

## Effect Concentration of Moringa (*Moringa oleifera* Lam) Leaf Extract in Citrate-Egg Yolk in Maintaining Motility and Viability of Spermatozoa of Kacang Goat

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### ABSTRACT

This study aimed to determine the effectiveness and the best concentration of Moringa leaf extract (MLE) in the citrate-egg yolk (C-EY) to maintain the motility and viability of spermatozoa kacang goat. Semen was collected from 3 goats aged two years; by using the artificial vagina method. The semen was evaluated macroscopically and microscopically. The semen that had >70% sperm motility and >250x10<sup>6</sup>/ml sperm concentration was divided into 4 equal tubes, each diluted with 100% C-EY (P1), 10% MLE+ 90% C-EY (P2), 20% MLE +80% C-EY (P3), and 30% MLE+70% C-EY (P4). The diluted samples were then stored in a refrigerator (3-5°C) and evaluated for motility and viability every 24 hours. The study was designed using a completely randomized design (CRD) consisting of four treatments and five replications. The results showed that the addition of MLE in C-EY significantly affected goat spermatozoa's progressive motility and viability. The data showed that the spermatozoa kept during four days in a diluent of P2 had higher ( $P < 0.05$ ) motility  $44.67 \pm 4.80\%$  and viability  $74.24 \pm 4.46\%$  than the other three diluents of P1 ( $36.00 \pm 4.70\%$ ;  $70.10 \pm 3.6\%$ ), P3 ( $33.67 \pm 0.42\%$ ;  $66.85 \pm 4.99\%$ ) and P4 ( $29.67 \pm 3.99\%$ ;  $63.96 \pm 5.44\%$ ). This study concluded that adding 10% MLE was the best concentration as source energy in 90% C-EY diluents, which effectively maintained the motility and viability of kacang goat spermatozoa for four days of storage at a temperature of 3-5°C.

**Keywords:** Moringa leaf, semen, diluent, temperature, stored

### INTRODUCTION

Goat is one livestock that produces meat and is a source of protein other than cattle like buffalo and poultry. Apart from being a meat producer, goats are also one of the most popular livestock, easy to maintain, and have a significant and good market opportunity to develop. The genetic potential of goats themselves can be increased by crossing them. In general, crossbreeding of livestock can be done by natural mating or by injection or artificial insemination (AI). The application of AI technology can further optimize the genetic potential of Kacang goats. Through AI applications integrated with semen processing technology, one ejaculate of a male sheep or goat can serve about 35 females, which by natural mating can only mate with one female (Rizal et al., 2003).

Semen quality is one factor that determines the success of AI technology applications. The quality of semen will decrease if it is not used as soon as possible. The quality of semen is also determined by other factors such as storage techniques and the type of diluent used (Ariantje, 2013). To maintain the quality of spermatozoa, it is necessary to dilute the semen so that it can be used for a relatively long time. The diluent used must provide food as a source of

energy for spermatozoa, protect spermatozoa from cold shock, act as a buffer to prevent a decrease in pH, and maintain osmotic pressure and electrolytic balance and reduce bacterial growth.

Egg yolk diluent is a diluent that can be used and functions as an energy source, protective agent, and buffer to protect and maintain the protein coat of spermatozoa. Egg yolk can protect spermatozoa because it contains phospholipids as a source of energy and protects spermatozoa from cold shock due to the presence of lecithin and lipoproteins that work to maintain and protect the integrity of the lipoprotein sheath of spermatozoa (Yildiz et al., 2000). In addition, the quality of semen can be improved by adding various types of additive compounds to the semen diluent. Moringa leaf extract (MLE) is one of the natural ingredients that can improve the quality of preserved semen. Moringa leaves contain high antioxidants (Kasolo et al., 2010) and antibacterial compounds (Das et al., 2012). According to Kumala et al. (2016), Moringa leaves contain flavonoids that act as antioxidants to bind free radicals. Moringa leaf is one part of the Moringa plant that has been widely studied for its nutritional content and uses. Moringa leaves are very rich in nutrients, including calcium, iron,

protein, vitamin A, vitamin B and vitamin C (Misra et al., 2014). According to Yameogo et al. (2011), Moringa leaves contain higher iron than other vegetables, 17.2 mg/g. Moringa Leaf Extract (*Moringa oleifera* LAM) contains antioxidants, including vitamin C 17.3 mg and vitamin E 113g/100g, which protect the body against the damaging effects of free radicals by neutralizing them before they can cause cell damage and disease (Kurniasih, 2013).

The purpose of adding diluent is to provide an energy source, reduce density and maintain the viability of spermatozoa until a specific storage time limit under storage conditions both at the refrigerator and frozen temperatures, so a diluent is needed (Ducha et al., 2013). Several researchers have carried out the use of MLE in the semen preservation process in related studies. According to Sokunbi et al. (2015), adding 4–16% Moringa leaf extract into the diluent can maintain the quality of bovine spermatozoa preserved at 6°C for three days. Meanwhile, Fafo et al. (2016) stated that egg yolk citrate diluent added with 5% Moringa leaf extract effectively maintained the motility and viability of the sperm of Landrace pigs preserved at a temperature of 18–20°C. In this study, the diluent was modified by combining Moringa leaf extract (MLE) with citrate egg yolk (C-EY). MLE is made using fresh Moringa leaves taken and cleaned from the small stems and then dried (aired).

After that, puree using a blender then sieved to produce fine Moringa leaf powder and analyzed for nutritional content (proximate analysis) then MLE can be used as an ingredient diluent. Any suitable diluent can show its ability to reduce the rate of decrease in the value of motility (progressive motion). In the end it prolongs the post-dilution storage time. The MLE used is expected to have nutrients that can minimize damage to spermatozoa cells during preservation so that the quality can be maintained and meet the requirements for use in the AI program. Therefore, it is necessary to research The Effect of Moringa Leaf Extract (*Moringa oleifera* LAM) in Citrate Egg Yolk Diluent in Maintaining Spermatozoa Motility and Viability of Kacang goats at 3-5°C. The aims of this research are 1). It is the percentage of motility and viability of goat spermatozoa 2). The best concentration of Moringa leaf extract in citrate-egg yolk diluent during storage is known.

## MATERIALS AND METHODS

The research was conducted at the Animal Health Laboratory of the Livestock Service Office of East Sumba Regency from August to November 2020

### Semen Source

The liquid semen used in this study came from 3 adult male Kacang goats aged 2-3 years, bodyweight of 25-30 kg and in healthy condition, proportional body, and having normal reproductive organs. Cattles are housed in individual cages equipped with feed and drinking containers.

### Collection and Evaluation of Semen

Kacang goat semen was collected twice a week using the female angler technique equipped with goat semen collection devices (artificial vagina). The semen obtained was then evaluated both macroscopically (volume, color, consistency, and pH) and microscopically (mass movement, spermatozoa motility, spermatozoa concentration, live spermatozoa, spermatozoa morphology and intact plasma membrane/IPM and intact acrosome hood/IAH) (Arifiantini 2012).

The semen used in this study had to meet criteria such as having more than 70% spermatozoa motility, a concentration of more than  $250 \times 10^6$  cells/ml, and spermatozoa abnormality of less than 10% (processed for liquid semen). Semen that meets the requirements is diluted using a diluent.

### Preparation of Diluent

The materials used as a semen diluent in this study included: Moringa leaf extract, sodium citrate, egg yolk, 2% eosin, eosin-negrosin, 70% alcohol, aquabidestilata, tissue, penicillin and streptomycin (antibiotics). The primary diluent used in this study was citrate and egg yolk diluent (C-EY) with Moringa leaf extract (MLE).

### Semen processing and storage

The semen was divided into four tubes, where Treatment I (P1) was 100% C-EY, Treatment II (P2) was 90% C-EY+10% MLE, Treatment III (P3) was 80% C-EY+20% MLE, and treatment VI (P4) was 70% C-EY+30% MLE. The diluted semen was stored at a refrigerator temperature (3-5°C) controlled with a thermometer and evaluated every 24 hours. The semen of each treatment was evaluated for quality every day (until the percentage of motile spermatozoa reached 40%). Semen quality was

evaluated after storage (fresh semen), dilution, and storage.

### Evaluation of Semen Quality Post Storage

The quality of the semen tested was the percentage of progressive motility and viability performed every 24 hours at a temperature of 3-5°C.

### Percentage of Spermatozoa Motility

This is assessed by dripping one drop of semen into one drop of 0.9% physiological NaCl. The two solutions were homogenized. One drop was taken, then transferred to an object-glass, covered with a cover glass, and then observed using a light microscope with a magnification of 10x40. The motility percentage can be assessed subjectively by comparing the motile spermatozoa that move forward (progressive) and non-progressive. Ratings are given from 0% (non-motile) to 100% (all motile).

*Spermatozoa concentration:* per ml was calculated using the Neubauer chamber with a dilution of 500 times (2  $\mu$ l in 998  $\mu$ l in formo saline).

*Percentage of Viability:* using eosin-nigrosine dye (Arifiantini, 2012), then do a quick review and dry. The examination was carried out under a light microscope with a magnification of 10x40. Spermatozoa were counted at least 200 cells from 10 fields of view. Live spermatozoa do not absorb color (transparent), and the dead absorb color on the head.

*Spermatozoa morphology:* evaluated by eosin-nigrosine staining, followed by examination under a light microscope with a magnification of 10x40.

### Research Design and Data Analysis

The study was designed using a completely randomized design (CRD) consisting of four treatments and five replications. The data were analyzed using variance or analysis of variance (ANOVA), and if there were differences between treatments, it was continued with Duncan's test. Data is presented in the mean and standard deviation.

## RESULT AND DISCUSSION

### Quality of Fresh Semen

Appropriate or not semen to be used can be seen from the quality of the fresh semen produced. The results obtained from this study were that the quality of the Kacang goat semen produced was still in the sound and normal range

so that the fresh semen could be diluted. Semen evaluation was carried out macroscopically and microscopically. The average value of the quality characteristics of fresh Kacang Goat semen can be seen in table 1.

Tabel 1. The average value of the quality characteristics of fresh Kacang goat semen

Characteristics Macroscopy	Average
Volume (ml)	1.43±1.03
pH	6.52±0.15
Colour	
Consistency	Thick Medium
Microscopy	
Mass Movement	+++
Motility (%)	75.00±0.00
Concentration (10 <sup>6</sup> /ml)	289.33±21.86
Viability (%)	85.23±2.92
Abnormality (%)	9.57±0.84
Whole Plastic Membrane (%)	85.39±1.42
Whole Acrosome Hood (%)	85.18±1.10

Source: Observational data (2020); +++ (good): visible waves fast and a lot

The results in table 1 above show that the average volume of Kacang goat semen obtained is 1.43±1.03 ml. The results obtained are higher than the results of Hariadi's research (2018). The average volume of Kacang goat semen is 1.2±0.12 ml, while the results of Masyito et al. (2018) stated that the average volume of Sapera goat semen was 0.9±0.39 ml. The difference in the amount of semen volume in goats in each ejaculation is caused by feed, age, frequency of storage, and other factors (Hastono, 2013). The color of the semen observed was cream with the consistency of thick semen (the thicker the cement, the higher the concentration of spermatozoa).

The results obtained indicate similarities with Hidayati's research (2017) which states that the color of Sapera goat semen is cream with a thick consistency. The smell of the semen obtained has a characteristic odor. The distinctive aroma in question indicates that the semen is produced well and under normal conditions or is not contaminated by microorganisms (Suyadi et al., 2012). The average degree of acidity (pH) obtained from this study was 6.52±0.15 (measured using a pH meter). This result is not much different from Masyito et al. (2018), which is 6.5±0.52. However, the pH value of the research results obtained is lower than the research results of Kusumawati et al. (2016) that

Peranakan Etawa goat semen has a pH value of 7.

The observations also showed that the average motility of the spermatozoa obtained was  $75.00 \pm 0.00\%$ . The results obtained are lower than those of Bezerra et al. (2011) the average motility of spermatozoa was  $95.00 \pm 2.00\%$ . Although the results obtained are low, these results still meet the requirements for further semen processing. The percentage of normal progressive spermatozoa motility can range from 70%-90% (Ax et al., 2000). For examination of spermatozoa concentration, the average obtained was  $289.33 \pm 21.86\%$ . The results of this examination are lower than those of Riyadhhi et al. (2017) stated that the concentration of PE goat semen was  $3.220 \times 10^6$  cells/ml. However, the study results showed a low concentration of spermatozoa obtained; the concentration of spermatozoa was still in average condition. Spermatozoa concentration values in the range of  $2000-6000 \times 10^6$  cells/ml (Syawal, 2010). Spermatozoa viability (live and dead) is also an indicator of semen quality (because it is related to the viability of spermatozoa).

The observations also showed differences in the colour of live and dead spermatozoa. Spermatozoa cells that were not motile and considered dead sucked colour. In contrast, motile and living spermatozoa cells were considered colourless (figure 1) and were evaluated using a microscope with a magnification of  $10 \times 40$ . Live and dead spermatozoa could be distinguished by reacting to the material eosin negrosin dye (Susilawati, 2011).



Figure 1. Live and dead spermatozoa (eosin-nigrosine staining)

Description: Live Spermatozoa (H); Dead Spermatozoa (M)

In Figure 1 above, the examination results show that live spermatozoa are marked on the transparent colored head with an average percentage of  $85.23 \pm 2.92\%$ . The average results

obtained are not much different from Souhoka et al. (2009), equal to  $84.00 \pm 1.00\%$ . The high percentage of live spermatozoa compared to motile spermatozoa indicates that the number of viable spermatozoa is not necessarily progressively motile (Kostaman and Sutam, 2006). The observation of spermatozoa abnormalities showed that the average obtained was  $9.57 \pm 0.84\%$ . This result is higher than the research result of Savitri et al. (2014), which is  $7.51 \pm 2.88\%$ , and lower than the results of Bezerra et al. (2011), which is equal to  $23.90 \pm 1.70\%$ .

The abnormal mean spermatozoa obtained were still within the specified criteria. Measurement of spermatozoa abnormalities is important because high abnormalities will generally interfere with male fertility. The percentage of spermatozoa abnormalities is not more than 10% (According to Ax et al., 2000). The results of the observations show that the average IPM obtained is  $85.39 \pm 1.42$ . This result is higher than Ariantie's (2013) research, which is  $77.72 \pm 3.52\%$ . At the same time, the results of TAU's observations show that the average value obtained from the examination results is  $85.18 \pm 1.10\%$ . The whole Acrosome Hood (IAH) is also an essential part of spermatozoa and plays a role in fertilization (Arifiantini, 2012).

### Effect of MLE Concentration in C-EY Diluent on Spermatozoa Motility

The motility of individual spermatozoa is one factor that affects the fertility of spermatozoa and is one of the essential indicators for determining semen quality in general. Spermatozoa motility is the progressive forward movement of spermatozoa. The average percentage of the motility of the goat's sperm motility that has been diluted in the four types of diluents can be seen in Table 2.

Looking at the results in table 2, it can be stated that the observations during semen storage showed a progressive decrease in the movement (motility) of spermatozoa. This is thought to be caused an increasing number of spermatozoa being damaged and dying due to cold temperatures, the availability of energy in the diluent decreases, and the level of acidity (pH) of semen is increasing. The average rate of decrease in the percentage of the progressive movement of spermatozoa in each treatment was not the same. In table 2 above, the results of the variance (ANOVA) showed that each treatment was significantly different ( $P < 0.05$ ), where the P2

treatment still showed the percentage of progressive movement above IB-worthy motility (above 40%) until the fourth day of storage, which was 44.67±4.0%.

Table 2. The average motility of the spermatozoa of Kacang goat in egg yolk citrate diluent with Moringa leaf extract supplementation at a temperature of 3-5°C.

Storage Days to-	Treatment			
	P1	P2	P3	P4
Post Dilution	72.67±2.5 <sup>b</sup>	74.33±1.76 <sup>a</sup>	70.67±1.76 <sup>c</sup>	70.00±0.00 <sup>c</sup>
1	68.33±3.09 <sup>b</sup>	71.67±2.44 <sup>a</sup>	63.67±2.97 <sup>c</sup>	60.67±1.76 <sup>d</sup>
2	57.33±4.17 <sup>b</sup>	63.33±2.44 <sup>a</sup>	53.67±4.22 <sup>c</sup>	49.67±2.29 <sup>d</sup>
3	46.00±4.70 <sup>b</sup>	54.67±3.99 <sup>a</sup>	43.67±4.22 <sup>b</sup>	39.67±3.52 <sup>c</sup>
4	36.00±4.70 <sup>b</sup>	44.67±4.80 <sup>a</sup>	33.67±4.42 <sup>b</sup>	29.67±3.99 <sup>b</sup>
5	27.67±3.71 <sup>b</sup>	35.00±4.63 <sup>a</sup>	23.33±4.50 <sup>c</sup>	17.67±5.62 <sup>d</sup>

Notes: Different notations on the same line indicate significantly different (P<0.05)

In treatments P1 and P3, each showed an average value of spermatozoa motility, which was 46.00±4.70% and 43.67±4.22%, which lasted until the third day of storage. Meanwhile, the P4 treatment showed that spermatozoa motility was 49.67 ± 2.29%, which lasted until the second day of storage. The difference decrease in spermatozoa motility between treatments was due to the difference in the concentration of MLE given in the diluent, causing a high level of acidity in the diluent. This study showed a high percentage of progressive motility in P2 treatment on the fourth day of storage due to the

modification between MLE as an energy source and C-EY diluent as a source of phospholipids, cholesterol, and low cholesterol density lipoprotein.

### Effect of MLE Concentration in C-EY Diluent on Spermatozoa Viability of Kacang Goat

Spermatozoa viability examination needs to be done because it affects the motility of spermatozoa. The results of observing the average spermatozoa viability in the research can be seen in Table 3 below.

Table 3. Average viability of Kacang goat spermatozoa in citrate-egg yolk diluent with moringa leaf extract supplementation at a temperature of 3-5°C

Storage Days to-	Treatment			
	P1	P2	P3	P4
Post Dilution	82.66±1.67 <sup>ab</sup>	84.14±2.72 <sup>a</sup>	81.26±2.52 <sup>bc</sup>	80.33±3.15 <sup>c</sup>
1	80.02±0.93 <sup>b</sup>	82.44±3.05 <sup>a</sup>	78.72±2.36 <sup>b</sup>	76.35±2.80 <sup>c</sup>
2	77.19±1.31 <sup>b</sup>	80.10±3.22 <sup>a</sup>	75.20±2.33 <sup>c</sup>	73.18±3.04 <sup>d</sup>
3	73.04±3.26 <sup>b</sup>	77.57±3.40 <sup>a</sup>	70.91±3.50 <sup>bc</sup>	68.77±4.11 <sup>c</sup>
4	70.10±3.62 <sup>b</sup>	74.24±4.46 <sup>a</sup>	66.85±4.99 <sup>bc</sup>	63.96±5.44 <sup>c</sup>
5	65.73±5.24 <sup>b</sup>	71.08±4.72 <sup>a</sup>	62.50±5.96 <sup>bc</sup>	59.16±6.59 <sup>c</sup>

Notes: Different notations on the same line indicate significantly different (P<0.05)

The results obtained in table 3 showed a decrease in the percentage of spermatozoa survival every day in each given treatment. The observations showed that there was no significant difference between P1 and P2 treatments after dilution on the percentage of spermatozoa viability of Kacang goats. However, on the first day until the fifth day of storage, the results of variance (ANOVA) showed that each treatment was significantly different (P<0.05). Treatment P2 was able to maintain the percentage of spermatozoa viability, which was 71.10±4.72% higher than treatment P1 (65.73±5.24%), P3

(62.50±5.96%), and P4 (59.16±6.59%). The difference in the percentage of spermatozoa viability was influenced by the physical and chemical properties of the diluent, diluent levels, temperature and light in the treatment, storage of spermatozoa, pH, electrolytes and non-electrolytes.

When compared with the percentage of spermatozoa motility, survival was still higher, indicating that many spermatozoa were still alive but not motile. The results also showed that the P2 treatment with a concentration of 10% MLE in 90% C-EY effectively maintained the viability of

goat spermatozoa. This illustrates that with the right concentration and the presence of many nutrients in MLE, it is optimal to maintain the viability of the spermatozoa of Kacang goat. By modifying the addition of a balanced concentration of MLE in the C-EY diluent, it can provide energy and protect spermatozoa from the effects of free radicals and cold shock during storage.

## CONCLUSION

The results of the study concluded that the addition of 10% Moringa Leaf Extract (MLE) in 90% citrate-egg yolk (C-EY) diluent was the best concentration that was effective in maintaining motility and viability of Kacang Goat spermatozoa for four days of storage at a temperature of 3-5°C with the percentage of progressive motility that is equal to 44.67±4.80% (motility suitable for IB is above 40%).

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