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Survivability of Kedu Rooster Sperm at Different Storage Times and Diluent Supplemented with Vitamin E and Fertilizing Ability on Kampung Chicken

Research Article

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ABSTRACT

In the present study, the effect and interaction of the addition of vitamin E in diluents during storage were investigated. Semen samples were collected from ten Kedu roosters. Analysis of spermatozoa quality used analysis of variance based on a randomized block factorial design. The first factor was lactate ringer (D_1) and skim milk + 50 mM glucose (D_2), the second factor was a dose of vitamin E (V_{0-3}= 0, 1, 2, 3%) and the third factor was storage time at 4 ˚C (T_{0-3}= 0, 2, 4, 6 hours). The analysis of variance showed that there was an interaction between type of diluents and vitamin E ($P<0.01$) on sperm viability and abnormalities, with the best combination at D_1V_2. The type of diluent had a significant effect ($P<0.05$) on sperm motility. The vitamin E dose had a significant effect on membrane integrity. Storage time had a high effect on sperm motility. There were eight combinations among the type of diluent, vitamin E dose, and storage time, which had a significant effect ($P<0.01$) on fertility and the fertile periods of kampung hen eggs. The combination of $D_2V_2T_0$ showed the best fertility and the $D_2V_0T_0$ showed the best fertile periods. In conclusion, the addition of vitamin E 2% in lactate ringer diluents was better for maintaining the quality of spermatozoa. Artificial insemination tests in kampung hens showed fresh semen in a combination diluent of skimmed milk + 50 mM glucose and vitamin E 2% resulting in the highest fertility and fertile period.

KEY WORDS fertile period, fertility, motility, plasma membrane integrity, viability.

INTRODUCTION

Red Kedu rooster is a common local breed in the Temanggung region, Central Java, Indonesia. The advantages of red Kedu chickens include relatively high endurance, adaptation to the environment, relatively high body weight and egg production, so it has the potential to be developed in Indonesia (Ismoyowati *et al*. 2012). It is expected that Kedu chicken breeding can help local Indonesian chicken farms to address the persistent issue of low population development and unavailability of high-quality chicken breeds with guaranteed quality. The quick method for improving the genetic quality of livestock is artificial insemination because it mates hens with superior roosters with tested genetics to produce offspring with superior genetic quality (Mussa *et al*. 2023). Semen quality and fertility of roosters in tropical countries, particularly those in open-air cages, are significantly influenced by environmental temperature (Rochmi and Sofyan, 2019). Therefore, spermatozoa for artificial insemination must be kept in storage with cold temperatures to slow down the cell damage. In general, the spermatozoa storage system in poultry can only fertilization

ability in vitro for 24 hours, thus less inefficient than storing spermatozoa in the oviduct *in vivo* where fertilization ability can last for weeks.

To ensure the survival of spermatozoa, artificial insemination requires diluent agents to meet nutritional requirements and provides an optimal environment. Diluents also contribute to increasing the number of calculated doses for artificial insemination from one ejaculation, thus promoting more efficient use of cocks. Diluent can maintain the quality of spermatozoa, which in turn stabilizes motility and viability and eventually increases fertility in artificial insemination (Vasicek *et al*. 2015). The prevalent semen diluents are ringer lactate and skim milk, but still underperformed in protecting spermatozoa cell membranes from oxidative damage after semen is ejaculated quality. Therefore, skim milk diluent needs to combine with other ingredients, namely antioxidants and glucose. One of the antioxidants that can be used in sperm diluents was vitamin E. Vitamin E serves to protect plasma membranes due to free radicals and lipid peroxides during storage (Tabatabaei *et al*. 2011; Partyka *et al*. 2012). Glucose provides nutrients as an energy source for spermatozoa, there are also sodium bicarbonate and sodium citrate which act as buffers that can maintain pH stability to extend sperm viability during storage (Gibb and Aitken, 2016; Nahak *et al*. 2022). This study aims to examine the effect of the interaction of vitamin E supplementation on different types of diluents and storage times on the quality of spermatozoa and fertility tests in kampung hen eggs.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Research ethics commission, Faculty of Veterinary Medicine, Gadjah Mada University (record no. 021/EC-FKH/Eks. /2022).

Management conservancy of chicken

Ten red Kedu roosters, one year old, are kept intensively in individual battery cages, dimensions 60 cm × 45 cm × 45 cm. Roosters are given feed 120 g/head/day, with nutrient content according to the feed standards of breeding chickens (ME 2825 kcal/kg; CP 17.2%; Ca 3.6%; lysin 1.08% and methionine 0.35%) and water was administered by *ad-libitum*. Thirty-two hens aged 48 weeks were used for fertility tests and the fertile period. The hen was caged individually, fed as much as 100 g/head/day, and drank *ad libitum*.

Spermatozoa quality research (stage 1)

The material used was ten red Kedu roosters aged one year with an average body weight of 2100 g, vitamin E (alpha-tocopherol (Sigma Aldrich), lactate ringer (produced by PT Widatra Bhakti, Pasuruan, Indonesia), physiological NaCl 3%, skim milk with the brand tropicana slim, D (+)-anhydrous glucose, ethanol 70%, eosin-nigrosin dyes, aquabidest, sodium citrate, and fructose.

Diluent preparation

Skim milk was made from 2 g of skimmed milk powder mixed with 20 mL of aquabidest. The solution was put into an Erlenmeyer and stirred. Heat the water then add Erlenmeyer and wait for the temperature of the skimmed milk to 92 ˚C for 10 minutes. Next, it was filtered using filter paper. Finally, add glucose 50 mM.

Dissolving vitamin E was carried out by mixing vitamin E and 70% ethanol in a ratio of 1:5 = 0.4 g:2 mL (Abdillah *et al*. 2021).

Semen collection and dilution

The semen was collected from 10 red Kedu roosters using the abdominal massage method (Malik *et al*. 2013). The roosters were massaged from the neck to the base of the tail until the they were aroused and releasing semen, which were then collected in a microtube. Semen samples were collected three times per week. The collected semen was diluted with diluent in a ratio of 1:2. The semen dilution treatment was as follows:

1. $D_1V_0T_0$= 0.375 mL semen + 0.84 mL lactate ringer diluent
2. $D_2V_0T_0$= 0.375 mL semen + 0.84 mL (100% skimmed milk+50 mM glucose)
3. $D_1V_1T_0$= 0.375 mL semen + 0.84 mL (99% lactate ringer+1% vitamin E)
4. $D_2V_1T_0$= 0.375 mL semen + 0.84 mL (99% skimmed milk+50 mM glucose)+1% vitamin E)
5. $D_1V_2T_3$= 0.375 mL semen + 0.84 mL (98% lactate ringer+2% vitamin E)
6. $D_2V_2T_3$= 0.375 mL semen + 0.84 mL (98% skimmed milk+50 mM glucose)+2% vitamin E)
7. $D_1V_3T_0$= 0.375 mL semen + 0.84 mL (97% lactate ringer+3% vitamin E)
8. $D_2V_3T_0$= 0.375 mL semen + 0.84 mL (97% skimmed milk+50 mM glucose)+3% vitamin E)

After dilution, semen was divided into eight parts and put into microtubes, then stored at different times observation of 0 hours was carried out after dilution was complete while 2, 4, and 6 hours were stored in a refrigerator with a temperature of 4 ˚C.

Observation of the quality of spermatozoa each treatment was carried out three times, corresponding to the period of semen collection.

Sperm motility assessment

Three hundred spermatozoa from five different microscopic fields were observed under a phase-contrast microscope using a 400x objective by dripping one drop of semen on the object glass and lactate ringer solution on it, then covered with a cover glass. Sperm with good motility move progressively straight in one direction (Chauychu-noo *et al.* 2021).

Sperm viability assessment

The sperm viability was assessed using the eosin-nigrosin staining for review. One drop of semen was mixed with five drops of eosin nigrosin on an object glass, then homogenized, and a review preparation was made. The results were observed under a 400x magnification microscope and calculated as a percentage of live versus dead sperm. The number of unstained heads of spermatozoa (live) and stained / partially stained heads of spermatozoa (dead) were counted (Arifiantini, 2012). Dead spermatozoa are unstable, so it easily absorbs color (Bansal and Bilaspuri, 2008).

Sperm abnormality assessment

Sperm abnormality was observed under a microscope then reviewed. The method of calculating was the same as viability, from 10 fields of view using a check counter. Abnormalities of spermatozoa can be seen in circular tails, tail only, and severed only there was a head (Kusumawati *et al.* 2020).

Plasma membrane integrity assessment

It was assessed using a modification of the hypo-osmotic swelling test (HOST) method described by (Revell and Mrode, 1994). Preparing a hypoosmotic solution (0.9 g of fructose+0.49 g of sodium citrate+100 mL of aquabidest). 10 mL hypoosmotic solution added 0.1 mL of semen and mixed until homogeneous, then incubated at 37 ˚C for 45 minutes.

A thin review preparation was made on the object glass using eosin nigrosin and then evaluated based on observations with a 400x magnification microscope with a minimum of 200 sperm cells/sample. Spermatozoa that have an intact plasma membrane was characterized by a circular or bulging tail, while damaged ones are characterized by a straight tail.

Fertility test by artificial insemination in kampung hen (Stage 2)

The material used in this stage was the semen of red Kedu roosters that have been treated, 32 kampung hens aged 48 weeks with an average weight of 1.500 g as fertility test objects and fertile periods.

Artificial insemination

Semen was diluted according to 8 treatments that meet the standards for artificial insemination: $D_1V_0T_0$, $D_1V_2T_0$, $D_1V_0T_2$, $D_1V_2T_2$, $D_2V_0T_0$, $D_2V_2T_0$, $D_2V_0T_2$, $D_2V_2T_2$ obtained from the combination of the best treatments in semen quality tests *in vitro*. Diluted semen was stored in the refrigerator at 4 ˚C with a storage time according to the treatment, after which it is inseminated to kampung hens with a dose of 0.1 mL and a concentration of 100 × 10^6/breeding unit. The hen was held in a position where the posterior part was slightly elevated from the axis of the body thus higher than the anterior part. The hen should massage and press the upper and lower parts of the abdomen to release her vagina from the cloacal cavity. Artificial insemination was carried out at 2-4 pm to avoid the presence of eggs in the uterus that can inhibit the progressive movement of spermatozoa (Mariani and Kartika, 2018).

Collection and incubation of artificially inseminated eggs

Eggs were collected at 4-5 pm on the second until 20th day after artificial insemination. The eggs were cleaned from dirt using wet wipes and dry wipes. The collected eggs were put into an automatic hatching machine every five days, and incubated at 37 ˚C with 60% humidity (Saleh *et al*. 2022).

Sperm fertility assessment

The collected eggs were put in the hatching machine. Candling was done on the fifth, 10th, and 15th days to determine whether the egg is fertile. Fertile eggs will show the spread of blood vessels. Eggs that do not look fertile when candling will be broken down to determine whether the egg was fertilized or not.

Fertile period assessment

The fertile period was calculated from the second day after insemination until the egg does not produce buds, observed in 20 days. The average fertile period was calculated from the number of days the hen produces fertile eggs and then divided by the total number of eggs produced. Calculated in days to determine the ability of spermatozoa to fertilize eggs in the kampung hen reproductive tract (Mariani and Kartika, 2018).

Statistical analysis

Analysis of spermatozoa quality used analysis of variance based on a randomized block factorial design. The first factor was ringer lactate diluent + skim milk + 50 mM glucose, and the second factor was three levels of vitamin E: 0%, 1%, 2%, and 3%.

The third factor was different storage time at 4 ˚C, namely 1, 2, 4, and 6 hours. The semen quality test was replicated three times at the tapping period as a group. If the treatment had a significant effect (P<0.05) on the variable, it proceeded with the orthogonal polynomial test. Fertility and fertile period data were subjected to analysis of variance, in a completely randomized design (CRD). If the treatment had a significant effect (P<0.05) on the measured variable, it proceeded with the Honestly Significant Difference (HSD) test.

RESULTS AND DISCUSSION

Assessment and evaluation of fresh semen of red Kedu roosters include macroscopic and microscopic observations can be seen in Table 1. Based on the results of the study (Table 1) the average volume of red Kedu roosters' semen resulting obtained was normal and these results are in line with the research of Garner and Hafez (2008) that local chickens generally have an average volume 0.2-0.5 mL. The degree of acidity or pH was measured using BTB pH indicator paper. The red roosters' semen in this study produced indicated the pH was normal. These results align with the research of Yendraliza *et al.* (2015) that the normal pH of fresh semen in roosters' state in the form of lactate acid, the higher or lower the pH of semen will cause spermatozoa to die faster.

The viscosity of red Kedu roosters semen showed that the semen was normal. The viscosity was similar to that reported by Peters *et al.* (2008), namely viscous or highly viscous. According to Putranto *et al.* (2020), the concentration of spermatozoa can be determined based on color and viscosity, semen with low concentrations generally has dilute viscosity and clear color, whereas semen with high concentrations has viscous viscosity and dense white color. The color of the red kedu roosters semen showed normal and the statement of Ax *et al.* (2008) that good semen was creamy white. Semen with a mixture of other colors indicates that semen has been contaminated (Kusumawati *et al.* 2020). The smell of red Kedu roosters semen produces a characteristic smell that was normal. These results were in line with those of Junaedi *et al.* (2017) that chicken semen had a typical smell. Triardi *et al.* (2022) stated that semen has a characteristic odor, such as a fishy smell typical of sperm and the smell of the animal itself.

The average concentration of spermatozoa of red Kedu roosters was within the normal range, and according to Garner and Hafez (2008), there were 3-7 billion/mL chicken semen. The mass motility of spermatozoa of red Kedu roosters showed an upward trend progressive movement.

The percentage of motility in this study was higher than that of Hidayat *et al.* (2020), namely 83.75 ± 25%. The motility of spermatozoa is highly dependent on energy supply of adenosine triphosphate (ATP) from metabolism (Danang *et al.* 2012). The viability of spermatozoa of red Kedu roosters is influenced by the storage time because the amount of energy in the diluent decreases (Haq *et al.* 2020). The percentage of abnormalities in red Kedu roosters' semen in this study was normal and thus viable for artificial insemination because, according to Nugroho and Saleh (2016), artificial insemination only tolerates no more than 20% abnormalities. The results of plasma membrane integrity assessment using a hypo-osmotic solution with the hypo-osmotic swelling test (HOST) technique on red Kedu roosters semen roosters were higher than those of intact plasma membranes in partridges reported by Bebas and Laksmi (2015) with the same method, namely 30.00 ± 1.00%.

Motility assessment was the main parameter that indicates the ability of spermatozoa to fertilize the ovum at the time of fertility (Bakst and Dyamond, 2013; Moradpour, 2019). The plasma membrane serves as the first defense from outside environment that potentially damages the cell. Assessment of the plasma membrane integrity was one of essential parameters besides motility, viability, and abnormality because damaged plasma membrane will disrupt energy supply Plasma membrane also plays a vital role in regulating all processes in the cell. Spermatozoa whose plasma membrane has damaged is characterized by straight tails, while spermatozoa with intact plasma membranes have circular or bulging spermatozoa tails (Karja *et al.* 2017). The viability of spermatozoa is one indicator of testing living spermatozoa with intact membranes. The viability of spermatozoa was assessed by examining motility and life/death ratio (Barth and Oko, 1989). The average results and analysis of each variable on the interaction of the type of diluent and the addition of vitamin E as well as different storage periods at 4 ˚C can be seen in Table 2.

Based on the results of the study Table 2, the interaction among the type of diluent, vitamin E dose, and storage time had no real effect (P>0.05) on motility, plasma membrane integrity, viability, and spermatozoa abnormalities. This showed that semen inserted in lactate ringer diluent or skim milk + 50 mM glucose and added vitamin E produced the same response between treatment combinations. The combined treatments showed relatively similar responses which may be because diluent and vitamin are complemental. Skim milk and glucose provide nutrition for spermatozoa, while ringer lactate is the buffer in maintaining pH balance for spermatozoa motility (Vasicek *et al.* 2015).

Table 1 Fresh semen characteristic of red Kedu roosters

Parameters	Result	Standard	Reference
Macroscopic			
Volume (mL/ejaculate)	2.50	0.20-0.50	(Garner and Hafez, 2008)
pH	7	6-8	(Peters *et al*. 2008)
Viscosity	Viscous	High/viscous	(Putranto *et al*. 2020)
Color	White creamy	White creamy	(Ax *et al*. 2008)
Smell	Typical	Typical	(Junaedi *et al*. 2017)
Microscopic			
Concentration (cells/mm^3) x 10^6	4840	3000-7000	(Garner and Hafez, 2008)
Mass sperm motility (%)[1]	+++	++/+++	(Toelihere, 1993)
Individual sperm motility (%)	85	> 70	(Toelihere, 1993)
Abnormal sperm	7.50	10-15	(Garner and Hafez, 2008)
Viability sperm (%)	91.50	> 85	(Toelihere, 1993)
Plasma membrane integrity sperm (%)	85.50	> 30	(Bebas and Laksmi, 2015)

[1] (-): bad; (+): medium; (++): good and (+++): excellent.

Table 2 The average value and standard deviation of interaction among type diluent, vitamin E dose, and storage time on sperm quality of red Kedu roosters at 4 ˚C

Treatments[1]	Motility (%)[ns]	Plasma membrane integrity (%)	Viability (%)	Abnormality (%)
$D_1V_0T_0$	80.33±5.13	82.83±5.62	92.33±2.52	9.17±2.02
$D_1V_0T_1$	79.33±1.15	80.83±11.43	92.50±1.32	10.83±1.61
$D_1V_0T_2$	81.33±1.15	83.50±6.38	93.00±2.00	6.17±3.33
$D_1V_0T_3$	81.33±0.58	83.50±11.69	90.17±1.26	8.67±3.51
$D_1V_1T_0$	82.83±2.93	83.67±14.77	91.33±1.53	9.67±2.02
$D_1V_1T_1$	80.33±1.53	81.33±9.65	90.50±1.32	9.67±1.53
$D_1V_1T_2$	83.00±2.65	82.83±8.02	91.50±0.50	8.00±4.77
$D_1V_1T_3$	82.67±2.52	84.33±9.52	90.17±1.89	9.50±3.28
$D_1V_2T_0$	80.33±2.31	81.00±4.58	91.33±0.29	10.17±2.75
$D_1V_2T_1$	81.00±1.00	82.17±15.37	91.83±0.29	12.17±2.02
$D_1V_2T_2$	84.33±2.08	86.17±12.89	93.67±1.53	9.17±2.75
$D_1V_2T_3$	82.00±1.73	83.50±20.20	91.50±1.50	11.00±1.00
$D_1V_3T_0$	81.67±3.06	84.33±14.68	93.33±1.53	10.00±2.00
$D_1V_3T_1$	82.50±2.29	85.33±19.02	94.00±1.73	11.00±1.50
$D_1V_3T_2$	82.33±2.08	83.83±5.48	94.17±1.26	6.83±2.02
$D_1V_3T_3$	82.00±1.00	84.83±3.18	92.60±0.66	7.50±1.32
$D_2V_0T_0$	81.83±4.54	82.17±18.10	92.17±1.61	10.33±1.76
$D_2V_0T_1$	79.00±2.00	80.83±15.91	91.83±1.76	9.00±1.80
$D_2V_0T_2$	81.67±1.53	82.50±17.51	92.00±2.00	7.00±2.65
$D_2V_0T_3$	80.33±0.58	81.50±34.83	91.83±0.76	8.83±2.75
$D_2V_1T_0$	81.83±2.75	82.83±10.28	92.33±1.53	10.67±3.21
$D_2V_1T_1$	81.00±2.00	81.33±6.60	90.83±1.76	11.33±2.25
$D_2V_1T_2$	82.00±0.00	82.67±5.53	91.83±0.29	8.00±2.65
$D_2V_1T_3$	81.67±2.52	82.00±10.58	90.17±1.26	9.50±3.28
$D_2V_2T_0$	80.00±0.00	81.50±14.31	90.83±0.76	15.50±1.32
$D_2V_2T_1$	79.67±1.15	80.00±18.03	89.67±0.76	15.17±2.02
$D_2V_2T_2$	82.17±0.29	84.83±8.40	92.17±0.29	9.83±2.36
$D_2V_2T_3$	81.33±1.53	83.33±15.78	91.67±1.53	12.67±1.44
$D_2V_3T_0$	79.00±1.00	80.17±15.89	90.00±1.32	9.50±1.80
$D_2V_3T_1$	79.00±1.00	80.17±15.02	89.50±0.50	9.50±0.50
$D_2V_3T_2$	80.67±0.58	82.67±6.53	92.17±0.29	6.33±2.08
$D_2V_3T_3$	80.33±1.53	82.33±8.02	90.50±1.32	8.17±2.02
P-value	0.937	0.979	0.821	0.323

D_1: lactate ringer; D_2: Skim milk + 50 mM glucose; V_0: 0% vitamin E; V_1: 1% vitamin E; V_2: 2% vitamin E; V_3: 3% vitamin E; T_0: 0 hours; T_1: 2 hours; T_2: 4 hours and T_3: 6 hours storage.
NS: non significant.

Vitamin E is the antioxidant to maintain the membrane cell integrity and protect spermatozoa cell membrane from free radicals (Khan *et al.* 2021).

The results of variance analysis showed that the interaction of diluent type and vitamin E dose had no real effect (P>0.05) on motility and integrity of plasma membrane integrity, but very different (P<0.01) on spermatozoa viability and abnormality. The combination of lactate ringer diluent with 2% vitamin E results in the highest viability (Figure 1).

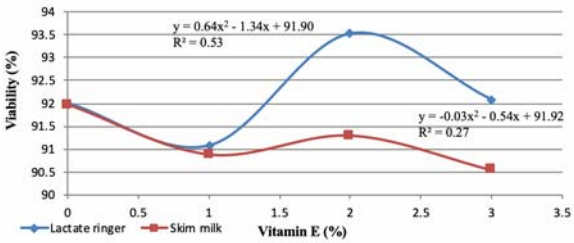

Figure 1 Graph interaction diluent type and vitamin E on viability spermatozoa of red Kedu roosters

Based on the orthogonal polynomial test in Figure 1, the equation was obtained in the lactate ringer diluent which means that every 0.1% vitamin E in the lactate ringer diluent will increase viability by 0.53% and in the skim milk diluent + 50 mM glucose which means that every 0.1% vitamin E in the skim milk diluent + 50 mM glucose will increase viability by 0.27%.

Figure 1 showed that the addition of vitamin E to the diluent ringer lactate and skim milk + 50 mM glucose both decreased the viability trend in the addition of vitamin E by more than 2%. Incorporating 2% vitamin E is the most optimum to maintain spermatozoa motility because at this level, vitamin E exhibits the optimum antioxidant properties to protect the sperm from free radicals during storage. However, doses higher than 2% will reduce spermatozoa viability because alpha tocopherol is alkaline, so without oxygen. It was in line with Ansgarius *et al.* (2022) that vitamin E is alkaline and contains plenty of antioxidant which could be exhibit peroxide properties and, in turn, decrease motility. This is because the nutrient content is too high, namely lactose in skim milk, and the addition of glucose in diluents can cause a decrease in sperm metabolism that is high during cold storage temperatures (Clarke *et al.* 1982).

The addition of 50 mM glucose in skim milk diluent was thought to cause a decrease in viability because the addition of extracellular cryoprotective compounds in large quantities can result in increased osmotic pressure of diluent solutions and is less well adapted by spermatozoa resulting in adverse spermatozoa metabolic processes (Mayesta *et al.* 2014).

This would disrupt the normal biochemical processes in the cell, which in turn would decrease the viability of spermatozoa during storage temperature 4 ˚C. Lactate ringer diluent was better than skim milk diluent + 50 mM glucose in maintaining spermatozoa survival, because it is a solution whose properties were close to semen plasma.

Fitriyah *et al.* (2019) explained that lactate ringer solution is a solution consisting of various kinds of mineral salts, namely sodium lactate, sodium chloride, potassium chloride, calcium chloride, osmolarity, Na^+, K^+, lactate and sodium chloride content which is the same as electrolyte elements from chicken semen plasma itself such as sodium, chloride, calcium and magnesium which have buffers and isotonic that can support spermatozoa viability in time longer storage, while skim milk contains protein, carbohydrates, potassium, vitamins A, B1, B2, B3, and B6 which are less able to support the endurance of spermatozoa.

This was also by the opinion of Danang *et al.* (2012) that lactate ringer diluents contain Na-lactate to maintain the acidity of the solution and the osmotic pressure of the solution. The combination of diluents and vitamin E that effectively maintains spermatozoa abnormalities is lactate ringer with 2% vitamin E.

The results of the orthogonal polynomial test in Figure 2, obtained the equation in skim milk diluent + 50 mM glucose which means every 0.1% vitamin will reduce abnormalities by 0.60%, and in lactate ringer diluent which means every 0.1% vitamins will reduce abnormalities by 0.24%.

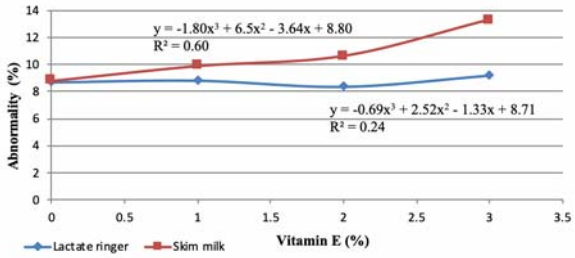

Figure 2 Graph interaction diluent type and vitamin E on the abnormality of spermatozoa red Kedu roosters

According to Gordon (1990) the large concentration of antioxidants added can affect the rate of oxidation, and adding at high concentrations will cause antioxidant activity to disappear even if the antioxidants become per-oxidants. Spermatozoa abnormality in skim milk diluent + 50 mM glucose occurred; it increased at the addition of 0-3% vitamin E. Similarly, ringer lactate diluent also showed abnormality, but the increase was not as high as skim milk diluent, in fact, the abnormality value declined at the addition of 2% vitamin E, then increased again at 3% vitamin E.

Incorporating vitamin E into ringer lactate diluent was better than skim milk + 50 mM glucose to prevent spermatozoa damage. The damage of spermatozoa cells' plasma membrane is due to binder protein in rooster's semen, meanwhile, protein plasma is the main component of semen plasma that interacts with milk protein and this interaction is important for spermatozoa protection (Khaeruddin *et al.* 2020).

Figure 2 showed that in skimmed milk diluent + 50 mM glucose there was an increasing trend in the addition of 0-3% vitamin E, while in lactate ringer diluent there was the same trend as in skim milk diluent + 50 mM glucose but the increase was not too high and the abnormality value decreased with the addition of vitamin E 2% then increased in vitamin E 3%. The higher the abnormality value, the worse the quality of spermatozoa. The addition of vitamin E to the lactate ringer diluent is better than the skim milk diluent + 50 mM glucose in preventing spermatozoa damage.

Damage to the plasma membrane of spermatozoa cells is caused by binder proteins in chicken semen, semen plasma proteins are the main component of semen plasma that interacts with milk proteins, this interaction is important for the protection of spermatozoa (Khaeruddin *et al.* 2020). Lactate ringer and skim milk + 50 mM glucose thinners used in this study are good in maintaining the integrity of spermatozoa because skim milk contains lactose which serves as an energy source for spermatozoa, while ringer lactate is a solution whose osmosis pressure is almost the same as chicken semen and antioxidants help maintain semen pH during storage. The results of this study are almost the same as Nugroho and Saleh (2016) research report that the use of lactate ringer diluent resulted in abnormality of kampung roosters spermatozoa by 8.50% during one hour of storage.

The results of variance analysis showed the interaction between diluent type and storage time, as well as the interaction between vitamin E dose and storage time, had no real effect ($P>0.05$) on motility, the integrity of plasma membrane integrity, viability, and abnormality of spermatozoa (Table 3, 4, and 5).

The effect interaction had no significant on the quality of spermatozoa is possible because the combination of treatments only exerts a separate influence but does not exert a joint influence on the quality of spermatozoa. The type of diluent had a significant effect ($P<0.05$) on spermatozoa motility.

The motility produced by ringer lactate diluent was higher than that of skim milk diluent + 50 mM glucose. This is because ringer lactate contains electrolytes that are almost identical with semen plasma, thus enabling spermatozoa to move freely like in their original environment, while skim milk which has fat particles that can interfere with spermatozoa movement. Ringer's lactate contains water and electrolyte sources, produces metabolic alkalinization effects, and contains several chemical compounds, water, pH value, osmolarity, and energy sources (Telnoni *et al.* 2021). Lactate ringer is a physiological diluent, Na-Lactate in lactate ringer is needed to meet the needs of bicarbonate ions to maintain the acidity of the solution or as a buffer for the solution and maintain the osmotic pressure of the solution. Lactate ringer solution also contains glucose which is substitute energy for fructose in semen plasma which is needed for metabolic activities during semen storage (Pandia *et al.* 2021). The results of this study are slightly higher than the results of the study of Azzam *et al.* (2022) with motility of 80.20 ± 1.30% using lactate ringer diluent with a storage time of 2 hours, the difference is the difference in chicken strains and the addition of 10% egg yolk in ringer diluent. According to Telnoni *et al.* (2021).

In contrast, reported lower motility using skim milk diluent than that by Saleh *et al.* (2022), namely 82 ± 2.74%, with a difference in the chicken strain and storage time (1 hour). According to Chakraborty and Saha (2022), semen must have an 80% motility for artificial insemination purposes. High motility of spermatozoa provides a higher chance of fertilization because only motile spermatozoa can fertilize an egg. The direction of forward movement of spermatozoa (progressive motility) is the main criterion of semen quality. The addition of vitamin E with different concentrations had no real effect ($P>0.05$) on spermatozoa motility. The results of this study are better than the report of Amaefule *et al.* (2020), which resulted in a motility value of 77.15 ± 5.81% with the addition of vitamin E 125 mg.

The addition of vitamin E had a significant effect ($P<0.05$) on the plasma membrane integrity of spermatozoa. Orthogonal polynomial test results are in Figure 3. A linear equation was obtained which means that every 0.1% of vitamins will reduce abnormalities by 0.07%. The greater addition of vitamin E will decrease the value of the plasma membrane. Masrifah *et al.* (2017) explained that the addition of vitamin E at high concentrations resulted in lost antioxidant activity even if these antioxidants became pro-oxidants or toxic. According to Dorota and Kurpirsz (2004) impaired oxidation phosphorylation leads to an increase in reactive oxygen species (ROS) semen. High levels of ROS in cells can oxidize lipids, proteins, and DNA where semen plasma membrane lipids are very susceptible to ROS. ROS attack during storage causes a decrease in oxygen which causes lipid peroxide thereby damaging the structure of the lipid matrix in the sperm membrane and resulting in decreased motility, viability, plasma membrane integrity, intracellular enzyme activity, and sperm DNA damage (Vahedi *et al.* 2018).

Table 3 The average value and standard deviation interaction of diluent type and storage time on sperm quality of red Kedu roosters at 4 ˚C

Treatments[1]	Motility (%)[ns]	Plasma membrane integrity (%)[ns]	Viability (%)[ns]	Abnormality (%)[ns]
D_1T_0	81.29±1.34	82.96±20.02	92.08±1.35	9.75±2.42
D_1T_1	80.79±1.34	83.67±3.18	92.21±1.61	10.92±2.24
D_1T_2	82.75±2.52	84.67±13.82	93.08±1.32	7.54±1.32
D_1T_3	82.00±1.73	84.29±10.79	91.11±1.50	9.17±2.02
D_2T_0	80.67±0.58	82.17±13.01	91.33±1.44	11.50±2.25
D_2T_1	79.67±1.15	80.33±6.60	90.46±1.32	11.25±1
D_2T_2	81.63±1.58	84.83±8.85	92.04±1.74	7.79±2.65
D_2T_3	80.92±2.88	82.54±15.78	91.04±1.28	9.79±2.42
P-value	0.965	0.511	0.207	0.297

D_1: lactate ringer; D_2: Skim milk + 50 mM glucose; T_0: 0 hours; T_1: 2 hours; T_2: 4 hours and T_3: 6 hours storage.
NS: non significant.

Table 4 The average value and standard deviation interaction of vitamin E and storage time on sperm quality of red Kedu roosters at 4 ˚C

Treatments[1]	Motility (%)[ns]	Plasma membrane integrity (%)	Viability (%)[ns]	Abnormality (%)[ns]
V_0T_0	81.08±2.23	82±8.02	92.25±1.32	9.75±2.36
V_0T_1	79.17±2	80.83±10.35	92.17±1.61	9.92±3.04
V_0T_2	81.50±3.06	82.50±17.51	92.50±1.32	6.58±2.08
V_0T_3	80.84±4.54	82±13.84	91±1.50	8.75±3.51
V_1T_0	82.34±2.08	83.75±21.62	91.83±1.65	10.17±2.75
V_1T_1	80.67±0.58	81.83±10.94	90.67±1.32	10.50±3.21
V_1T_2	82.50±2.29	84.25±17.51	91.67±1.53	8±2.65
V_1T_3	82.17±2.08	83.17±9.52	90.17±1.89	9.50±0.50
V_2T_0	80.17±1.77	82.25±15.89	91.08±1.76	12.83±2.85
V_2T_1	80.33±2.31	81.58±12.85	90.75±1.33	13.67±2.02
V_2T_2	83.25±2.39	84.50±15.02	92.92±0.66	9.50±3.28
V_2T_3	81.67±1.15	82.42±5.48	91.58±0.50	11.83±2.85
V_3T_0	80.34±2.31	81.25±12.38	91.67±0.29	9.75±2.36
V_3T_1	80.75±1.82	82.75±4.58	91.75±1.61	10.25±2.75
V_3T_2	81.5±1.15	83.25±10.79	93.17±1.53	6.58±2.08
V_3T_3	81.17±1.53	83.08±12.65	91.55±0.50	7.83±1.32
P-value	0.713	0.813	0.493	0.930

V_0: 0% vitamin E; V_1: 1% vitamin E; V_2: 2% vitamin E; V_3: 3% vitamin E; T_0: 0 hour; T_1: 2 hours; T_2: 4 hours and T_3: 6 hours storage.
NS: non significant.

Table 5 The average value and standard deviation interaction of diluent type, vitamin E and storage time on sperm quality of red Kedu roosters at 4 ˚C

Treatment factors	Motility	Plasma membrane integrity	Viability	Abnormality
D_1	81.71±2.29^{a}	81.69±12.85	92.12±1.74^{a}	9.34±2.62^{b}
D_2	80.72±1.82^{b}	82.68±13.82	91.22±1.41^{b}	10.08±3.04^{a}
P-value	0.017	0.118	0.002	0.02
V_0	80.65±2.44	81.96±16.18^{ab}	91.98±1.65	9.75±2.57^{bc}
V_1	81.92±2	82.19±13.84^{a}	91.08±1.37	9.54±2.58^{b}
V_2	81.35±1.89	83.50±8.85^{bc}	92.03±2	8.60±2.14^{a}
V_3	80.94±1.99	81.08±11.93^{c}	91.58±1.38	11.96±2.85^{c}
P-value	0.145	0.02	0.069	0.001
T_0	80.98±2.88^{bc}	82.07±12.65	91.71±1.61^{b}	10.63±2.66^{a}
T_1	81.46±1.44^{c}	81.00±14.45	91.33±1.81^{b}	549.48±2.44^{a}
T_2	82.19±1.69^{a}	83.25±10.94	92.56±1.38^{a}	7.67±2.76^{c}
T_3	80.23±1.77^{ab}	83.22±15.42	91.08±1.43^{b}	11.08±2.36^{b}
P-value	0.01	0.439	0.002	0.004

D_1: lactate ringer; D_2: Skim milk + 50 mM glucose; V_0: 0% vitamin E; V_1: 1% vitamin E; V_2: 2% vitamin E; V_3: 3% vitamin E; T_0: 0 hours; T_1: 2 hours; T_2: 4 hours and T_3: 6 hours storage.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).

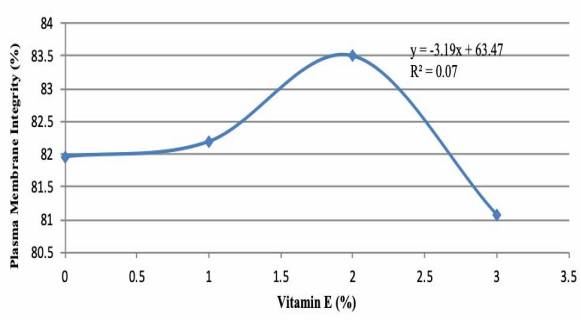

Figure 3 Graph of the effect vitamin E addition on of the plasma membrane the integrity (PMI) spermatozoa of red Kedu roosters

The results of this study were lower than the research report of Farid *et al.* (2021), which resulted in an intact plasma membrane integrity value of 88.50 ± 1.73% with the addition of 5 µg/mL of vitamin E for 1 hour of storage. The thing that distinguishes it is the concentration of vitamin E used. In contrast to the report of Vahedi *et al.* (2018) but showed a significant increase, resulting in the best plasma membrane integrity value of 56.11 ± 4.07% with supplementation of 8 mL/dL *Thymus vulgaricus* with tris diluent in sheep semen. This is due to differences in antioxidant compounds used and types of species, *Thymus vulgaricus* used is a polyphenol compound that has natural antioxidant properties so that it can protect against oxidative damage and take free radicals in cells (Loziene *et al.* 2007; Vahedi *et al.* 2018). According to Tabatabaei *et al.* (2011) sperm plasma membrane contains high amounts of unsaturated fatty acids, so it is susceptible to peroxidative damage indicated by subsequent loss of membrane integrity and failure of sperm cell function will decrease spermatozoa motility.

The metabolic process of spermatozoa will be produced free radicals in the form of oxygen derivatives, including single oxygen (O_2), oxygen triplet ($3O_2$), superoxide anion (O^{-2}), hydroxyl radical (.OH) and nitric oxide (.NO^-) all of which are called reactive oxygen species (ROS). Single oxygen can damage the double bonds in fatty acids that can damage and proteins (Devi *et al.* 2000). According to Dorota and Kurpirsz (2004) that oxidation phosphorylation in the metabolism of spermatozoa cells is disrupted causing an increase in reactive oxygen species (ROS) semen. High levels of ROS in cells can oxidize lipids, proteins, and DNA where semen plasma membrane lipids are very susceptible to ROS.

Free radicals will take electrons from unsaturated fatty acids that make up the phospholipids of the plasma membrane, resulting in a peroxide reaction. The effects of phospholipid peroxide on avian spermatozoa include damaging the morphology of spermatozoa, decreasing motility, and causing low fertility (Long and Kramer, 2003). Vitamin E acts in the phospholipid layer of the cell membrane and serves to protect polyunsaturated fatty acids and other cell membrane components from free radical oxidation by breaking the lipid peroxide chain. Vitamin E acts by donating hydrogen ions to neutralize or reduce levels of fat peroxide (Hariyatmi, 2004).

The results analysis of the variance of the shelf time factor had a signifficant effect (P>0.01) on sperm motility. The results of the orthogonal polynomial test obtained a cubic equation which means that every 1 hour will reduce motility by 0.11% (Figure 4). The results of this study were in line with the research report of Farid *et al.* (2021) the addition of vitamin E as much as 5 µg/mL in KUB chicken semen decreased motility followed by the longer storage time shown the results T1 (2 hours)= 90.75 ± 1.708, T2 (4 hours)= 85.50 ± 4.203, T3 (8 hours)= 83.50 ± 2.88, T4 (48 hours)= 76.25 ± 5.058, this is thought to be related to energy reserves in the form of ATP when storage has begun to decrease. This is supported by the opinion of Yaman *et al.* (2021) that the longer the shelf life, the lower the motility of spermatozoa due to cold stress, osmotic imbalance, and the presence of lactic acid due to anaerobic metabolism.

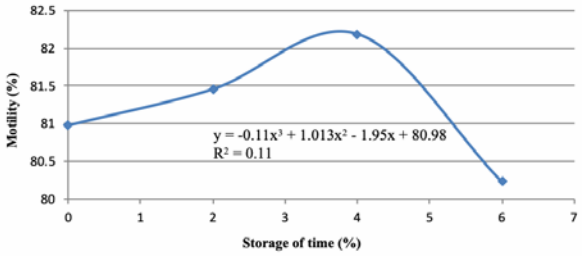

Figure 4 Graph of the effect storage time on motility spermatozoa of red Kedu roosters

The measure of success of the artificial insemination program is the fertility of eggs from hens (Bakst and Dyamond, 2013). Fertility depends on the quality and quantity of spermatozoa deposited (Brillard, 2003). Determination of egg fertility through candling on the 7th day of incubation. Egg fertility was calculated from the total fertile eggs divided by the total incubated eggs multiplied by 100% (Modupe *et al.* 2013) can be seen in Table 6. Based on the results of variance analysis showed a combination of among diluent type vitamin E dose, and storage time had a significant effect (P<0.05) on fertility and fertile period. The highest fertility rate in the treatment of skim milk diluent + 50 mM glucose with 2% vitamin E, without storage (fresh semen/0 hours storage). This is because skim milk contains lactose as a food source for spermatozoa to produce ATP on fresh semen. The quality of spermatozoa declined during storage, thus proving that some dilutions could not maintain spermatozoa quality.

Table 6 The average value and standard deviation of the combination treatment fertility and fertile period

Treatments	Fertility (%)**	Fertile period (day)*
$D_1V_0T_0$	50.70±28.05^{b}	10.67±3.06^{bc}
$D_2V_2T_0$	93.63±5.53^{c}	11.67±1.53^{bc}
$D_1V_2T_0$	58.90±8.40^{b}	11.33±2.08^{bc}
$D_2V_0T_0$	70.50±26.19^{b}	14.67±0.58^{c}
$D_1V_0T_2$	30.53±4.79^{a}	7.67±6.11^{b}
$D_2V_2T_2$	26.50±9.69^{a}	7.33±2.52^{b}
$D_1V_2T_2$	82.23±16.76^{bc}	4.00±1.00^{a}
$D_2V_0T_2$	65.47±6.64^{b}	8.67±4.93^{b}
P-value	0.001	0.032

D_1: lactate ringer; D_2: Skim milk + 50 mM glucose; V_0: 0% vitamin E; V_2: 2% vitamin E; T_0: 0 hours and T_2: 4 hours.
** (P<0.01) and * (P<0.05).

In fact, diluent cannot thoroughly maintain spermatozoa quality because at a low-temperature storage, sperm metabolism is not fully stopped but only undergo some major changes, namely declining motility, morphology integrity, and unchangeable spermatozoa fertility (Dumpala *et al.* 2006).

In contrast to the Shanmugam and Mahaptra (2023) report, using sasaki diluent supplemented by 4% dimethyl sulfoxide (DMSO) and 5 mM period reduces fertility value by 6.67 ± 6.67% but can increase progressive motility and viability.

Hoesni (2016) stated that the use of skim milk as a diluent serves as a protector of spermatozoa from the influence of cold shock as well as a source of food for spermatozoa because of the content of lipoproteins and lecithin which acts on the lipoprotein sheath of sperm cells that protect them from cold shock. Glucose in the diluent solution acts as a cryoprotectant, maintaining the osmosis pressure of the diluent solution and as an energy source for spermatozoa during storage.

Vitamin E with a concentration of 2% worked optimally as an antioxidant that serves to protect spermatozoa from the influence of free radicals to maintain the quality of spermatozoa. According to Putra *et al.* (2019) that vitamin E is one of the antioxidants used to inhibit lipid peroxidation reactions, which was a substance that can bind free radical compounds by transferring hydrogen phenolics to free radicals from polyunsaturated fatty acids that have undergone peroxidation. The results of this study are higher than the report of Saleh *et al.* (2022), which was 90 ± 1.41% using skim milk diluent + 50 mM glucose in kampung roosters semen. Fertility results in this study are high, supported by the opinion of Mohan *et al.* (2018) that the percentage of fertility resulting from artificial insemination in poultry generally ranges from 73-87%.

A storage time of 0 hours is the best time to increase fertility. This is related to energy reserves in the form of ATP when storage has begun to decrease. This is supported by the opinion of Yaman *et al.* (2021) that the longer the shelf life, the lower the motility of spermatozoa due to cold stress. Osmotic imbalance and the presence of lactic acid due to anaerobic metabolism. During the storage process at 4 ˚C spermatozoa rapidly lose their motility and there was a lot of inhibition of activity to metabolic activity physically and chemically with a marked decrease in the metabolism of adenosine triphosphate and adenosine 3,5 monophosphate (Apell and Evans, 1997). Acidity is one of the factors that affect the vitality of spermatozoa, thus affecting motility and fertility (Susilawati and Hernawati, 1992). This is by the opinion of Telnoni *et al.* (2021) states that the low percentage of fertility was due to the failure of spermatozoa to reach and enter the sperm storage site, the ability to reach the fertilization site in the infundibulum, penetrate the perivitelline layer of the ovum and the failure of spermatozoa to form a pronucleus which causes fertilization to not occur.

The semen deposition method used is intravaginal, meaning that sperm deposition is injected into the border area between the vagina and a depth of ± 3-4 cm. The sperm will be moved to the main place of sperm in the sperm storage tubules (SST). Sperm will come out of the SST and transported to the infundibulum as a place of fertilization and serve as a storage place for the second sperm. The fertilized ovum will be transferred to the magnum as a place of albumin secretion, and continue to the isthmus as a place of shell membrane formation and eggshell formation until ovulation. Chickens need 24-26 hours for follicle formation (first follicle) and will ovulate (Bakst and Dymond, 2013).

Skim milk diluent + 50 mM glucose without the addition of vitamin E stored for 4 hours produces the best fertile period value. This is because skim milk with glucose had a high nutrient content including lactose, protein, carbohydrates, potassium, vitamins A, B1, B2, B3, and B6 which serve as a food source for spermatozoa so that the survival of spermatozoa is maintained for a longer time than lactate ringer diluent. The results of this study are lower than the results of Mariani and Kartika (2018) research that the length of the fertile period in chickens was obtained on average 15 days.

The addition of a concentration of 0% vitamin E results in a longer fertile period value than the addition of vitamin E. It is suspected because the addition of vitamin E as an antioxidant that prevents free radicals in diluents will better maintain the motility value of spermatozoa compared to without the addition of vitamin E. So, the vitamin E addition on diluent will increase the metabolic process of spermatozoa which causes energy to run out quickly and spermatozoa will not be able to survive longer. This is in line with the report of Hidayat *et al.* (2019), namely the lowest fertile period produced in the treatment of giving 80 µg/mL

of vitamin E in Magelang ducks of 6.3 ± 3.4 days and the highest in the Mojosari duck group without vitamin E of 19.5 ± 0.6 days. Hidayat *et al*. (2019) explained the low fertility period due to the addition of vitamin E is caused by the energy sources used by spermatozoa to maintain motility are running out more quickly and lactic acid is accumulating more quickly so that spermatozoa are unable to survive for a long time. Spermatozoa move by utilizing energy from metabolism.

However, the by-products of this metabolism are lactic acid has a bad impact on spermatozoa because it can lower the pH and cause damage spermatozoa cell membrane. The high metabolic rate of spermatozoa causes energy reserves to be depleted more quickly and lactic acid deposits increase. The addition of vitamin E would shorten the fertile period because spermatozoa cannot survive long in the storage place of female spermatozoa which will be brought to the infundibulum as a place of fertilization and serves as a second sperm storage site in the reproductive system of hens.

A storage time of 0 hours is the optimal time to produce a long fertile period in contrast to 4 hours of storage which results in a faster fertile period value. This is related to energy reserves in the form of ATP when storage has begun to decrease. This is supported by the opinion of Yaman *et al.* (2021) that the longer the shelf life, the lower the motility of spermatozoa due to cold stress, osmotic imbalance, and the presence of lactic acid due to anaerobic metabolism.

CONCLUSION

The interaction among the type of diluent, the dose of vitamin E, and the length of storage had no significant effect on the quality of spermatozoa of red Kedu roosters. The combination of lactate ringer diluent and 2% vitamin E was able to maintain the highest viability with the lowest percentage of spermatozoa abnormalities. Lactate ringer diluents were better able to maintain motility compared to skim milk + 50 mM glucose, while the addition of 2% vitamin E had an impact on the durability of the percentage plasma membrane integrity. Artificial insemination tests in kampung hens showed fresh semen in a combination diluent of skim milk + 50 mM glucose and vitamin E 2% resulting in the highest fertility of the fertile period.

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