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| RESEARCH ARTICLE

Quality of Bali Cattle Spermatozoa During Cryopreservation in Egg Yolk Tris Diluent with Vitamin E

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| ABSTRACT
Freezing during cryopreservation causes lipid peroxidation in the plasma membrane of spermatozoa. Vitamin E contains antioxidants that can inhibit the formation of free radicals. The study aimed to determine the effect of vitamin E in egg yolk tris diluent to improve sperm quality during the cryopreservation process with different freezing times on Bali cattle spermatozoa's motility, viability, and recovery rate. Semen was collected using the artificial vagina. The study used an experimental method with a factorial complete randomized design. The first factor was the dosing of vitamin E in 100 ml of egg yolk tris diluent, namely control (without vitamin E), doses of 0,2 g; 0,4 g; and 0,6 g with 3 replicates. The second factor was freezing time on day 0, 2, and 8. Data results were analyzed with Anova One Way, if the significant effect in further test with DMRT level 5 %. The results showed that the dosing of vitamin E and freezing time had a significant effect ($P<0.05$) on motility and viability of spermatozoa. In the recovery rate of spermatozoa with vitamin E dosing had a significant effect ($P<0.05$) and freezing time had no significant effect ($P<0.05$). The interaction of vitamin E dosing and freezing time had a significant effect ($P<0.05$) on motility and viability. The interaction of Vitamin E dosing on spermatozoa recovery rate was significant ($P<0.05$). The highest quality value of Bali cattle spermatozoa was in the dose of vitamin E 0.6 g/100 ml of egg yolk Tris diluent on day 8 on motility of 89.10%, viability of 93.33%, and recovery rate of 105.81%. Suspected, the antioxidant content of alpha-tocopherol in vitamin E can affect the quality of Bali cattle spermatozoa during cryopreservation.

| KEYWORDS
Bali cattle, spermatozoa, vitamin E (alpha-tocopherol)

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1. Introduction

Bali cattle are the result of domestication of wild bulls which have the advantage of being able to breed and reproduce in hot and cold environmental stress conditions (Aswandi & Latabar 2017). Male Bali cattle have high spermatozoa fertility with a pregnancy percentage of 81% and a birth rate of 78-90% (Saili *et al.* 2017). The high fertility of Bali cattle spermatozoa is used to produce frozen cow semen using cryopreservation techniques for artificial insemination.

The cryopreservation process of spermatozoa is done by storing spermatozoa in a frozen state with a temperature of -196 ^{0}C in liquid nitrogen. The freezing process will reduce the quality of semen by up to 50% of fresh semen and result in 30% spermatozoa death (Arifiantini *et al.* 2018; Zelpina *et al.* 2012). The process of freezing spermatozoa results in (*Cold Shock*) cold shock. Where, this is due to the formation Lipid peroxidation in the plasma membrane of spermatozoa. The plasma membrane of spermatozoa contains many polyunsaturated fatty acids which are susceptible to free radical oxidation. Oxidation of membrane lipids with free radicals forms a lipid peroxidation reaction, producing new free radical compounds. Damaging free radical

compounds will cause oxidative stress in spermatozoa cells, resulting in decreased motility, viability, and even death of spermatozoa (Amitran *et al.* 2020).

Semen cryopreservation often uses egg yolk Tris diluent to dilute the spermatozoa. Tris is a tris aminomethane that functions as (*Buffer*) to maintain pH, osmotic pressure, and electrolyte balance. Egg yolk is useful as an extracellular cryoprotectant, nutrient and food for spermatozoa during freezing (Suharyati & Hartono 2011). However, data from inspections of the quality of frozen semen at the Pekanbaru Livestock Artificial Insemination UPT showed that spermatozoa that were diluted using only Tris egg yolk experienced a decrease in motility of up to 16%. This is not in accordance with the SNI ISO:9001:2015 standard, the motility of frozen semen spermatozoa is > 40% (Armunanto 2021). Decreased motility is caused by damage to the plasma membrane due to lipid peroxidation reactions by free radicals during freezing. One way that can inhibit spermatozoa damage during freezing from free radicals is by administering antioxidant supplements in the diluent such as vitamin E (Alpha-Tocopherol).

Vitamin E contains antioxidants, namely the compound alpha-tocopherol, soluble in fatty acids and has a phenol group which can ward off organic peroxyl free radicals. Vitamin E which is soluble in fatty acids protects polyunsaturated fatty acids in the spermatozoa plasma membrane from oxidation by free radical compounds. Vitamin E wards off free radicals by providing free hydrogen atoms from the hydroxyl (OH) group in the ring structure as electrons which will be captured by free radical compounds to form new compounds that are more stable. Thus stopping the production of free radical compounds and inhibiting oxidative stress in spermatozoa, spermatozoa motility and viability can be maintained (Fitriani, 2014).

Research Prastiya et al. (2021) effect of supplementation α-tocopherol in Tris egg yolk diluent on the quality of Sapera goat spermatozoa produces 61% motility and 78% viability. Amitran Research *et al.* (2020) The effect of administering vitamin E in Tris egg yolk diluent on the quality of Duroc pig spermatozoa resulted in motility of 76% and viability of 82%. Sartika Research *et al.* (2022), the addition of commercial vitamin E in the andromed diluent to the quality of Simental cow spermatozoa resulted in 56% motility and 79% viability. Vitamin E can apparently improve the quality of spermatozoa in cows, goats and pigs. Therefore, it is necessary to further investigate the quality of Bali cattle spermatozoa during cryopreservation by administering vitamin E in Tris egg yolk diluent.

2. Materials and Methods

Tools and materials

The tools used are *hot plate, magnetic stirrer*, 10 µl micropipette, 10 µl pipette tip, analytical balance, Erlemenyer, pH indicator paper, measuring cup, beaker, paper *whatman*, spatula, AV pump/air valve, sperm tube, sperm tube protector, oven, mercury thermometer, AV funnel, AV funnel protective jacket, jelly lubricant stick, dispenser, *inner liner*/artificial vagina, tissue, microscope AndroVision:CASA software, pH indicator paper, scissors, micropipette 0.5 µl and 10 µl, electronic micropipette 100 µl and 1 ml, pipette tip 0.5 µl and 10 µl, electronic pipette tip 100 µl and 1ml, semen chamber 20 µm, slide glass, cover glass, sperm tube, microtube, PCR tube rack, *electric warmer*, *water bath*, measuring cup, conical measuring cup, aluminum foil, Bali cattle cement straw, straw container, ozone cupboard, needle and rubber *filling-sealing*, hose and pipette *filling-sealing*, machine *filling-sealing*, vacuum bottle, *stabilizer*, straw stacking rack, *cold cabinet*, *cool box*, iron straw rack support *fre-freezing*, goblet tube, goblet clamp, tweezers, and tube *container*.

The ingredients used are hot water, lubricant jelly/lubricant, egg yolk of laying hens, aquabides, tris aminomethane, citric acid, fructose, glycerol, 100,000 IU penicillin, 10 mg streptomycin, 2% eosin, cow semen, liquid N2, semen thinner (tris- egg yolk + vitamin E), and pure Vitamin E powder, the active ingredient dl-alpha tocopherol 50 grams.

Method

The research was carried out in January 2023. Examination of spermatozoa quality was carried out at the Semen Production and Testing Laboratory of the Animal Artificial Insemination UPT, Livestock and Animal Health Service in Riau Province. This research uses an experimental method with a Completely Randomized Factorial Design which has 2 factors, namely the vitamin E dosage factor based on Hatono (2008) is control/no vitamin E (P0); dose 0.2 g (P1); 0.4 g (P2); and 0.6 g (P3) in 100 ml of Tris egg yolk diluent, each with 3 repetitions, and a factor in the length of freezing time on days 0, 2, and 8. The experimental livestock used were 8 year old Bali cattle weighing 571 kg raised at the Pekanbaru Livestock Artificial Insemination UPT. Cows are given 10% fresh forage, 1% concentrate, 1.5 kg/BW of bean sprouts and drinking water *at will* sesuai SNI ISO: 9001:2015.

Research Procedures

Making semen thinner (Tris-Egg Yolk with Vitamin E)

Tris egg yolk semen diluent was made from 4 diluents, 1 diluent P0 (control/without vitamin E) and 3 diluents treated with vitamin E (alpha-tocopherol) according to the treatment dose (P1 0.2 g), (P2 0.4 g), (P3 0.6 g). Tris egg yolk semen diluent contains

25.17 ml of egg yolk, 7.99 glycerol, 100 ml of aquabidest, 3.028 grams of tris, 1.7 grams of citric acid, 1.25 grams of fructose, 0.3 ml of penicillin 100,000 IU, 0.1 ml of streptomycin, and pure Vitamin E powder, the active ingredient dl-alpha tocopherol 50 grams. The pH of the semen diluent is checked both before and after administration of vitamin E to ensure the pH value remains within the normal semen pH range.

Preparation of Artificial Vagina for Semen Retention

Semen samples from Bali cattle were collected using an artificial vagina. The artificial vagina is assembled by attaching the AV funnel to the end of the inner liner near the hot water air valve hole. The sperm tube is installed at the base of the AV funnel and then a sperm tube protector is attached. AV mouthpiece protective jacket installed. Add 600 ml of 50-55 hot water ^{0}C into the inner liner through the air valve hole and closed again using an AV pump. The final temperature of the artificial vagina was checked with a 40–45 thermometer ^{0}C. Jelly lubricant is applied to the inner liner hole up to 1/3 of the artificial vagina using a stick. An artificial vagina is given to the collector so that semen can be collected.

Spermatozoa Quality Check (Fresh Semen)

After being collected, the quality of fresh sperm spermatozoa is checked macroscopically, including volume, color, pH, consistency and microscopically, including concentration, motility and viability. Fresh cement must be above the SNI:ISO:9001:2015 standard, namely >70% motility to be processed into frozen cement.

Stages of Bali Cow Semen Cryopresevation

1. Cement Dilution and Equilibration

The volume of diluent needed to dilute semen is calculated using the AndroVision microscope when examining fresh semen or calculated using the formula:

$$(VP) = \frac{\text{Fresh semen volume x fresh semen concentration}}{\text{Cement concentration in straw}} \times \text{volume semen dalam straw.}$$

0.5 ml of fresh semen was taken to be diluted according to treatment (P0), doses 0.2 g (P1), 0.4 g (P2), 0.6 g (P3) each with 3 repetitions and observations were made on day 0 before freezing including spermatozoa motility and viability with an AndroVision microscope. Then, the cement is covered with aluminum foil and placed in the machine *filling-sealing* to be packaged into straws that have been coded according to the treatment and repetition code and have been sterilized in an ozone cupboard for 60 seconds. The straw is connected to a cement filler needle so that the cement can be filled into the straw. After process *filling-sealing* When finished, the straw containing cement is put into the cold cabinet machine for the equilibration stage at a temperature of 5 ^{0}C for 4 hours.

***2. Fre-Freezing* and Semen Straw Storage in N_2 Liquid**

The rack containing the cement straw is placed on an iron support in a cool box that has been poured with N_2 Liquid -190 ^{0}C and cool box closed for 10 minutes. Then the straw containing cement from the cool box is inserted into the goblet tube containing N_2 liquid -196 ^{0}C and transferred into a frozen semen storage container tube. The straw in the goblet is placed until it is submerged in N_2 The liquid is stored during the 2nd and 8th days of freezing. Examinations for observations on days 2 and 8 include motility, viability, and *recovery rate* spermatozoa. Frozen cement with good quality is above the SNI:ISO:9001:2015 standard, namely motility > 40%.

Observed parameters

1. Quality of Fresh Semen Spermatozoa

Semen volume is measured by the scale value on the sperm tube, and semen pH is measured by pH indicator paper. The color of the semen is observed in the sperm tube and the consistency is measured by shaking the sperm tube repeatedly, then looking at the remaining semen that falls on the tube wall. The rate at which the remaining cement falls indicates the thickness of the cement.

2. Spermatozoa Motility (Fresh Semen and Frozen Semen) and Spermatozoa Concentration

Examination of motility and concentration of fresh semen spermatozoa using an AndroVision microscope. 3.0 µl of cement was taken using a micropipette and inserted into the cement chamber and observed under a microscope with 400x magnification. The motility and concentration values of fresh semen spermatozoa can be observed on the AndroVision microscope screen.

Examination of the motility of frozen semen spermatozoa is carried out after the semen has been removed *thawing* on the 2nd and 8th days of observation. The straw containing frozen cement is taken from the container tube using tweezers, and placed into the water bath to be processed *thawing* with a temperature of 37 ^{0}C for 40 seconds. Straw is taken in a water bath, cut and cement is placed into a microtube. 3.0 µl of cement was taken and placed in an object glass and covered with a cover glass. Spermatozoa were observed on an AndroVision microscope with 400x magnification.

3. Spermatozoa Viability

Examination of the viability of fresh semen and frozen semen spermatozoa was carried out using the smear preparation method. 1 drop of cement and 2 drops of 2% eosin (1:2) were placed in an object glass and homogenized. Drops of a mixture of cement and eosin are made into smear preparations. The semen smear preparation was dried for 30 minutes and observed under a microscope with 100x magnification. Live spermatozoa do not suck color and dead spermatozoa suck color. The total number of spermatozoa is counted as 200 cells or 10 fields of view and presented as a percentage value. Calculation of spermatozoa viability using the following formula (Manehat *et al*. 2021).

$$\%\, Viability = \frac{Number\ of\ live\ spermatozoa}{Total\ Number\ of\ spermatozoa\ \ which\ is\ calculated}\ x\ 100\ \%$$

4. *Recovery Rate* Spermatozoa

Calculation *recovery rate* spermatozoa uses the following formula: (Kurniawan & Nurhawidah 2021)

$$\%\, RR = \frac{Frozen\ Semen\ Motility\ (After\ thawing)}{Motility\ of\ fresh\ semen}\ x\ 100\ \%$$

Data analysis

The research data obtained is tabulated in table form. Data was analyzed qualitatively and quantitatively with *Anova One Way*, if the dosing treatment and clotting time show a significant effect (P<0.05), further testing is carried out with a test *Duncan Multiple Range Test* (DMRT).

3. Results and Discussion

Characteristics of Fresh Semen from Bali Cows

The results of research testing the quality of fresh semen from Bali cattle before being processed into frozen semen can be seen in Table 1.

Table 1. Characteristic Parameters of Fresh Semen from Bali Cows

Parameter	Results
Macroscopic	
Volume (ml)	7,3 ml ± 0,10
Color	Milk White
Consistency	Currently
pH	6,4 ± 0,10
Microscopic	
Concentration (10^6/ml)	1429 10^6/ml ± 231,9
Progressive Motility of Spermatozoa %)	84,20 % ± 3,42
Viability (Live Spermatozoa %)	87,16 % ± 3,06

The volume of semen produced was 7.3 ml. This is in accordance with previous research, the volume of normal Bali cattle semen is around 4.56-8.7 ml (Ciptadi *et al*. 2021; and Indriastuti *et al*. 2020). The volume of fresh semen from Bali cattle in this

study was normal. The color of Bali cattle semen in this study was milky white. Similar research was carried out by Akmal *et al*. (2019), that normal Bali cattle semen is milky white and creamy.

Cement consistency is divided into three categories, namely medium, thick and thin. The cement consistency in this study was medium. The consistency depends on the color of the semen and the concentration of spermatozoa. Regarding color, consistency and concentration of spermatozoa are related to each other, meaning that if the semen is thinner, the concentration of spermatozoa is lower and the color of the semen is paler and clearer (Candrawati *et al*. (2020). Dilute semen contains a concentration of spermatozoa <1000 10^6/ml, medium semen contains a spermatozoa concentration of 1000-1500 10^6/ml, and thick semen contains a spermatozoa concentration of >1500 10^6/ml (Ismaya 2014).

Bali cattle semen in this study had a pH of 6.4. The pH level of Bali cattle semen as a result of this research is still within the normal range of cattle semen pH. This is similar to research by Garner & Hafez (2016), the pH of normal cow semen is 6.4-7.8. The concentration of fresh semen spermatozoa in this study was 1,429 10^6/ml. The spermatozoa concentration of fresh Bali cattle semen obtained was categorized as normal. Garner & Hafez (2008), stated that normal cow semen concentration ranges from 1,000-1,800 million/ml. Different spermatozoa concentrations can be influenced by age, reproductive organ health, and male ejaculation frequency.

The results of the fresh semen motility examination in this study were 84.20%. This is similar to Ratnawati's research *et al*. (2018), fresh semen from Bali cattle has spermatozoa motility of 71.0 ± 2.2%, but Prastowo's research *et al*. (2018) fresh semen produces lower spermatozoa motility, namely 67.02 ± 6.92 %. Standard Regulations SNI ISO :9001:2015 UPT Artificial Insemination for Livestock Pekanbaru Fresh semen spermatozoa must have a motility above 70%. The motility value of fresh cement in this research is in accordance with the SNI ISO: 9001:2015 standard which can be processed into frozen cement. The viability of fresh semen spermatozoa in this study was 87.60%. The results of fresh semen viability in this study showed good spermatozoa viability. These results are not much different from the results of Blegur's research *et al*. (2016) fresh semen from Bali cattle has spermatozoa viability of 81.07 ± 3.23%, and Fadilah's research *et al*. (2016), namely 77.74 ± 4.34%. Garner & Hafez (2000) stated that the viability of spermatozoa for making frozen semen is at least 75% live spermatozoa.

Motility Quality of Bali Cow Spermatozoa in Tris-Egg Yolk Diluent with Vitamin E (Alpha-Tocopherol)

The motility of Bali cattle spermatozoa in Tris egg yolk diluent with vitamin E in this study can be seen in Table 2.

Table 2. Results of the average motility of Bali cattle spermatozoa added with vitamin E in yellow tris diluent

Freezing Time (%)	Administration of Vitamin E (Dose) (%)			
	P0 (Control)	P1 (0.2 g)	P2 (0,4 g)	P3 (0,6 g)
Day 0	87,86±7,58^{not}	90,84±4,29^{chapter}	89,84±8,61^{chapter}	**94,01±2,71^{bB}**
Day 2	75,76±15,38^{aA}	80,21±12,48^{aAB}	80,19±7,51^{aAB}	86,29±2,14^{aB}
Day 8	72,61±3,04^{aA}	85,05±5,09^{aAB}	80,25±6,98^{aAB}	**89,10±1,03^{aB}**

Note: (a,b) (P<0.05) on the length of clotting time and (A,B) (P<0.05) on dose administration.

The results of statistical analysis in Table 2 show that the dose of vitamin E and freezing time had a significant effect (P<0.05) on the motility of Bali cattle spermatozoa. On day 0, the freezing time for spermatozoa motility was significantly different (P<0.05) compared to days 2 and 8. On day 2 and day 8, the freezing time of spermatozoa motility was not significantly different (P<0.05). On days 0, 2, and 8, for the length of freezing time with different doses of vitamin E P0, P1, P2, and P3, spermatozoa motility was not significantly different (P<0.05). During the freezing time, Bali cattle spermatozoa motility was highest, namely 94.01 ± 2.71% on day 0 (before the freezing process) at a vitamin E dose of 0.6 g (P3).

When administering different doses of vitamin E on days 0, 2 and 8, the clotting time for P0 spermatozoa motility was significantly different (P<0.05) compared to P1, P2 and P3. At dose P1, spermatozoa motility was not significantly different (P<0.05) from dose P2. At dose P3, spermatozoa motility was significantly different (P<0.05) from doses P1 and P2. In the difference in doses of vitamin E after the freezing process (days 2 and 8), the highest motility at the P3 dose (0.6 g) was 89.10 ± 1.03% on day 8. The sperm motility of frozen semen from Bali cattle in this study is above the SNI ISO:9001:2015 standard for frozen semen quality procedures at the Pekanbaru Livestock Artificial Insemination Unit, the motility value of frozen semen is >40%.

Giving vitamin E can increase the motility value of Bali cattle spermatozoa during freezing (cryopreservation) until the 8th day. It is thought that the antioxidant alpha-tocopherol content in vitamin E helps increase spermatoza motility. Mubarak's research results *et al*. (2017), that antioxidant levels (IC_{50}) vitamin E (alpha-tocopherol) of 7.15 µg/ml is a very strong antioxidant. Salim (2018), also stated that the criteria for a compound to be said to be an antioxidant have value (IC_{50}), very strongly valued (IC_{50}) <50, strongly valued (IC_{50}) 50-100, being worth (IC_{50}) 100-150, and weakly valued (IC_{50}) 150-200. The smaller the value IC_{50} the stronger the antioxidant content of a compound.

Table 3. Interaction between administration of vitamin E dose and freezing time on Bali cattle spermatozoa motility

Parameter	Significance Levels		
	Interaction		
Motility	D	H	DH
	*	*	NS

Note: D: Dosage P0 (control); P1 (0.2 g); P2 (0.4g); and P3 (0.6 g). H: Freezing Time on day 0, day 2, and day 8. DH: Interaction between treatment dose and length of clotting time, significant*P<0.05 and NS (not significant)

Table 3 shows that the interaction between different doses of vitamin E and different lengths of freezing time shows a significant influence (P<0.05) on the motility of Bali cattle spermatozoa. The interaction between different doses of vitamin E and different lengths of freezing time showed no significant effect (P<0.05) on Bali cattle spermatozoa motility.

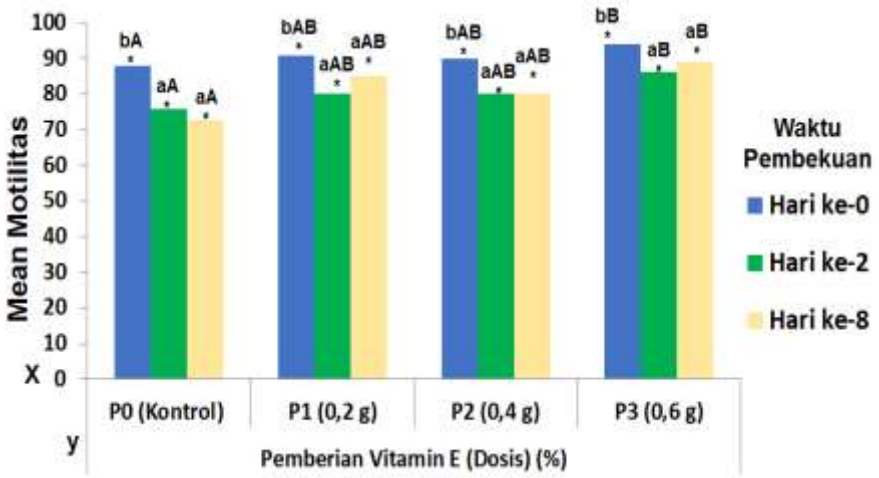

Figure 1. Histogram of Bali cattle spermatozoa motility in egg yolk Tris diluent with added vitamin E (Alpha-Tocopherol). (a,b) (P<0.05) on length of clotting time and (A,B) P<0.05) on dose. *P<0.05.

In Figure 1, the histogram of Bali cattle spermatozoa motility shows that at P0 there was a decrease in spermatozoa motility successively from day 0, 2, to day 8. It is suspected that this was due to the absence of vitamin E which contains the antioxidant compound alpha-tocopherol in the egg yolk Tris diluent. So the nutrients and antioxidants contained in the semen diluent are not enough to maintain spermatozoa motility during freezing and the spermatozoa are more at risk of experiencing (*Cold Shock*) cold shock resulting from a decrease in freezing temperature. In the absence of antioxidants, the spermatozoa plasma membrane experiences damage more quickly due to free radicals.

In Figure 1, the histogram of the motility of Bali cattle spermatozoa with a dose of vitamin E shows that at doses P1, P2, and P3 there was only a decrease in motility on day 2 and an increase in motility on day 8. The decrease in spermatozoa motility on day 2 was possibly due to new spermatozoa adapting to the egg yolk Tris diluent and the decrease in temperature from the freezing process. Decreased spermatozoa motility can be caused by damage to the spermatozoa plasma membrane *Cold Shock* from a decrease in freezing temperature. The increase in spermatozoa motility on day 8 is probably due to the spermatozoa having

adapted to the Tris egg yolk diluent and the spermatozoa being able to adapt to the freezing temperature of -196 ℃ in N_2 liquid during cryopreservation. So by administering antioxidants it can increase the motility of Bali cattle spermatozoa.

Viability Quality of Bali Cow Spermatozoa in Tris-Egg Yolk Diluent with Vitamin E (Alpha-Tocopherol)

Figure 2. Results of observing the viability of Bali cattle spermatozoa a) dead spermatozoa b) live spermatozoa.

On Figure 2 shows the difference in spermatozoa viability between dead spermatozoa cells and live spermatozoa cells. The part that shows live spermatozoa (a), is because the spermatozoa plasma membrane is still functioning properly and does not absorb eosin dye. Meanwhile, the part that shows dead spermatozoa (b) will absorb the eosin color and become red, because the spermatozoa plasma membrane has been damaged due to *Cold Shock* during freezing. Research by Aini & Pemayun (2015) states that the affinity absorption of dyes in different spermatozoa can indicate dead and live spermatozoa. Several factors influence the survival of spermatozoa, namely the dilution level, physical and chemical properties of the diluent, and temperature conditions.

Table 4. Results of the average viability of Bali cattle spermatozoa added with vitamin E in the yellow tris diluent

Freezing Time (%)	Administration of Vitamin E (Dose) (%)			
	P0 (Control)	P1 (0.2 g)	P2 (0,4 g)	P3 (0,6 g)
Day 0	89,67±5,69^{not}	92,50±4,36^{bB}	93,17±7,18^{chapter}	**96,50±1,00^{bB}**
Day 2	78,83±13,37^{aA}	84,50±9,85^{aB}	83,17±7,57^{aAB}	90,50±2,65^{aB}
Day 8	76,50±1,80^{aA}	89,50±5,00^{aB}	85,83±6,11^{aAB}	**93,33±0,76^{aB}**

Note: (a,b) (P<0.05) on the length of clotting time, and (A,B) (P<0.05) on dose administration.

The results of statistical analysis in Table 4 show that the dose of vitamin E and freezing time had a significant effect (P<0.05) on the viability of Bali cattle spermatozoa. On day 0, the freezing time for spermatozoa viability was significantly different (P<0.05) compared to days 2 and 8. On day 2 and day 8, the freezing time for spermatozoa viability was not significantly different (P<0.05). On days 0, 2, and 8, for the length of freezing time with different doses of vitamin E P0, P1, P2, and P3, spermatozoa viability was not significantly different (P<0.05). With the longest freezing time, the viability of Bali cattle spermatozoa was highest, namely 96.50 ± 1.00% on day 0 (before the freezing process) at a vitamin E dose of 0.6 g (P3).

When administering different doses of vitamin E on days 0, 2 and 8, the clotting time for P0 spermatozoa viability was significantly different (P<0.05) compared to P1, P2 and P3. At the P1 dose, spermatozoa viability was not significantly different (P<0.05) from the P3 dose. Based on the difference in doses of vitamin E after the freezing process (days 2 and 8), the highest viability was at the P3 dose (0.6 g) of 93.33 ± 0.76% on the 8th day. The results of the viability of Bali cattle spermatozoa in this study were higher than the results of the Azura study *et al*. (2020), by administering a dose of 1.5 mM vitamin E in skim milk egg yolk diluent, the viability of Simental cow spermatozoa was 65.90% during 72 hours of freezing. The viability of frozen semen spermatozoa in this study is above the frozen semen SNI standard 4869.1-2017 National Standardization Agency 2017 (BSN) viability of ±82.65% (Handayani *et al*. 2021). This means that by administering vitamin E in Tris egg yolk diluent, it is suspected that

the antioxidant content of alpha-tocopherol in vitamin E can increase the percentage of viable spermatozoa cells in Bali cattle during cryopreservation.

Vitamin E contains the compound α-tocopherol which is a lipophilic antioxidant. Alpha-tocopherol breaks the covalent bonds formed *Reactive Oxygen Species* (ROS) due to lipid peroxidation, namely a chain reaction between free radicals and polyunsaturated fatty acids in spermatozoa plasma membrane lipids. Alpha-tochopherol counteracts free radicals through the propagation stage of free radical formation by lipid peroxidation by transferring the H electron atom from the OH- hydroxyl group to peroxyl radicals. Hydrogen will react and pair with peroxyl/peroxide radicals (ROO). This reaction produces a more stable tocopheryl radical compound. The new compounds formed as a result of antioxidant reactions and free radicals do not damage the plasma membrane of spermatozoa cells. So that lipid peroxidation events no longer occur, the spermatozoa plasma membrane remains intact and spermatozoa viability can be maintained (Simamora 2003).

Table 5. Interaction of dose and freezing time on the viability of Bali cattle spermatozoa

Parameter	Significance Levels		
	Interaction		
Viability	D	H	DH
	*	*	NS

Note: D: Dosage P0 (control); P1 (0.2g); P2 (0.4g); and P3 (0.6 g). H: Freezing Time on day 0, day 2, and day 8. DH: Interaction between treatment dose and length of clotting time, significant*P<0.05 and NS (not significant)

Table 5 shows that the interaction between different doses of vitamin E and different lengths of freezing time showed a significant influence (P<0.05) on the viability of Bali cattle spermatozoa. The interaction between different doses of vitamin E and different lengths of freezing time showed no significant effect (P<0.05) on the viability of Bali cattle spermatozoa.

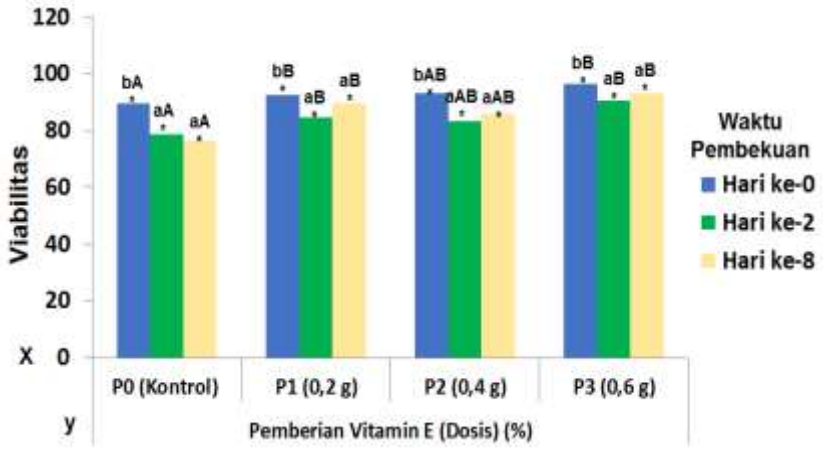

Figure 3. Histogram of viability of Bali cattle spermatozoa in egg yolk Tris diluent with added vitamin E (Alpha-Tocopherol). (a,b) (P<0.05) on length of clotting time and (A,B) P<0.05) on dose. *P<0.05.

In Figure 3, the histogram of Bali cattle spermatozoa viability shows that at P0 there was a decrease in spermatozoa viability successively from days 0, 2, and 8. It is suspected that this is due to the longer the freezing time, the condition of the egg yolk Tris diluent decreases the availability of energy, nutrients and antioxidants needed for survival and maintaining the viability of spermatozoa. So the energy requirement for spermatozoa to metabolize is also reduced. During cryopreservation, spermatozoa continue to metabolize both aerobically and anaerobically. The end result of anaerobic metabolism is lactic acid which can reduce the pH of the semen diluent so that it becomes acidic and is toxic to spermatozoa.

In Figure 3, the histogram of the viability of Bali cattle spermatozoa with a dose of vitamin E shows that at doses P1, P2, and P3 there was only a decrease in viability on the 2nd day and an increase in viability on the 8th day. The decrease in spermatozoa viability on day 2 was possibly due to new spermatozoa adapting to the Tris egg yolk diluent and the temperature reduction process from the freezing process. Decreased viability of spermatozoa can be caused by damage to the spermatozoa membrane

during the process of decreasing temperature due to (*Cold Shock)* cold shock during freezing results in the formation of ice crystals in the cells which damage the structure of the spermatozoa plasma membrane (Sari *et al*. 2014). The increase in spermatozoa viability on the 8th day is possibly due to the spermatozoa having adapted to the diluent in the egg yolk Tris and the spermatozoa being able to adapt to temperatures of -196 ℃ in N_2 liquid during cryopreservation. And the antioxidant compound alpha-tocopherol in vitamin E can increase the percentage of viable spermatozoa during cryopreservation.

Quality *Recovery Rate* Bali Cow Spermatozoa in Tris- Egg Yolk Diluent with Vitamin E (Alpha-Tocopherol)

Inspection *recovery rate* spermatozoa aims to determine the resistance and ability of spermatozoa cells to recover after freezing spermatozoa by comparing the percentage value of frozen semen motility with fresh semen motility. The results of administering vitamin E in egg yolk Tris diluent against the *recovery rate* Bali cattle spermatozoa can be seen in Table 6.

Table 6. Average results *recovery rate* Bali cattle spermatozoa added with vitamin E in yellow tris diluent

Freezing Time (%)	Administration of Vitamin E (Dose) (%)			
	P0 (Control)	P1 (0.2 g)	P2 (0,4 g)	P3 (0,6 g)
Day 2	89,97±18,80^{A}	95,26±14,82^{AB}	95,23±8,92^{AB}	102,48±2,54^{B}
Day 8	86,23±3,61^{A}	101,00±6,04^{AB}	95,31±8,28^{AB}	**105,81±1,22^{B}**

Note: (A,B) P<0.05) on differences in Vitamin E dosage.

The results of the statistical analysis in Table 6 show that giving a dose of vitamin E has a significant effect (P<0.05) on *recovery rate* Bali cattle spermatozoa. Meanwhile, the length of freezing time had no significant effect (P<0.05). *recovery rate* Bali cattle spermatozoa. When administering different doses of vitamin E on days 0, 2, and 8, the clotting time for P0 *recovery rate* spermatozoa were not significantly different (P<0.05) compared to P1, P2, and P3. At dose P1 *recovery rate* spermatozoa were not significantly different (P<0.05) with the P2 dose. At dose P3 *recovery rate* spermatozoa were significantly different (P<0.05) with doses P1 and P2. The difference in vitamin E dosage after the freezing process (days 2 and 8) *recovery rate* The highest dose was P3 (0.6 g) at 105.81 ± 1.22 % on day 8. Results *recovery rate* Bali cattle in this study were higher than the results of the Sunami study *et al*. (2017) *recovery rate* Limousin cattle by 67% and Saifullah research *et al*. (2020) *recovery rate* Simental cattle amounted to 84.20%. The spermatozoa recovery rate of frozen semen from Bali cattle in this study was above the standard SNI 4869.1-2017 National Standardization Agency 2017 *recovery rate* frozen semen spermatozoa of at least 50%.

Providing vitamin E in egg yolk Tris diluent can increase the value *recovery rate* Bali cattle spermatozoa. Allegedly, this is because vitamin E contains the antioxidant alpha-tocopherol which can provide protection against the spermatozoa plasma membrane from free radicals during freezing. So spermatozoa have the ability to recover well after the spermatozoa have been frozen. Mark *recovery rate* shows the ability of spermatozoa to recover after freezing. The higher the value *recovery rate* This means that the recovery ability of spermatozoa after freezing is getting better. High value *recovery rate* shows that the clotting process is taking place well which is related to the high level of integrity of the spermatozoa plasma membrane which supports the metabolic process resulting in progressive movement of spermatozoa (Suherlan *et al*. 2017). Mark *recovery rate* can be used as an indicator of the ability of a diluent to become a cryoprotectant that is able to protect spermatozoa cells during the cryopreservation process (Arifiantini *et al*. 2018).

Table 7. Interaction of dose and clotting time on *recovery rate* Bali cattle spermatozoa

Parameter	Significance Levels		
	Interaction		
Recoery Rate	D	H	DH
	*	NS	NS

Note: D: Dosage P0 (control); P1 (0.2 g); P2 (0.4g); and P3 (0.6 g). H: Freezing Time on day 0, day 2, and day 8. DH: Interaction between treatment dose and length of clotting time, significant*P<0.05 and NS (not significant)

Table 7 shows that the interaction between different doses of vitamin E shows a significant effect (P<0.05) on *recovery rate* Bali cattle spermatozoa. The interaction between differences in the length of clotting time and the interaction between differences in vitamin E dosage and differences in clotting time showed no significant influence (P<0.05) on *recovery rate* Bali cattle spermatozoa.

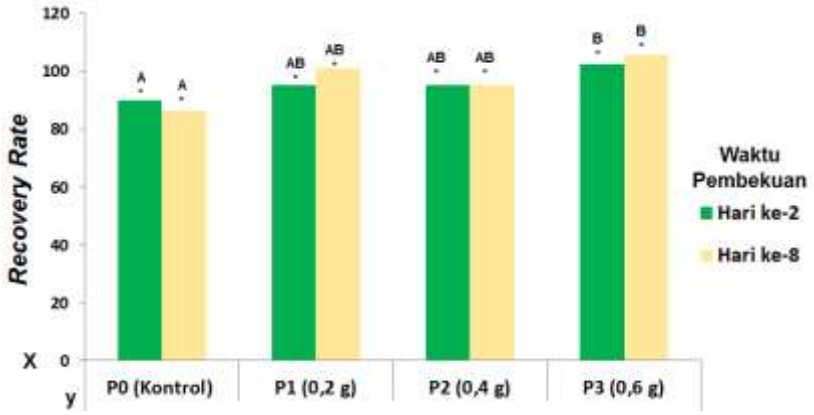

Figure 4. Histogram *recovery rate* Bali cattle spermatozoa in Tris egg yolk diluent were added with vitamin E (Alpha-Tocopherol). (A,B) P<0.05) at the dose. *P<0.05.

In Figure 4. histogram *recovery rate* Bali cattle spermatozoa showed a decrease at P0 *recovery rate* on the 8th day. Allegedly, this is due to the absence of vitamin E which contains antioxidants, so there are no reserves of other nutrients and antioxidants in the egg yolk Tris diluent to maintain the life of spermatozoa during freezing. The longer the freezing time the available nutrients decrease. If nutrients are reduced, spermatozoa will run out of energy which causes a decrease in spermatozoa movement. Decreased movement of spermatozoa during the freezing process will affect the ability of spermatozoa to recover

In Figure 4. histogram *recovery rate* Bali cattle spermatozoa with a dose of vitamin E showed an increase in value at doses P1 and P3 *recovery rate* spermatozoa on day 8. At P2 dose it does not increase *recovery rate* spermatozoa until the 8th day. Improvement *recovery rate* spermatozoa on the 8th day, it is possible that the spermatozoa have adapted to the diluent contained in the Tris egg yolk diluent and the spermatozoa have been able to adapt to the environmental temperature in N_2 liquid -196 ℃ during the freezing process. In addition, it is possible that administration of vitamin E can protect the sperm plasma membrane from oxidative reactions caused by free radicals during freezing. So that spermatozoa have recovery (*recovery rate*) which is good after experiencing freezing.

4. Conclusion

The administration of Vitamin E (alpha-tocopherol) in Tris egg yolk diluent has an effect on improving the quality of Bali cattle spermatozoa during cryopreservation. The interaction of vitamin E dose and clotting time had a significant effect (P<0.05) on motility and viability. Interaction of Vitamin E dosage with *recovery rate* spermatozoa had a significant effect (P<0.05). When administering a dose of vitamin E (P3) 0.6 g/100 ml of Tris egg yolk diluent, the highest spermatozoa quality score for motility was 89.10%, viability was 93.33%, and *recovery rate* amounted to 105.81% on the 8th day of freezing during cryopreservation. It is suspected that the alpha-tocopherol content in vitamin E can affect the quality of Bali cattle spermatozoa during cryopreservation.

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