Short Communication

Separation of Bull Spermatozoa Bearing X- and Y-Chromosome by using Albumin Gradient and Swim-up Technique in Pesisir Cattle

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Abstract | The aim of this research was to evaluate the quality characteristics of X- and Y-chromosome-bearing sperms separated by using albumin gradient and swim-up methods in Pesisir bull. The semen samples from three bulls were collected and evaluated for macroscopic and microscopic quality parameters including the percentage of motility, plasma membrane integrity (PMI) and acrosome intact (AI). The result revealed that the average motility, PMI and AI of sperms bearing X- and Y-chromosome in the albumin gradient method was 58.33±10.32% and 60.83±13.57%, 53.63±16.54% and 52.76± 8.88%, and 53.00±7.93% and 54.08±8.76%, respectively. In swim-up method the average motility, PMI and AI of sperms bearing X- and Y-chromosome was recorded 60.83±7.54% and 70.00±8.94%, 54.85±7.40% and 58.43±8.08%, and 53.37±8.76% and 58.96±8.41%, respectively. The percentage of sperm motility, PMI and AI was higher (P < 0.05) in swim-up than in the albumin gradient technique. From the results, it could be concluded that the motility, PMI and AI was slightly higher for Y-sperm than X-sperm regardless of semen sexing technique.

Keywords | Pesisir bull, Albumin gradient, Swim up, Motility, PMI, AI

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INTRODUCTION

Pesisir bull is a genetic resource for local beef cattle in West Sumatera, Indonesia. The population of Pesisir cattle tends to decrease over the last 10 years due to the high demand for beef, negative selection, and crossing. These local cattle are raised on rural farms with mature body weights ranging from 200 to 400 kg under poor management, reproduction, and breeding program. Pesisir cattle are reared on the small scale ranging from 2 to 4 heads per farmer. An evaluation of the fertility of local cattle is essential for improving the breeding efficiency, future strategy, and selection of bulls for the production of frozen semen. Nowadays Indonesian government is paying attention to local Pesisir cattle as a genetic resource of livestock and selected it for the breeding stock in 2014. In earlier studies by Budhiyadnya et al. (2021) in Pesisir cattle the high intensity mating criteria for bulls were 36-48 months, height 134±1.73 cm, weight 348±3.18 kg, scrotal boundary 32±0.87 cm, testosterone 12.62±3.77 ng/ml and good semen quality. Furthermore Zaituni et al. (2022) reported that maintaining and optimizing the fertility of local cattle and crossbred is likely through ovulation synchronization and fix-time artificial insemination with the synch protocol program to improve food security in Indonesia.

The application of artificial insemination in local cattle is aimed to preserve available genetic resources to utilize for improved cow fertility and beef production. The quality of frozen semen is affected by handling procedures such

as before freezing. Artificial insemination is widely used for the genetic improvement of cattle and to maximize the number of inseminated cows per year. This can be achieved by inseminating cows with sufficient progressively motile spermatozoa from a given ejaculate without reducing their fertilizing capacity. For the artificial insemination program, high-quality semen from the selected bull is needed for cryopreservation and insemination of the cows. According to Holden and Butter (2018), the availability of sexed semen would allow the selection of the best bulls and cows. In addition, 0.2% level of bovine serum albumin (BSA) could maintain the highest viability of sperm during eight days of storage (89.36±2.62%%) following Indonesian National Standards (Rachmawati et al., 2020). Furthermore, Rasad et al. (2021) found that the quality of Etawah crossbreed sperm after sexing was affected by the BSA concentrations. According to Rawat and Sharma (2020) that percoll density gradient method (7 layer of 10-70%) could improve the semen quality, however this claim need to be validated by further large-scale trials. Moreover, to enhance the semen quality and to observe its effect on sex ratio and conception rate after artificial insemination with enriched semen, are largely remained unexplored.

Semen sexing is allowed to produce the offspring of the desired sex, either male or female, and selecting the sex of the progeny using sex-sorted sperm. The offspring sex in cattle was determined by using the sperm bearing X- and Y- chromosomes. Methods of sperm sexing using 5% and 10 % BSA have been widely applied to both dairy and beef cattle (Kaiin et al., 2013). In local Pesisir cattle, sperm sorting has never been done. It could be used to produce more male offspring and can be applied in artificial insemination technology. So this method can be used to increase the efficiency of the artificial insemination program to produce the calf that is needed in the local Pesisir cattle farms for beef production. According to Boro et al. (2016), sexing of semen is based on the principle of difference in DNA content between X- and Y-chromosomes, as X-spermatozoa contains more DNA. Sexed semen is obtained by the application of many sorting techniques. Sex sorting of sperms is reproductive biotechnology which capable to control the sex of calves born for specific purposes by separating sperm-bearing X- and Y-chromosomes. Sperm with X- and Y- chromosome have several characteristic differences such as DNA content, morphology, motility, mass weight, and specific genes contained therein (Sharma and Sharma, 2016). The current research focused on the qualitative differences of spermatozoa bearing X- and Y-chromosomes separated by using albumin gradient and swim-up methods in local Pesisir cattle in West Sumatra, Indonesia.

MATERIALS AND METHODS

Materials

Semen samples were collected from local Pesisir bulls (n=3) of 3 years of age that were obtained from the Lengayang district in Pessel of West Sumatra. The Pesisir bulls were selected based on the Standard National Indonesia (SNI, 2015) to produce the semen with superior traits.

Methods

Semen was collected by using an artificial vagina twice per bull in the Animal Science Faculty of Andalas University. The collected semen was immediately brought to the laboratory of biotechnology to evaluated the fresh semen according to SNI protocol that includes various macroscopic and microscopic quality checks of fresh semen (SNI, 2015). The procedure of semen sexing was carried out according to Kaiin et al. (2013) using 5 and 10 percent BSA column method and swim-up method. For BSA column method, semen containing 300 million cells was incubated for 1h in 5 and 10% BSA column at 37°C. Thereafter, each sperm fraction was collected and transferred into a 15 ml centrifuge tube and add with bracket-oliphant (BO) medium. The tube was then centrifuged at 1800rpm for 10 minutes, at 26-27°C. The 5% column fraction was predicted as X-sperm fraction and the 10% column fraction as Y-sperm. Swim-up method is based on the differences in swimming speeds between the X- and Y-sperm. The medium used in swim-up was NaCl physiological solution, that used to add 0.5 ml of fresh semen. It was placed in the rack in an upright position to the swim-up time of 10 minutes at room temperature. Then, the upper layer was taken to determine the Y-chromosome and the lower layer for the X-chromosome (Kaiin et al., 2013).

The percentage of sperm bearing X- or Y-chromosome in two sexing methods was observed in terms of sperm motility, plasma membrane integrity, (PMI) and acrocome intact (AI). The sperm motility was calculated by using a glass object dripped with 10-15 µl of semen and the examination was carried out under a binocular microscope at 400X magnification, as the motile spermatozoa were observed to be moving forward. The minimum observed spermatozoa were 100-200 cells in 5 fields of view. The number of motile spermatozoa was divided by the total number of visible sperms and expressed in percentage (%).

The evaluation of PMI was done by adding 100µl of sperm sample into 1 ml of 150 mOsm/kg hypoosmotic solution (0.735g sodium citrate and 1.352 g fructose dissolved in 100ml double-distilled water). The sperm was incubated for 60 minutes at 37°C. Sperm cells with an intact plasma membrane were indicated by curled tail, while the defected or dead sperm were marked by a straight tail (Kaiin et al., 2017). Evaluation of AI was carried out by using AI

solution (2.54 g potassium dihydrogen phosphate, 5.41 g sodium chloride, 6.19g disodium hydrogen phosphate dehydrate, 125 ml formaldehyde solution). The AI was observed under light microscope and the acrosome intact was indicated by ½ to 2/3 of the head with dark color (Arifiantini and Yusuf, 2006).

Statistical analysis

The data were analyzed using ANOVA in randomized block design for quality characteristics of bull semen and results were expressed as mean values and standard deviation.

RESULT AND DISCUSSION

Motility Of Spermatozoa

The average motility (%) of sperms bearing X-chromosome in the semen of local Pesisir bull were 58.33± 10.32 and 60.83±7.54 in albumin gradient and swim-up methods, respectively. While the percentage of Y-sperms in the albumin gradient and swim-up methods were 60.83±13.57 and 70.00± 8.94 respectively. This result showed a significant effect (P<0.05) of the sexing method (BSA column vs. swim-up) on the motility of both sperms X and Y. There was also a significant (P<0.05) difference in motility in sexed and fresh semen (Table 1). This indicated that the motility of the local Pesisir bull was fairly high. The difference in the percentage of motility of sperms bearing sperm X- and Y-chromosome is probably due to the processing methods. Moreover, other factors like differences in the amount of DNA, size, density, and motility of the sperm bearing the X- and Y-chromosome are also vital for the overall motility of sperms (Manzoor et al., 2017). The sperm sorting method by using 5% and 10% bovine serum albumin (BSA) column has been widely applied to both dairy and beef cattle (Kaiin et al., 2013). The swim-up method is based on the differences in speed between X- and Y-sperm. The smaller and lighter Y-sperm head results in a much faster movement of Y-sperm than X-sperm.

Our results showed that the sperm-bearing Y-chromosome in the local Pesisir bull is higher than the sperm-bearing X-chromosome. According to Hamano (2007), X- and Y-sperm separation using a high-speed sorter could be the basic methods for more efficient and commercial techniques of sex preselection in the bovine. This method was also capable to be used in local Pesisir bulls in West Sumatra to produce offspring with the desired sex. This result showed that the percentages of motility in sperm bearing X- and Y-chromosomes were more in swim-up as compared to the BSA column method. Furthermore, the percentage sperm bearing Y-chromosomes was higher than sperm bearing X-chromosomes in the swim-up method of the local Pesisir bull. The different techniques have been used for the separation of the X- and Y-spermatozoa, which were based on variation in mass, immunological structure, size, swimming pattern, and the charges on the surface of sperm (Yadav et al., 2018). Finally, several procedures allow distinguishing X- and Y-sperm such as the motility pattern of X-chromosome has lower than Y-chromosome and the surface antigen are also different between X- and Y-chromosome (Garner and Seidel, 2008). Furthermore, Alomar et al. (2006), reported that motility is one of the most important sperm characteristics for the maintenance of fertility, and its evaluation is essential in any sperm analysis.

The percentage of sperm motility in our study was higher than Utomo et al. (2021) which exhibited 44.1% and 45.0% motility in X- and Y-sperm; whereas, Kaiin and Tappa, (2008) reported 45% post thawing motility in sperm sexing. The percentage of motility in the study of Kaiin et al. (2017) was reported as 60-70% in X- and Y-sperms. However, our result showed both the semen separation methods can be used to separate the X- and Y-chromoseme in local Pesisir bull semen. According to Manzoor et al. (2017), sex-sorted semen is not modified but is a natural product based on the principle of differences in DNA content between X- and Y- spermatozoa where X-spermatozoa contains more DNA. Otherwise, the methods to separate bull sperm (sexing) using BSA columns 5% and 10% can be verified molecularly by duplex PCR to distinguish between the X-chromosome and Y-chromosome of bull sperm. Furthermore, the female sex ratio of calves significantly (P<0.05) increased to 66.66% with X-sperm enriched semen compared to the male percentage of 33.33% with semen, and the female percentage of 46.15% with non-sexed semen (Bath and Sharma, 2020). Our findings regarding motility of sexing semen using the BSA column and swim-up methods verified that both the methods could be used in local Pesisir bull to separate X- and Y-chromosome-bearing sperms.

Sperm Plasma Membrane Intact (PMI)

The percentage of PMI of sperm bearing X- and Y-chromosomes of local Pesisir bull in albumin gradient method of semen sexing were 53.63±16.54 and 52. 76 ± 8.86 and in the swim-up method were 54.85 ±7.40 and 58.43±8.08, respectively. The PMI of non semen sexing was higher (P<0.05) than semen sexing (Table 2). The result showed that the average of PMI bearing X- and Y-chromosomes ranged in good quality of semen to be applied for cryopreservation cells such as frozen semen for artificial insemination of cows by using antigen and their sorting with less damage and sperm number. Quelhas et al. (2021) reported that the uniquely-expressed plasma membrane in X- and Y-sperm enable the generation of specific antibodies. According to Aini et al. (2016), sexed spermatozoa separated

Table 1: The Motility (%) of fresh semen (non-sexed), and X- and Y-sperm in albumin gradient and swim-up semen sexing methods of local Pesisir bull.

Sperm type	Albumin gradient	Swim-up
Non-sexed semen	80.83±6.64*	80.83±6.64*
X-sperm	58.33±10.32[b]	60.833±7.54[a]
Y-sperm	60.83±13.57[b]	70.00±8.94[a]

[a,b] Different superscripts letters on the mean results showing significant difference (P<0.05) between semen sexing methods i.e., albumin gradient and swim–up.
* Significantly higher (P<0.05) as compared to sexed semen (viz., X-sperm and Y-sperm).

Table 2: The percentage of plasma membrane intact (PMI) of X- and Y-sperm in albumin gradient and swim–up semen sexing methods of local Pesisir bull.

Sperm type	Albumin Gradient	Swim-up
Non-sexed semen	81.67±4.16*	81.67±4.16*
X-sperm	53.63±16.54	54.85±7.40
Y-Sperm	52.76±8.86[b]	58.43±8.08[a]

[a,b] Different superscripts letters on the mean results showing significant difference (P<0.05) between semen sexing methods i.e., albumin gradient and swim–up.
* Significantly higher (P<0.05) as compared to sexed semen (viz., X-sperm and Y-sperm).

Table 3: The percentage of Acrosome Intact of X- and Y-sperm in albumin gradient and Swim-up semen sexing methods of local Pesisir bull.

Sperm type	Albumin Gradient	Swim-up
Non-sexed semen	81.58±4.50*	81.58±4.50*
X-Sperm	53.00±7.93	53.37±8.76
Y-Sperm	54.08±8.76[b]	58.96±8.41[a]

[a,b] Different superscripts letters on the mean results showing significant difference (P<0.05) between semen sexing methods i.e., albumin gradient and swim–up.
* Significantly higher (P<0.05) as compared to sexed semen (viz., X-sperm and Y-sperm).

by the BSA gradient column had comparable fertilization ability with unsexing spermatozoa and had the ability to support early embryonic development.

This result indicated that two methods of semen sexing can be used to separate X- and Y-chromosomes of local Pesisir bull ultimately applying to beef production. According to Yadav et al. (2018), a modified swim-up method using real-time PCR may be one of the effective methods for sperm sexing in different species. Susilawati (2011) reported that if a sperm with a membrane intact is placed on a hypoosmotic medium, can increase the water content in its cytoplasm to make the same concentration between the solution and the cell, thereby causing the sperm tail to curl, this is signing a motile sperm. This indicated that the PMI of sperm is correlated with sperm motility. In addition, the PMI quality of sperm is important to maintain fertility after sperm motility evaluation. The use of X and Y plasma membrane content is a useful approach to developing efficient and low-cost semen sexing such as albumin gradient and swim-up methods. According to Quelhas et al. (2021), the identification of plasma membrane proteins uniquely expressed in X- and Y-sperm will enable the generation of specific antibodies that will recognize the unique antigens, allowing their separation with less damage and sperm loss.

This research found lower PMI than those reported by Kaiin (2017), the X-sperm with an intact plasma membrane was as much as 77.29% while the Y-sperm was 76.30%. However, this result was higher than those reported by Carvalho et al. (2010) that the intact membrane of bovine of X- and Y-sperm were 36.0±2.9% and 36.4 ± 2.9%. This indicated that our Pesisir bull semen has good quality for use in artificial insemination programs. In addition, the quality of sexed semen is needed to get the best bull and cows (Holden and Butler, 2018). Our study exhibited that semen sexing of local Pesisir cattle using the BSA column and swim-up methods is feasible.

Acrosome Intact Of Spermatozoa

The average percentage of AI of X- and Y-chromosomes was 53.00±7.93 and 54.08±8.76 respectively in the albumin gradient method of semen sexing. In the swim-up method, the average percentage of AI was 53.37±8.76 and 58.96±8.41 in X- and Y-chromosome-bearing sperms

respectively. The average percentage of AI in non-sexed semen was higher (P<.0.05) than in sexed semen (Table. 3), which might be due to processing protocols. This result showed that two methods of semen sexing used in local Pesisir bull were similar and capable to separate the X- and Y-chromosomes. Sex-sorted sperm produced also reduced viability and overall quality after cryopreservation and thawing (Seidel and Gamer, 2002). According to Bath and Sharma (2020), there was no significant effect on percent spermatozoa with intact acrosomal membrane and fully damaged acrosomal membrane at all three stages of centrifugation however, particularly damaged acrosomal membrane sperms were significantly (P<0.05) decreased at stage II and III compared to stage I centrifugation.

For beef production, semen quality is vital in all cattle breeds including the local Pesisir cattle in West Sumatra. Mikkola and Taponen (2017) reported the decreased fertilization potential and embryonic development of sex-sorted semen, compared to non-sexed semen and attributed this phenomenon to sperm damages caused during sorting procedures. The sex-sorting procedure by flow cytometry affected some structure-related charactheristics of bovine sperm but did not reduce their capacity to produce embryo in vitro (Carvalho et al., 2010). A modified swim-up method using real-time PCR may be one of the effective methods for sperm sexing in different species. A promising technique for sperm sexing would be an exploration of differentially expressed proteins present on the membrane of spermatozoa and thus identification of X- and Y-spermatozoa would be possible (Yadav et al., 2018). Selecting the sex of progeny using the separation of X- and Y-chromosome has been an advantage for the animal breeders (Johnson, 2000).

The average percentage of AI in this study was higher than reported by Carvalho et al. (2010) who found that the AI in bovine was 37.1±3.3% and 37.3±3.5% for X- and Y-chromosomes respectively. Furthermore, the higher AI in semen sexing indicated that the acrosome intact was fairly high in Pesisir Bull. However, the AI was lower than the fresh semen. According to Aini et al. (2016), the sex spermatozoa separated by BSA gradient column had comparable fertilization ability with unsexing spermatozoa and had the ability to support early embryonic development. In addition, the immunological methods based on sperm cells surface protein differentially expressed between X- and Y-chromosome could be an interesting and promosing approach to semen sexing (Quelhas et al., 2021). Besides that Manzoor et al. (2017) reported that sexed semen should be used in highly fertile herds and in healthy cycling females bearing good body condition scores.

Our research showed that the quality of semen sexing was higher in the swim-up method than in the albumin gradient and the sperm-bearing Y-chromosome in both methods used in local Pesisir bull. This result is supported by Rodriguez-Martinez (1997) that described that the swim-up method is based on the difference in swimming speed between X- and Y-bearing spermatozoa. Sperm recovered from the top portion of the column was predominantly Y-bearing (60%, P<0.05), which were capable of fertilizing matured oocytes and produced significantly more male embryos (Azizeddin et al., 2014). According to Kobayashi et al. (2004), the proportion of X- and Y-bearing bovine spermatozoa can deviate after disrupted Percoll gradients, although the proportion of X- and Y-bearing bovine spermatozoa was affected by sperm motility of the sample applied. Furthermore, the disrupted sucrose density gradients can be considered as low-cost tool for sperm sexing of bovine semen in developing countries (Kanessharatnam et al., 2012). The quality of semen sexing is varied between the two methods of semen sexing is dependent on the DNA of X- and Y-chromosomes. According to Wolf et al. (2008), semen selected by swim-up and 76.5% continuous Percoll density gradient centrifuged for 10 min increased the percentage of *in vitro* produced male female bovine embryo, respectively.

CONCLUSION

The results of the current study exhibited that both methods of semen sexing used in the present study produced the fairly good quality X- and Y-chromosome-bearing sperms thus found suitable to use in the local Pesisir bull. Moreover, percentages of motility, plasma membrane intact, and acrosome intact were slightly higher for Y-sperm than X-sperm in both methods of semen sexing.

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CONFLICT OF INTEREST

All authors state that there is no conflict of interests.

NOVELTY STATEMENT

To produced frozen semen and sexing semen from superior pesisir bull for AI program of local pesisir cattle in West Sumatra.

AUTHORS CONTRIBUTION

Tinda AFRIANI (TA) and Zaituni Udin (ZU) and James H (JH) conducted reseach Adisti and Dwiki Wahyudi (DW) collected the data, ZU wrote the manuscript, Endang Purwati (EP) and ZU critized and revised, TA completed the manuscript.

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