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Quality of Polled Bali Bull Spermatozoa Results from Sexing Using the Bovine Serum Albumin (BSA) Column Method

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Abstract

Polled Bali cattle are a variant of Bali cattle whose horns do not grow naturally. The development of Bali polled cattle is crucial in reducing the risk of injury to livestock due to mutual antlers. Increased and efficient productivity of Bali polled cattle can increased by implementing Artificial Insemination (AI) technology using sexed semen. One factor in the success of artificial insemination is the quality of the semen used. This research aimed to determine the quality of polled Bali bull spermatozoa resulting from sexing using the bovine serum albumin (BSA) column method. This research used semen from three polled Bali bulls, which were then sexed using the 5% and 10% BSA column method. Spermatozoa resulting from sexing were evaluated microscopically in terms of viability, abnormalities, and intact plasma membrane (IPM) using an Olympus CX23 microscope as well as motility, progressive motility, and kinematics of spermatozoa using Computer Assisted Semen Analysis (CASA). The data obtained was analyzed using the independent T-test. The results showed that the motility, viability, abnormalities, and intact plasma membrane (IPM) of spermatozoa in the upper fraction were 37.64±5.26%, 56.60±4.24%, 14.83±3.90%, and 55.97±1.43%, respectively, while in the lower fraction, they were 46.39±1.56%, 61.13±2.10%, 16.21±3.37%, and 60.99±2.67% respectively, which are statistically significantly different ($P<0.05$) in motility, viability, and IPM parameters. Meanwhile, the progressive motility and kinematics of upper fraction and lower fraction spermatozoa obtained were statistically significantly different in the parameters of progressive motility, VAP, VCL, and VSL. The quality of Bali Polled bull spermatozoa resulting from sexing in the upper fraction is lower than in the lower fraction.

Keywords: Polled Bali bull, sexing spermatozoa, BSA column.

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1. Introduction

Bali Polled Cattle is a type of cattle developed and preserved as a native Indonesian livestock resource. Polled Bali cattle are a variant of Bali cattle, which naturally do not have horns, so have advantages in rearing management [1]. The development of Bali polled cattle is essential in reducing the risk of injury to livestock due to mutual antlers and safer for farmers [2]. Increasing the Bali polled cattle population can done using reproductive biotechnology, one of which is artificial insemination (AI), with the main aim of increasing the livestock population and improving efficiency in livestock management. One technology that can help farmers manage livestock birth according to the desired gender can be applied with AI using semen that separates spermatozoa with X and Y chromosomes [3]. Sex sorting in semen with the help of genetic selection of livestock can be a solution for breeders in reducing production costs and giving breeders options on the best way to improve the productive characteristics of their livestock [4]. Determining the sex of spermatozoa is often done by separating the X and Y chromosomes based on

differences in deoxyribonucleic acid (DNA) content, physical characteristics, macro proteins, and weight and motility of spermatozoa.

One method for determining the sex of spermatozoa is the bovine serum albumin (BSA) column method. The BSA column method can separate X and Y spermatozoa based on the shape, size, and motility of the spermatozoa and tends not to change the quality or morphology of the spermatozoa excessively. This step aims to prevent a decrease in the quality of spermatozoa after sex determination [5]. The quality of spermatozoa is an important factor determining the success of AI. The quality of spermatozoa is also influenced by factors such as age, animal health, feeding, and environmental conditions [6]. Previous research on the quality of fresh semen from Bali polled bull reported by Hasbi and colleagues, showed 73.33% motility, 92.73% viability, 4.01% abnormalities, and 76.58% membrane integrity [7]. However, data presenting the quality of Bali polled bull spermatozoa resulting from the separation of spermatozoa with X and Y chromosomes have not been widely reported. This research aims to determine the quality of spermatozoa of polled Bali bull resulting from sexing using the 5% and 10% BSA column methods. It is hoped that the results will provide information regarding the quality of Bali polled bull spermatozoa resulting from the separation of spermatozoa with X and Y chromosomes, which can be used as reference material in developing AI.

2. Materials and Methods

2.1. Ethical approval

The Animal Ethics Commission of Hasanuddin University approved this study (approval number: 302/UN4.6.4.5.31/PP36/2021).

2.2. Research Materials

The material used in this research was semen originating from three Bali polled bulls aged 5-8 years kept at the Technical Implementation Unit for Artificial Insemination and Semen Production Services (UPT PIBPS) Pucak, Maros, Indonesia. The bulls kept in individual cages measuring 2.5×2 m with feed and drink containers. Feeding carried out in the morning and evening as fresh forage 10% of body weight and concentrates 2 kg/head/day with drinking water ad libitum.

2.3. Research Methods

2.3.1. Sample Collection and Evaluation

Semen collected every week in the morning using an artificial vagina. The bull is first allowed to false mount 3-5 times before being deposited; then, the semen is collected using an artificial vagina and taken at the first ejaculate. After collecting samples, they are then evaluated in the laboratory according to the SOP at UPT PIBPS, macroscopically including smell, volume, color, consistency, and pH, while microscopically including mass motility (+, ++ and +++), individual motility (>70%) using Olympus CX23 microscope (Japan) and semen concentration using an SDM 6 spectrophotometer (Minitube®, Tiefenbach, Germany) [8].

2.3.2. Sexing Spermatozoa

Sexing of spermatozoa carried out using the 5% and 10% BSA column method. A total of 2 mL of 10% BSA medium (lower fraction) put into a test tube, and then 2 mL of 5% BSA medium (upper fraction) was added slowly until the total volume was 4 mL. 1 mL of semen was slowly inserted into a tube containing sexing medium and then incubated for 40 minutes. Each fraction was separated in a different centrifuge tube and centrifuged at 1800 rpm for 10 minutes. The remaining pellets from centrifugation were washed using 1 mL of Andromed diluent and then centrifuged again. The supernatant was discarded, and the pellet was added with 2 mL of Andromed diluent and then homogenized. The sample placed in a 0.25 mL mini straw and frozen in liquid nitrogen at -196°C [9].

2.3.3. Evaluation of Spermatozoa Quality

Evaluation of the quality of spermatozoa resulting from sexing includes evaluation of motility, viability, abnormalities, intact plasma membrane (IPM), progressive motility, and kinematics. Motility, progressive motility (p.mot), and kinematics of spermatozoa were observed using computer-assisted semen analysis (CASA) with the Sperm Vision Program application (Minitube®, Tiefenbach, Germany). 3-5 µL of the sample was dropped on a glass object, covered with a cover glass, and then observed under a microscope (200× magnification in 4 fields of view). Kinematic parameters of spermatozoa include distance average path (DAP: µm/second), straight linear velocity (DSL: µm/second), distance curve linear (DCL: µm/second), average path velocity (VAP: µm/second), distance straight line (DSL: µm/second), curvilinear velocity (VCL: µm/second), linearity of forward progression (LIN: VSL/VCL). Straightness (STR: VSL/VAP), average lateral head displacement (ALH: µm), beat cross frequency (BCF: Hz). In addition, wobble (WOB: VAP/VCL) [10]. The viability and abnormalities of spermatozoa were observed using the smear preparation method with eosin-nigrosin staining.

A sample of 2 µL was added with 10 µL of eosin nigrosin dye and homogenized; smear preparations were made, dried on a heating table, and observed under a microscope (400× magnification at 10 fields of view/minimum 200 spermatozoa cells). Evaluation of spermatozoa viability characterized by the spermatozoa heads absorbing color, indicating dead spermatozoa. Evaluation of spermatozoa abnormalities characterized by abnormalities in the head, neck, and tail of the spermatozoa [8]. The Hypo-Osmotic Swelling Test (HOST) observed the intact plasma membrane of spermatozoa. The sample and HOST solution were put into a microtube in a ratio of 1:10, homogenized and incubated at 37°C for 30 minutes. The sample was dropped on a glass object, covered with a cover glass, and then observed under a microscope (200× magnification in 10 fields of view/minimum 200 spermatozoa cells). Coiled or swollen tails characterize the evaluation of spermatozoa with normal membranes, while spermatozoa with damaged membranes are characterized by straight tails [11].

2.4. Statistical Analysis

This research uses independent T-test statistical analysis with the IBM SPSS Statistics for Windows program, Version 16.0 (IBM Corp., NY, USA). The results obtained for all parameters are presented as mean ± standard deviation, with a P value of less than 0.05 indicating statistical significance.

3. Results and discussion

Based on the data presented in Table 1 shows that the percentage of spermatozoa motility of polled Bali bull resulting from sexing in the upper fraction was 37.63±5.26%, while in the lower fraction, it was around 46.39±1.56%, which was statistically significantly different (P<0.05). These results show that spermatozoa motility in the upper fraction is lower than in the lower fraction. The low percentage of motility in the upper fraction compared to the lower fraction in this study shows that the lower fraction contains more Y spermatozoa, and the upper fraction contains more X spermatozoa. Y spermatozoa have higher motility than X, which causes Y spermatozoa to move upper and lower fraction layers. X spermatozoa contain 4% more DNA than Y spermatozoa, and the shape and head size of Y spermatozoa are smaller and rounder than X spermatozoa, which are large and elongated [12]. Oyeyipo and colleagues, added that Y spermatozoa have a smaller length, area, head circumference, neck, and tail length than X spermatozoa, which makes Y spermatozoa able to swim faster and more progressively [13]. Several previous studies that carried out sexing spermatozoa using an albumin gradient identified that >85% of Y spermatozoa reached the lower fraction first, of which around 90-95% were motile [27].

The percentage of motility in this study was lower than the 52.79% motility of Bali polled bull spermatozoa without sexing [7]. This study's low percentage of motility was caused by the sexing process, which took a long time. The spermatozoa released a lot of energy, which over time caused the energy of the spermatozoa resulting from sexing to decrease, resulting in reduced motility or even death. The percentage of viability in the upper fraction was 56.60±4.24%, while in the lower fraction, it was 61.13±2.10% (Table 1), which was statistically significantly different (P<0.05). Viable spermatozoa are characterized by the head not absorbing color, while dead ones absorb color (Figure 1B). The results of this study showed that the percentage of spermatozoa viability in the lower fraction was higher than in the upper fraction. The high viability percentage in the lower fraction positively correlated with the percentage of spermatozoa motility in the lower fraction, which also showed a high rate. Azzahra and colleagues, stated that viability is associated with motility, which is determined by the strength of the spermatozoa plasma membrane [28]. The death of spermatozoa is closely related to damage to the plasma membrane [14].

An intact spermatozoa plasma membrane will help spermatozoa to survive and prevent increased reactive oxygen species (ROS) production [15]. The freezing process can cause damage/death of spermatozoa. Freezing spermatozoa aims to suppress the activity of spermatozoa cells so they can survive longer. However, the freezing process of spermatozoa resulting from sexing can cause cold shock or damage and even death of spermatozoa due to a decrease in temperature, with a percentage of cell death between 20-80% [16]. Apart from death due to cold shock, thawing is also one of the causes of spermatozoa death due to the large amount of lactic acid from the metabolism of spermatozoa, which cannot be oxidized. The buildup of lactic acid increases the acidity level of the solution, which is terrible for spermatozoa because it is toxic [17]. Data in Table 1 shows that the percentage of sperm abnormalities resulting from sexing in the upper fraction was 14.83±3.90%, and in the lower fraction, it was 16.21±3.37%, which was not statistically significantly different (P>0.05).

The percentage of abnormal spermatozoa in this study was still within the normal range because it was <20%. Sabeti and colleagues, stated that spermatozoa abnormalities <20% can still be used for insemination [18]. Abnormal spermatozoa morphology will be active in producing ROS. ROS produced by abnormal spermatozoa will be the leading cause of infertility [19]. Abnormalities are closely related to chromosomal abnormalities, chromosome structure, and low DNA quality, which can be observed in spermatozoa's head, neck, and tail (Figure 1D). Abnormalities in the head of the spermatozoa will affect the male's fertility level because they are related to the genetic material in the head of the spermatozoa. Meanwhile, abnormalities in the spermatozoa tail will influence spermatozoa motility because the spermatozoa tail helps the spermatozoa move progressively [20]. The percentage of the intact plasma membrane in the upper fraction was lower, namely 55.97±1.43%, compared to 60.99±2.67% in the lower fraction (Table 1), which was statistically significantly different (p<0.05). This shows that spermatozoa in the lower fraction have better metabolism and membrane integrity than spermatozoa in the upper fraction.

This study's results align with research reported by [29] that spermatozoa in the lower fraction have high motility, indicating that these spermatozoa have good membrane integrity and metabolism. The plasma membrane can influence the physiological function and metabolism of spermatozoa. Spermatozoa with coiled or swollen tails characterize spermatozoa with normal membranes, while spermatozoa with damaged membranes are characterized by straight tails (Figure 1C). Damage to the plasma membrane disrupts the metabolic and physiological processes of spermatozoa, causing the death of spermatozoa. The intact of the plasma membrane is also highly correlated with the motility of spermatozoa. If the spermatozoa plasma membrane is damaged, the metabolism of the spermatozoa will be disrupted, causing the spermatozoa to lose its power of movement and resulting in cell death. Spermatozoa metabolism can affect spermatozoa viability. High metabolic activity in spermatozoa can produce high levels of lactic acid, killing spermatozoa [14].

Sexing spermatozoa logically disrupts cell function physically and biochemically, which triggers stress in sperm cells [4-24]. Apart from that, the cryopreservation of spermatozoa resulting from sexing creates an additional burden on the spermatozoa cells, triggering high ROS production [4]. ROS produced in mitochondria and spermatozoa plasma membranes. A large number of mitochondria are found in the center of spermatozoa cells, which function in delivering energy to support spermatozoa motility. Mitochondrial dysfunction due to increased ROS production will affect mitochondrial integrity, and a reciprocal cycle will occur, which causes increasingly severe mitochondrial damage [19]. Based on the results of research on spermatozoa quality using CASA (Table 2), the percentage of progressive motility in the upper fraction was 30.73%, and the lower fraction was 42.22%, which was statistically significantly different (P<0.05). The progressive motility of spermatozoa in the lower fraction was higher than in the upper fraction. Spermatozoa motility is the number of spermatozoa cells that are alive and moving forward or progressively (Figure 1A).

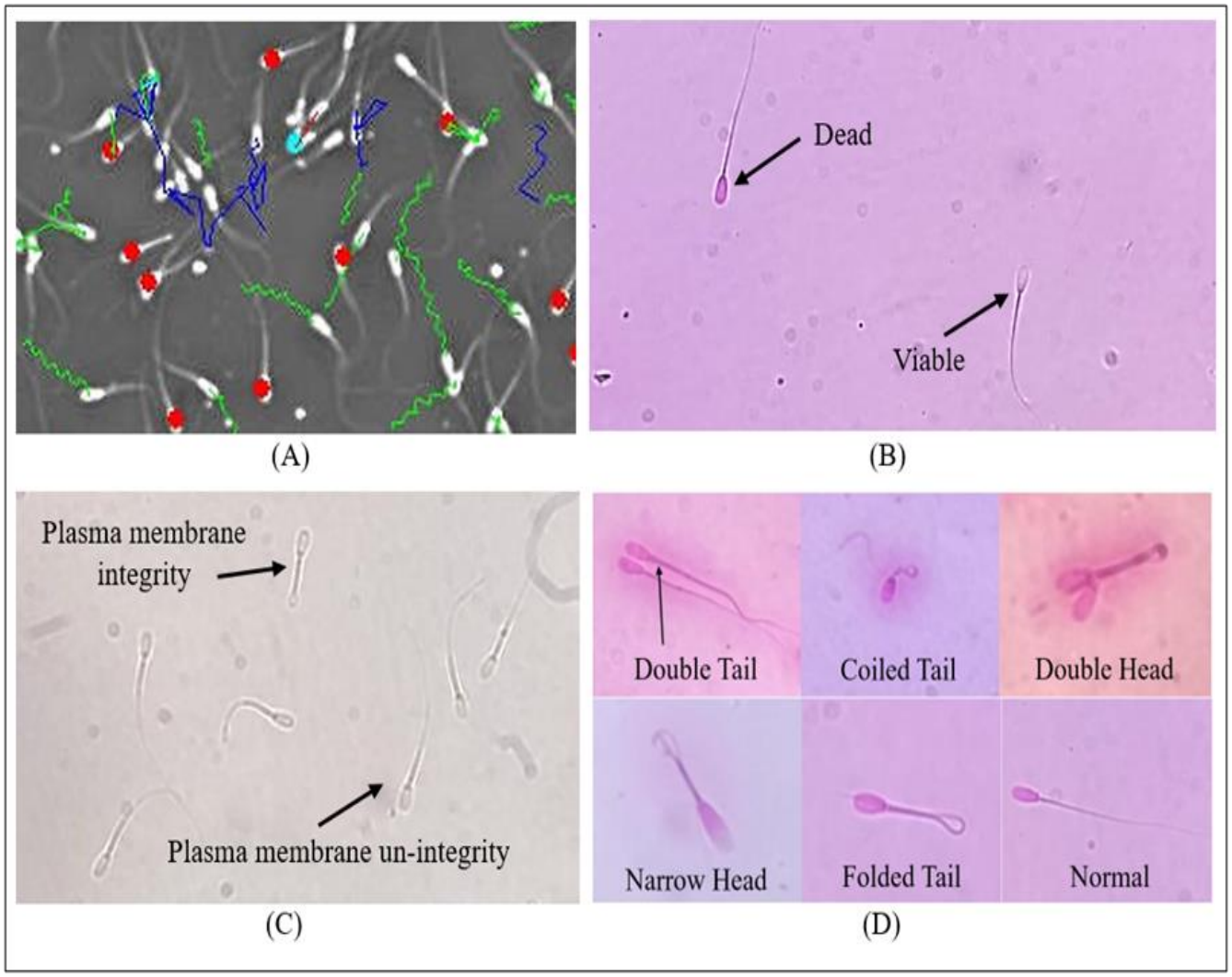

Figure 1. Polled Bali bull spermatozoa as a result of sexing (A) Kinematics of CASA spermatozoa; Green line= progressive motility, cyan line= local motility, red line= immotile and blue line= hyperactive; (B) Viability of spermatozoa; (C) Intact plasma membrane of spermatozoa; (D) Abnormalities of spermatozoa

Table 1. Polled Bali bull semen quality resulting from sexing

Parameter	Upper fraction (Mean ± SD)	Lower fraction (Mean ± SD)
Motility	37.63±5.26[a]	46.39±1.56[b]
Viability	56.60±4.24[a]	61.13±2.10[b]
Abnormality	14.83±3.90	16.21±3.37
Intact plasma membrane	55.97±1.43[a]	60.99±2.67[b]

[a,b,c] different superscripts on the same line indicate differences (P<0.05)

Table 2. Kinematics of polled Bali bull spermatozoa as a result of sexing

Parameter	Upper fraction (Mean ± SD)	Lower fraction (Mean ± SD)
P.MOT (%)	30.73±6.04[a]	42.22±2.32[b]
VAP (µm/s)	66.65±8.11[a]	80.60±15.66[b]
VCL (µm/s)	97.18±15.89[a]	113.13±14.92[b]
VSL (µm/s)	51.40±9.44[a]	65.88±17.82[b]
ALH (µm)	3.40±0.71	3.41±0.70
STR (%)	0.76±0.05	0.80±0.06
LIN (%)	0.52±0.04	0.57±0.08
WOB (%)	0.68±0.04	0,70±0.06
BCF (Hz)	27.90±3.62	29.78±2.60

[a,b,c] different superscripts on the same line indicate differences (p<0.05)

The movement of spermatozoa is influenced by mitochondrial function, which produces adenosine triphosphate (ATP), an important energy source in increasing spermatozoa motility. However, mitochondrial function will hampered due to temperature changes, which will cause a decrease in the motility of spermatozoa [25]. Spermatozoa motility is essential because it is needed to move forward in the female genital tract, which then fertilizes the ovum. Progressive motility indicates normal spermatozoa that move straight forward and are more fertile [26]. The VAP, VCL, and VSL values of polled Bali cattle spermatozoa resulted from sexing in the results of this study. Which are presented in Table 2, are statistically significantly different ($P<0.05$) meanwhile ALH, STR, LIN, WOB, and BCF in the upper and lower fractions were statistically significantly different ($P>0.05$). The success of spermatozoa fertilization can determined by motility in speed and kinematics, so assessing spermatozoa kinematics helps predict spermatozoa fertility [21]. Soler and colleagues, stated that the VAP, VCL, VSL, ALH, and BCF values are the main parameters of spermatozoa kinematics, while the LIN, STR, and WOB values are ratio measures of the main parameters [22]. The results of measuring spermatozoa kinematics in this study showed the percentage of spermatozoa motility classified as motile. Aghazarian and colleagues, stated that the percentage of motile spermatozoa has a VAP value of >25 µm/s and STR>80% with a limit value for slow motility, namely VAP<5 µm/s and VSL<11 µm/s [23]. Measuring spermatozoa kinematics shows a significant relationship with spermatozoa vitality and can predict DNA fragmentation and spermatozoa membrane integrity.

4. Conclusions

Based on the results obtained in this study, it can concluded that the quality of Bali polled bull spermatozoa decreased after the sexing process carried out. However, the quality of the spermatozoa in the lower fraction is higher than that in the upper fraction, which indicates that the lower fraction contains more Y spermatozoa, and the upper fraction contains more X spermatozoa.

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