The quality of Sumba Ongole sperms after sexing using bovine serum albumin column in Bracket Oliphant extender at different temperatures

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Abstract. The objective of this study was to determine the effect of using Bracket Oliphant medium (BO) extender at different temperatures on motility, viability, abnormalities and plasma membrane integrity of sexed sperms using fresh semen of Sumba Ongole (SO). Sexing sperms was carried out using a BO extender in the BSA column. The parameters observed in this study were motility, viability, abnormalities and plasma membrane integrity and were compared using the Paired-Sample t-Test with two treatments and six replications. P1 was BO extender with a temperature of 27°C and P2 was BO extender with a temperature of 37°C. The results of this study showed that there were no significant differences (P>0.05) between the two treatments of the sperms X for motility (65.8%, 53.3% and 41.3% vs. 66.3%, 55.0% and 43.3%), viability (65.9%, 68.0% and 67.0% vs. 67.7%, 61.9% and 69.6%) and plasma membrane integrity (70.1% and 59.8% vs. 64.9% and 64.2%) after sexing, equilibration and thawing, but differed significantly (P<0.05) in abnormalities after sexing (15.5%, 15.6% and 16.2% vs. 15.5%, 17.0% and 19.5%). Likewise, the motility (66.7%, 55.8% and 42.1% vs. 65.8%, 55.0% and 42.1%), viability (68.8%, 59.0% and 64.5% vs. 67.4%, 59.4% and 71.7%), abnormalities (13.6%, 19.4% and 16.6% vs. 18.9%, 18.6% and 17.2%) and plasma membrane integrity (68.1% and 57.5% vs. 62.8% and 62.8%) of sperms Y at P1 and P2 did not differ significantly (P>0.05). The difference in the value of abnormalities of the sperms after separation is still within the limits of abnormalities. It can be concluded that the use of the BO extender method can be used at a temperature of 27°C and at 37°C in the process of sexing sperms.

1. Introduction

Along with technological advances, especially in the field of reproduction, one of reproductive technologies has been known as sperm sexing. Sperm sexing technology is conducted by separating the X and Y chromosomes. Spermatozoa X chromosome when fertilizing the egg will produce female embryos while Y chromosome spermatozoa when fertilizing the egg will produce a male embryo. Spermatozoa X and Y have different characteristics both from their DNA content and from their morphometric [1].

Separation of spermatozoa has many benefits, besides producing a calf according to what is desired, it can also support the role of artificial insemination (AI) in the context of efficiency in...
livestock business [2]. Another benefit of using sexing semen is that it can reduce maintenance costs so that the costs incurred decrease. Besides that, the use of sexed semen can support breeding programs in the context of selecting superior cattle [3].

One of the methods for separating the X and Y chromosomes is an albumin column albumin such as Bovine Serum Albumin (BSA) or egg white albumin. The use of BSA has widely been applied in artificial insemination centers. The study of Gunawan, et al. [4] showed that the separation of X and Y bearing spermatozoa using BSA columns of 5% and 10% with the suitability of desired sex after artificial insemination (AI) was more than 85%.

Separation of spermatozoa using the albumin column method has several disadvantages including a decrease in the quality of frozen semen after thawing, a decrease in the concentration of sperm produced, low motility and a decrease in pH, due to energy use during the separation process. To reduce the loss of quality, it is necessary to use a medium that can maintain the quality of spermatozoa. One of them is extender medium of Bracket Oliphant (BO). The obstacle faced in the use of medium BO is should be carried out at 37°C so that it requires a special room to perform. Based on these problems, it is necessary to conduct a study on the quality of spermatozoa separated by BSA columns using BO extender at different temperatures.

The use of BO medium in the process of spermatozoa separation uses more temperature at 37°C, so it requires special space to separate it. The effort that can be done is to modify the temperature of 37°C to 27°C so that it does not require special space in its use. Based on the above review, it is necessary to do research on the use of BSA columns with BO solvents at different temperatures.

2. Materials and methods

2.1. Materials

The materials used in the present study were artificial vagina (minitube®), centrifugation tubes (corning®), glove, rubber funnel, thermometer, jelly (KY gel), pH indicator (universal), object glass (sail brand), cover glass 20x20mm, assteat), micropipette 10-100 µl, 200 µl, 1mL, 5 mL (eppendorf®) tips, tubes, para film (labmate®), warming plate (HT 400, minitube®), isotyp (fisher scientific), hand counter, microscope (Olympus BH-2®), laminar air flow (telstar BH-100®), incubator (thermo scientific), filling sealing (minitube®), straw, rack straw, cool box, tweezers, straw scissor, beaker glass 50 and 100 mL (iwaki®), Erlenmeyer 50 and 100 mL (iwaki®), centrifuge (hettich EBA 20®).

In the study, one bull Sunba Ongole (SO) was used to collect the semen. The other materials were Bovine Serum Albumin (BSA) fraction V (roche) at concentration of 5% and 10%, medium extender of BO (Bracket Oliphant), eosin 2%, Hypo Swelling Test (HOS-Test, optixcell 2, aquabidest, tissue, aluminium foil, labelling paper, and alcohol 70%.

2.2. Materials

The study was conducted in four stages. The stages were collection and evaluation of semen, BSA column and extending the semen using BO, separation of X and Y bearing sperms, and motility test.

a) Collection and evaluation of semen: Semen collection was carried out using artificial vagina with a temperature of 40 - 45°C. The collected semen was then subjected to macroscopic and microscopic evaluation. Macroscopic evaluation including volume (ml), consistency, color and pH. While microscopic evaluation was carried out following the method of Maulana [5]. Sperms motility was evaluated subjectively with two observers using 5 points of view, with the help of an objective microscope with a magnification of 400x. The mass movement was observed by placing the sample on a glass object then viewed using an objective microscope with a magnification of 100x. Concentrations of the sperms were calculated using a Neubauer counting chamber method.

b) BSA column and extending the semen using BO: Fresh semen was diluted using BO medium. A sample of 1 milliliter was put into a tube containing BSA% and 10% columns according to treatment. The final concentration of sperms was 200 million cells per milliliter.
c) Separation of X and Y bearing sperms; Sperm sexing was performed using the BSA column method. The BSA column was dissolved in a test tube with a concentration of 10% at the bottom and 5% at the top. Semen that has been examined through microscopic evaluation was then added with BO medium, then added 1 mL to each BSA column tube of semen for 30 minutes. After that, each tube was then placed in a tube rack and stored in a water bath at the temperature of 37°C and in a laminar cabinet at a room temperature of 27°C. Each BSA column was separated, then centrifuged at 1800 rpm for 10 minutes [6]. The upper BSA column with a concentration of 5% was predicted to contain X chromosome sperm and the lower part with a concentration of 10% was predicted to contain the Y chromosome sperm. Semen that has been diluted using extender were packaged in mini-straw and then equilibrated at 5°C for four hours in the refrigerator. Freezing process was conducted by placing the straws in a box containing liquid N2 for 10-15 minutes. Then the straws were stored in a container containing liquid N2 [7].

d) Motility test; Sperm motility test was carried out after freezing for at least 24 hours. The straw was picked up using tweezers from a container, then thawing in water at 37°C for 3-5 minutes and placed on the object glass to observe its motility. Observations were carried out using a 400x magnification microscope with 5 views.

2.3. Parameters of the study
The parameters measured in the present study were motility, viability, and whole plasma membrane.

2.4. Study design and statistical analyses
The study was experimental design in which two treatments were performed. The treatments were 1) BO extender with temperature of 27°C (P1) (room temperature) and BO extender with temperature of 37°C (P2) (in water-bath). All data obtained in the study were tabulated in Microsoft Excel program. The difference among parameters between the two treatments was compared using paired t-Test [8].

3. Results and discussion

3.1. The quality of Sumba Ongole (SO) bull semen
Determining the quality of fresh semen includes macroscopic and microscopic quality. Macroscopic quality tests include volume, degree of similarity (pH), mass motion, consistency, and colour, while microscopic quality tests include motility, viability, abnormalities and intact plasma membrane. The results of the evaluation of SO cattle fresh semen are macroscopically presented in Table. 1

| Table 1. The Macroscopic Quality of SO Cattle Fresh Semen |
|----------------------------------------------------------|
| Parameters (Macroscopic and Microscopic) | Average (± SD) |
| Volume (ml) | 5.16 ml± 1.86 |
| (pH) | 6.5± 0.00 |
| Mass Movement | + + |
| Color | Cream-colored |
| Consistency | Medium-dense |

(+ +) = Mass movements like cloud waves.
SD = Standard Deviation

Table 1 shows that the volume of fresh semen in this study was 5.16 ± 1.86 ml. The volume of fresh semen produced was still normal. The differences in the volume of ejaculated are affected by bull, age, season, nutrition, frequency of ejaculated, and libido [9].

The degree of acidity (pH) of bull semen obtained in this study was still within the normal pH limit, which was equal to 6.5 ± 0.00. If the pH is too acidic and too alkaline it cannot be processed
further. This is in accordance with the quarterly explanation of Campbell et al. [10] that the pH range that can be processed is 6.58 - 7.00. The study of Garmer and Hafez [9], showed that the average pH of cattle semen ranges from 6.4 to 7.8 or 6.8.

The consistency of semen from this study was still relatively good, with a rather thick consistency. There are several factors that affect the consistency of semen produced, one of them is nutrition. Dewi, et al. [11], reported that one of the factors that influence the thickness of semen is the quality of feed.

The colour produced from this study was normal; creamy. This is consistent with the statement of Ismaya [12] that normal fresh semen is milky or creamy white.

| Parameters                         | Average ± SD |
|-----------------------------------|--------------|
| Motility sperm (%)                | 75 ± 2.58    |
| Viability sperm (%)               | 65.55 ± 5.22 |
| Abnormality sperm (%)             | 11.20 ± 5.26 |
| Concentration sperm (10⁶/ml)      | 1600 ± 465.56|
| Plasma Membrane Integrity sperm (%) | 70 ± 6.20  |

SD = Standard Deviation

The results of the study (Table 2) shows that the quality of SO bull fresh semen were good. Motility tests and concentrations of fresh semen of the sperms were 75 ± 2.58% and 1600 ± 465.56 million/ml, respectively, indicating that the semen was suitable for further processing. According to SNI for frozen semen in 2017, the motility of fresh semen to be processed into frozen semen is 70%, if the motility is less than 70%, it is can still be used if the recovery value is ≥ 50% [13].

The percentage of viability and abnormalities of the sperms in this study were 65.5 ± 5.22% and 11.2 ± 5.26%, respectively. Plasma membrane integrity of the sperms was 70 ± 6.20%. The MPU value shows a good, the percentage of MPU value affects the fertility rate of spermatozoa.

The intact plasma membrane (MPU) has a role as one of the determinants of spermatozoa survival. The sperm membrane serves as a suggestion for the transportation of energy in ATP produced by enzymes in the mitochondria through the Kreb cycle, so progressive motile sperm must have a complete membrane. On the other hand, the integrity of plasma membranes is very important for sperm, because if the sperm membrane is damaged it cannot be repaired [3].

3.2. The quality of Spermatozoa X after separation with Addition of BO Diluent (Oliphant Bracket) at Temperature of 27°C and 37°C

3.2.1. Motility of spermatozoa X. The motility of spermatozoa X after separation, equilibration, and thawing at temperature of 27°C and 37°C decreased slightly (p> 0.05). The percentage of fresh semen motility, after separation, after equilibration, and after thawing at a temperature of 27 °C it were 75 ± 2.58, 65.83 ± 3.76, 53.33 ± 7.52 and 41.25 ± 7, respectively, while after separation, after equilibration, and after thawing at a temperature of at 37 °C were 66.25 ± 4.40, 55.00 ± 5.47 and 43.33 ± 4.08 respectively. Decreasing the quality of spermatozoa during the separation process can be caused by damage to the plasma membrane. Damage to the plasma membrane during the freezing and thawing process causes a decrease in fertility of frozen semen [14].

3.2.2. Viability of spermatozoa X. The percentage of viability of spermatozoa X after separation, after equilibration and after thawing using BO diluents at temperatures of 27°C and 37°C, did not differed significantly (p> 0.05). The average percentage of viability at 27°C after separation, after equilibration and after thawing were 65.86 ± 7.85, 68.03 ± 9.68, and 66.98 ± 9.13 while at 37°C were 67.65 ± 9.86, 61.88 ± 7.17, and 69.60 ± 9.06. This shows that the use of temperature 27°C when the separation of
spermatozoa does not affect the level of viability of spermatozoa. Study of Takdir et al. [15] in sheep spermatozoa using a temperature of 28°C using egg white albumin can increase the motility and viability of spermatozoa after separation.

3.2.3. Abnormality of spermatozoa X. The abnormalities of spermatozoa X after separation, after equilibration and after thawing with the addition of BO extender at 27°C and 37°C did not differed significantly (p> 0.05). The percentage of fresh semen was 11.20 ± 5.26 after separation, after equilibration and after thawing at 27°C were 15.45 ± 4.13, 15.63 ± 2.78 and 16.15 ± 5.53, respectively, while at 37°C were 15.50 ± 3.41, 17.28 ± 4.06 and 19.46 ± 9.16, respectively. An increase in the value of abnormalities after separation might be resulted from the presence of mechanical effects such as centrifugation. This is in accordance with the study of Ervandi et al. [16] which stated that the percentage of abnormalities after separation increases due to the centrifugation process when washing spermatozoa. Centrifugation results in friction between the spermatozoa with the separating medium and the tube wall.

3.2.4. Plasma membrane integrity of spermatozoa X. The percentage of spermatozoa X MPU on fresh semen 70.00 ± 6.20, after separation, and after thawing at a temperature of 27°C were 70.05 ± 5.48 and 59.83 ± 9.76, respectively, while the temperature of 37°C were, 64.88 ± 8 and 64.16 ± 9.17, respectively. The MPU value generated from the study was not significantly different (p> 0.05) in the two treatments. The decrease in MPU value is caused by the mechanical influence of the centrifugation force such as the friction of the surface of the membrane of the spermatozoa with the tube wall during washing. This is consistent with the statement of Donnelly, et al. [17] that separation of spermatozoa by centrifugation can induce damage to the spermatozoa plasma membrane resulting in increased abnormalities. The intact plasma membrane has a role as one of the determinants of spermatozoa survival. The sperm membrane serves as a suggestion for the transportation of energy in ATP produced by enzymes in the mitochondria through the Kreb cycle, so progressive motile sperm must have a complete membrane. On the other hand, the integrity of plasma membranes is very important for sperm, because if the sperm membrane is damaged it cannot be repaired [3].

3.3. The quality of Spermatozoa Y after separation with Addition of BO Diluent (Oliphant Bracket) at Temperature of 27°C and 37°C

3.3.1. Motility of spermatozoa Y. The motility ratio of Y spermatozoa at 27°C and 37°C after separation, after equilibration, and after thawing the motility value was decreased but did not differed significantly (p> 0.05). The average percentage of motility after separation, after equilibration, and after thawing at 27°C were 66.66 ± 4.08, 55.83 ± 4.91 and 42.08 ± 2.45, respectively, while at 37°C were 65.83 ± 3.67, 55.00 ± 6.32 and 42.08 ± 4.00. The motility of the spermatozoa tends to decrease during the separation process. This decrease might be as a result of reduced nutrition possessed by spermatozoa. This is in line with the statement of Triwulanningsih et al. [18].

3.3.2. Viability of spermatozoa Y. The viability of Y spermatozoa at 27°C and 37°C did not differed significantly (p> 0.05). The average viability at temperature of 27°C after separation, after equilibration and after thawing were 6.81 ± 7.85, 59.01 ± 3.81 and 64.50 ± 8.69, respectively, while the percentage of viability at 37°C were 68.81 ± 9.60, 59.43 ± 8.10 and 71.70 ± 7.82. The final results of the percentage of spermatozoa viabilities in both treatments were still relatively good with a percentage value still above 60%. Toelihere [19] stated that good semen has a viability percentage above 50%.

Decreasing the quality of spermatozoa after the separation generally occurs because spermatozoa need a lot of energy to still be able to maintain physiological stability. Separation of spermatozoa from incubation for 30 - 40 minutes until washing can result in a reduction in plasma semen concentration [5].
3.3.3. Abnormality of spermatozoa Y. The abnormalities of sperm after separation, after equilibration and after thawing with the addition of BO (Bracket Oliphant) diluents at temperatures of 27 °C and 37 °C can be seen in Figure 10. Figure 10 shows a significant difference between the two treatments (p <0.05) after separation, but after equilibration and after thawing there was no significant difference (p> 0.05). The percentage value of fresh semen abnormalities was 11.20 ± 5.26, after separation, after equilibration and after thawing at 27 °C respectively 13.63 ± 2.24, 19.41 ± 4.67 and 16.55 ± 6.66, while in temperatures of 37 °C 18.91 ± 2.94, 18.58 ± 2.74 and 17.20 ± 7.18. An increase in the value of abnormalities after separation is caused by the presence of a mechanical force of separation, but the abnormalities produced are still normal. The abnormality produced is still carried 20%. This is in accordance with the opinion of Toelihere [19] which states that as long as the spermatozoa abnormality has not reached 20% of the semen sample, then the semen can still be used for insemination. However, the results of research conducted by Campbell et al. [10] and Arifiantini [20], stated that semen with high quality has an abnormality value of around 5-15%. The results in the present study showed similar with those two studies. This indicated that the abnormalities of the sperms during study period were acceptable to be further processed.

3.3.4. Plasma membrane integrity of spermatozoa Y. The percentage of Y spermatozoa on fresh semen was 70.00 ± 6.20, after separation, and after thawing at a temperature of 27°C were 68.08 ± 5.91 and 57.45 ± 8.76, respectively, while at 37°C were 62.75 ± 9.02 and 62.76 ± 8.76, respectively. The plasma membrane integrity values of the two treatments were higher at a temperature of 27°C than at a temperature of 37°C but did not show a significant difference (p> 0.05). Decreasing the value of plasma membrane integrity from fresh to thawing might be caused by the treatment during the separation process starting from the initial testing to the freezing stage. This is in accordance with the study of Situmorang [21] that the result of freezing occurs a decrease in phospholipid and cholesterol. Phospholipid functions to protect spermatozoa cells from cold shock while cholesterol plays an important role in maintaining spermatozoa cell integrity from different membrane system variations during the cooling process.

The freezing process causes damage to the plasma membrane so that the formation of lipid peroxide which results in changes in the structure and function of the membrane and when thawed causes changes in protein activity and changes in permeability to water and solutes. The percentage of plasma membrane integrity varies due to the biophysical and biochemical characteristics of sperm membranes [22].

4. Conclusion

Based on the results and discussion, it can be concluded that the use of the BO extender was suitable to be used at a temperature of 27°C and 37°C in the process of separating spermatozoa. Motility, viability, and plasma membrane integrity after separation, after equilibration and after thawing at a temperature of 27°C and a temperature of 37°C can be maintained on the quality of frozen semen. The Y sperms abnormalities after separation were higher at 37°C, but the abnormalities produced were still normal.

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