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Effectiveness of Nanoparticle-based Young Palmyra Fruit Water–egg Yolk in Liquid Semen Diluent of Sumba Ongole Bulls

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ABSTRACT

This research aims to determine the quality of liquid semen of Sumba Ongole (SO) bulls during cold storage in palmyra fruit water and egg yolk-based nanoparticle diluent. The research was conducted at the Animal Husbandry Service Laboratory of East Sumba Regency. Four SO Bulls heads aged between 3 and 4 years, weighing 272 to 525kg, were used in the study. They were maintained to meet healthy body conditions and normal reproduction. Semen was collected twice a week using an artificial vagina. This research method was through an experiment in the laboratory, which consisted of four treatments and 10 replications (semen collection). The treatments are T0: CEP-3 diluent (control); T1: 90% of young palmyra fruit water (PFW)+10% of egg yolk (EY); T2: 85% PFW+15% EY; T3: 80% PFW+20% EY; T4: 25% PFW+25% EY. The PFW diluent was filtered three times using membrane filters to obtain an average particle size of 580±35 nm. The measured variables were individual motility, concentration, total motile sperm, viability, abnormalities, intact plasma membrane, and acrosomal cap. The research used a randomized block design. Duncan's Multiple Range test was carried out to determine the difference between treatments. The results showed that the nanoparticle diluent from egg yolk and palmyra fruit water effectively maintained the quality and integrity of SO Bull's spermatozoa membranes. In conclusion, nanoparticle-based palmyra fruit water and egg yolk diluent effectively maintain the semen quality of Sumba Ongole Bulls.

Key words: Palmyra fruit water, Egg yolk, Nanoparticle, Liquid semen, Sumba Ongole bulls

INTRODUCTION

Sumba Ongole (SO) is a local livestock commodity important in supporting animal-based food security and a source of income for farmers. These local cattle are known for adapting to harsh environments such as hot weather, poor feed quality, and resistance to parasites and infectious diseases (Bakae et al. 2022). Using appropriate technology, such as artificial insemination (AI), can be one solution to improve the sustainability of SO in society. AI is a livestock reproduction technology successfully applied to local livestock (Rungroekrit et al. 2019). In some areas, obstacles in implementing the AI include the limited sources of frozen semen and liquid nitrogen.

To address the issue, one possible solution for performing AI is using the liquid semen of superior bulls selected in each region. According to Wiebke et al. (2023), liquid semen is an alternative to overcome the problems of frozen ones. To ensure a successful AI using liquid semen would require a diluent that meets some conditions as follows: it provides nutrition for spermatozoa; it does not contain any toxic materials; it includes buffers and can support spermatozoa motility; and it can act as a cryoprotectant agent to protect spermatozoa from the effects of cold shock (Rungroekrit et al. 2019).

Unfortunately, the commonly used diluents are relatively expensive and only sometimes available in certain areas, for regions outside Java, such as Sumba Island, AI using frozen semen can be particularly difficult due to the scarce availability of liquid nitrogen. Therefore, a diluent made from palm fruit water and egg yolk presents a practical and affordable alternative to liquid semen diluent. It is readily available in the local area at an affordable price, making it a viable option for regions like Sumba Island. The Palmyra palm, or lontar palm, is a genus of five species of fan palm native to tropical regions.

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Sumba, a tropical island, is abundant in the Palmyra palm. The Palmyra fruit water contains carbohydrates such as glucose and fructose; these carbohydrate sources can be used as a source of energy and capacitation for spermatozoa (Ferramosca and Zara 2014). Egg yolk contains phospholipids, cholesterol, and low-density lipoprotein so that it can protect the quality of spermatozoa, maintain the balance of diluents during the dilution process, and increase conception rates in livestock (Saad et al. 2022).

Previous studies have reported the successful use of nanoparticle-based semen diluents, demonstrating the potential of this technology in maintaining spermatozoa quality during the storage process (Farhadi et al. 2022). The small size of the nanoparticles results in a better surface-to-volume ratio (Rakib-Uz-Zaman et al. 2022), thus making it easier to reach the outer cell membrane, interact with the extracellular matrix (Behzadi et al. 2018), and penetrate the semen (Falchi et al. 2018). Based on the rationale, this research aims to determine the quality of liquid semen of Sumba Ongole (SO) bulls in palmyra fruit water and egg yolk-based nanoparticle diluent, opening up new possibilities for AI in cattle breeding.

MATERIALS AND METHODS

Ethics approval

This study was conducted following the Animal Care and Use Committee, Universitas Brawijaya, Malang, East Java, Indonesia, with ethical clearance number 090-KEP-UB-2024.

Bulls semen collection

This research used SO bulls following the Indonesian National Standards (SNI) of RSNI4 7651.8:2016. They were then selected with an age ranging from 3-4 years with a body weight ranging from 372-525kg. Meanwhile, the quantitative requirements for the SO breed used in the class I category are an average shoulder height of 147.25cm, body length of 147.25cm, chest circumference of 180.50cm, and scrotum circumference of 28.75cm. The selected bulls were then trained to collect sperm, which was performed twice a week.

Ingredients and equipment

Ingredients

SO bulls semen, aquabidest, 70% alcohol, eosin-nigrosine, penicillin and streptomycin, 3% NaCl, Host Test solution, formalin solution, egg yolk, Vaseline, cauda epididymal plasma-3 (CEP-3) (modified) consists of (NaCl, KCl, $CaCl_2(H_2O)_2$, $MgCl_2(H_2O)_6$, $NaHCO_3$, NaH_2PO_4, KH_2PO_4, fructose, tris aminomethane, citric acid), PFW, $NaHCO_3$ and free-range chicken eggs aged less than three days.

Research methods

The production of CEP-3 diluent was performed at the Animal Reproduction Laboratory, Faculty of Animal Husbandry, Brawijaya University. The author then analyzed the chemical composition of palmyra fruit water at the Biochemistry Laboratory of FMIPA Brawijaya University. Particle size analyzer (PSA) was conducted at the Integrated Laboratory of the Faculty of Agricultural Technology, Brawijaya University. The manufacturing process of nanoparticles, storage, and dilution of semen, as well as preparation of accessible PFW, occurred at the Laboratory of the Animal Husbandry Service of East Sumba Regency, East Nusa Tenggara.

This research uses an experimental laboratory. The sperm was collected from fresh SO semen and diluted using a nanoparticle diluent made from PFW and EY stored at a temperature of 3-5^{o}C, which was then observed every 24 hours until the motility reduction reached 40%. This research used a randomized block design of 10 replications with five treatments being tested, including T0: CEP-3 diluent + 20% egg yolk (EY) (control); T1: 90% young palm fruit water (PFW) + 10% EY; T2: 85% PFW + 15% EY; T3: 80% PFW + 20% EY; T4: 75% PFW + 25% EY.

CEP-3 diluent was prepared according to the treatment, and then 0.4% egg white or four mL was added. The media was added with 20% EY and centrifuged at 252 x g three times for 30min; then, the supernatant was taken. The palmyra water was obtained by cutting off the head of the young fruit. Using a sterile syringe, the fruit's juice was sucked out and put into a measuring cup for processing as follows: 1). The PFW diluent was previously inactivated at 56^{o}C for 20min; 2). add 0.1g $NaHCO_3$ (buffer) and antibiotics to prevent the growth of germs as much as 1000IU penicillin; 1000mg streptomycin then add egg yolk according to treatment and homogenize with a magnetic stirrer for 10-15min; then 3). It was centrifuged at 252 x g 3 times for 30 min, then the supernatant was taken; 4). The PFW is then filtered using a filtration apparatus based on the size of the filter membrane so that an average value of 580±35nm to obtain the nanoparticle size (Fig. 1). Next, dilution was carried out according to the treatment, stored at a temperature of 3-5°C, and observed every 24 hours until 40% motility was reached.

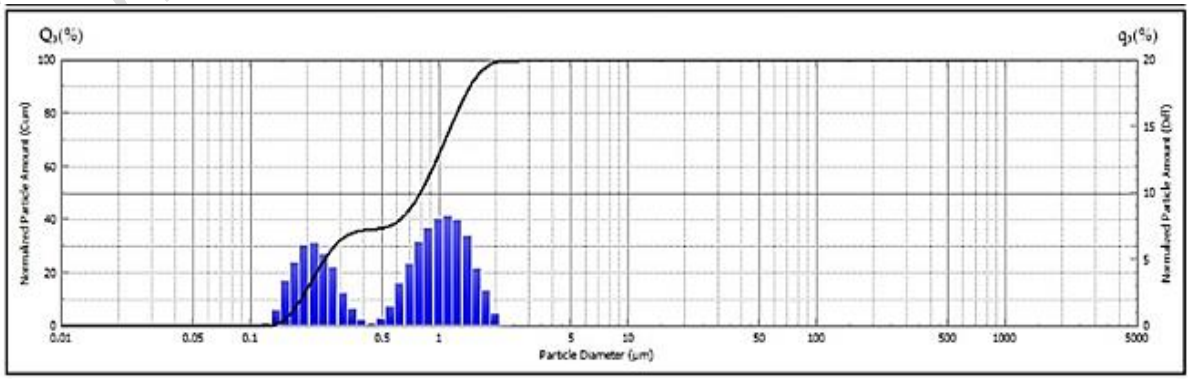

Fig. 1: The analysis results of palmyra fruit water particle size with a particle size analyzer.

Parameters observed

The following variables were variables measured:

1. Individual motility (%). The percentage of individual motility of progressively moving spermatozoa was assessed by taking one drop of semen using an Ose, then dripping it on an object glass, covering it with a cover glass, and observing it using a microscope with a magnification of 400 times. Spermatozoa that move backward and circularly are not counted (Ax et al. 2000).
2. Viability (%). Viability observations were made by taking one drop of semen placed on an object glass and then adding it with eosin-negrosin solution, then homogenizing and making a thin and dried screw preparation, and then observing using a 400 times magnification microscope without cover glass. The sperm is declared alive if the head is white or does not absorb color, and dead spermatozoa is dead if it absorbs color so that the spermatozoa head is red (Ax et al. 2000).
3. Analysis of total motile sperm (million/mL). The optimal number of progressive motile spermatozoa in an ejaculate in liquid semen is used to support fertility in cattle.
4. Observation of intact plasma membrane (IPM) (%). IPM assessment can use the hypoosmotic swelling (HOS) Test. The test was performed by adding 5µL of semen to 50µL of hypo-osmotic solution and then incubated in a water bath at 37ºC for 30min (Prochowska et al. 2022). From the incubation results, one drop of the sperm sample was taken with an Ose, which was then dripped on a glass object. A review was made using the tip of another glass object. Without being covered with a cover glass, the sample was observed using a microscope light with a magnification of 400 times. A circular tip of the tail will indicate sperm with an intact plasma membrane, while spermatozoa with a straight tail indicate that the plasma membrane has been damaged (Nalley et al. 2019).
5. Observation of intact acrosomal cap (IAC) (%). The observation process of the intact acrosome cap was done by placing 10µL of semen and 990µL of formalin solution into a microtube and homogenizing it by vertexing twice (Mahendra et al. 2018) and then incubated in a water bath at 37°C for 30min. Using an Ose, one drop of the semen solution was dripped on a glass object and covered with glass. An Intact Acrosomal Cap (IAC) was observed using a microscope with 400 times magnification. Spermatozoa with intact acrosome hoods are characterized by the anterior part of the head being darker than the posterior part or having a black color at the tip of the head. With damaged IAC, there is no black color on the head of the sperm.

Data analysis

The research data were tabulated and analyzed using a randomized block design with ten replications and five treatments, grouped based on semen storage time. The data obtained were presented as the mean±SD and analyzed general linear models univariate at $P<0.05$. Differences between treatments were continued using the Duncan's test. Data were estimated using SPSS version 26 (IBM® Corp., Armonk, NY, US).

RESULTS AND DISCUSSION

Fresh semen quality

Macroscopic evaluation of the semen obtained, including its volume, color, consistency, and pH. Meanwhile, microscopic evaluation includes mass movement, motility, viability, sperm concentration, and morphology (Arif et al. 2020). Data on the characteristics of fresh semen from SO bulls is presented in Table 1. The research obtained an average sperm volume of 7.30±1.59mL/ejaculation. As for the sperm's pH level, the research reported an average pH of 6.3, mass motility of 3+, and individual motility of 77.15%.

Table 1: Data on the Quality of fresh semen from SO bulls

Evaluation	Variables	Mean±SD
Macroscopic	Volume (mL/ejaculation)	7.30±1.59
	Consistency	Moderate
	Color	Creamy white
	pH	6.50±0.46
Microscopic	Mass motility	+++
	Individual motility (%)	77.15±3.24
	Concentration (10 million/ml)	286.50±198.01
	Viability (%)	81.73±5.41
	Abnormality (%)	8.22±4.50
	Total motile sperm (million/ml)	218.46±146.58
	Intact plasma membrane (%)	83.48±4.98
	Intact acrosomal cap (%)	89.21±4.09

Meanwhile, the total average of motile sperm, intact plasma membrane, and intact acrosomal cap was 218.46±146.58million/mL, 83.48±4.98%, and 89.21±4.09. In general, the average bull's semen volume ranges from 4-8mL per ejaculation (Carvalho et al. 2023). According to (Srivastava et al. 2022), the average pH of bulls' semen is 6.4-7.4. Mass motility is in the good (++) and very good (+++) categories (Mittal et al. 2022). The obtained motility of SO spermatozoa can be further processed as liquid semen because it has a motility above 70%. Normal semen motility in SO bulls ranges from 75-85% (Kaka and Ina 2021). According to (Pieper et al. 2023), semen with less than 70% motility cannot be used. Meanwhile, (Ratnawati et al. 2023) stated that motility can be diluted if the motility ranges between 70-90%.

Sperm motility

The percentage of spermatozoa motility is a crucial parameter in evaluating sperm quality in SO bulls. Sperm motility is crucial in fertilization and influences embryo development (Rodríguez-Villamil et al. 2016). It has been stated that decreased semen quality could decrease AI's success rate (Singh et al. 2013). Table 2 presents the research results of CEP-3 + 20% EY diluent and PFW-based nanoparticle diluent on the motility of SO spermatozoa and Table 3 shows data on initial and final pH decreases.

The percentage of SO spermatozoa motility in CEP-3 + 20% EY diluent and nanoparticle diluent made of PFW and EY decreased gradually, both in the type of treatment and storage time. A decrease in motility for each treatment was seen on days 1 to 7 of storage at a temperature of 3-5°C. These results indicate that, in general, each treatment slowly decreased the sperm motility of SO bulls. The decrease in motility was caused by the composition of the diluent, low-temperature storage, long storage time, and changes in pH. Although low-temperature storage can slow down metabolic processes and maintain the viability of SO bulls' sperm, low temperatures can cause damage to cell membranes and cell structures, which in turn can affect sperm motility (Pramesti and Ducha, 2023). In addition, metabolic activity, both anaerobic and aerobic, continues, thus consuming sperm energy and consuming diluent nutrients during storage.

Table 2: Mean and Standard Deviation Spermatozoa Motility (%)

Storage (days)	Treatment				
	T0	T1	T2	T3	T4
1	74.35±4.19^{a}	66.55±8.41^{c}	68.60±6.17^{bc}	71.15±4.30^{ab}	74.15±4.40^{a}
2	69.40±5,57^{a}	52.75±8.32^{c}	55.55±7.69^{bc}	60.75±6.86^{b}	68.75±6.18^{a}
3	63.40±7.89^{a}	42.45±7.80^{d}	48.85±6.14^{c}	55.00±7.45^{b}	63.85±7.26^{a}
4	57.85±8.21^{a}	29.45±8.93^{d}	38.85±7.09^{c}	47.50±5.93^{b}	58.45±7.17^{a}
5	52.75±7.86^{a}	16.25±9.22^{d}	27.50±9.50^{c}	42.40±4.84^{b}	53.10±7.72^{a}
6	46.35±5.25^{a}	9.50±6.85^{d}	16.00±8.76^{c}	34.75±7.12^{b}	47.55±6.44^{a}
7	40.50±6.52^{a}	7.00±5.38^{c}	11.00±9.37^{c}	24.00±9.37^{b}	40.20±7.93^{a}

Values (mean±SD) in the same row with different superscripts differ significantly (P<0.05).

Table 3: Data on initial and final pH Diluent

Observation	Treatment				
	T0	T1	T2	T3	T4
Initial pH	7	7	7	7	7
Final pH	6.4	6.2	6.2	6.4	6.4

Note: Observation pH performed after lower motility 40%.

Metabolism also produces lactic acid, which is toxic to sperm. Data on initial and final pH decreases are shown in Table 3. In this research, the decrease in motility percentage was followed by a low decrease in pH, such as in treatments T1 and T2; the average initial pH was from 7 down to 6.2 on the third day of storage. In the T3 treatment, the pH decreased to 6.4 on the fifth day of storage. Meanwhile, in treatments T0 and T4, the average pH decreased from 7 to 6.4 until the seventh day of storage. Storing diluents with a pH below six can inhibit motility and change the integrity of spermatozoa membranes (Contri et al. 2013). At a high pH above 8, bulls' spermatozoa remain alive. However, they are not motile, and a reduction in mitochondrial function causes the inability of spermatozoa to move because changes in mitochondrial structure and function affect motility (Piomboni et al. 2012)A decrease in the diluent's pH is also accompanied by physical and chemical damage, such as a change in color from light yellow to slightly white, an increase in viscosity to slightly thicker, and a rancid odor. This condition is caused by anaerobic metabolic activity in the sperm.

The results of variance analysis showed that the treatment had a significant effect (P<0.05) on SO spermatozoa motility. Duncan's test results showed that treatments T0 and T4 significantly differed from T1, T2, and T3. Meanwhile, treatments T0 and T4 did not show any significant differences. Referring to these requirements, treatments T1 and T2 can only be used in the AI program until the third day of storage with motility of 42.45% and 48.85%, respectively. Treatment T3, 42.40% motility was obtained on the fifth day of storage. Meanwhile, in treatments T0 and T4, motility was achieved on the seventh day of storage at 40.50% and 40.20%, respectively (Table 2). These results suggest that the higher percentage of EY in the PFW diluent may provide the more balanced spermatozoa need, therefore maintaining the motility of SO spermatozoa until the seventh day of storage at a temperature of 3-5°C.

PFW-EY-based nanoparticle diluent is also effective in maintaining the motility of SO spermatozoa and can replace CEP-3+20% EY diluent. This is due to the carbohydrates (fructose, glucose, and sucrose) contained in PFW, which provides the primary nutrients in the diluent and becomes an energy source for the sperm. Sperm can use fructose, glucose, and sucrose, which are metabolized into energy for movement through the formation of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the mitochondria through glycolysis. The addition of EY and PFW-based diluent can be an excellent substrate to support the motility and viability of spermatozoa, where egg yolk also contains carbohydrates, vitamins, and minerals that maintain the life of spermatozoa. Egg yolk also contains lipoprotein and lecithin compounds, which protect spermatozoa from cold shock (Tarig et al. 2017); glucose and vitamins in egg yolk also dissolve easily in water, making them beneficial for spermatozoa.

The energy contained in the diluent is required for motility. It is obtained from ATP stored in the cell (Ratnawati et al. 2023). ATP provides energy to support the primary function of spermatozoa, formed by two metabolic pathways: glycolysis in the head and the central part of the flagellum and oxidative phosphorylation (OXPHOS) produced in mitochondria (Plessis et al. 2015). Meanwhile, (Mukai and Travis, 2012), ATP maintains the intracellular environment and cellular processes such as motility, capacitation, hyperactivation, and acrosome reactions.

The preliminary research showed that 75% palmyra fruit water diluent with the addition of 25% egg yolk without nanoparticles was only able to maintain motility, reaching 40.30% until the fifth day of storage. This condition shows that nanoparticle-based diluents allow spermatozoa to pass through membranes easily so that spermatozoa can use nutrients properly to maintain motility. It aligns with (Naing et al. 2010), suggesting that diluents with small molecular weights can pass through the sperm plasma membrane and provide energy for sperm life and movement.

Sperm viability

Testing spermatozoa viability is crucial in supporting other variables and the fertility of SO bulls. The percentage of SO spermatozoa viability in this research is presented in Table 4. The data in Table 4 shows the percentage decrease in sperm viability during storage. The percentage reduction in spermatozoa viability in each treatment was different, so it was seen that the average percentage of sperm viability after seven days of storage in 75% PFW+25% EY diluent was higher than in other treatments. The difference between the living and dead sperm can be seen in Fig. 2.

Table 4: Mean and Standard Deviation Spermatozoa Viability (%)

Storage (days)	Treatment				
	T0	T1	T2	T3	T4
1	86.13±4.03[a]	81.65±4.34[b]	82.05±4.48[b]	83.13±4.85[b]	85.29±4.67[a]
2	82.08±3.06[a]	74.66±3.54[d]	76.36±3.48[c]	78.71±4.21[b]	81.70±3.79[a]
3	77.99±2.97[a]	67.21±3.69[d]	69.94±2.27[c]	72.59±3.39[b]	77.00±4.01[a]
4	73.39±3.41[a]	59.83±4.86[d]	63.87±3.63[c]	68.38±3.82[b]	73.31±3.97[a]
5	68.23±3.63[a]	53.15±5.71[d]	56.56±5.82[c]	61.22±4.46[b]	68.94±4.82[a]
6	62.82±4.65[a]	45.57±5.59[d]	50.20±5.73[c]	54.01±5.11[b]	63.14±6.07[a]
7	55.70±2.83[a]	35.40±8.28[d]	41.94±6.05[c]	46.77±4.28[b]	55.90±6.68[a]

Values (mean±SD) in the same row with different superscripts differ significantly (P<0.05).

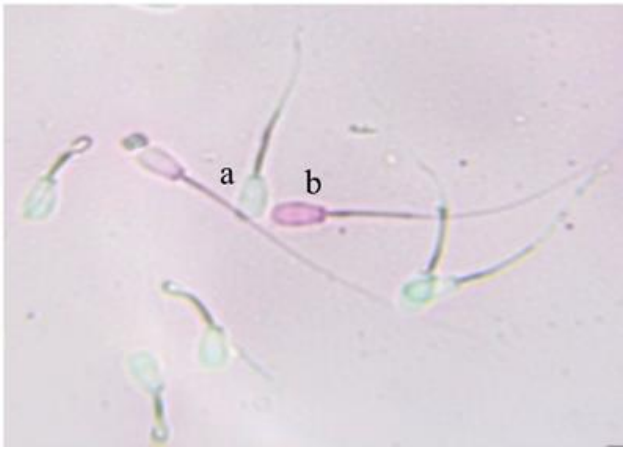

Fig. 2: a. Living sperm; b. Dead sperm.

The viability percentage is generally higher than the motility percentage because some spermatozoa are alive but unable to move. However, referring to the motility percentage in Table 2, the percentage of viability of SO bull's spermatozoa in treatments T0 and T1 until the third day of storage, the average viability reached 67.21% and 69.94%. With T3 treatment on the fifth day of storage, viability was obtained at 61.22%. Meanwhile, treatments T0 and T4 were achieved on the seventh day of storage with viability of 55.70% and 55.90% respectively. According to (Ghareeb et al., 2017), normal sperm viability can reach 90% and be as low as 43% viability (Felipe-Perez et al. 2008). The results obtained in this research were still above normal on average. However, it still refers to the motility percentage as required in AI.

One of the causes of this difference is the metabolic activity of spermatozoa, which can produce reactive oxygen species (ROS). According to (Qamar et al. 2023), ROS is produced during cellular metabolic processes, which are highly reactive and result from oxygen-derived oxidation. This condition shows that the diluents CEP-3+20% EY and PFW-EY can adequately protect the life of SO liquid semen spermatozoa during storage. The protective ability of liquid semen diluent is influenced by energy availability, antioxidant activity in protecting sperm membranes and reducing metabolic activity, thus minimizing the buildup of lactic acid, which can be detrimental to sperm. Physiologically, ROS levels are required by sperm for the achievement of different cell functions, including proliferation, maturation, capacitation, acrosomal reaction, and fertilization. Furthermore, it states that excessive ROS production creates an imbalance between ROS production and can result in oxidative stress (OS).

Statistical analysis results showed that treatment had a significant effect (P<0.05) on spermatozoa viability. The follow-up tests on the first day of storage showed significant differences (P<0.05) between treatments T0 and T4 and T1, T2, and T3. Meanwhile, there was no significant difference (P>0.05) between treatments T0 and T4. The differences between treatments T1, T2, T3, and T4 were due to the different percentages of PFW and EY in each treatment, impacting the ability to maintain pH and provide nutrients during storage.

Total motile spermatozoa

Total Motile Spermatozoa (TMS) in bulls refers to the total number of sperm capable of active movement, both progressive (moving forward) and non-progressive motility (moving but not moving forward) (Table 5). Table 6 shows that treatments T0 and T4 had a significant effect (P<0.05) between treatments T1, T2, and T3 on total motile sperm after being stored at 3-5ºC for seven days of storage. However, T0 and T4 treatments did not significantly affect TMS (P>0.05). These results indicate that each treatment can guarantee that the spermatozoa population can move en masse up to 7 days of storage. The TMS value correlates with the concentration and percentage of spermatozoa motility. The higher the percentage of spermatozoa motility and the concentration value, the higher the total value of motile spermatozoa (Yekti et al. 2023).

The TMS values in treatments T0 and T4 on the seventh day of storage were relatively high, with an average of 303.73 million/straw and 298.40 million/straw. Meanwhile, T3, T2, and T1 showed average TMS values of 184.25 million/straw, 84.00 million/straw, and 51.25 million/straw (Table 5). This difference is due to the availability of different energy sources in each treatment to ensure spermatozoa obtain sufficient energy to move. In addition, the diluent used maintains osmolarity and has diluent buffering properties so that it can maintain the acidity of the diluent due to sperm metabolism, electrolyte balance, and osmotic pressure during the 7-day storage period. The total number of motile sperm is still above 100 million/straw in treatments T0 and T4. The defense mechanism of buffer solutions against sperm motility, namely sucrose and fructose, continuously supplies energy for anaerobic metabolism and prevents membrane damage due to cold shock, thus allowing sperm to move in large quantities during the storage period (Sieme et al. 2015).

Intact plasma membrane

The spermatozoa's intact plasma membrane (IPM) is the outer layer of sperm cells, and it plays a vital role in maintaining sperm integrity and stability. Table 6 shows that the obtained IPM decreased on the seventh day of storage. Fig. 3 shows the difference between intact and incomplete spermatozoa membranes.

Table 5: Mean and Standard Deviation Total Motil Spermatozoa (million/straw)

Storage (days)	Treatment				
	T0	T1	T2	T3	T4
1	543.70±124.58^{a}	484.55±110.66^{c}	500.65±110.39^{bc}	519.25±110.75^{a}	543.55±171.17^{a}
2	507.40±119.78^{a}	383.80±90.82^{c}	405.20±97.84^{c}	445.05±111.92^{b}	505.00±128.2^{a}
3	465.20±125.25^{a}	309.10±78.73^{d}	354.60±75.72^{c}	401.50±99.70^{b}	469.80±126.42^{a}
4	426.05±121.60^{a}	214.55±71.20^{d}	280.35±60.10^{c}	347.78±88.18^{b}	430.35±117.14^{a}
5	390.00±118.39^{a}	120.00±67.28^{d}	193.75±62.24^{c}	310.90±81.49^{b}	386.68±101.51^{a}
6	342.25±96.79^{a}	70.50±48.56^{d}	118.00±70.56^{c}	259.25±87.50^{b}	345.65±87.43^{a}
7	303.73±105.94^{a}	51.25±36.00^{c}	84.00±78.23^{c}	184.25±95.23^{b}	298.40±89.18^{b}

Values (mean±SD) in the same row with different superscripts differ significantly (P<0.05).

Table 6: Mean and Standard Deviation Intact Plasma Membrane (%)

Day Storage	Treatment				
	T0	T1	T2	T3	T4
1	76.57±2.85^{a}	66.80±5.23^{b}	70.84±4.29^{b}	73.93±5.10^{ab}	75.10±3.83^{a}
2	72.17±3.51^{a}	54.79±5.04^{c}	59.04±4.49^{bc}	63.29±7.76^{b}	70.72±4.94^{a}
3	65.23±3.06^{a}	51.38±3.07^{c}	52.47±5.53^{c}	60.55±5.08^{b}	64.24±4.27^{ab}
4	61.53± 4.01^{a}	39.69±2.21^{d}	44.79±3.57^{c}	53.54±6.48^{b}	60.93±4.48^{a}
5	56.01± 3.51^{a}	27.93±4.14^{d}	32.87±4.04^{c}	45.80±6.62^{b}	55.01±4.64^{a}
6	51.76±4.40^{a}	16.94±6.13^{c}	19.47±2.99^{c}	38.01±3.54^{b}	50.77±2.11^{a}
7	46.67±4.45^{a}	10.63±4.67^{d}	14.82±2.08^{c}	26.70±3.98^{b}	45.56±2.63^{a}

Values (mean±SD) in the same row with different superscripts differ significantly (P<0.05).

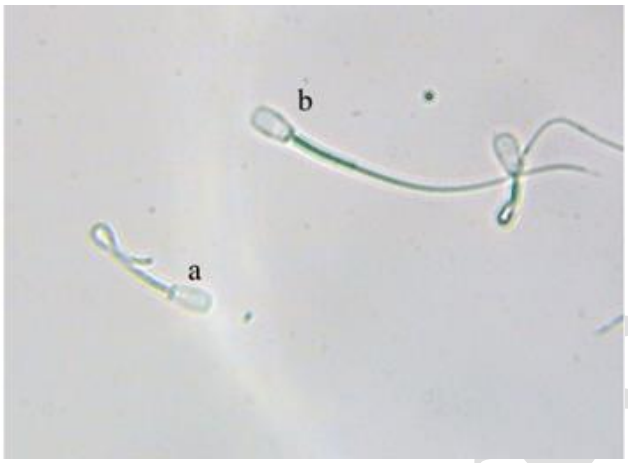

Fig. 3: a. Intact Plasma Membrane; b. Damage Plasma Membrane

The spermatozoa's intact plasma membrane (IPM) is the outer layer of sperm cells, which plays a vital role in maintaining sperm integrity and stability. Table 6 shows that the obtained IPM decreased on the seventh day of storage. The highest IPM value was in treatment T0 (46.67%), followed by T4 (45.56%), T1 (10.63%), T2 (14.82%) and T3 (26.70%). The results of variance analysis showed that T0 and T4 treatments did not show a significant effect (P>0.05) on intact plasma membranes. However, it showed a significant difference (P<0.05) when comparing treatments T0 and T4 with T1, T2 and T3.

During sperm storage, the PFW and EY-based nanoparticle diluent can maintain the integrity of the plasma membrane so that spermatozoa remain alive and move well, supporting AI's success. The combination of 75% PFW with 25% EY can fuse with the sperm membrane before storage at 3-5^{o}C, increasing resistance to cold shock. In addition, spermatozoa can properly utilize the nutrients in PFW diluent to maintain IPM. An intact plasma membrane is critical in sperm metabolism because it utilizes nutrients entering the cell and the acrosome reaction. Meanwhile, egg yolk plays a role in protecting spermatozoa membranes because of its lecithin (Arvioges et al. 2021). According to (Wu et al. 2015), the lecithin in egg yolk protects the spermatozoa plasma membrane, so a higher amount of egg yolk will result in a higher concentration of lecithin in the diluent. The plasma membrane generally regulates calcium, sodium, and potassium ions necessary for mitochondrial function and sperm motility (Khalil et al. 2018). Cell membranes comprise lipid molecules such as cholesterol, glycolipids, and phospholipids (Arvioges et al. 2021). The ratio of lipids and cholesterol affects changes in membrane structure, including in the sperm head (Indriastuti et al. 2020). The integrity of the plasma membrane affects cell organelles, causing spermatozoa to be able to move progressively (motile) and remain alive (Kaeoket et al. 2021). This aligns with (Kang et al. 2020), who suggests a relationship exists between motility and viability in intact plasma membranes.

Intact acrosomal cap

The intact acrosomal cap (IAC) is the structure that covers the head of the sperm cell, which is essential in fertilization. The acrosomal cap plays a vital role in determining the success of fertilization, so its integrity must be protected until sperm capacitation and the acrosome reaction process occur (Khalil et al. 2018). Table 7 shows that the obtained IAC decreased on the seventh day of storage, and Fig. 4 shows the difference between IAC and incomplete.

The acrosomal hood contains essential enzymes required to weaken the lining of the egg cell so that sperm can enter the egg cell and initiate fertilization. Plasma membrane integrity is essential for sperm function because it directly influences metabolic processes and is closely related to sperm motility and viability (Iskandar et al. 2022). Table 7 shows that IAC decreased every day until the seventh day of storage, where the percentage of IAC was obtained in treatments T0 (56.25%), T4 (56.80%), T1 (37.94%), T2 (44.90%), and T3 (47.50%).

The addition of 25% egg yolk in the PFW diluent tends to have a higher IAC percentage value than the addition of 10, 15, and 20% PFW. This suggests that PFW and egg yolk are essential in providing the best protection of

Table 7: Mean and Standard Deviation Intact Acrosomal Cap (%)

Day Storage	Treatment				
	T0	T1	T2	T3	T4
1	86.76±5.05^{a}	82.95±4.90^{a}	83.03±2.35^{a}	83.28±1.89^{a}	86.12±5.14^{a}
2	83.45±2.36^{a}	77.43±3.94^{b}	77.84±3.44^{b}	80.03±3.31^{ab}	82.57±5.01^{a}
3	79.65±4.64^{a}	69.89±2.60^{c}	70.52±3.88^{bc}	74.16±4.14^{b}	78.68±5.59^{a}
4	75.66±2.79^{a}	62.68±4.47^{c}	64.12±4.48^{c}	70.82±4.33^{b}	74.94±4.32^{a}
5	70.19± 2.75^{a}	54.44±6.55^{c}	56.31±8.16^{bc}	60.30± 3.25^{b}	68.66±3.44^{a}
6	63.36±4.53^{a}	48.15±6.99^{c}	50.71±4.92^{bc}	54.10±1.85^{b}	63.44±4.29^{a}
7	56.25±3.22^{a}	37.94±7.00^{c}	44.90±5.12^{b}	47.50±2.88^{b}	56.80±4.65^{a}

Values (mean±SD) in the same row with different superscripts differ significantly (P<0.05).

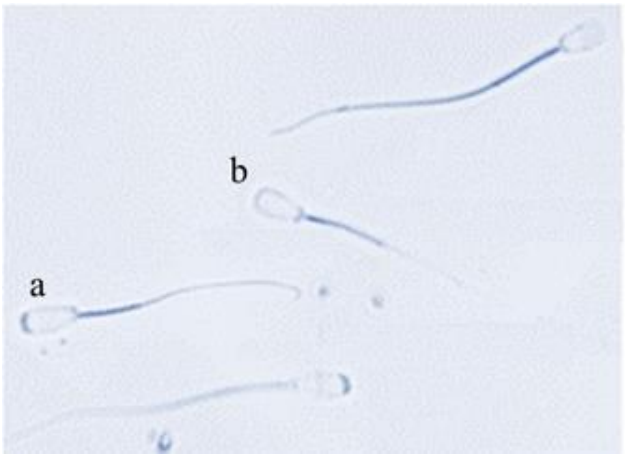

Fig. 4: a. Intact Acrosomal Cap; b. Deterioration acrosome cap

acrosome integrity during storage at low levels. Egg yolk contains lecithin and unsaturated fatty acids to reduce the effects of free radicals, stabilize cell membranes, and increase sperm metabolism.

Nevertheless, the PFW-EY-based nanoparticle diluent effectively protected sperm from oxidative damage. During sperm storage, spermatozoa experience several sources of oxidative stress, such as storage at low temperatures and general oxidative metabolism in mitochondria (Bucak et al. 2007). Oxidative stress can damage the acrosome cap and the enzymes contained therein. Protection against oxidative stress may help maintain the integrity of the acrosomal cap.

Conclusion

Based on the research results, it can be concluded that the nanoparticle diluent made from palm fruit water can effectively maintain the quality of SO bull spermatozoa during storage at a temperature of 3-5ºC. For preservasi liquid semen implementation, we can use nanoparticle diluent made from 75% PFW and the addition of 25% EY. This research suggests that it is necessary to analyze the chemical composition of palm fruit water and its antioxidant content and test the fertility of the nanoparticle diluent based on EY and PFW.

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Author's contribution

All authors designed the study, Alexander Kaka performed the practical procedures, analyzed and interpreted the data, and wrote the manuscript. This manuscript content was authored, reviewed, and approved by Aulia Puspita Anugra Yekti, Sucik Maylinda, Sri Rahayu and Trinil Susilawati for publication.

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