The quality of sexed sperm separated using bovine serum albumin column and extended using tris aminomethane at different temperatures

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Abstract. Sexing sperm is one of the reproductive technologies that is considered as a promising alternative in the effort of reproductive efficiency to produce offspring according to the desired sex. The method for maintaining and extending the sperms can be done by using bovine serum albumin (BSA) column with Tris Buffer at room temperature and waterbath without damaging the quality of the sperms. The purpose of this study was to determine the effect of BSA - Tris Buffer extender on the quality of sexed sperms after thawing at 27°C and 37°C. The study was divided into two treatments; P1 was Tris extender in the temperature of 27°C and P2 was Tris extender in the temperature of 37°C with 6 replications. Those treatments were compared using a Paired Sample test (T-Test). The parameters observed in the present study were motility, viability, abnormalities, and intact plasma membrane. The results showed that motility, viability, abnormalities, and intact plasma membrane of the sperms X and Y at 27°C and 37°C after sexing did not differ significantly (P>0.05). Likewise, after equilibration, the motility of sperms X at 27°C and 37°C did not differ significantly (P>0.05), however, sperm Y at 27°C and 37°C showed a significant difference (P<0.05). Viability and abnormalities of sperms X at 27°C and 37°C differed significantly (P<0.05) but sperms Y at 27°C and 37°C did not show any significant differences. After thawing process, motility, viability, abnormalities and intact plasma membrane of the sperms X and Y at 27°C and 37°C did not show any significant differences. However, it can be concluded that the quality of sexed sperms in the present study is still good because it has motility above 40% and abnormalities below 20%.

1. Introduction
Artificial Insemination (AI) is one of the appropriate technologies to increase livestock population and production qualitatively by using bulls’ semen that are disease free and have high genetic quality. Genetically qualified bulls will make a major contribution to their offspring as well as high quality semen. Therefore, selection is needed to choose the bulls with good performance. This is related to the ability of bulls to mate a number of cows, produce sperms and high fertility.

Utilization of spermatozoa X and Y separation technology or more commonly called sexing sperm has become an appropriate alternative in the application of AI related to efforts to increase reproductive efficiency and increase the efficiency of animal husbandry business. Sexing is an attempt to change the
natural proportion of spermatozoa X and Y (50%: 50%) to the desired proportion using a certain method. This technology is believed to be able to increase the value of AI applications, because it is able to produce superior calves according to desired sex and maintenance the goals of raising livestock animals. Spermatozoa separation technique with BSA gradient is considered not to manipulate spermatozoa excessively, besides that spermatozoa are exposed to BSA medium which is also often added to semen extender, so it is expected to be able to prevent the decline in quality of spermatozoa after the separation process. Afiati [1] reported that the percentage of spermatozoa resulting from albumin gradient sexing is predicted to carry an X chromosome of 80.88% and Y of 58.82% with motility after the sexing process reach 75.00%.

The method of separating spermatozoa using BSA columns with Tris Buffer is possible to be carried out at room temperature or at water bath temperature without damaging the quality of spermatozoa. This study intended to know the effect of BSA on spermatozoa quality after sexing.

2. Materials and methods

2.1. Time and location of the study
This study was conducted in January to February 2019, located at the Field Laboratory of PT. Anugerah Rumpin, Bogor and the Laboratory of Reproduction, Reproduction, Breeding and Animal Cell Culture, Biotechnology Research Center, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor, West Java.

2.2. Materials
The main component of this study was two fresh semen of Sumba Ongole (SO) bull. Supporting materials included: Medium Tris, Bovine Serum Albumin (BSA) fraction V (roche) concentration of 5% and 10 %, OpticXell Diluent, Label Paper, Aquabidest, NaCl 0.9%, Eosin 2%, Alcohol 70%, Jelly (KY Gel), aluminum foil, tissue, and Hypoosmotic Swelling Test (HOS-Test).

The equipment used were artificial vagina, water bath, object Glass (sail brand), Cover glass 20x20 mm, assteat), ilncubator (thermo scientific), photometer (SDM-6, minitube®) Centrifuge, warming plate (HT 400, minitube®), laminar air flow (telstar BH-100®), Toshiba TVs, refrigerators, spoileliers, centrifugation tubes (Corning®), funnels, thermometers, pH indicators (Universal Indicators), micropipettes 100-100µl, 1000µl, 5 ml (eppendort® ) tips, glass test tubes, parafilms (labmate®), hand counters, isotemp (fisher scientific), microscopes (Olympus BH-2®), filling sealing (minitube®), printing machines (leibinger jet 2 SE, minitube®), straw racks, cool boxes, tweezers, scissors, gublets, beaker glass; 50 mL and 100 mL (iwaki®), Erlemeyer; 50 mL and 100 mL (iwaki®) and centrifugation (hettich EBA 20®) and straws.

2.3. Methods

2.3.1. Semen collection and evaluation. Collection of semen was carried out in the morning using an artificial vagina at temperatures around 40-45°C. The bull was brought to the collection place with the teaser inside to be able to provide libido stimulation to the bull. Artificial vagina that has been prepared was lubricated, and if there were signs that the bull will be riding the teaser, then the artificial vagina was directed at the penis to collect semen. Collection of semen was carried out 6 times as replications.

Evaluation of semen was conducted macroscopically and microscopically. Macroscopic evaluation consisted of volume, color, consistency, and pH. While microscopic evaluation included mass movement, motility, concentration, viability, morphology, and intact plasma membrane.

2.3.2. Preparation of BSA Column and dilution with Tris medium. Preparation of BSA column was conducted using four cylinder tubes, then placing the column to the bottom with a concentration of 10% and the top with concentration of 5%. Each tube was stored at 37°C and 27°C. Fresh semen was then diluted using Tris Aminomethan medium. A sample of 1 milliliter was put into a tube containing BSA column of 5% and 10% according to treatment. The final concentration of sperms was 200 million cells per milliliter. The semen was the kept for 30 minutes, then each tube was placed in a tube rack and stored in a water bath for 37°C and laminar cabinets for room temperature (27°C).
2.3.3. Separation of $X$ and $Y$ spermatozoa with BSA column and semen freezing. Each BSA column was separated, then centrifuged at 1800 rpm for 10 minutes [2]. 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.

The diluted semen was packaged in a mini straw and then equilibrated at 5°C for four hours in the refrigerator, then freeze the straw in a box containing liquid N2 for 10-15 minutes before stored in a N2 container [3].

2.3.4. Parameters, study design and data analyses. The parameters of the study were the sperms motility, viability, and intact plasma membrane. The study was design in two treatments; P1=Tris extender at temperature of 27°C and P2=Tris extender at temperature of 37°C. The data obtained in this study were tabulated in the SPSS application. 16 such as motility, viability, abnormality, and intact plasma membrane. The two treatments at different temperatures were analyzed using a comparative test of Paired Sample Test (T-Test) [4].

3. Results and discussion

3.1. The quality of fresh semen of Sumba Ongole bull

The quality of fresh semen of Sumba Ongole (SO) bull that evaluated macroscopically are shown in Table 1.

| Parameter       | Mean ± SD |
|-----------------|-----------|
| Volume (mL)     | 4.5 ± 1.86|
| Consistency     | Slightly thick |
| Odor            | Typical sperms |
| Color           | Creamy |
| pH              | 6.5 |

Table 2 shows that the volume of fresh semen produced from 6 replications was 4.5 mL. The volume of fresh semen produced is still in normal condition. Normal volume of fresh semen ranges from 4-6 mL per ejaculation. According to Afiati et al. [5], the volume of semen can be influenced by the breed, age, body size, feeding, frequency of collections and other factors. Bull semen volume varies between 1-15 ml. Butar [6] also stated that the semen volume of bulls ranged from 2-10 ml. The difference in the volume of fresh semen can be due to the size of testes [7].

The acidity (pH) level of bull semen obtained in this study was relatively good, that was 6.5. If the pH is too acidic or too alkaline then it cannot be processed further. This is consistent with Wahyuningsih et al. [8] 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 bull ranged from 6.4 to 7.8 or 6.8 in average. Putranti et al. [10] stated that sperm cells that are at neutral pH will increase the average metabolism rate (MR) and a decrease in metabolism when it becomes alkaline or acidic.

The color of fresh semen obtained was creamy, indicated normal. This is in accordance with the statement of Feradis [7] that normal bull semen is milky white or creamy and turbid. This is supported by Partodihardjo [11] that the color of semen produced is whitish, if yellowish green means it contains Pseudomonas aeruginosa, red semen contains blood.

The consistency of semen resulting of this study was still relatively good, which was rather thick. There are several factors that affect the consistency of semen, one of them is the nutrition provided, and besides that the frequency of collections can affect the consistency of semen. According to Dewi et al. [12], one of the factors that affect the thickness of the semen is the quality of the feed provided. Better quality of feed resulted the thicker semen and vice versa.

The odor of semen collected in this study can be considered normal; typical odor of semen. This is supported by the study of Kartasudjana [13] that normal semen generally has a characteristic odor.
accompanied by the odor of the animal. A foul odor can occur if the semen contains pus caused by infection of the bull reproductive organs.

The quality of fresh semen of Sumba Ongole (SO) bull that evaluated microscopically are shown in Table 2.

![Table 2. Microscopic quality of Sumba Ongole (SO) bull fresh semen](image)

Table 2 shows that the motility of Sumba Ongole (SO) bull fresh semen was relatively normal; 75%. This in line with the statement of Susilawati et al. [14] that, the motility of fresh semen of bull ranges from 70-90%. According to SNI frozen semen in 2017, in which that the motility of fresh semen to be processed into frozen semen should be ≥ 70% for a bull, if the motility is less than 70%, it can still be used if the recovery rate is ≥ 50% (BSN, 2017). This suggests that the bull used in this study had have high fertility.

Viability of fresh semen in this study was 65.6%. This viability is lower compared to the study of Arifiantini et al. [15] which shows the viability of fresh semen of bull was 89.32%. Nevertheless, the abnormality was quite good; 11.2%, less than 20%. Semen that has a high abnormality will affect fertility. Furthermore, the semen concentration was 1600 x 10^6 cells/mL. This concentration can be categorized as normal. According to Garner and Hafez [9] stated that standard concentration of bull semen is 800 to 2000 10^6 cells/mL. This standard are in line with our study, therefore the semen that were used in the present study could be further processed.

3.2. The quality of spermatozoa X post-equilibration and post-thawing

The quality spermatozoa X post-equilibration and post-thawing are shown in Table 3.

![Table 3. The quality spermatozoa X post-equilibration and post-thawing](image)

Table 3 shows that the quality of spermatozoa X in the two different temperatures (27°C and 37°C) at control, post-equilibration, and post-thawing. Motility of spermatozoa X after separation using a BSA column and Tris Aminomethane extender at 27°C was 62.50% and 60.83% at 37°C. After filling and sealing process and evaluation was continued during the equilibration process, the motility of spermatozoa X at a temperature of 27°C was 52.50% and a temperature of 37°C was 50.83%. These motilities were able to the sperms kept in the container. The motility of spermatozoa X after thawing at
room temperature of 27°C was 43.33% and at 37°C was 46.66%. Although the motility of each treatment continues to decrease but it could be processed because it was still above 40%. This is consistent with the statement of Fatimah [16] in a previous study which stated that spermatozoa are suitable for Artificial Insemination (AI), if the sperms motility is above 40%.

Viability of the sperms at temperatures of 27°C and 37°C were 68.75% and 65.60%, respectively. After equilibration at -5°C, the viability of the sperms at 27°C was significantly higher (P <0.05) compared to 37°C (59.36% vs. 55.55%). After thawing, viability at 27°C and 37°C were 64.71% and 65.31%, respectively. Study of Diliyana et al. [17] stated that viability can affect the motility and integrity of the spermatozoa membranes so that not all motile spermatozoa have good membrane integrity.

Abnormalities of the sperms X at temperatures of 27°C and 37°C were 14.61% and 15.36%, respectively. After the equilibration process, the abnormality at a temperature of 27°C was significantly (P <0.05) lower than at 37°C (14.43% vs. 16.85%). After thawing the sperms abnormalities at a temperature of 27°C was 18.78% and at 37°C was 18.63%. The percentage of sperms abnormalities using Tris Aminomethane extender was relatively good because it was no more than 20%. This is consistent with the study of Ervandi et al. [18] stated that the percentage of abnormalities in spermatozoa after sexing with the addition of extender can be considered to be good if not more than 20%. The high abnormalities can affect fertility [19]. Percentage of abnormality after sexing can be caused by centrifugation process which results in friction between the sperms or sperms with the separating medium or with the tube wall. This may cause the percentage of sperms abnormalities and spermatozoa membrane damage[20].

The intact plasma membrane of sperms X which were incubated in a Hypoosmotic Swelling Test (HOS-Test) solution for 30 minutes at temperatures of 27°C and 37°C were 70.66% and 69.20%, respectively. After separation, freezing process, and thawing for 30 seconds at 30°C, the intact plasma membrane at 27°C and 37°C were 64.43% and 60.75%, respectively. Spermatozoa membrane integrity was still good, in which showed that phospholipids could survive and maintain well on the collision between the tube and the medium when centrifugation. This is in agreement with the study of Dilyana et al. [17] which stated that the process of sexing using centrifugation can cause the release of a portion of spermatozoa membrane phospholipids due to mechanical influences, such the presence of centrifugal force. The release of a portion of membrane phospholipids can cause the integrity of the membrane to be disrupted, thereby affecting membrane viability.

3.3. The quality of spermatozoa Y post-equilibration and post-thawing

Table 4 shows that the quality of spermatozoa Y in the two different temperatures (27°C and 37°C) at control, post-equilibration, and post-thawing. The motility of spermatozoa Y at temperatures of 27°C and 37°C were 59.16% and 56.66%, respectively. After centrifugation and the addition of extender, the motility of spermatozoa at 27°C was significantly (P <0.05) lower than at 37°C (51.66% vs. 56.66%). After post-thawing, motility of the sperms at a temperature of 27°C was 43.33% and a temperature of 37°C was 46.25%. The motility spermatozoa Y after sexing, equilibration and post-thawing continue to decline. This is caused by the centrifugation process which causes spermatozoa to die. This is consistent with the study of Gunawan and Kaini [21] stated that the motility of spermatozoa Y post-thawing is around 39.14%.

Viability of spermatozoa Y at temperatures of 27°C and 37°C were 71.31% and 72.66%, respectively. After the addition of extender and equilibration, there was a decrease in viability, where at a temperature of 27°C was 64.46% and at 37°C was 60.68%. Viability after thawing at temperatures of 27°C and 37°C were 72.93% and 73.23%, respectively. In this study dead and living spermatoza can be distinguished using 2% eosin staining. This is in accordance with the statement of Mulyono [22] that colored sperm means the sperm is dead, while that is not colored means the sperm is alive. Discoloration of the dead spermatozoa is caused by damage to the spermatozoa plasma membrane and eosin is absorbed by the spermatozoa.

The quality spermatozoa Y post-equilibration and post-thawing are shown in Table 4.
Table 4. The quality spermatozoa Y post-equilibration and post-thawing

| Treatment        | Characteristic of the sperms |
|------------------|------------------------------|
|                  | Motility (%)                 | Viability (%)  | Abnormality (%) | Intact plasma membrane (%) |
| Control          |                              |                |                |                            |
| 27°C             | 59.16 ± 8.01                 | 71.31 ± 9.42   | 15.86 ± 2.01   | 71.48 ± 9.57               |
| 37°C             | 56.66 ± 7.52                 | 72.66 ± 8.72   | 14.70 ± 2.52   | 61.25 ± 8.17               |
| Post-equilibration |                              |                |                |                            |
| 27°C             | 51.66 ± 5.16a                | 64.46 ± 8.47   | 13.68 ± 5.40   | -                          |
| 37°C             | 56.66 ± 5.16b                | 60.68 ± 8.16   | 19.56 ± 5.53   | -                          |
| Post-thawing     |                              |                |                |                            |
| 27°C             | 43.33 ± 4.08                 | 72.93 ± 9.93   | 16.85 ± 2.35   | 57.96 ± 8.35               |
| 37°C             | 46.25 ± 3.06                 | 73.23 ± 8.88   | 14.76 ± 3.47   | 62.41 ± 9.20               |

abDifferent superscript, differed significantly (P <0.05)

The mean value of spermatozoa abnormalities both at 27°C and 37°C at under 20%. It is still considered to be feasible for storage. Sperm abnormality also determines bull fertility. Arifiantini et al. [15] stated that sperm abnormalities consist of two categories, which are primary abnormalities; abnormalities that occur during spermatogenesis and secondary abnormalities; abnormalities that occur after the spermiation stage. Indonesian National Standard (SNI) for frozen bull sperms products, requires sperm abnormalities below 20%. In this study, sperm abnormality was found to be below that value of 20%, so the quality of sperm produced from sexing was still in accordance with SNI.

The intact plasma membrane of spermatozoa Y at temperatures of 27°C and 37°C were 71.48% and 61.25%, respectively. After thawing, the intact plasma membrane decreases, at 27°C it was 57.96% and at 37°C it was 62.41%. According to Tappa et al.[23] that the tail of spermatozoa which are swollen in a Hypoosmotic medium is a sign that the integrity of the membrane is intact.

4. Conclusions
The quality of spermatozoa obtained in this study can be used for artificial insemination because motility is still above 40%, abnormality below 20%, and has good viability. The use of BSA column in Tris Aminomethane extender can be used for sperm sexing and treatment for sexing the sperms at 27°C and 37°C can produce good quality sperms.

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