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Characteristics of Bali Bull (*Bos Sondaicus*) Sexed Sperms with Freeze Dry Egg White at Different Incubation Time

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ABSTRACT

The development of reproductive technology for the separation of spermatozoa with X and Y chromosomes, which is better known as sexing, can be carried out in vitro using various methods, one of which is using freeze dry egg whites. This study aims to determine the quality of semen based on incubation time. This study used four types of treatment, namely P0 = No Incubation, P1 = Incubation 30 minutes, P2 = Incubation 45 minutes, P3 = Incubation 60 minutes with six replications. Parameters observed were the quality of spermatozoa macroscopically (volume, odor, color and consistency) and microscopically (motility, abnormality, concentration, intact plasma membrane, intact acrosome cap). Y using descriptive statistical test. The results of analysis of variance that sexing spermatozoa using freeze dry egg whites at different incubation times showed a significant effect (P<0.05) on intact plasma membrane (IPM), intact acrosomal membrane (IAM), but had no significant effect P>0.05) on concentration, motility, and abnormalities. Evaluation of fresh Bali cattle spermatozoa showed results that were in accordance with the standards for further examination of semen sexing. Incubation time of 30 minutes in the sexing process of spermatozoa caused a significant decrease in motility and concentration, meanwhile, IPM and IAM of sexed spermatozoa experienced a significant decrease in incubation time of 60 minutes, but the decrease was still within the normal range for spermatozoa. to be used in the artificial insemination process.

Keywords: Sexing, incubation, concentration, motility, abnormality, intact plasma membrane, intact acrosome caps.

INTRODUCTION

The development of livestock in Indonesia, especially in order to increase the livestock population, to meet domestic consumption needs, needs to be supported by various factors [1]; [2]; [3]; [4]. The application of AI technology in addition to being able to increase

productivity and accelerate population distribution with better genetic quality, is also expected to optimize the function of a male [5]; [6]; [7].

Artificial insemination can be increased in value by producing superior seeds with sex according to the purpose of maintenance [8]; [9], for example, for slaughter (meat production) males are required, while for milk production, seeds are required for females. The technology needed to regulate the sex of the child is by sexing spermatozoa [10]. This can be useful for getting a child of the desired gender. Sexing technology is the process of separating X and Y spermatozoa, which is one technology to obtain the desired calf birth [11]. Various techniques and methods of sperm sexing have been applied to various farm animals [12]; [13]; [14] [15]; [16]. The techniques used to separate X and Y spermatozoa, one of which is Egg White Freeze Dry or commonly called freeze dry egg whites, Freeze drying method is based on being able to produce products with relatively higher quality than with another drying. The product from freeze drying has a rigid structure due to the sublimation process, so it does not shrink when dry, and when rehydrated the condition is the same as in its fresh form [17].

One of the stages of sexing spermatozoa with the freeze dry method of egg whites is incubation. If the incubation time is too long, it can result in the re-mixing of X and Y spermatozoa in layers of different medium concentrations, besides that it can cause damage to sperm cells, thereby reducing their quality. Therefore it takes the right time to produce sexing spermatozoa with good quality.

Based on this description, it is necessary to conduct research related to spermatozoa of Bali cattle sexing with freeze dry egg whites. Therefore, this study examines the characteristics of spermatozoa of Bali cattle which are sexed with freeze-dyed egg whites at different incubation times.

Incubation time is an important factor in the success of sperm separation using freeze dry egg whites. How is the effect of incubation time on spermatozoa characteristics of Bali Cattle (*Boss Sondaicus*) sexed with freeze dry egg whites and the quality of spermatozoa both macroscopically (volume, odor, color and consistency) and microscopically (concentration motility, abnormality,intact membrane plasma, intact acrosome cap).

MATERIALS AND METHODS

This research was conducted in May - June 2021 inSamata Integrated Farming System, Samata Village, SombaOpu District, Gowa Regency, and the Livestock Reproduction Laboratory of the semen Processing Unit, Faculty of Animal Husbandry, Hasanuddin University, Makassar.

This study used a completely randomized design (RAL) with 4 treatments and 6 replications (semen storage). The treatment in this study was the incubation time for sexing spermatozoa which consisted of:

P0 = Fresh Semen + Diluent (Control)
P1 = Incubation Time 30 Minutes
P2 = Incubation Time 45 Minutes
P3 = Incubation Time 60 Minutes

While the replication in this study was 6 times. Semen collection is done 2 times in 1 week.

Research procedure

Spermatozoa sexing will be carried out using the freeze dry method of egg whites [11]. Make a medium concentration (upper fraction 10% and lower fraction 30%) by mixing freeze dry egg white with Andromed diluents. Each medium was put into a different test tube. Then, add 2 ml of 30% medium in the lower layer and 2 ml of 10% medium in the middle layer and 1 ml of semen + top layer diluents. The process of making this medium must be carried out carefully by entering each solution through the walls of the tube slowly, so that the two sexing mediums do not mix.

Measured Parameter

The parameters observed in this study were the quality of fresh semen and the quality of spermatozoa after sexing which included macroscopic and microscopic observations consisting of Macroscopic: Color, volume, Degree of Acidity/pH, Odor, consistency while Microscopic: Motility, Concentration, Viability, Abnormality, Proportion, intact plasma membranes, intact acrosome caps

Data analysis

The results of the study were analyzed using One Way Analyses of Variance. If the data analyzed is significant, it is continued with Duncan's Multiple Distance Test (UJBD) using the SPSS program, then the proportion of X and Y spermatozoa using descriptive statistical test with the first actor incubation time and the second factor being the type of spermatozoa.

RESULTS AND DISCUSSIONS

Characteristics of Fresh Bali Cow Semen

The results of the evaluation of fresh semen become an important indicator before carrying out further processing of the semen. The results of the evaluation of fresh semen both macroscopically and microscopically in this study can be seen in Table 1.

Table 1 shows the quality or characteristics of fresh Bali cattle semen obtained from six holdings. The average volume of ejaculate obtained is 5.17±0.37 ml/ejaculate. This indicates that the volume of fresh semen obtained in this study was in the normal range. This is in accordance with the opinion of Butar [18] which states that the semen volume of bulls ranges from 2-10 ml.

Table 1.Bali Cow Fresh Semen Quality

Parameter	Mark
Volume (ml/ejaculate) (±SD)	5.17±0.37
Color	Beige
Smell	Typical
Degree of Acidity pH (±SD)	6
Consistency	Currently
Concentration (109/ml) (±SD)	7.285±4.13
Mass Motility	+++
Individual Motility (%) (±SD)	92.66±2.57
Viability (%) (±SD)	91.80±2.77

Note: (±SD) Standard Deviation

Semen Color

In this study, the color of fresh cow semen was obtained which was in beige color, this indicates that the semen has a normal color. This is in accordance with the opinion of Feradis [19] which states that normal cow semen is milky white or cream and cloudy. Furthermore, the color of the semen produced was whitish cream color [20]. Furthermore, the color of the if it was yellowish green it meant it contained Pseudomonas aeruginosa bacteria, red semen means it contains blood and brown semen means the semen contains blood that has been contaminated with blood. rot.

Degree of Acidity (pH)

The degree of acidity (pH) obtained in this study ranged from 6 to 6.5, indicated the pH of the semen is in the normal range (Table 1). This is in accordance with Zhou et al., [21] that the pH of normal cow semen is 6.2 - 7.5. Furthermore, Garner and Hafes [7] stated that the average pH of cow semen ranged from 6.4 to 7.8.

Consistency

Consistency and concentration in semen are closely related, the consistency in the semen can indicate that the number of spermatozoa present in the semen. The concentration of semen in this study had an average number of 7.285±4.13 spermatozoa/ml^{9}. The concentration of spermatozoa in the semen was in the normal range. This is in accordance with the opinion of Feradis [19] which states that the consistency of semen is said to be thick if the semen has a concentration of 1000 million - 2000 million per ml. Further Dewi *et. al.*[22] stated that there are several factors that affect sperm consistency, namely the nutrition provided, and the frequency of storage.

Mass Motility

The motility of the fresh semen mass obtained from this study was very good (+++) with individual motility of about 92.66±2.57%. Motility describes the ability of spermatozoa to move progressively. The more spermatozoa were move progressively, meaning the better the quality of the spermatozoa. This is in accordance with the opinion of Susilawati *et al.*[23] which states that the motility of fresh beef semen ranges from 70-90. Mass motility and individual motility were obtained by observing in a CASA-connected microscope with a magnification of 200 times.

Viability

Viability which shows the percentage of viable spermatozoa based on the difference in permeability to fluid in spermatozoa that were stained with eosin and made smear preparations to distinguish live and dead spermatozoa. Spermatozoa those are reddish in color means that the spermatozoa are dead, while those that do not absorb color mean that they are alive. This is in accordance with the opinion of Susilawati [24], who stated that the membranes of living spermatozoa were still good, so that dye could not enter, while dead spermatozoa were membranes that did not function, so that dyes could enter the spermatozoa membrane. The viability of fresh semen obtained in this study after five observations was 95.94±1.38%. Hafez [25] stated that the percentage of spermatozoa viability should be more than 50%,

Spermatozoa Concentration Result of Sexing

Spermatozoa concentration is the number of spermatozoa contained in one ml/ejaculate semen. The concentration of spermatozoa is important to determine the success rate of sexing and can determine how good the quality of semen is. Assessment of spermatozoa concentration is very important because this factor describes the characteristics of spermatozoa that are used as one of the criteria for determining semen quality [26]. The concentration of spermatozoa after sexing can be seen in Table 2.

Table 2. Average Spermatozoa Concentration After Sexing

Spermatozoa Concentration (×109)	Treatment			
	P0	P1	P2	P3
Before Sex	0.7285ax	-	-	-
After Sexing				
Upper layer	-	0.4533b	0.3647b	0.3392b
Bottom Layer	-	0.2215y	0.2685y	0.2812xy

Notes: [a,b]Different superscripts showed significant differences (P<0.05);
[x,y]Different superscripts showed significant differences (P<0.05)
P0: control, P1: 30 Minutes, P2: 45 Minutes, P3: 60 Minutes.

Based on Table 2, the results of the study, namely treatment without sexing or P0: produced 728.5 million spermatozoa, P1: 453.3 million, P2: 364.7 million and P3: 339.2 million in the upper fraction and P1: 221.5 million, P2: 268.5 million and P3: 281.2 million in the lower

fraction. The results of the variation show that the incubation time will affect the concentration of the control in this study, namely between P0 and P1, P2 and P3 (P<0.05). The concentration of spermatozoa in the study of [27] was lowest in the lower layer, namely 70% at 18.8 million and at the top 10%-70% at 18.8 million. However, it is not in accordance with the results of [25] study, namely the concentration of semen after undergoing the separation process with the albumen column in the upper fraction, namely 427.50 million and the lower fraction, namely 335.63 million sperm/ml.

Bali Cattle Spermatozoa Motility after Sexing

Motility or motility of spermatozoa is the ability of spermatozoa to move forward indicating how well the spermatozoa are to fertilize the egg. Bali Cattle Spermatozoa Motility after Sexing can be seen in Table 3

Table 3. Average Motility of Spermatozoa Sexing Results

Motility (%)	Treatment			
	P0	P1	P2	P3
Before Sex	91,48ax	-	-	-
After Sexing				
Upper layer	-	83.36b	79.94b	78.78b
Bottom Layer	-	71.28y	64.05z	60.55z

Notes: [a,b]Different superscripts showed significant differences (P<0.05);
[x,y]Different superscripts showed significant differences (P<0.05)
P0: control, P1: 30 Minutes, P2: 45 Minutes, P3: 60 Minutes.

The results of the analysis of variance showed that there was a significant effect (P<0.05) between P0 and each treatment of spermatozoa in both the upper and lower layers. The average percentage of spermatozoa motility decreased after going through the sexing process with P1, P2 and P3 treatments. Based on Table 3, it can be seen that the percentage of motility P0 with a value of 91.48% was higher than the treatment P1: 83.36%, P2: 79.94% and P3: 78.78% in the upper layer, while in the lower layer P1 was 71.28 %, P2: 64.05 and P3: 60.55%. The average spermatozoa resulting from sexing in the upper layer had a higher number than the motility of the spermatozoa in the lower layer.

Spermatozoa Abnormalities Sexing Results

Abnormalities in spermatozoa greatly determine the quality of spermatozoa. The percentage of abnormalities obtained from observations using a microscope with a minimum number of 100 spermatozoa and a maximum of 200 from five fields of view. The results of abnormal measurements can be seen in Table 4.

The percentage of spermatozoa abnormalities resulting from sexing with different incubation times, respectively, P0: 5.99%, P1: 7.93%, P2: 9.56% and P3: 10.44% in the upper fraction and P1: 11.04%, P2: 12.97% and P3: 13.91% in the lower fraction (Table 4). The results

of this abnormality calculation are very low and indicate that the spermatozoa used are very good. Fatahillah *et al.*[28] said semen was very good because the lower the proportion of abnormal spermatozoa, the better the quality of the spermatozoa and if it is high, it will affect the fertility rate.

Table 4. Average Spermatozoa Abnormalities Results of Sexing

Abnormalities (%)	Treatment			
	P0	P1	P2	P3
Before Sex	5.99ax	-	-	-
After Sexing				
Upper layer	-	7.93ab	9.56b	10.44b
Bottom Layer	-	11.04y	12.97y	13.91y

Notes: [a,b]Different superscripts showed significant differences (P<0.05);
[x,y]Different superscripts showed significant differences (P<0.05)
P0: control, P1: 30 Minutes, P2: 45 Minutes, P3: 60 Minutes.

Intact Plasma Membranes (IPM) Spermatozoa Sexing Results

The plasma membrane of spermatozoa is a part that functions to regulate the traffic in and out of all substrates and electrolytes from cells that are needed in the metabolic process for spermatozoa. The integrity of the spermatozoa plasma membrane physiologically plays a role in protecting and maintaining spermatozoa motility in the female reproductive tract, capacitating, and fertilization [29]. The percentage of intact plasma membranes of sexed spermatozoa can be seen in Table 5.

Table 5. Percentage of Intact Plasma Membranes (IPM) of Spermatozoa Result of Sexing

MPU (%)	Treatment			
	P0	P1	P2	P3
Before Sex	96.64ax	-	-	-
After Sexing				
Upper layer	-	87.00ab	84.50ab	80.81c
Bottom Layer	-	80.64y	80.24y	71.45y

Notes: [a,b]Different superscripts showed significant differences (P<0.05);
[x,y]Different superscripts showed significant differences (P<0.05)
P0: control, P1: 30 Minutes, P2: 45 Minutes, P3: 60 Minutes.

Data in Table 5 explained that there was a downward trend in the percentage of intact plasma membrane (IPM) of sexed spermatozoa along with the increase in incubation time. The decrease in the percentage of intact plasma membranes (IPM) was significantly different (P<0.05) between P1 (30 minutes incubation) and P3 (60 minutes incubation), both for observations in the upper layer which was thought to contain X spermatozoa and observations in the lower layer which was thought to contain spermatozoa. Y. The highest percentage of

intact plasma membrane (IPM) was obtained at P1 (30 minutes incubation) with an average of 87.00% in the upper layer and 80.64% in the lower layer.

The decrease in the quality of the plasma membrane of spermatozoa during the sexing process can be caused by several factors, such as mechanical and chemical factors. Mechanical factors that can cause damage to the plasma membrane of spermatozoa are friction between spermatozoa and other spermatozoa, spermatozoa with the separating medium, and spermatozoa with the walls of the tube during the sexing process [30].

Intact Acrosome Caps (TAU) Spermatozoa Result of Sexing

The acrosome cap is a cap-shaped structure that covers the anterior two-thirds of the head. The acrosome cap is one of the important variables of spermatozoa quality which has a central role in determining the success of fertilization, so it must be maintained intact until capacitation occurs. The percentage of intact acrosome caps from sexed spermatozoa can be seen in Table 6.

Table 6. Percentage of intact acrosome Membrane (IAM) Spermatozoa Result of Sexing

MPU (%)	Treatment			
	P0	P1	P2	P3
Before Sex	91.06ax	-	-	-
After Sexing				
Upper layer	-	87.03ab	85.46ab	82.19b
Bottom Layer	-	77.09y	73.68y	72.11y

Notes: [a,b]Different superscripts showed significant differences (P<0.05);
[x,y]Different superscripts showed significant differences (P<0.05)
P0: control, P1: 30 Minutes, P2: 45 Minutes, P3: 60 Minutes.

Based on Table 6, it can be seen that there was a decreasing trend towards the percentage of IAM along with the increase in incubation time. The percentage of IAM decreased significantly (P<0.05) since 30 minutes of incubation, both in the upper layer which was estimated to contain X spermatozoa and in the lower layer which was estimated to contain Y spermatozoa. The highest percentage of IAM was obtained at P1 (30 minutes incubation) with an average of 87.03% in the upper layer and 77.09% in the lower layer. According to Triwulaningsih *et al.* [31], the percentage of IAM decreased with increasing processing time or storage of cement.

CONCLUSIONS

The conclusion from the results of this study stated that the evaluation of the characteristics of the spermatozoa of Bali cattle in fresh conditions showed results that were in accordance with the standard for further processing into sexing semen. Incubation time of 30 minutes in the sexing process of spermatozoa caused a significant decrease in motility and concentration, while abnormalities, intact plasma membranes (IPM) and intact acrosomal

membrane (IAM) of spermatozoa sexing results experienced a significant decrease at 60 minutes of incubation, but the rate of decrease was significant. This is still within the normal range for spermatozoa to be used in the Artificial Insemination process later.

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