Quality and Proportion of Balinese Bull Sperm Using Percoll Density Gradient Centrifugation Sexing Method at Different Gradients with AndroMed® Diluent

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Abstract. This study aimed to determine the quality and proportion of Bali Bull semen using the Percoll density gradient centrifugation (PDGC) method at different gradients with Andromed® diluent. The study was conducted at Singosari Artificial Insemination Center from December 2023 to February 2024. The experimental method used was a Randomized Group Design. The treatments in the study were T1: 10 Gradients (20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, and 65%), T2: 5 Gradients (20%, 30%, 40%, 50%, and 60%), and T3: 3 Gradients (20%, 40%, and 60%) with 10 replications. Statistical analysis showed highly significant differences (P<0.01) in differences gradient treatments in the lower layer of individual motility. The results showed that the percentage of motility resulting from sexing in the lower layer obtained the highest value in T1 77.86 %, while in the upper layer the highest percentage of motility was obtained in T3 which was 61.72% Meanwhile, in terms of viability, abnormalities, total motile spermatozoa and concentration, there were no differences (P>0.05) in differences gradient treatments. While the proportion of spermatozoa in sexed X and Y, T1=77.6% and 84.8%; T2=75% and 81.8%; T3=75.8% and 79.1%. In conclusion, the quality of sexed semen was categorized as good, in terms of the parameters of motility, concentration and total motile spermatozoa.

Keywords: Andromed, Bali Cattle, Percoll Density Gradient Centrifugation, Sexing, Quality.

1 Introduction

Fulfilling domestic meat needs was a challenge that must be faced by the government and the livestock industry, especially to reduce imports of meat and livestock. Beef consumption in Indonesia in 2023 was estimated to reach 816.79 thousand tons, while beef production in that year was estimated to only reach 524.76 thousand tons [1]. Fulfillment of beef availability from local production in Indonesia was still experiencing

a deficit of 374.1 thousand tons. This condition caused a gap in domestic beef supply and demand. According to the 2022 Livestock and Animal Health Statistics data (fixed figures), the population of beef cattle in Indonesia currently reaches 17.61 million head, down around 2.05% from the 2021 population of 17.98 million head. In general, the fulfillment of domestic beef needs, around 30% - 40% was still supplied by imports. The average volume of beef imports during 2022 was 19,065.90 tons per month, while the average volume of cattle imports per month was 10,059.91 tons of cattle weight [1]. The development of biotechnology in the field of livestock reproduction could be utilized to increase livestock productivity. Determining the sex of a calf before birth was much more economically beneficial, because in addition to reducing maintenance costs, it could also improve breeding programs in selecting superior breed [2]. The application of spermatozoa sexing biotechnology was one of the alternatives created to be able to predict the sex of the calf so that it could be adjusted to the goals of livestock farming [3].

The Percoll density gradient centrifugation sexing method was an effective and commonly used separation method. The principle of the Percoll density gradient centrifugation sexing method was the difference in weight and head size of X and Y spermatozoa [4]. Generally, the PDGC method sexing process was carried out using 10 gradients with density variations of 1.036, 1.038, 1.047, 1.052, 1.055, 1.057, 1.060, 1.065, and 1.070 [5]. The Percoll density gradient centrifugation sexing process caused a decrease in motility and viability, spermatozoa concentration, and damages the spermatozoa membrane. This was due to a very complex process starting from separation to gradient level, centrifugation, dilution, and freezing of semen. The success of the gradient centrifugation sexing process was influenced by several things including the quality of fresh semen, the diluent used, the duration and speed of centrifugation, the composition of the density gradient, and the number of gradients.

The diluent media used must contain components that support spermatozoa motility, such as buffers, energy sources, and antimicrobials. The use of inappropriate media could cause gradient instability, reduce separation effectiveness, and increase the risk of spermatozoa damage, such as decreased motility. AndroMed® was a diluent medium without egg yolk that could be used for freezing bovine semen with an effective antibiotic formula. Percoll density gradient centrifugation sexing 10 gradients had a higher complexity and takes longer. The success of sexing spermatozoa X and Y could be seen from the quality and proportion of X and Y produced. This study aimed to evaluate the quality and proportion of spermatozoa X and Y sexed by the Percoll density gradient centrifugation method using 3 different gradients, namely 10, 5, and 3 gradients.

2   Materials and Methods

2.1   Materials

Fresh semen samples came from 1 Balinese bull named Sabala at Singosari Artificial Insemination Center, aged 7 years with a weight of 508 kg and had fresh semen quality with progressive motility ≥ 70%. The materials used include Andromed® diluent

(diluted with aquabides with a ratio of 1:4), 3% NaCl, and eosin nigrosin dye (20 grams of nigrosin, 1.5 grams of sodium citrate, 3.3 grams of eosin yellow, 100 ml of aquades).

2.2   Methods

Semen Storage and Treatments. Balinese cattle semen was routinely collected using an artificial vagina, then evaluated macroscopically and microscopically. Fresh semen that meets the requirements was separated with several treatments including T1: 10 gradients (20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, and 65%), T2: 5 gradients (20%, 30%, 40%, 50%, and 60%) and T3: 3 gradients (20%, 40% and 60%).

Percoll Density Gradient Centrifugation Sexing Procedure. The sexing procedure using the Percoll density gradient centrifugation method refers to Susilawati [6]: Making a Percoll density gradient with AndroMed® diluent in each treatment. Furthermore, each treatment was arranged in each tube from the highest to the lowest density with the volume of each density at T1 (0.5 ml), T2 (1 ml), and T3 (1.5 ml). After the gradient was formed, 1 ml of good quality semen was inserted into each treatment. Furthermore, it was centrifuged at a speed of 2200 rpm for 5 minutes. The centrifugation results consist of 6 layers, the top layer was the seminal plasma that was discarded and the second layer was the one that contains a lot of Y spermatozoa, while the bottom layer that contains a lot of X spermatozoa was taken and inserted into a tube that already contains 3 ml of AndroMed®. Then centrifuged at a speed of 1600 rpm for 5 minutes, the supernatant was discarded and 1-2 ml of sediment containing spermatozoa was left to test the quality and proportion.

Sexing Semen Quality Evaluation. Evaluation of semen motility was carried out using a light microscope with a magnification of 400x through an average of five fields of view [7]. Viability and abnormality tests were carried out by observing the smear preparation (eosin nigrosine). Sperm concentration was calculated using Neubauer [8]. Sperm concentration was calculated using the formula Yekti et al. [8] as follows:

$$\text{The number of sperm/ml} = N \times 5 \times FP \times 10{,}000 \tag{1}$$

Information:
N   : Average number of spermatozoa in chambers A and B
5   : Correction factor for only calculating 5 boxes out of 25 boxes
FP   : Dilution factor (1:100)
10,000   : neubauer space depth 0.0001 ml/neubauer chamber
Total motile spermatozoa could be calculated by multiplying the percentage of individual spermatozoa motility by the spermatozoa concentration [9].

Identification of Sperm X and Y. Sperm identification was done by measuring the spermatozoa head, the measurement was done by making an eosin negrosin staining preparation, then the sample was observed using an Olympus CX-33 microscope. Morphometric measurements including the length and width of the spermatozoa head were carried out using LC-Micro Software. Observations were made on 1000 spermatozoa in fresh semen samples to determine the natural proportion (50:50), while in each treatment observations were made on 100 spermatozoa in each sample. If the area of the spermatozoa head was larger than the average control size, it was identified as spermatozoa X, while the size of the head area that was smaller than the average was identified as spermatozoa Y [10].

2.3   Data Analysis

The study was conducted using a Randomized Block Design using three treatments and 10 replications. The research data included mass motility, individual motility, viability, abnormality, concentration, TSM analyzed using Analysis of Variance (ANOVA). If there was a difference, further testing was carried out using the Duncan Multiple Range Test (DMRT).

3   Results and Discussion

3.1   Fresh Semen Quality

Fresh semen quality was an indicator of the success of semen sexing. Macroscopic evaluation included semen volume, color, pH, odor, and semen consistency, while microscopic evaluation included mass motility, individual motility, viability, abnormality, concentration, and total motile spermatozoa. Cow semen that was suitable for sexing must had motility above 70%. Based on the results of macroscopic and microscopic fresh semen evaluation, the Balinese cow semen was categorized as suitable for sexing. Nyuwita et al. [11] stated that factors that affect the production of fresh semen in cows produced include genetic factors, temperature, season, ejaculation frequency, feed, and body weight of livestock. The average quality of fresh Balinese cow semen both macroscopically and microscopically, as shown in Table 1.

3.2   Semen Quality After Sexing

The success of sexing could be seen from the quality of semen after sexing which still had a high value and there was a separation of X and Y spermatozoa. The quality of semen after sexing could be seen in Table 2. Based on the results of the analysis of variance, it showed that there was a very significant difference (P <0.01) in the percentage of individual motility of the lower layer and no significant difference (P> 0.05) in the percentage of individual motility of the upper layer, while in the parameters of viability, abnormality, concentration, and total motile spermatozoa there was

no significant difference (P> 0.05) in each layer. The average results of semen after sexing were listed in Table 2.

Table 1. Quality of fresh semen from balinese bull

Parameter	Average ± SD
Volume (ml)	8.44 ± 2.31
Color	Milky white
Smell	Typical
pH	6.5 ± 0.13
Consistency	Thin
Mass motility	++
Individual motility (%)	82.71 ± 4.46
Viability (%)	89.00 ± 3.39
Abnormality (%)	5.41 ± 5.41
Concentration (milion/ml)	944.78 ± 369.05
Total motile spermatozoa (milion/ml)	701 ± 274.53

Table 2. Semen quality after sexing

Treatments	Layer	Motility (%)	Viability (%)	Abnormality (%)	Concentration (milion)	Total Motile Sperm (million)
T1	Top	58.72 ± 11.47	69.40 ± 13.67	6.87 ± 1.49	78.10 ± 36.32	46.92 ± 28.37
	Bottom	77.34 ± 6.51	76.78 ± 7.63	4.20 ± 1.64	165.10 ± 39.84	128.29 ± 35.85
T2	Top	56.04 ± 11.06	65.43 ± 12.98	6.82 ± 2.86	64.50 ± 20.53	36.39 ± 13.02
	Bottom	72.30 ± 6.40	72.14 ± 10.25	4.77 ± 2.94	168.40 ± 50.54	123.34 ± 43.91
T3	Top	61.72 ± 8.76	65.25 ± 16.80	6.26 ± 2.33	60.80 ± 16.50	37.59 ± 12.26
	Bottom	77.86 ± 4.57	72.14 ± 10.25	4.22 ± 1.92	167.80 ± 45.99	131.10 ± 38.28

The difference in DNA mass of spermatozoa X and Y caused differences in spermatozoa weight and density. Based on these differences, spermatozoa X would be in the bottom layer and spermatozoa Y would remain in the top layer when centrifuged for separation [6]. The percentage of individual semen motility after sexing decreased compared to fresh semen. The decrease in motility in sexing treatment was caused by the centrifugation process, this was in accordance with Takdir et al. [10] that the decrease in sexing motility could be reduced by around 20% due to centrifugation treatment due to friction between spermatozoa and other spermatozoa and spermatozoa with the test tube. The results of the lower layer sexing had better values than the upper layer, due to the difference in the specific gravity of spermatozoa X which had a heavier mass than spermatozoa Y so that when centrifuged, spermatozoa X descends to the bottom faster than spermatozoa Y. Based on the average results in Table 2, the percentage of semen motility sexing in the upper and lower layers, the highest value was obtained at T3.

The percentage of semen viability after sexing T1 produced a higher value compared to other treatments both in the upper and lower layers. This was thought to be due to T2 and T3 having a thicker viscosity in each gradient, so that spermatozoa lose a lot of energy to penetrate the gradient which results in a lower percentage of viability. Nur et al. [12] explained that the decrease in the viability of spermatozoa that had undergone sexing was caused by reduced spermatozoa energy during the sexing process and was influenced by other factors in the form of environmental temperature and components contained in the medium used. Based on the cause, abnormalities were divided into 2, namely primary abnormalities and secondary abnormalities. Sexing semen abnormalities in each treatment did not show significant differences. The sexing process increases the percentage of semen abnormalities due to membrane damage. According to Novita et al. [13], factors that influence the high percentage of spermatozoa abnormalities apart from genetic factors include treatment during semen processing, handling of fresh semen, the review process, and the length of spermatozoa storage. In addition, the cause of high abnormalities could be caused by when making the smear preparation.

The average semen concentration after sexing T1 showed a higher value in the upper layer, while T2 showed a higher value in the lower layer but did not show any significant difference in each treatment. The decrease in spermatozoa concentration from fresh semen was caused by several factors such as being wasted with the supernatant, pipetting, dilution and remaining in the upper and lower diluent media. Total motile spermatozoa in T1 had a higher average compared to other treatments. This was because T1 had good motility and a high concentration of spermatozoa so that the total motile spermatozoa obtained was also higher. Total motile spermatozoa was influenced by spermatozoa that move progressively and the concentration of spermatozoa produced.

3.3   Proportion of X and Y Sperm

The proportion of X and Y spermatozoa was used to ensure the success of sexing using the PDGC method in addition to the sexing semen quality variable. The upper layer had a higher percentage of Y spermatozoa than the percentage of X spermatozoa, while the lower layer had a higher percentage of X spermatozoa than the percentage of Y spermatozoa. The average proportion of X and Y spermatozoa in each treatment was shown in Table 3.

Table 3. Average proportion of X and Y spermatozoa

Treatments	Layer	X (%)	Y (%)
T1	Top	22.40	77.60
	Bottom	84.80	15.20
T2	Top	15.00	75.00
	Bottom	81.80	18.20
T3	Top	24.20	75.80
	Bottom	79.10	20.90

Based on the average results of the separation of spermatozoa X and Y, T1 produced the highest proportion of separation in both the upper and lower layers. Factors that could affect the proportion of spermatozoa in the PDGC 10 gradient sexing method include the molecular weight of the density gradient (percoll, diluent), centrifugation time, centrifugation speed, and skill in using a micropipette (pippeting). In addition, the DNA content of spermatozoa X and Y had a difference of only around 3-4%, so there was very little difference in size between spermatozoa X and Y [14]. In the PDGC sexing method, the lower layer had a higher value compared to the upper layer because spermatozoa X was heavier than spermatozoa Y so that when centrifuged, spermatozoa X would settle to the bottom and spermatozoa Y would be on top.

4   Conclusion

The quality of sexing semen and the proportion of separation of X and Y spermatozoa using the PDGC 10 gradient method was better than the PDGC using 5 and 3 gradient sexing methods.

Disclosure of Interests. The authors have no competing interests to declare that are relevant to the content of this article.

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