Quality and Proportion of Friesian Holstein Bull Sperm Using Egg White Albumin Sedimentation Sexing Method with Different Gradients

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Abstract. This study aims to evaluate the effect of different gradients on the quality and proportion of sexed semen using the egg white albumin sedimentation method. This study was conducted at the Singosari Artificial Insemination Center in Malang, using Friesian Holstein semen with an average fresh motility of >70%. The study was conducted experimentally with 10 replicates and 2 treatments, namely T1 = egg white sedimentation sexing with 2 gradients (30% and 50%) and T2 = egg white sedimentation sexing with 3 gradients (30%, 40%, and 50%). The parameters observed included motility, viability, abnormality, membrane integrity, and the proportion of X and Y chromosomes. The quality evaluation results were analyzed using an independent t-test, while the determination of the spermatozoa proportion was analyzed using the Chi-Square test. The results showed that differences in gradients did not have a significant effect ($p < 0.05$) on any quality parameters in each layer. In addition, the proportion of X and Y spermatozoa also showed no difference between the upper and lower fractions in both layers ($p > 0.05$). In conclusion, spermatozoa separation using 3-gradient egg white albumin sedimentation tended to provide more efficient separation while maintaining spermatozoa quality, although no significant differences were observed compared to the 2-gradient method.

Keywords: Egg White Albumin, Friesian Holstein Bull, Gradient-Based Separation, Semen Quality, Sperm Sexing.

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1 Introduction

Friesian Holstein (FH) cattle are among the most widely bred dairy breeds in Indonesia due to their high milk production potential. However, domestic production remains insufficient to meet national milk demand optimally. National milk demand reaches approximately 4.3 million tons per year, while domestic production accounts for only about 1 million tons, meeting merely 18–20% of national demand [1]. This gap has driven efforts to increase dairy cattle populations and productivity to enhance the domestic milk supply.

One technology that has made an important contribution to increasing the population of female livestock is Artificial Insemination (AI) using sexed semen, which is semen that has been separated based on X and Y chromosomes. The use of sexed semen increases the proportion of female calf births, thereby supporting the long-term improvement of national milk production. As technology advances, various methods of sperm sexing have been developed, including the albumin column sedimentation method, Percoll density gradient centrifugation, Sephadex column, and flow cytometry [2].

The albumin sedimentation method is a relatively simple, economical technique that does not require expensive equipment. Generally, this method uses Bovine Serum Albumin (BSA) as a separation medium. Previous studies on Garut sheep showed that the BSA column method was effective as a sperm separation technique, with the capability to maintain sperm quality and achieve optimal fertility rates [3]. However, because BSA is an imported material at a high price, it is necessary to find a more efficient, readily available alternative. One potential substitute for BSA is egg white albumin, which has a similar protein content and physicochemical properties to BSA.

Research on using chicken egg white albumin as a separation medium for sperm has demonstrated its effectiveness in sorting sperm by sex and maintaining sperm quality in several livestock species. Research comparing the Percoll Density Gradient Centrifugation (SGDP) sexing method and albumin sedimentation using egg whites shows that the sedimentation method produces better sperm quality, with higher motility and lower abnormality rates [4]. The use of egg white albumin may reduce production costs while supporting local resource efficiency, without adversely affecting the effectiveness of the sperm separation process. The number of gradients used in the sedimentation process is a key factor influencing separation efficiency and spermatozoa quality. Therefore, further investigation into the effects of different numbers of egg white albumin gradients on the quality of sexed spermatozoa is required to determine the optimal separation conditions.

This study is limited to analysing sperm quality after sexing using the albumin sedimentation method with different gradients. The results are expected to provide recommendations for using egg white albumin as a substitute for BSA and to become a standard protocol in applying the albumin sedimentation method for sperm sexing. In addition, these results are expected to form the basis for further research to develop more efficient and applicable sexing technology in livestock reproduction.

2 Materials and methods

2.1 Materials

The study was conducted using fresh semen from Friesian Holstein (FH) bulls that met the following quality criteria: minimum mass motility of ++, individual motility ≥ 70%, and sperm concentration of 600-2000 million per mL^{-1}. The materials used included 3% NaCl solution, eosin-nigrosin stain, HOST solution, TAU solution, egg white albumin, Trico-egg yolk buffer, Trico-egg yolk diluent, and distilled water.

2.2 Methods

2.2.1 Semen Evaluation and Treatment

Fresh FH semen collected by the BBIB Singosari bull master was then evaluated macroscopically and microscopically. Fresh semen that meets the criteria will be further processed for sexing by dividing it into several treatments, including: T1 = Sexing by 2-gradient egg white sedimentation (30% and 50%) and T2 = Sexing by 3-gradient egg white sedimentation (30%, 40%, and 50%).

2.2.2 Albumin Sedimentation Sexing Procedure

The albumin sedimentation sexing method with different gradients refers to the procedure by Susilawati[5], as follows:

1. Prepare the sexing medium, which is egg white, in test tubes according to the treatment.
2. Add the diluent with a volume corresponding to the gradient in the test tube.
3. Homogenize and then arrange the gradient layers from the highest to the lowest gradient using a micropipette slowly.
4. Pour 1 mL^{-1} of semen into the test tube containing the gradient.
5. Incubate for 30 minutes at room temperature.
6. Prepare a test tube containing 3 mL^{-1} of diluent.
7. After incubation, discard 0.5 mL^{-1} from the top, then take 2 mL^{-1} of the bottom and top fractions.
8. Centrifuge at 1600 rpm for 10 minutes.
9. Take 3.5 mL^{-1} of the supernatant (upper part) and sediment (lower part) for testing the quality and proportion of spermatozoa resulting from the separation.

2.2.3 Sexing Semen Evaluation

Semen that has been sexed is then evaluated microscopically for motility, viability, abnormalities, concentration, membrane integrity, and the proportion of X and Y chromosomes. Motility is assessed by placing a drop of the sample on a slide and observing it under a microscope. Viability and abnormality assessments use the same preparation with eosin-nigrosin staining. Concentration assessment is calculated using a hemocytometer, and spermatozoa count is based on the calculation method described by Susilawati[6] :

$$\text{Sperm count/ml} = N \times 5 \times FP \times 10.000$$

Plasma membrane evaluation was performed using a Hypoosmotic Swelling Test (HOST) solution with a 1:10 ratio. Membrane integrity was calculated using the formula[7].

$$\text{IPM (\%)} = \frac{\text{Total reactive spermatozoa count}}{\text{Total sperm count}} \times 100\%$$

2.3 Data analysis

The results of motility, viability, abnormality, concentration, and membrane integrity evaluations were analyzed using an *independent t-test.* In contrast, the determination of the proportions of X and Y spermatozoa was analyzed using the *Chi-Square test.* Results and Discussion.

3 Results and discussion

3.1 Fresh Semen Quality

The evaluation of fresh semen (Table 1) showed that the average semen volume was 7.99 ± 2.43 mL^{-1} with a milky white color and a typical semen smell. The pH value of 6.48 ± 0.28 indicated normal semen quality and fell within the ideal range for the bull's semen (6.4–6.8).

Table 1. Fresh Semen Quality

Parameter	Average ± SD
Volume (mL^{-1})	8.77 ± 3.14
Color	Milky white
Smell	Thypical
pH	6.49 ± 0.23
Consistency	Thin
Individiual motility (%)	70.97 ± 1.40
Viability (%)	86.72 ± 2.22

Abnormality (%)	6.02 ± 2.00
Concentration (milion/mL^{-1})	1529 ± 349.145
IPM (%)	83.11 ± 7.47

The motility of individual spermatozoa in fresh semen was 70.25 ± 2.43%, indicating acceptable motility quality. Sperm viability was 80.6 ± 1.70%, reflecting a high proportion of live spermatozoa. The low abnormality rate (6.14 ± 0.44%) indicates normal spermatozoa morphology. The sperm concentration was 1513.2 ± 249.12 million/mL^{-1}, indicating adequate fertility potential, while the IPM value of 83.76 ± 7.43% reflected good plasma membrane integrity. According to the Indonesian National Standard [8], the minimum motility required for further processing is 70%, and post-processing spermatozoa must exhibit motility above 40%.

3.2 Semen Sexing Quality

The results of semen quality evaluation after sexing using two and three egg white gradients are presented in Table 2. Overall, semen quality parameters decreased compared to fresh semen but remained within acceptable limits for artificial insemination. Previous studies have reported that semen-handling processes, such as dilution, cryopreservation, and oxidative reactions during cooling, as well as environmental temperature, can affect sperm motility [9]

Table 2. Semen Sexing Quality

Treatment	Layer	Motility (%)	Viability (%)	Abnormality (%)	Concentration (Juta/mL^{-1})	IPM (%)
T1	Top	51.9 ± 7.6	78.4 ± 2.84	7.9 ± 1.57	85 ± 45.18	78.5 ± 2.75
	Buttom	55.5 ± 8.1	77.1 ± 1.87	5.9 ± 1.14	46 ± 12.76	72.8 ± 2.65
T2	Top	55.1 ± 9.1	78.0 ± 1.79	6.0 ± 1.13	89.25 ± 13.10	78.4 ± 2.18
	Buttom	53.6 ± 6.7	79.0 ± 2.86	6.7 ± 1.24	46.63 ± 17.03	71.1 ± 4.15

The results show that differences in gradients in both the upper and lower layers had no significant effect ($P \geq 0.05$) on motility, viability, abnormality, concentration, and IPM parameters. Sperm motility after sexing ranged from 51.9 ± 7.6% to 55.5 ± 8.1%, with the highest value obtained in the lower layer of treatment T2. The relatively higher motility observed in the lower layer of T1 suggests that spermatozoa retained in this layer may possess better motility characteristics as a result of the sedimentation process. Spermatozoa viability after sexing ranged from 77.1 ± 1.87% to 79.4 ± 1.79%, with no significant differences between treatments. These values remained above 70%, indicating that the egg white sedimentation method did not substantially impair sperm viability. Spermatozoa abnormalities remained low (5.9–7.9%), indicating that spermatozoa structure remained stable after the sexing process. Although the albumin sedimentation method produces good quality, it requires proper incubation and centrifugation protocols to minimize sperm quality loss during the sexing process[10]. Studies on Bali cattle show that the use of albumin, both in fresh and freeze-dried, maintains sperm quality parameters such as motility, viability, membrane integrity, and acrosome without significant differences between both media [11].

Sperm concentration after sexing ranged from 46.6 ± 17.03 to 89.5 ± 13.10 million/mL^{-1}, with the highest value observed in the upper layer of treatment T2. This indicates that spermatozoa are distributed differently in Pesisir cattle. Sexing using bovine serum albumin (BSA) columns resulted in an average sperm concentration of 1.58 ± 1.05 × 10^9 mL^{-1} in fresh semen, followed by a 20–30% reduction in sperm quality and concentration after sexing [12]. The intact plasma membrane (IPM) value ranged from 71.1 ± 4.15% to 78.5 ± 2.75%, indicating that plasma membrane integrity remained good. Overall, these results indicate that both the two- and three-gradient egg white sedimentation methods maintain the quality of sexed semen. Previous studies have shown that plasma membrane integrity can be maintained following the separation process, although a slight decrease may occur [13]. The upper layer (generally containing X spermatozoa) was 55.97%, and the lower layer (generally containing Y spermatozoa) was 60.99%, with a statistically significant difference between the two layers. Egg white albumin contains proteins, essential amino acids, phospholipids, and antioxidants that protect the sperm cell membrane from oxidative damage and osmotic stress. These components help maintain membrane integrity, prevent lipid peroxidation, and provide energy for sperm during the sex separation process [14].

3.3 The Proportions of X and Y Spermatozoa

The proportions of X and Y spermatozoa obtained using the egg white albumin sedimentation method are presented in Table 3. In T1 (two gradients), the upper layer was dominated by X spermatozoa (87%), whereas the lower layer was dominated by Y spermatozoa (65%). In T2 (three gradients), the upper layer contained 78% X spermatozoa, while the lower layer contained 77% Y spermatozoa.

Table 3. Proportion of Spermatozoa X and Y

Treatment	Layer	X (%)	Y (%)
T1	Upper	87	13
	Lower	35	65
T2	Upper	78	21

	Upper	23	77

The observed differences in the distribution of X and Y spermatozoa between layers suggest that the egg white sedimentation method can separate spermatozoa based on density differences. Y spermatozoa, which are generally characterized by a smaller DNA content and higher motility, tend to migrate toward the lower layer. In contrast, X spermatozoa, with relatively higher DNA content, tend to remain in the upper layer [5]. However, *Chi-Square* analysis showed that the differences in X and Y spermatozoa proportions between the two gradient systems were not statistically significant ($P \geq 0.05$)

Previous studies have demonstrated that two- or three-gradient egg white albumin sedimentation can enhance the separation of X and Y spermatozoa. However, the level of statistical significance achieved remains limited [15]. This indicates that increasing the number of gradients from 2 to 3 does not significantly improve the effectiveness of X and Y spermatozoa separation. However, in terms of semen quality preservation, T2 tends to provide a more balanced sperm distribution with relatively stable quality parameters.

4 Conclusion

In conclusion, fresh semen from Friesian Holstein bulls met the requirements for sexing. The use of two- and three-layer egg white albumin showed equal potential to maintain semen quality and enable the biological separation of X and Y spermatozoa. Therefore, the egg white albumin sedimentation method can be considered a simple and practical alternative to support artificial insemination programs.

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