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Motility, Abnormality and Intact Plasma Membrane of Sexed Bali Bull Sperm in Different Equilibration Time

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ABSTRACT

The purpose of this study was to obtain the optimal equilibration time to produce quality frozen semen of sexed Bali bulls in Tris-yolk diluent. Semen was collected from two male Bali cows from artificial insemination center, Tenayan Raya. Semen was collected for 8 wk and used as a replicate of the study. The motility of the semen used was 70%. Sperm X and Y were separated with 10% and 30% bovine serum albumin (BSA). The diluent used was tris egg yolk. The research treatment was equilibration time (2, 3, 4, and 5 h). Parameters were measured after sperm separation, dilution and thawing after freezing. Parameters consisted of motility, abnormality and intact plasma membrane. Data analysis was performed using a group randomized design. The results showed that the equilibration times of 2, 3, 4 and 5 h were significantly different on motility, abnormality and intact plasma membrane. The value of motility and intact plasma membrane is significantly different from after separation, dilution and freezing of Bali sperm. The values of motility, abnormality and intact plasma membrane of sperm X and sperm Y were not significantly different. Conclusion, 2 h equilibration time can maintain motility, abnormality and intact plasma membrane of sperm X and sperm Y of Bali cattle.

Keywords: *Abnormality, Bali bull sperm, Motility, Plasma membrane intact*

Introduction

Beef production in Indonesia in 2023 is 500.4 tons/yr, while beef demand in Indonesia is 695.4 tons/yr (BPS, 2024). To cover this gap between supply and demand, technology is needed that can increase the cattle population. Artificial insemination is one of the efforts made to increase the beef cattle population. The availability of calves in large quantities to meet national meat needs is also a problem for farmers. Therefore, sexing sperm is a solution to obtain calves according to the needs of farmers (Garner and Seidel, 2008). Sperm separation technique using albumin has been carried out on Bali sperm with a separation time of 10 min with the diluent used tris egg yolk citrate (Yendraliza *et al*., 2019). The separation technique using BSA has been performed on buffalo sperm with a separation time of 45 min and the addition of 0.4% sucrose in tris egg yolk diluent resulted in the best sperm quality (Yendraliza *et al.*, 2023). The optimum time for Balinese cattle sperm before freezing to produce good quality sexed sperm according to Indonesian national standards in frozen semen, has not been investigated Manjunath (2012) states that the equilibration time is the period required by sperm before freezing to adjust to the diluent. The compatibility of sperm with diluent will prevent sperm death during freezing and thawing and can streamline the time and costs incurred *(Prakash et al*., 2014). This study aimed to determine the optimal equilibration time for sexed Bali cattle sperm using an egg yolk Tris diluent.

Materials and Methods

Semen used collected from two male Balinese bulls from the Artificial Insemination center of the Livestock and Animal Health Service Office (DPKH). The Bali bulls used were 8 years old with a body weight of 512 – 609 kg. Cattle were housed individually and maintained intensively. Each male received grass according to body weight. Grass feeding was done in the morning and evening. Each male gets 6 kg/d. Drinking water is given adlibitum. Semen is collected once a week every Tuesday using an artificial vagina for 8 wk.

Preparation of diluent materials

The diluent used was egg yolk tris. The composition of the diluent was in accordance with the standards of the UPT. Artificial Insemination of Livestock (Table 1).

Table 1. Composition of research tris-egg yolk diluent

No	Materials	Total
1	Tris (Hydroxymethyl) Aminomethan	3.028 g
2	Asam Citrat	1.7 g
3	Fruktosa	1.25 g
4	Penicilin	0.5 mL
5	Streptomycin	0.4 mL
6	Gliserol	6 mL
7	Kuning Telur	20 mL
8	Aquabidest	80 mL

Preparation of buffer solutions; This solution was carried out in a 250 mL glass Erlenmeyer. The mixture consisted of tris, citric acid, fructose and aquabidest. The mixture was homogenized with a magnetic stirrer for 15 min. Then the first mixture was covered with aluminium foil.

Preparation of tris-egg yolk diluent; 74 mL of buffer solution was put into a 250 mL beaker glass. then added 20 mL egg yolk and 6 mL glycerol, then homogenized using a magnetic stirrer for 60 min. Added 0.5 mL penicillin and 0.4 mL streptomycin. The diluent was stored in a 5^{o}C refrigerator until used.

Preparation of sexing sperm medium diluent

The composition of the separation medium refers to the standard of UPT. Artificial Insemination of Livestock (Table 2).

Table 2. Composition of 5% BSA and 10% BSA sperm separation medium

No	bahan	Jumlah
1.	Liquid Medium sexing	80 mL
2.	BSA 5%	2 g
3	BSA 10 %	4 g

Preparation of sperm separation medium was carried out in two 250 mL erlenmeyer glasses. each glass A and B was added with 40 mL of sexing medium. In glass A, 4 g of 10% BSA was added and in glass B, 2 g of 5 % BSA was added. This mixture was stored in a refrigerator at 5^{o}C without being homogenized and used the next day.

Separation of sperm X and spermY

100 mL of sexing medium was warmed in a water bath at 37^{o}C for 5 min. Fresh semen was diluted by adding liquid sexing medium until the volume reached 20 mL. Separation was done in a 10 mL test tube. 1 mL of 10% BSA liquid was put into a 10 mL test tube, after which 1 mL of 5% BSA liquid was added. Finally, add 1 mL of fresh semen that has been diluted earlier. This mixture was incubated in a water bath for 45 min. After incubation, the supernatant will be seen on the top layer. The top layer was taken 1 mL and discarded. The middle layer is x sperm separated in another test tube. The bottom layer is y sperm, separated into another test tube. The results of this separation were centrifuged for 10 min at 1800 rpm with a temperature of 25^{o}C. After centrifugation, the supernatant was discarded, and each sperm sample was transferred to a new test tube for microscopic evaluation.

Dilution of the sexed sperm.

Sexed semen is diluted with egg yolk tris that has been made for the first time. Before use, egg yolk tris was warmed in a water bath for 5 min at 37^{o}C. Semen that has been diluted with egg yolk tris is covered with aluminium foil before being inserted into the straw. Next, it was inserted into a 0.25 mL straw using filling and sealing. The straw that has been filled with sexed semen is put into a cool top machine with a temperature of 5^{o}C. This process is called the equilibration process. The treatment of this study is the time used in the equilibration process consisting of 2, 3, 4, and 5 h. Furthermore, microscopic evaluation was carried out.

Freezing of the sexed semen.

After equilibration, the semen is placed on a 2 – 3 cm high box containing liquid nitrogen at -110^{o}C for 10 – 12 min. The straws were then transferred to liquid nitrogen (-196^{o}C). After 24 h, the straw was thawed using warm water at 37^{o}C for 30 s. Furthermore, it was evaluated microscopically again.

Parameters measured

Motility. motility is forward sperm movement. motility evaluation using an object glass and cover glass and then viewed under a light microscope with a magnification of 10 x 45. The motility value is taken from the number of sperm moving forward divided by the total number of sperm counted multiplied by 100%.

Sperm abnormality. Sperm abnormalities are seen using a review preparate made from mixing 1 mL of eosin dye mixed with 0.2 mL of semen, air-dried and then viewed under a microscope (Motic BA310®) magnification of 10 x 45. Abnormalities seen are the total sperm that have a curled tail, broken tail, the middle of the tail folds divided by the total sperm counted multiplied by 100%.

Plasma membrane intact. Plasma membrane intact use evaluated instead using Hypoosmotic Swelling test (HOST) solution. The composition of the Host-test solution was 1.35 g fructose, 0.735 g sodium citrate plus 100 mL aquabidest. 200 µ of host solution was added to 20 µL of semen, homogenized and incubated in a 37ºC water bath for 45 min. 10 µL of the mixture after incubation was placed in an object glass and covered glass was observed under a light microscope (Motic BA310®) at 400x magnification. sperm with plasma membrane damage were characterized by a straight tail. Circular or inflated tails are sperm that have intact plasma membrane. the value of intact plasma membrane is seen from the number of sperm that have intact plasma membrane divided by the number of sperm counted multiplied by 100%.

Data Analysis

Data were analyzed using a Randomized Group Design (RCD) according to Steel and Torrie (1991). Differences in treatment center values were tested with DMRT test. Data analysis was performed with Microsoft excel 2023.

Results and Discussion

Fresh semen quality of Bali cattle

The average fresh semen volume of Balinese cows in this study was 8.43 ± 1.59, pH 6.5 ± 0.15, motility 84.67 ± 1.15, abnormality 6.83 ± 1.70 and plasma membrane intact 87.54 ± 2.56. The fresh semen volume of Balinese cows in this study was higher than that of Balinese cows in the previous study *(Yendraliza et al*., 2019) and lower than Balinese cattle from the study Yendraliza *et al*. (2020). The pH of Bali cattle semen in the study was within the range of 6.4 - 7.8 (Lemma, 2011). Vishwanath and Moreno (2018) stated that age, frequency of ejaculation, environmental conditions and individual conditions affect semen volume. According to Standard Nasional Indonesia (SNI), the requirements for dilutable semen are 70% motility and <10% sperm abnormality (SNI, 2017). Balinese cattle semen used in this study had motility, sperm abnormality above SNI. The average quality of sexed Balinese cattle semen can be seen in Table 3. The average motility, abnormality and MPU values of sperm X and sperm Y from sexing are still above SNI standards. So that dilution of sexed semen is feasible. Compared to motility, abnormality and MPU of fresh semen, the quality of sexed semen has decreased. This is due to the series of processes that sperm have gone through starting from movement between layers, washing and centrifugation processes that require a lot of energy to keep normalizing their physiological conditions (Holt, 2000). Semen plasma concentration will decrease due to the washing process, so that sperm motion will decrease after being separated and continued with dilution. In addition, the decrease in motility after sexing is also due to the presence of albumin which can bind cholesterol and Fe ions which are useful for stabilizing the plasma membrane (Best, 2015).

Table 3. Quality of sexed bovine semen and quality of sexed semen after dilution

No	Variable	After sexing		After diluent	
		X	Y	X	Y
1	Motilitas	76,2 ± 3,85	74,3 ± 4,52	92 ± 10,39	92,4 ± 10,27
2	Abnormalitas (%)	10,52 ± 1,30	10,22 ± 1,70	10,37±3,06	9,05±1,15
3	MPU (%)	80,30 ± 2,48	84,18 ± 2,60	85,30 ± 0,92	84,36±1,81

Quality of sperm X and sperm Y after equilibration and after freezing

Motility

The mean motility quality of sperm X and Y after equilibration and after freezing was significantly different at p>0.05 at all equilibration times (Figure 1). The value of sperm X motility of Balinese cattle after equilibration (83.97-92.57%) was higher than sperm X of motility after thawing (63.23-67.83 %). The motility value of sperm Y after equilibration (84.03-92.57) was higher than the motility value of sperm Y after thawing (59.8-76.27 %). The motility value of sperm X and sperm Y is still within the range of frozen semen requirements according to SNI (SNI, 2017). Sperm X motility is higher than sperm Y both after equilibration and after thawing. This is because the energy required by sperm Y is more than sperm X (Quelhas *et al*., 2021).

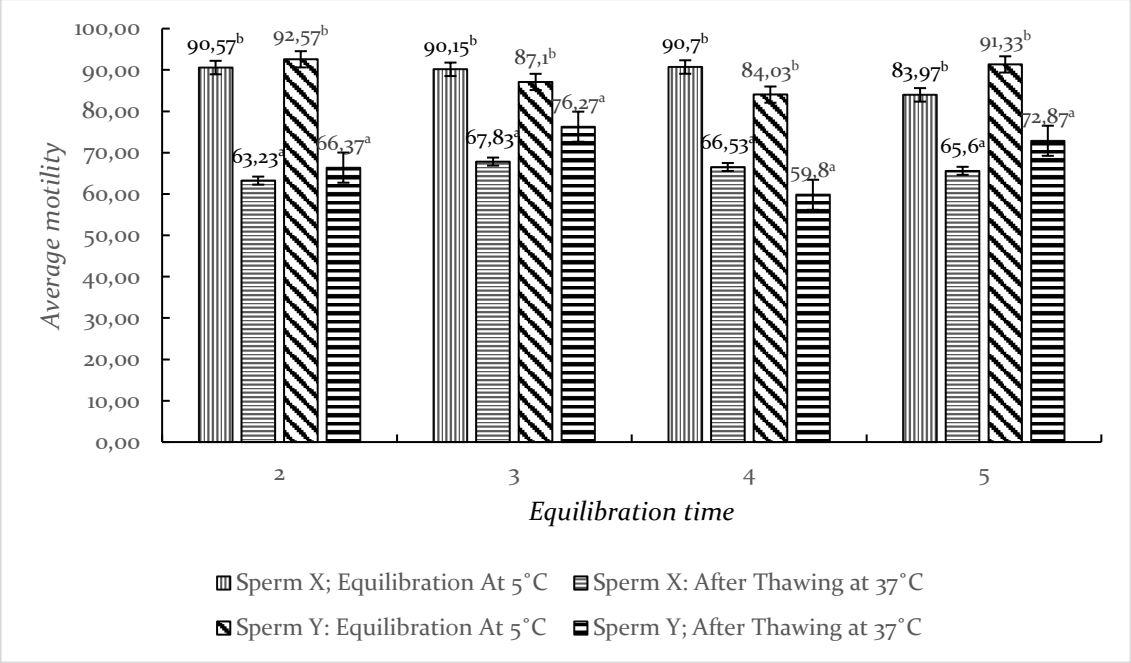

Figure 1. Average Motility sperm X an Sperm Y of Bali Bull Semen on Equilibration at 5˚C and After thawing at 37˚C with Different Diluents

The egg yolk tris diluent used has a composition that is suitable for the Bali cattle sperm in this study. Lecithin and lipoproteins present in the diluent serve to protect and maintain the integrity of the lipoproteins that make up the spermatozoa plasma membrane (Bergeron *et al*., 2007). Furthermore, added by Manjunath (2012) that lecithin and lipoproteins present in egg yolk have a large molecular membrane, so as to protect the plasma membrane. The main component of lipoprotein is low density lipoprotein which is bound to the plasma membrane to maintain the integrity of the plasma membrane. In addition, the BSA used to separate sperm X and sperm Y contains lysozyme protein compounds that can lyse bacterial cells by breaking the glycosidic bond between N-acetylglucosamine and cell wall chitin (Qadeer *et al*., 2015). Equilibration time of 3 h produced the best motility values for sperm X and sperm Y of Bali cattle in this study. Equilibration time in this study is different from the best equilibration time in Bali cattle semen by Yendraliza *et al*., (2020) (4 h). This difference is due to differences in livestock breeds, diluent composition and sperm separation composition and diluent method (Manjunath, 2012).

Abnormality

The average quality of abnormalities of sperm X and Y that have been equilibrated and frozen can be seen in Figure 2. The mean abnormality values of sperm X and sperm Y before freezing and after thawing were not significantly different ($p>0.05$). abnormality between sperm X and sperm Y was significantly different ($p<0.05$). Abnormalities seen were secondary abnormalities due to treatment such as broken tails and heads. The process of collecting, diluting, separating sperm and freezing sperm affects the plasma membrane of unstable spermatozoa, so sperm have the opportunity to be abnormal (Vishwanath and Moreno, 2018). The sperm abnormality of sexed Balinese cattle in this study was close to that of buffalo sperm in Sulawesi (9.7%) (Kaiin *et al*., 2017) and higher than Limousin cattle sperm (6.78%) (Mahfud *et al*., 2019). This difference is due to the type of livestock, age of livestock and the type of diluent and sperm separation method carried out is also different. Manjunath (2012) and Holden *et al*. (2017) stated that cold stress during freezing will cause an imbalance of osmotic pressure because metabolism continues during storage will cause sperm abnormalities.

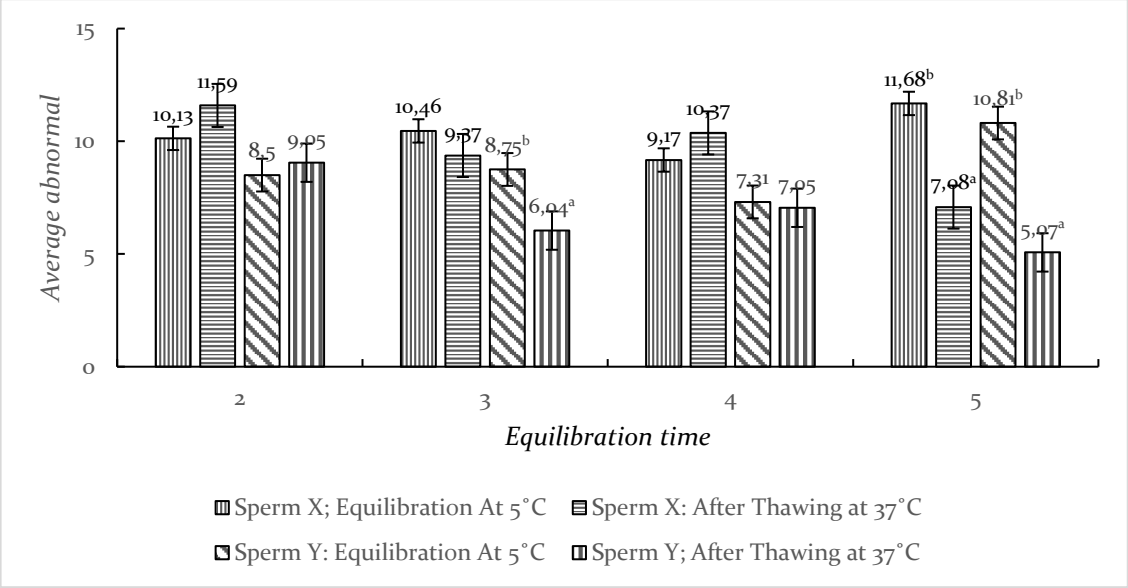

Figure 2. Average Abnormal Sperm X and sperm Y of Bali Bull Semen Samples on Equilibration At 5˚C and After Thawing at 37˚C with Different Diluents

Intact Plasma Membrane

The mean quality of intact plasma membrane of X and Y sperm that have been equilibrated and thawed after freezing is significantly different ($p<0.05$) (Figure 3). This difference is due to the motility between sperm X and sperm Y is also different. In addition, the motility of Bali cattle sperm after equilibration and after thawing in this study was also significantly different. Compared to sexed sperm in mud buffalo (70% – 71%) (Yendraliza *et al*., 2023), the intact plasma membrane of Bali cattle sperm in this study was higher (85-87% for sperm X; 82% – 89% for sperm Y). This difference is due to motility values before sperm separation, dilution and sperm freezing are also different.

The motility of Bali cattle sperm before separation in this study was 84.67% while the motility of buffalo sperm before separation was only 72.5%. The nature of membrane hydraulic permeability is related to the resistance of spermatozoa to cold shock. MPU values in this study (85% – 87% for sperm X; 82% – 89% for sperm Y) were higher than the MPU values of Balinese cattle in Tuah Sakato (60% for sperm Y and 65% for sperm X) (Yendraliza *et al*., 2019). The durability of spermatozoa during freezing in each individual animal has a different level of tolerance to the membrane phase and the arrangement of membrane domains. Each membrane has a different membrane lipid transition phase (Bergeron *et al*., 2007). Equilibration time of 2 h resulted in high values of intact plasma membrane of Bali cattle spermatozoa, both sperm X and sperm Y. The presence of egg yolk in the diluent containing LDL will adhere to the surface of the sperm membrane to replace phospholipids that are lost or damaged during freezing. In addition, the addition of glycerol in the diluent also helps prevent the formation of ice crystals during freezing. Thus, different equilibration times have no effect on the quality of sexed Balinese sperm.

Conclusion

Equilibration time of 2 h in freezing sexed Balinese sperm can maintain the value of motility (63% – 90.57% sperm X, 66% – 92% sperm Y), abnormality (10.13% – 11.59% sperm X; 8.05% – 9.05% sperm Y) and intact plasma membrane (85% – 87% for sperm X; 87% – 89% for sperm Y).

Conflict of interest

The authors have no conflict of interest to declare. All authors have seen and agree with the contents of the manuscript.

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