluents, including tris-egg yolk, AndroMed®, and OviXcell®, on semen quality. The fresh semen samples from three male Saanen goats aged 1.5-2 years were collected and the mean individual motility of samples was recorded at 70%. The cryopreservation quality of the semen was evaluated based on motility, viability, abnormality, and total sperm motility (TSM) indexes. The present laboratory experiment was performed with 3 treatments and 10 repetitions. The treatments in this study were T0 (tris-egg yolk), T1 (AndroMed®), and T2 (OviXcell®). The results showed no significant difference in the parameters of motility, viability, abnormality, and TSM among the treatment group. The kinematic parameters' average path length, velocity curved linear, and linearity showed a significant difference in all treatment groups. However, there were no significant differences among the three groups in terms of motility, progressiveness, distance curved line, distance straight line, average velocity path, velocity straight line, straightness, amplitude lateral head, beat cross frequency, and wobble kinematic parameters. Motility was higher in T2 than in T0 and T1, viability was higher in T1 than in T0 and T2, and abnormality was lower in T1 than in T0 and T2. In conclusion, the use of various diluents, such as tris-egg yolk, AndroMed®, and OviXcell®, can maintain the quality of frozen spermatozoa for over 24 hours, including motility, viability, abnormality, and TSM. Kinematic parameters obtained using CASA IVOS II can provide relevant information for various parameters using these diluents.

Keywords: Computerized Assisted Sperm Analyzer, Goat's sperm, Saanen goats, Semen quality, Sperm cryopreservation

INTRODUCTION

Saanen goats are a type of dairy goat originating from Switzerland with the primary product of milk (Susilorini, 2019). Male Saanen goats have higher levels of calcium, sodium, phosphorus, and magnesium, compared to females, making them superior in terms of nutrient content (Vargas et al., 2018). Saanen goat is a popular breed for milk production and can produce an average of 750 kg of milk, making them adaptable to various regions of the world (Sadjadian et al., 2012). The adaptability of Saanen goats and their high milk production make them a promising area for further research.

Since the origin of the Saanen goats is in subtropical regions, additional research is necessary to improve their management for optimal productivity in Indonesia (Anggraeni et al., 2020). Productivity improvement can be optimized by improving genetic quality. Genetic quality improvement in livestock can be done by applying Artificial Insemination (AI). Artificial insemination is a reproductive technology that is often successful and widely accepted by the wider community, especially breeders (Rege et al., 2011). The AI is more widely applied in large ruminants than in small ruminants. This is in accordance with previous studies indicating a success rate of 47.05% for AI in small ruminants, especially dairy goats (Ciptadi et al., 2014). Factors that influence the success of AI include livestock conditions, feed, environmental conditions, semen quality, and inseminator skills.

In Indonesia, frozen semen is commonly used for AI in goats. The production of frozen semen involves several stages, including preparation, semen storage, semen evaluation, dilution, packaging, freezing, and inspection after freezing (Moore and Hasler, 2017). The success of freezing semen largely depends on the type of diluent used. The process of producing frozen semen requires a diluent to maintain the quality of semen (Arif et al., 2022). Diluents are added to semen to protect and energize the spermatozoa and prevent cold shock during cryopreservation (Tethool et al., 2021). The most commonly used diluents for freezing semen in Indonesia are tris-egg yolk, AndroMed®, and OviXcell®. Egg yolk is added as an extracellular cryoprotectant and energy source, containing lecithin and lipoprotein (Tethool et al., 2022). To determine the effectiveness of semen dilution, it is important to examine the quality of the

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diluent used. The high-quality diluent can protect the membrane cell and prevent cold shock (Arif et al., 2022). Cold shock occurs due to adaptation during storage and gradual cooling from 30°C to 4°C for 2 hours (Falchi et al., 2018).

Semen used for insemination must be tested for its quality. Semen quality tests can be performed at macroscopic and microscopic stages (Arif et al., 2020). The quality analysis of goat semen during freezing is carried out to determine the frozen semen's suitability for insemination. Parameters of motility, viability, and abnormality are usually used to determine the quality of goat semen during storage (Davendra and Krishnappa, 2019). The motility of sperm can be measured visually or using Computerized Assisted Sperm Analyzer (CASA). Motility assessment results with CASA are more objective, accurate, repeatable, and standardized. The CASA is based on the movement and trajectory of spermatozoa (Anand and Yadav, 2016). The aim of this study was to analyze the quality of the frozen semen of Saanen goats using different diluents, namely tris-egg yolk, AndroMed®, and OviXcell®.

MATERIALS AND METHODS

Ethical approval

The research was conducted at the Singosari Center for Artificial Insemination (SCAI), Malang, East Java, Indonesia, according to standard procedures of SNI ISO 9001:2015 NO.G.01-ID0139-VIII-2019 and supervised by veterinarians. Ethical guidelines and approvals on research procedures have been provided by the SCAI ethics committee. This research has been approved by the ethics committee of the University of Brawijaya number 009-KEP-UB-2023.

Study design

The current study was performed on three male goats aged 1.5-2 years from the Singosari Center for Artificial Insemination (SCAI) Singosari, Malang, East Java, Indonesia. The fresh semen samples Saanen goats aged 1.5-2 years. The goats were kept in a pen at a temperature of 28-30°C under normal conditions. The goat pen had enough lighting using an intensive system with grazing in the paddock in the morning. Each goat had its own pen with a size of 2.5 x 2 m^2. The feed composition consists of 2 kg/head of forage, 2.5 kg/head of legumes, and 1 kg/head of concentrate. A laboratory experimental method was used for this study with 3 treatments with 10 repetitions. The three goats had body weights of 32.5 kg, 43 kg, and 44.5 kg. The semen aseptically was collected once a week for 10 weeks using an artificial vagina (IMV, France). A teaser was used to induce libido and facilitate semen collection.

Diluent preparation

The dilution process involves the use of three different diluents, namely tris-egg yolk (T0), AndroMed® (T1), and OviXcell® (T2). The T0 was made up of1.363 g tris aminomethane (Merck, Germany), 0.762 g citric acid (Serva, USA), 15 g lactose (Serva, USA), 0.5 g fructose (Kanto Chemical, Japan), 20 g egg yolk, 2.7 g raffinose (Serva, USA), 0.1 g streptomycin (Meiji, Indonesia), 80 ml aquabidest 0.1 g penicillin (Meiji, Indonesia), and 13% glycerol (Merck, Germany). The T1 using AndroMed® (Minitube, Germany) contained aquabidest, fructose, glycerol, citric acid, buffer, phospholipids, spectynomycine, 15 mg lyncomycine, 5 mg tylocin, and 25 g gentamycin (Susilawati, 2011). Finally, T2 entailing OviXcell® (IMV, France) had ultra-pure water, salt, sugar, electrolytes, glycerol, antibiotics protein, and some ingredients that were not listed by the manufacturer (Kakati et al., 2019).

The tris-egg yolk diluent was divided into three parts, namely volume A, volume B, and volume C. Volume A had no glycerol with a ratio of volume and semen of 1:1. Volume B, without glycerol, was added after the semen has equilibrated at 4-5°C and volume C contained 13% glycerol. AndroMed® was diluted with aquabides and could be used immediately in a ratio of 1:4. For example, 20 ml of AndroMed® was added to 80 ml of aquabides (in a 100 ml preparation). The semen mixed with AndroMed® was immediately put in the cool top until the temperature reached 4-5°C, then the dilution process with semen continued. OviXcell® did not require any additional dilution with aquabides before being mixed with semen. It could be directly mixed with semen without the need for any intermediary dilution step. The dilution formulas applied to Singosari Center Artificial Insemination for frozen semen are as follows (Shukla, 2020).

$$\text{Total volume} = \frac{\text{Semen Volume} \times \text{Concentration} \times 10^6}{200 \times 10^6} \qquad \text{Formula 1 (All diluents)}$$

$$\text{Volume A} = 1{:}\,1 \text{ (semen: diluent)} \qquad \text{Formula 2 (All diluents)}$$

$$\text{Volume B} = \frac{\text{Total Volume}}{2} - \text{Volume A} \qquad \text{Formula 3 (All diluents)}$$

$$\text{Volume C} = \frac{\text{Total Volume}}{2} \times \text{glycerol } 13\% \qquad \text{Formula 4 (tris-egg yolk diluent)}$$

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To maintain semen quality during cold storage, it was important to dilute the semen using a water bath with a temperature of 37°C and then stored it at 4-5°C. The diluent and glycerol conditions must be the same as the treatment so that the semen quality during cold storage can be maintained. In order to proceed to the freezing stage, the semen must be of high quality. If the motility of the semen before freezing is less than 55%, which is the minimum acceptable standard according to the Indonesian National Standard (2014), it should be rejected.

Semen collection

Semen was collected once a week in the morning at 10 using an Artificial Vagina (IMV, France) filled with 450-500 ml of 50-60°C warm water. The artificial vagina was pumped to a certain consistency and coated with lubricating jelly. The volume of Saanen goat's semen obtained was around 0.5-1 ml. The collected semen was immediately brought to the research site for semen dilution. The minimum dose of spermatozoa in diluent was 200 million sperm cells in each tube at the time of the study.

Cryopreservation and semen dilution procedures

After carrying out the shelter of semen, a quality test was carried out before dilution. This test included analyzing the motility, viability, abnormality, and total sperm motility (TSM) of fresh, liquid, and frozen semen using a radiance microscope (Olympus BX-53, Japan) with 400x magnification. After freezing, a more detailed test was carried out by observing kinematic parameters using a Computerized Assisted Sperm Analyzer (CASA, Hamilton Thorne, USA). Cryopreservation of goat semen using cold storage and length of time according to the type of diluent, tris-egg yolk was diluted at a ratio of 1:1 with a diluent temperature of 37°C (Susilawati, 2011). Equilibration was carried out for 2 hours (Volume A), then Volume B was added and stored for 18-22 hours at 4-5°C for tris-egg yolk diluent (Tethool et al., 2022) before adding 13% glycerol (Volume C). For AndroMed® and OviXcell® diluent process of filling and sealing was immediately carried out if the test of the quality of the liquid semen was decent. At the time of freezing, a 0.25 ml straw was filled with semen and then stored in liquid nitrogen (N_2) at a temperature of -196°C.

Statistical analysis

The obtained data were analyzed using SPSS software (version 29 IBM) with one-way ANOVA and a follow-up Duncan Multiple Range Test (DMRT) when the p-value was significant ($p < 0.05$). All data are presented as a mean ± standard error (SE).

RESULTS AND DISCUSSION

Semen cryopreservation in goats involves the use of cooling and freezing methods to dilute and preserve spermatozoa. However, it can decrease sperm quality due to temperature changes during storage and freezing (Memon et al., 2012), the formation of ice crystals, and changes in electrolyte concentrations (Tekin and Daşkin, 2019). It was stated that during the cooling and freezing phases, this damage can result in the production of ROS, lack of energy, ionic imbalance, changes in cell volume, hyperosmolarity, and protein denaturation (Ugur et al., 2019). To prevent this, a semen diluent is required to maintain the quality of the spermatozoa during cryopreservation.

The motility results showed no significant difference in semen motility of the treatments ($p > 0.05$). However, the lowest motility percentage was obtained in T0 and the highest motility percentage was indicated in T2 (Table 1). Motility is the power or movement of spermatozoa that moves forward or progressively. Motility is an important factor in determining sperm viability and its ability to fertilize an oocyte (Setiyono et al., 2020). As indicated in Table 2, the highest motility was obtained in T2 (59 ± 1.00), while the lowest motility was in T0 (49.50 ± 2.29). The decrease in motility in T0 during thawing may be due to the freezing process, which can cause damage to spermatozoa. The addition of egg yolk into the freezing extender increases the proportion of motile sperm (Aboagla and Terada, 2004). The results obtained in T2 were greater than those of Kakati et al. (2019) at 36 ± 1.21. However, the results of the T0 study were lower than those reported by Crespilho et al. (2012) at 58.52 ± 2.98. The difference in the percentages of T0 and T2 was due to the different diluents used, namely tris-egg yolk and OviXcell®. Tris-egg yolk contains egg yolk and OviXcell® contains soy lecithin so it has a different lipid composition and fatty acid content (Roof et al., 2012). Tris-egg yolk has a lower boiling point (<100°C) than the boiling point of tris solution (100°C), resulting in the formation of small granules of fructose during observation and consequently low motility (Susilawati, 2011). On the other hand, the fast progressive movement of OviXcell® diluent is because it contains soy lecithin and the diluent has a patent based on the original composition of the manufacturer (Kakati et al., 2019). OviXcell® contains soy lecithin can reduce damage due to cold shock during the freezing and thawing process (Nadri et al., 2019). As stated, the addition of 1% soy lecithin was successful in the cryopreservation of goat semen (Salmani et al., 2014). In the same line, Vidal et al. (2013) reported that the addition of soy lecithin at 0.04%, 0.08%, and 0.16% could be effective in freezing goat semen.

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Table 1. Semen quality of Saanen goats after freeze-thawing in various diluents

Parameters \ Treatment (Mean ± SE)	T0	T1	T2
Motility (%)	41 ± 2.87	46 ± 4.64	48.5 ± 2.89
Viability (%)	65.25 ± 8.09	67.53 ± 3.34	59.22 ± 5.54
Abnormality (%)	4.02 ± 1.34	1.87 ± 0.38	3.21 ± 0.41
TSM (million/straw)	16.75 ± 1.83	15.50 ± 1.82	17.25 ± 1.69

T: Treatment, T0: Tris-egg yolk, T1: AndroMed® and T2: OviXcell®

Table 2. Semen cryopreservation quality of Saanen goats before and after freezing

Parameters	Fresh semen	Semen quality before freezing (Mean ± SE)			Semen quality after freezing (Mean ± SE)		
		T0	T1	T2	T0	T1	T2
Sperm Motility (%)	73.5 ± 2.42	49.50 ± 2.29	55 ± 2.36	59 ± 1.00	41 ± 2.87	46 ± 4.64	48.50 ± 2.89
Sperm Viability (%)	72.69 ± 11.12	63.08 ± 6.84	79.41 ± 3.46	74.59 ± 4.00	65.25 ± 8.09	67.53 ± 3.34	59.22 ± 5.54
Sperm Abnormality (%)	2.78 ± 2.36	2.32 ± 0.76	2.4 ± 0.79	2.27 ± 0.49	4.02 ± 1.34	1.87 ± 0.38	3.21 ± 0.41
Total Sperm Motility (million/straw)	2706.90 ± 818.71	45.00 ± 1.15	50.00 ± 1.18	52.73 ± 0.41	16.75 ± 1.83	15.50 ± 1.82	17.25 ± 1.69

T: Treatment, T0: Tris-egg yolk, T1: AndroMed® and T2: OviXcell®

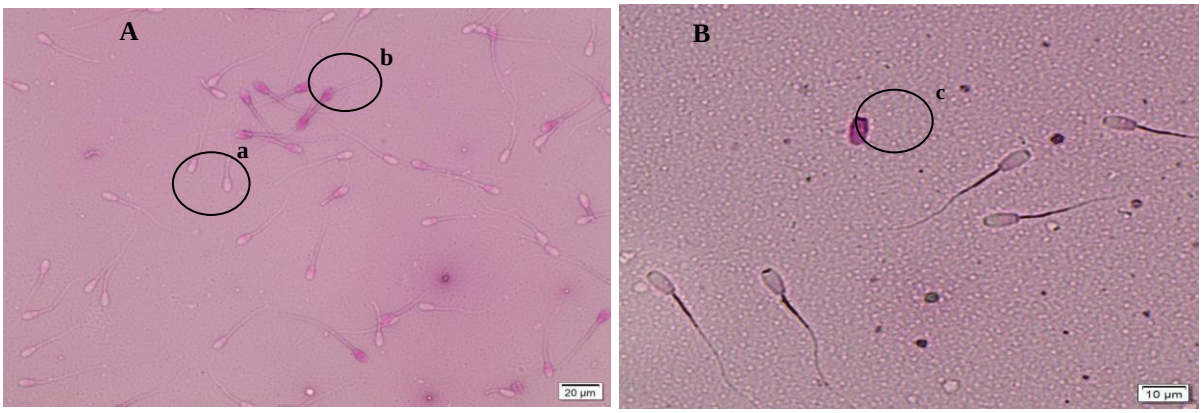

Figure 1. The viability and abnormal spermatozoa after thawing of the Saanen goats (eosin-nigrosine, x 400 magnification). **A**: Viable (the head of spermatozoa does not absorb [a]), Nonviable (b); **B**: abnormality of sperm (c).

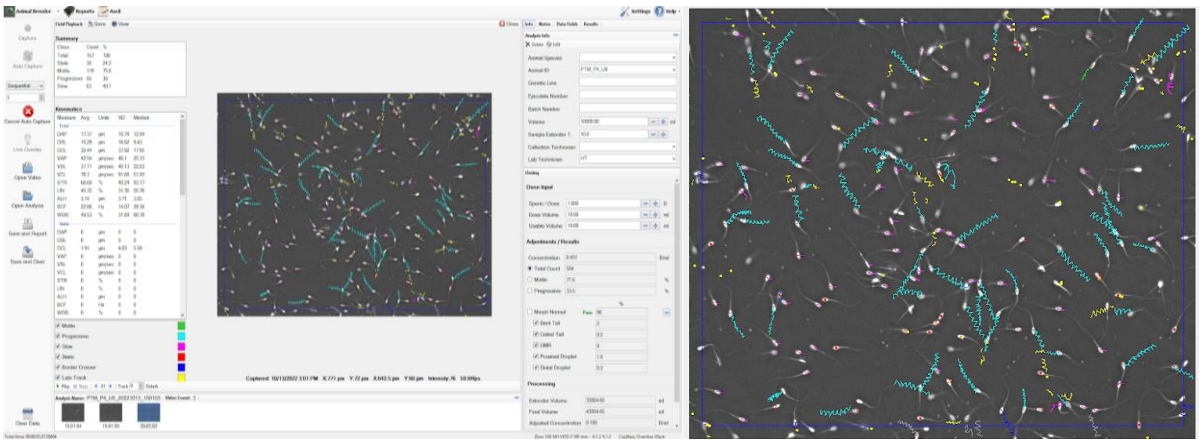

Figure 2. The kinematic parameters using CASA IVOS II (Hamilton Thorne, USA) with information green: Motile, aqua: Progressive, pink: Slow, red: Static, blue: Border crosser, yellow: Late track

The viability or survival of spermatozoa can be determined by assessing their ability to absorb the eosin-nigrosine stain on spermatozoa (Susilawati, 2011). Viability can be determined by mixing semen with eosin-nigrosine in a ratio of 10:10 µl (Yodmingkwan et al., 2016). Based on the results of the study, there was no significant difference in the viability of spermatozoa among the treatments ($p > 0.05$). However, the highest percentage of viability was observed in T1 and the lowest was in T2 (Table 1). The outcomes of this research are presented in Figure 1, where dead spermatozoa appear stained with pigment, while live spermatozoa do not absorb the stain. The viability of semen decreases during the process of cryopreservation, which is due to the storage of liquid semen in a frozen state (Shah et al., 2022). This

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decrease in viability is caused by damage to the cell membrane, which is a complex composition including two layers of phospholipids, proteins, glycoproteins, and carbohydrates (Peris-Frau et al., 2020). This is caused by a decrease in temperature during the cold storage process, which causes the lateral movement of phospholipids to change between the liquid and frozen phases leading to the membrane being stiff and brittle (Card et al., 2013). As can be seen in Table 2, viability in T1 was (79.41 ± 3.46), which was higher than in T0 (63.08 ± 6.84) before freezing. This was caused by the lecithin content in the T1 diluent, which could maintain and provide nutrition to spermatozoa or extracellular cryoprotectants. However, cryoprotectants could damage cells because they are toxic, so special protection is needed for cryoprotectants during the crystallization process, which was determined based on the type and concentration of the cryoprotectant (Wahjuningsih et al., 2019). AndroMed® is composed of ingredients needed during the cryopreservation process consisting of sodium and potassium, which are essential for maintaining the integrity of the plasma membrane (Pratiwi et al., 2014). However, the cryopreservation process can damage the plasma membrane due to the formation of lipid peroxides, which can cause peroxidation damage to unsaturated fatty acids in the membrane (Wahjuningsih et al., 2021). After the freezing process, the viability of the spermatozoa at T1 was (67.53 ± 3.34), which was higher than T0 and T2. These results are lower than the research performed by Bintara et al. (2015) at 92 ± 3.33.

Based on Table 2, the abnormality on T1 (2.40 ± 0.79) was higher than that of T2 (2.27 ± 0.49) before freezing. Lipid peroxidation can lead to changes in the structure of spermatozoa, low motility, very fast metabolic processes, and intracellular components (Grötter et al., 2019). The results of the study on abnormal spermatozoa showed no significant difference in all treatment groups ($p > 0.05$). The abnormality of sperm in T1 was lower than in T0 and T2 (Table 1). Figure 1 shows abnormalities in spermatozoa, such as a head that is distinct from the tail and a tiny head. These abnormalities can be categorized as primary abnormalities associated with the head or acrosome, secondary abnormalities that occur when cytoplasmic droplets are present in the midpiece or tail, and tertiary abnormalities that affect the tail (Susilawati, 2011). At the time of measurement after freezing, abnormality on T0 (4.02 ± 1.34) was higher than that of T2 (3.21 ± 0.41). This discrepancy may have occurred because the head and tail of the spermatozoa were cut off during the measurement. The concentration of glycerol used during the freezing process can also affect normal morphology since glycerol serves as an intracellular cryoprotectant that can prevent damage during the freezing process (Öztürk et al., 2020). The addition of glycerol with different percentages affects the percentage of abnormality so the greater the glycerol concentration, the lower the rate of reduction of abnormality (Hikmawan et al., 2016).

The TSM can be determined by multiplying the spermatozoa concentration by the volume of semen (Susilawati et al., 2020). The TSM results showed no significant difference among the treatment groups ($p > 0.05$). The percentage of total spermatozoa motile was the highest in T2 and lowest in T1 (Table 1). The TSM before freezing at T2 was 52.73 ± 0.41 million/straw, which was higher than in T1 (50.00 ± 1.18 million/straw) and T0 (45.00 ± 1.15 million/straw). This difference in TSM is attributed to lipid peroxidation during dilution and preservation (Bucak et al., 2013). Cold storage at a temperature of 4-5°C can result in cold shock and increase lipid peroxidation (Bucak et al., 2012). The higher value of TSM indicates high frozen semen quality (Tethool et al., 2021). However, However, after freezing, TSM decreased in all treatment groups, with T2 having the highest value of 17.25 ± 1.69 million/straw followed by T0 and T1. The decrease in TSM can be attributed to the low TSM value before freezing and the formation of ice crystals that can cause intracellular damage and cell death during the freezing process (Tethool et al., 2022). To prevent ice crystal formation, egg yolk phospholipids, and soy lecithin can be used to form a protective layer on the membrane, but it is important to note that these compounds may not penetrate the membrane (Kakati et al., 2019).

Table 3. Kinematic parameters after freeze-thawing of Saanen goats' semen using different diluents

Kinematic parameters	T0	T1	T2
Motile (%)	53.95 ± 3.88	57.70 ± 3.66	60.00 ± 3.48
Progressive (%)	37.13 ± 3.66	31.93 ± 3.62	40.22 ± 4.70
APL (µm)	24.17 ± 4.12[a]	23.55 ± 4.05[a]	30.44 ± 5.77[b]
DCL (µm)	30.66 ± 8.19	30.20 ± 8.15	34.46 ± 8.67
DSL (µm)	30.91 ± 2.78	30.17 ± 2.79	34.90 ± 4.48
AVP (µm/sec)	67.12 ± 10.28	63.07 ± 12.56	80.20 ± 16.61
VSL (µm/sec)	58.45 ± 8.61	56.93 ± 11.30	73.92 ± 15.58
VCL (µm/sec)	111.82 ± 17.11[b]	101.89 ± 19.67[a]	108.93 ± 21.79[b]
STR (%)	57.23 ± 7.03	66.71 ± 5.33	63.83 ± 7.09
LIN (%)	37.34 ± 4.84[a]	45.76 ± 3.43[b]	48.29 ± 5.42[b]
ALH (µm)	4.45 ± 0.75	3.75 ± 0.71	3.41 ± 0.61
BCF (Hz)	21.41 ± 2.49	24.32 ± 2.51	23.70 ± 3.52
WOB (%)	42.12 ± 5.39	46.85 ± 3.80	48.07 ± 4.19

[ab]Different superscripts with rows indicate significant differences at $p < 0.05$. T: Treatment, T0: Tris-egg yolk, T1: AndroMed® and T2: OviXcell®. APL: Average path length, DCL: Distance curved line, DSL: Distance straight line, AVP: Average velocity path, VSL: Velocity straight line, VCL: Velocity curved line, STR: Straightness, LIN: Linearity, ALH: Amplitude of lateral head, BCF: Beat cross-frequency

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The data in Figure 2 is used as input for the Computer-Assisted Sperm Analysis (CASA) software, specifically the CASA IVOS II by Hamilton Thorne in the USA. The software analyzes various sperm parameters such as straight-line velocity (STR), the amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and curvilinear velocity (WOB). In addition to these factors, the software also tracks various types of sperm motion, such as motile (green), progressive (aqua), slow (pink), static (red), border crosser (blue), and late track (yellow).

Based on the results of the study, the kinematic parameters consisted of 13 parameters using a different diluent. Results showed that sperm motility using OviXcell® diluent (T2) was higher than using tris-egg yolk (T0) and AndroMed® (T1), with no significant difference between treatments. Motility correlates with the ability of the sperm to fertilize the ovum (Wahjuningsih et al., 2021) and is crucial for success in penetrating the oocyte, the lower the sperm motility, the lower the success in penetrating the oocyte (Mocé et al., 2022). The motility of fresh semen used was 70% (Isnaini et al., 2019). The fertilization stages include capacitation of spermatozoa and maturation of spermatozoa, attachment of spermatozoa to the zona pellucida, acrosome reaction, penetration of the zona pellucida, meeting of sperm and oocyte (Yekti et al., 2017). Regarding motility, T2 had a higher percentage than T1 and T0, indicating that motility was affected by the concentration of spermatozoa and the diluent used (Yeste et al., 2018). The highest motility was obtained using OviXcell® diluent. The content in OviXcell® diluent contains soy lecithin so that it can provide nutrition to maintain its motility (Fernandes et al., 2021). This is consistent with the results of research that diluent containing soy lecithin takes longer to maintain spermatozoa motility (Khalifa et al., 2013). Soy lecithin in OviXcell® diluent could replace egg yolk lecithin during freezing with a higher motility percentage of OviXcell® diluent than tris-egg yolk (Akourki et al., 2018). In addition, spermatozoa stored at 4-5°C had greater motility than goats at 17-20°C (Xu et al., 2009). Motility decreases if the concentration and duration of storage are at 5°C (Wahjuningsih et al., 2012).

Motile and progressive are level 1 parameters in kinematic analysis using CASA (Ratnawati et al., 2017). The results of the motile percentage indicated the highest value in T2 (40.22 ± 4.70%), which was not significantly different from T0 (37.3 ± 3.66) and T1 (31.93 ± 3.62, $p > 0.05$). The highest progressive at T0 was due to the higher percentage of motility among the treatments, namely T0 and T1. However, T0 showed higher than T1 due to differences in the time of equilibration in T0 and T1. Progressive detection using CASA in research results was higher than in previous reports which indicated that progressive motility was 3.5 ± 0.4% (Bezerra et al., 2011). These results are lower than the research by Vázquez et al. (2015) at 34.6 ± 21.4. This could be due to the use of different glycerol (only 6%), while in this study it was 13%. The function of glycerol was as an intracellular cryoprotectant to prevent cold shock so that progressive spermatozoa could be protected (Sikarwar and Ramachandran, 2020). It was revealed that the addition of glycerol can cause osmotic damage and is toxic to spermatozoa if the percentage used is not appropriate (Sariözkan et al., 2010).

Based on the APL results obtained, there was a significant difference between the treatments ($p < 0.05$) with the highest yield being 30.44 ± 5.77 in the T2 treatment and the lowest in T1 of 23.55 ± 4.05 µm using the AndroMed® diluent followed by the T0 diluent of 24.17 ± 4.12. Average path length is the average direction of the path taken by the spermatozoa head (Kaewkesa et al., 2016). Significant differences between treatments in APL were caused by different levels of antioxidants resulting from diluent components, cryopreservation time, and freezing procedures (Naijian et al., 2013). The antioxidant content in egg yolks is β-carotene which gives egg yolks a yellow color pigment (Kutluyer et al., 2014). The function of antioxidants is to minimize the oxidation process of fats and oils (Yousr and Howell, 2015). Furthermore, DCL results indicated no significant difference between treatments ($p > 0.05$) with the lowest in T1 at 30.20 ± 8.15, followed by T2 (34.46 ± 8.67) and T0 (30.66 ± 8.19). The DSL results in the T2, T0, and T1 treatments were 34.90 ± 4.48, 30.91 ± 2.78, and 30.17 ± 2.79, respectively. The research results of Maylem et al. (2017) revealed that the results of APL, DCL, and DSL were 54.29 ± 21.48, 96.15 ± 39.87, 45.72 ± 21.6, respectively. The reported values were higher than those presented in a study by Wang et al. (2021).

The results of the AVP study showed that there was no significant difference among all treatment groups ($p > 0.05$). The results for T2 were higher than T0 and T1. The highest VSL results were obtained at T2 and the lowest at T1 and T0. Velocity curved linear showed significant results with differences between treatments, namely T0 was higher than T1 and T2 ($p < 0.05$). Velocity curved linear is an assessment indicator for power in the movement of spermatozoa so that it does not describe the direction of movement but provides complementary information (Susilawati et al., 2018). The results of AVP, VSL, and VCL were 30.35 ± 0.478, 18.50 ± 0.288, and 63.75 ± 0.47 respectively, in post-thawing of tris-egg yolk diluent (Anand and Yadav, 2016). This can lead to the production of reactive oxygen species (ROS), changes in membrane permeability, and the formation of intracellular ice crystals. The presence of ice crystals can result in increased osmotic pressure and alteration of sperm function, leading to changes in dissolved substances and damage to the plasma membrane. These changes can decrease sperm motility and increase damage, ultimately affecting capacitation and acrosome reactions (Wahjuningsih, 2019; Prastiya et al., 2021).

The results of the study showed that there was no significant difference ($p > 0.05$) in STR of spermatozoa between T0, T1, and T2, with STR values of 57.23 ± 7.03, 66.71 ± 5.33 and 63.83 ± 7.09 respectively ($p > 0.05$). The STR is an indicator of moving in a straight line on the average spermatozoa (Ratnawati et al., 2017). The lowest STR value was obtained using tris-egg yolk diluent at 57.23 ± 7.03%, which was lower than the results reported by Monteiro et al.

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(2022) at 85.97 ± 3.26. The use of egg yolk as a cryoprotectant can reduce sperm quality, hence the addition of extracellular cryoprotectants like soy lecithin in OviXcell® and AndroMed® diluents resulted in higher STR values, as reported by Sariözkan et al. (2010). Wang et al. (2021) reported STR values of 76.99 ± 8.67, which were lower than the results of this study.

The LIN of spermatozoa showed a significant difference ($p < 0.05$) between T0, T1, and T2, with the highest value obtained in T2 at 48.29 ± 5.42 and the lowest in T0 at 37.34 ± 4.84 and T1 at 45.76 ± 3.43. Linearity is an indicator of the straightness of the spermatozoa trajectory (Ratnawati et al., 2017). Research by Sadeghi et al. (2020) indicated that LIN value was 59.4 ± 1.2 in 24 hours of storage and 60.5 ± 0.9 in 48 hours of storage. This means that the storage of spermatozoa at 4-5˚C can decrease LIN values but increase motility. Efforts can be made to prevent cold shock by slowing down enzymatic reactions to maintain shelf life by reducing metabolic activity reversibly (Freitas-Ribeiro et al., 2019).

The ALH was highest in T0 at 4.45 ± 0.75, followed by T1 at 3.75 ± 0.71 and T2 at 3.41 ± 0.61 with no significant difference between the groups ($p > 0.05$). The ALH is the average width of the vibration oscillation or vibration of the spermatozoa head when moving (Ratnawati et al., 2017). The highest ALH value obtained in this study was lower than the value reported by Vázquez et al. (2015) at 5.624. Kathiravan et al. (2011) stated that ALH is a parameter that can indicate the average width of the oscillations when the spermatozoa heads swim. The ALH in the current study was <5 µm, which was still relatively stable after freezing.

The results of the BCF study showed that T1 was higher at 24.32 ± 2.51 compared to T2 at 23.70 ± 3.52 and T0 at 21.41 ± 2.49. In each treatment, the results showed no significant difference ($p > 0.05$). The BCF refers to a total of spermatozoa passages at an average rate per second (Ratnawati et al., 2017). The values obtained in this study were higher, compared to the results reported by Arangasamy et al. (2018), which were 10.2 ± 0.3 in goat semen. The values in this study indicate that the average flow per second has a path in a stable condition with a range of values > 20 Hz in each treatment. These results illustrate the strong frequency of spermatozoa movement. This relationship can be connected if the high ALH and low BCF values indicate a low kinetic speed.

The WOB results showed no significant difference among the treatments ($p > 0.05$) with the highest value observed in T2 at 48.07 ± 4.19 followed by T1 at 46.85 ± 3.80 and T0 at 42.12 ± 5.39. The WOB is a measurement of trajectory oscillation with a strength of one second (Ratnawati et al., 2017). The highest results were obtained using OviXcell® diluent and the lowest using tris-egg yolk diluent. The content of soybean lecithin in the diluent provides nutrition to the spermatozoa so that it strengthens the oscillations and kinematic efficiency can be known to be high or low. The results of research by Monteiro et al. (2022) showed a WOB result of 74.92 ± 8.29 using the tris-egg yolk diluent and 72.54 ± 5.04 using the skim milk diluent. Comparable results were obtained in another study at 70.01 ± 0.17 after freezing using a commercial tris-egg yolk diluent (Barbas et al., 2018).

CONCLUSION

In conclusion, the use of various diluents, such as tris-egg yolk, AndroMed®, and OviXcell®, can maintain the quality of frozen spermatozoa for over 24 hours, including motility, viability, abnormality, and TSM. Kinematic parameters obtained using CASA IVOS II can provide relevant information for various parameters using these diluents. The visual motility can be aligned to the ability of speed, distance, average width, and path length of spermatozoa which can be clearly detected. These results can be applied in further research involving direct artificial insemination.

DECLARATIONS

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Authors’ contributions

Lailatun Nisfimawardah, Aulia Firmawati, Muhammad Nur Ihsan, Trinil Susilawati, and Sri Wahjuningsih contributed manuscript writing, data analysis, and study design. Lailatun Nisfimawardah collected data analysis from the field and laboratory. All authors contributed review data from this research, statistical analysis, and approved final draft of the manuscript.

Ethical consideration

The authors declare that this manuscript has been checked by all authors, and it was originally submitted to the journal so that it is publishable for the first time in this journal.

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Competing interests

The authors state that there is no personal interest and financial interest in this research.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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