The Effect of Incubation Time on The Quality of Sexing Simmental Cattle Semen with The BSA (Bovine Serum Albumin) Column Method

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Abstract. *Simmental has the advantage of good reproduction and meat production. In addition, Simmental cow semen has a better viscosity level than other cow semen, so it can be used in spermatozoa sexing technology. One method of sex separation of bovine spermatozoa is the BSA column method which is able to produce 75-80% Y sperm. This study aims to determine the quality of fresh Simmental bovine semen (macroscopic and microscopic tests) and sexing (motility, viability and proportion tests). The results of the macroscopic test of Simmental cattle's fresh semen in general, the cement used in this study had good quality. The color of normal cow semen has a yellowish white color. The smell of cow semen has a distinctive smell. The volume of semen in this study was 6 ml, medium consistency, and pH 6.8. The results of the microscopic test had a mass movement of 2 (++), a motility of 70 and a concentration of 1650±259.81. The motility of the X spermatozoa sexing results, the best incubation time is 45 minutes, the motility is 55.00±5.00, while the motility of the Y spermatozoa sexing results is the best 30 minutes, the motility is 45.00±11.18. The viability of the sexed X spermatozoa was 54.85±10.25 with an incubation time of 45 minutes, while the best sexed Y spermatozoa was 32.6±13.98 with an incubation time of 30 minutes..*

Keywords : *BSA, motility, simmental cattle, sperm sexing, viability*

1. INTRODUCTION

Demand for beef is expected to continue to increase in line with national economic growth and public awareness of the importance of animal protein. One effort that can be done to meet these needs is to increase the cattle population through artificial insemination. Increasing the value of artificial insemination can be done by producing superior seeds with sex according to the purpose of maintenance through sexing technology. Sexing technology is the process of separating X and Y spermatozoa, to obtain the desired calf birth (Susilawati, 2014).[1]Simmental cattle have the advantage of good reproduction and meat production. Simmental bovine semen has a better viscosity than other bovine semen, so it can be used in spermatozoa sexing technology.[2]One method of sex separation of bovine spermatozoa is the BSA

column method which is able to produce 75-80% Y sperm.[3]

The success of the sexing process using the BSA method can be influenced by several factors, one of which is the length of time for the incubation of spermatozoa. Incubation time that is too short will result in a small proportion of X and Y sperm, while too long an incubation time can cause X and Y spermatozoa to re-mix in layers of medium with different concentrations, in addition, there can be an increase in damage to spermatozoa cells so that it can reduce quality.[4] The longer the incubation time, the more free radicals produced by sperm.[5]

Research on the effect of incubation time on the quality of spermatozoa has been done previously; Ferlianthi[6] examined the semen of Ettawah goats, Anwar et al.[4] researching the semen of Boer goats, Rasad et al.[7] examined the sperm of Pasundan cows examined Bali cattle with the egg white sedimentation method. Research on the effect of incubation time on sperm quality of Simmental cattle using the BSA column method has never been done. This study aims to determine the quality of fresh Simmental cattle semen (macroscopic and microscopic tests) and sexing (motility, viability and proportion tests).

2. METHODS

This research was conducted at the Laboratory of Regional Artificial Insemination Center (BIBD) Sembawa, Banyuasin District, South Sumatra. Simmental bovine semen collection was carried out with an artificial vagina, once a week for one repetition.

Experimental design

The research method used a completely randomized design, consisting of 3 treatments with each treatment repeated 4 times. The treatments were time difference with 5% and 10% BSA concentrations. P1: 30 minutes incubation time; P2: 45 minutes incubation time and P3: 60 minutes incubation time.

Semen evaluation

Evaluation of fresh semen includes macroscopic tests (color, odor, volume, consistency and pH) and microscopic tests (mass movement, motility, concentration and viability). Evaluation of semen sexing includes motility and viability.

Spermatozoa separation and washing

Separation was carried out by inserting 2 ml of 10% and 5% BSA solution respectively into the tube, then adding 1 ml of cement for each concentration of the BSA column. The tubes were then incubated in a water bath at 37ºC for 30, 45 and 60 minutes. After incubation, 1 ml of the upper solution was removed and the next solution was separated based on the boundary between 5% and 10% solution concentration, the upper layer was labeled Y and the lower layer was labeled X. Next, centrifugation was carried out at 1500 rpm for 5 minutes. Then the supernatant fluid was discarded and continued with the observation of live spermatozoa with a microscope.

Data analysis

The data in the form of mean are presented in a table and analyzed by ANOVA, the significant difference was followed by Duncan's 5% test.[4].

3. RESULT AND DISSCUSSION

Fresh semen quality

The results of the macroscopic test of Simmental cattle's fresh semen in general, the cement used in this study had good quality. The color of normal cow semen has a yellowish white color. The smell of cow semen has a distinctive smell. The volume of semen in this study was 6 ml, medium consistency, and pH 6.8 (Table 1). This is in accordance with previous research which states that good cow semen has a yellowish white or cream color,[7,8]a distinctive odor,[7,9] semen volume 10−15 ml per ejaculation.[10] The volume of semen produced from a bull in one ejaculation is quite variable and depends on age, weight, reproduction, health, feed quality, frequency of holding and breed of cattle.[10]

Table 1. Evaluation of Simmental's fresh semen

Parameter	Rataan
Uji Makroskopis	
Warna	Yellowish white
Bau	Local typical
Volume	6.00±0.00
Konsistensi	medium
pH	6.88±0.22
Uji Mikroskopis	
Mass	++

Motility	70.00±0.00
Concentration	1650.00±259.81

The consistency and concentration of cement in this study were 6.88±0.22 and 1650±259.81. Toelihere[10] stated that cow semen with cream consistency had a concentration of 1000×10^6–2000×10^6 cells/ml. This is in line with the statement of Garner and Hafez[11] which stated that the concentration of bovine spermatozoa ranged from $800x10^6$–$2000x10^6$ cells/ml. The pH in this study was normal (6.88±0.22) although it was slightly higher than the results of the research by Rasadet al.[7]which was believed to be caused by differences in cattle breeds. Feradis[12]states that each breed of cattle has a different acidity or pH of semen. Ashariet al.[13]stated that the pH of good quality cement was between 6.8–6.7. Fatah[14] stated that fresh semen had a pH between 6.4–7.8, while stated that the pH of fresh cow semen was 6.8±0.45. According to Garner and Hafez[11] stated that normal cow semen has a pH of 7.

The average mass movement and motility of spermatozoa in this study were classified as good (++) and had met the standard as a liquid cement making material. The more spermatozoa that move progressively, the better the quality of the spermatozoa.[14]Ervandiet al.[15] stated that the standard in the manufacture of liquid cement for mass movement is ++ to +++ and motility 70%.

Sexing semen quality

The average motility of the X spermatozoa sexing results, the best incubation time is 45 minutes, the motility is 55.00±5.00, while the motility of the Y spermatozoa with the best sexing results is 30 minutes, the motility is 45.00±11.18 (Table 2). When compared with the motility of fresh semen, the motility of spermatozoa resulting from sexing decreased and was much smaller than the results of research by Rasadet al.[7]. In addition, the entire incubation time did not show a significant difference in the motility of spermatozoa.

Motility is one of the main indicators to determine the quality of spermatozoa. Motility is the ability to move spermatozoa. Sexing with the albumin column method is based on the difference in motility between spermatozoa X and spermatozoa Y.[16-18]

Table 2**.** Rataan motilitas dan viabilitas spermatozoa X dan Y hasil sexing

Waktu Inkubasi	Motilitas		Viabilitas	
	X (%)	Y (%)	X (%)	Y (%)
30 Minute	30.00±12.25[a]	45.00±11.18[a]	27.35±13.71	32.60±13.98
45 Minute	55.00±5.00[a]	32.50±14.79[a]	54.85±10.25	24.80±10.74
60 Minute	22.50±16.39[a]	22.50±8.29[a]	26.00±21.06	10.70±4.50

Superscript the same on the same line shows that the effect is not significantly different (P>0.05).

According to Susilawatiet al.[1] the decrease in spermatozoa motility was due to the influence of incubation time, temperature during the sexing process and the influence of the mechanism during centrifugation. During the incubation process there is an increase in hydrogen peroxide (H_2O_2) levels which have toxic properties for sperm cells and damage the plasma membrane, thereby reducing sperm motility.[19] Longer incubation time causes an increase in metabolism, so that spermatozoa lose energy which affects the decrease in motility.[20] A lot of energy is used by spermatozoa after treatment to continue to normalize their physiological conditions.[16] According to Susilawati[5] the energy needed by sperm during the sexing process comes from both aerobic and anaerobic cell metabolism, high energy requirements can increase the intensity of cell metabolism which ultimately causes an increase in sperm oxygen consumption. Decreased motility is also caused by various changes that occur simultaneously followed by physiological acrosomal reactions with more energy used by spermatozoa to always move.[21]

Anwar et al.[4] stated that an incubation time that is too short will result in a small proportion of X spermatozoa and Y spermatozoa, while too long an incubation time can cause damage to sperm cells, thereby reducing their quality. The longer the incubation time, the more free radicals produced by sperm. Free radicals are by-products of sperm metabolism.Centrifugation of spermatozoa during washing during the sexing process causes a decrease in motility.[17,22]Added Ferlianti[6] that centrifugation causes friction between the spermatozoa and the media used during separation which can damage the structure of the spermatozoa cell membrane.

The average viability of the sexed X spermatozoa was 54.85±10.25 with an incubation time of 45 minutes, while the best sexed Y spermatozoa was 32.6±13.98 with an incubation time of 30 minutes (Table 2). Viability observations were carried out to determine whether spermatozoa

were alive or dead. The viability of sperm is closely related to its motility, if the motility decreases, the viability also decreases. Yadavet al.[23]stated that there is a relationship between the viability and motility of spermatozoa. According to Feradis[12] the smaller the viability of the spermatozoa, the smaller the motility of the spermatozoa. Sunartiet al.[20]stated that the quality of semen is determined by the size of the number of live spermatozoa, the larger the live spermatozoa, the better the quality of the semen. The percentage of spermatozoa motility in this study was relatively small. The decrease in the quality of spermatozoa is believed to occur due to damage to the membrane structure due to a series of treatment processes. As stated by Susilawati[1] that the procedure for separating X and Y chromosomes can cause damage to the structure of the sperm cell membrane which in turn will reduce the quality of the spermatozoa.

According to Hafez[3] the value of spermatozoa viability must be more than 50% and still declared normal and feasible to use. Live and dead spermatozoa can be distinguished by their reaction to certain colors, non-motile and dead spermatozoa cells suck color and motile and live spermatozoa cells are colorless (Figure 1). The dye commonly used is eosin negrosin. Spermatozoa that are alive have good membranes, so dye cannot enter, while dead spermatozoa are membranes that don't work, so dye can enter the membrane of spermatozoa.

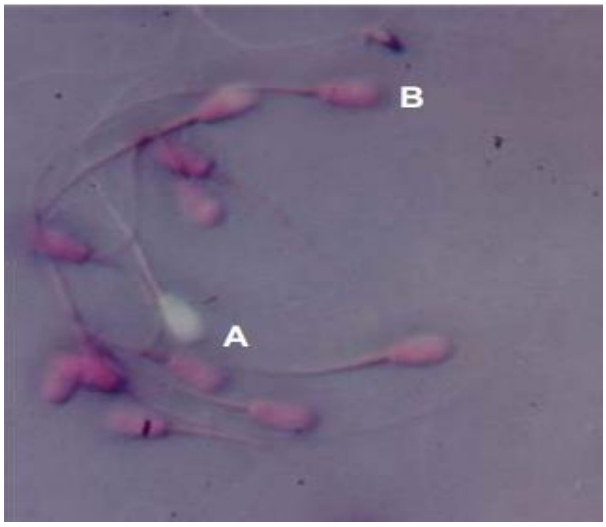

Figure 1. Bovine spermatozoa appearance with eosin negrosin staining. (a) live spermatozoa, (b) dead spermatozoa.

Protection of the acrosomal reaction requires the right medium at the time of sexing. Bovine Serum Albumin is a macromolecule that plays a role in preventing and binding the entry of excessive Ca^{2+} into the cytosol and protecting the spermatozoa membrane so as to minimize spermatozoa that undergo capacitation and early acrosomal reactions.[24] According to Purwoistri et al.[18] BSA also allows the membrane to be more effective in regulating the movement of calcium across the membrane and inhibits the accumulation of intracellular Ca^{2+} to levels that are toxic to spermatozoa so that the motility of uncapacitated spermatozoa can be maintained high. In vitro capacitation media needs to be done to increase the level of capacitation, acrosome reaction and fertilization. The media in question is one of them BSA (Bovine Serum Albumin).[23]

The sexing process with BSA did not reduce the ability of spermatozoa to fertilize so it was judged that BSA as the medium used in the sexing process could not damage the spermatozoa. The technique of separating spermatozoa with BSA is considered not to manipulate spermatozoa excessively so that it is expected to be able to prevent a decrease in the quality of spermatozoa after the separation process .[25] However, according to Uysal and Bucak[26] that spermatozoa in media containing BSA for more than four hours are feared to cause capacitation and premature acrosome reactions.

Susilawati[5] suggested choosing a diluent to reduce the decrease in viability. Susilawati[1] also stated that sexing requires a diluent that is able to protect and provide an optimal environment for spermatozoa, so that the quality of spermatozoa can be maintained. According to Mishraet al.[27]BSA contains 20 amino acids that can maintain the stability of spermatozoa cell membranes while also containing growth factors that are important for the life of spermatozoa. According to Uysal and Bucak[26], the function of BSA in diluent is to protect the integrity of the spermatozoa membrane and the environment, for example in hot conditions or in oxidative reactions. With the addition of BSA in the diluent, it is expected to be able to maintain the stability of the spermatozoa cell membrane.[28] Semen plasma proteins mainly play a role in maintaining the viability of spermatozoa and the fertilization process.[29] Meanwhile, according to Hafez and Hafez[29] stated that semen plasma proteins can stabilize the membrane until a capacitation reaction and acrosome reaction occur.

5. CONCLUSION

The results of the macroscopic test of Simmental cattle's fresh semen in general, the cement used in this study had good quality. The color of normal cow semen has a yellowish white color. The smell of cow semen has a distinctive

smell. The volume of semen in this study was 6 ml, medium consistency, and pH 6.8. The results of the microscopic test had a mass movement of 2 (++), a motility of 70 and a concentration of 1650±259.81. The motility of the X spermatozoa sexing results, the best incubation time is 45 minutes, the motility is 55.00±5.00, while the motility of the Y spermatozoa sexing results is the best 30 minutes, the motility is 45.00±11.18. The viability of the sexed X spermatozoa was 54.85±10.25 with an incubation time of 45 minutes, while the best sexed Y spermatozoa was 32.6±13.98 with an incubation time of 30 minutes.

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Author contributions

LP and TAP designed the research, IPS, HS, FNLL, AS performed laboratory work, analyzed the data and wrote the manuscript,ODP performed laboratory work and phylogenetic analyses and reviewed the manuscript.

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