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Sex Ratio of Calves Resulted from Artificial Insemination Implementation using Sexed Semen with Percoll Gradient Density Centrifugation Method in Ongole Crossbred Cows

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ABSTRACT

Background: Productivity of existing cattles in Indonesia is necessarily to be increase to balance meat consumption in this country. Determination of offspring of certain sex can be obtained from Percoll gradient density centrifugation.The purpose of this research was to elucidate the proportion of male calves that can result from artificial insemination using single and double doses of sexed semen in Ongole crossbred cows.

Methods: The sexed semen samples were obtained through Percoll gradient density centrifugation performed by the Artificial Insemination Center. The artificial insemination method adopted here was deep insemination. As much as 10 ml of BIO ATP® (Rheinvet) was injected in each cow before immediately insemination. Further, as much as 3 kg/day of additional feed was given over three days after insemination, with a protein level of about 12%.

Result: Our results showed the proportion of Y-bearing sperm among non-sexed semen was 52.77% and among sexed semen, was 80.79%. Further, 54.17% of the non-sexed semen, 42.11% of the single-dose sexed semen and 78.95% of the double-dose sexed semen treatments yielded male calves.

Key words: Artificial insemination, Crossbred ongole, Percoll gradient density centrifugation, Semen sexing.

INTRODUCTION

The natural increase in the beef cattle population is not balanced with the growing interest on meat consumption in Indonesia, leading to high import rates of cattle and beef from year to year. Therefore, it is necessary to increase the population and productivity of the existing cattle in the country. One solution that has been previously implemented by the governments of Indonesia and other countries is the usage of artificial insemination (AI) to increased the population and genetic quality of the cattle. The beef cattle business is composed of both breeding and fattening; however, the beef cattle fattening area of the business is more profitable than the breeding area. As such, the production of more male calves is needed because these animals experience faster rates of weight gain and fetch higher prices in comparison with female calves. The current method to ensure offspring of certain sex is gained is AI with semen sexing, with male calves resulting from the usage of Y-bearing. The value of AI can be increased through semen sexing. Many livestock farms in Indonesia maintain Ongole crossbred cattle for breeding to produce calves for beef. Based on the previous research (Prakash *et al*., 2014; Boro *et al*., 2016; Pindaru *et al*., 2016), AI using semen sexing can increase the income of livestock industry and semen sexing can produce offspring of the desired sex (Susilawati *et al*., 2014).

Some of the semen sexing tools and techniques employed to date include the sedimentation method, albumin column, Percoll gradient density centrifugation, electrophoresis, H-Y antigen, flow cytometry and filtration with Sephadex® column (Sigma-Aldrich, St. Louis, MO, USA) (Hafez and Hafez, 2008). Percoll gradient density centrifugation in particular (Prakash *et al*., 2014) employs a medium that consists of colloid particles coated with polyvenilpyroldane and is able to increase spermatozoa motility. During semen sexing, Percoll gradient density centrifugation is able to separate sperm with an accuracy of more than 80% based on the identification rate of spermatozoa heads (Susilawati *et al*., 2014). According to Ferreira-Silva *et al*. (2017), semen sexing could employ Percoll gradient density centrifugation potentially uses for Ruminantia. However, Kusumawati *et al*. (2017) found that, after performing semen sexing *via* the Percoll gradient density centrifugation method, the percentage of Y-bearing spermatozoa decreased by up to 48.55%, although the technique is still viable in conjunction with AI given that the minimum motility standard is 40%.

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Mean while, Susilawati *et al*. (2017) mentioned that semen sexing caused decreased spermatozoa quality. This is a fundamental reason for performing AI along with double-dose semen sexing. In addition, there is paucity of information that reviewed the level of accuracy in achieving the desired calf sex following semen sexing. The purpose of this study was therefore, to determine the proportion of male calves achieved by using AI with single-dose and double-dose semen sexing, respectively.

MATERIALS AND METHODS

Research design and subjects

This research was performed in the Palang Subdistrict, Tuban Regency, Indonesia. Eighty-two Ongole crossbred calves born from females that had been inseminated using non-sexed semen (n=24 calves), single-dose sexed semen (n=38 calves) and double-dose sexed semen (n=19 calves) were involved. The semen used for this research was frozen semen produced directly by the Artificial Insemination Center of Singosari using Percoll gradient density centrifugation.

Semen sexing with Percoll gradient density centrifugation

Percoll gradient density centrifugation was prepared as Kusumawati *et al*. (2017) stipulated using a discontinuous density-gradient as follows: Percoll (Sigma-Aldrich, St. Louis, MO, USA) was mixed with 1:4 diluted AndroMed® extender (Minitüb GmbH, Tiefenbach, Germany) extender and Aqua Des™ (AquaTactics, Kirkland, WA, USA). The AndroMed® extender was generated in 10 concentrations as follows: 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60% and 65%. A Percoll gradient was layered in a tube from the high (65%) to low (20%) concentrations. Each concentration consisted of 0.5 ml, with a total volume of 5 ml.

For the sexing process, 2 ml of semen was placed in a tube containing the Percoll gradient concentration, which was then centrifuged at 2,250 rpm for five minutes. Two milliliters of liquid from the centrifugation result was taken from the top fraction (Y-bearing sperm population). Then, they were washed in 3 ml of AndroMed® extender and centrifuged at 1,500 rpm for five minutes. After this centrifugation, the supernatant was carefully removed. Next, 1 ml of the bottom fraction of the sorted semen, which contained more spermatozoa than the top fraction, was collected and 2 ml of AndroMed® extender was added to the tube containing the sexed semen. This liquid concoction was inserted into a tube containing warm water (30°C), then into a cool tube (4°C). The diluted semen was inserted into a straw (0.25 ml). The freezing processing was then initiated and the semen was stored at -196°C in liquid nitrogen. Single-dose semen sexing was performed by applying warm water (30°C) to the straw for 28 seconds prior to insemination (Hopkins and Evans, 2003). Double-dose semen sexing (two straws at once) was performed in the deep insemination position (position 4+) (Dalton *et al*., 2004; Susilawati, 2005). Prior to AI, BIO ATP® (was injected intramuscularly and about 3 kg of additional feed with a protein level of 12% was provided for three days after insemination.

Data collection

Direct observation of crossbred Ongole calves' sex was used to collect this information. Other relevant data included the identification of X- and Y-bearing sperm in the non-sexing and sexing frozen semen that had been used for AI based on the measuring of the size of the spermatozoa heads. X-bearing sperm tends to be longer with larger heads, while Y-bearing sperm is shorter with smaller heads (Susilawati, 2014). Five hundred of sperm in sexed semen were measured for determining the proportions of X- and Y-bearing sperm.

Parameters that were focused on in order to understand the accuracy level of AI and semen sexing in ensuring a certain calf sex outcome were including (1) The percentage of male calves born; (2) The proportions of X- sperm and Y-bearing sperm (Susilawati, 2014); (3) The percentage of motility and concentration of sperm before AI to know the quality of sperm.

Statistical analysis

The chi-square test was used for data analysis to distinguish between the proportions of X- and Y-bearing sperm.

RESULTS AND DISCUSSION

X- and Y-bearing sperm proportions in the sexing and nonsexing frozen semen samples

Percoll gradient density centrifugation performs semen sexing according to the differences between X- sperm and Y-bearing sperm heads. According to Hafez and Hafez (2008) and Manzoor *et al*. (2017), X-bearing sperm presents larger heads. Using this information, Susilawati (2014) previously performed X- and Y-bearing sperm identification based on the size of the head and the length of the head sperm. Table 1 presents the proportions of X- and Y-bearing sperm in the non-sexed and sexed semen in the present study.

Table 1 shows that, in the non-sexed semen sample, X-bearing sperm composed 47.23% and Y-sperm composed 52.77% of all sperm. The results of chi-squared testing indicated that these percentages were not significantly different from one another ($p>0.05$). Meanwhile, for the sexed semen, the proportion of X-bearing sperm was 19.21% and that of Y-bearing sperm was 80.79%. Here, the results of chi-squared testing showed that there was a significant difference ($p<0.05$). Based on these percentages, it can be confirmed that this method was appropriate. After the sexing process using Percoll gradient density centrifugation, the percentage of Y-bearing sperm increased. Pindaru *et al*. (2016) highlighted Percoll gradient density centrifugation as a suitable method for semen sexing. Meanwhile, according to Susilawati *et al*. (2014), the proportion of Y-bearing sperm in semen sexed using Percoll gradient density centrifugation was 81.3% in the upper layer and centrifugation with a speed of 2,250 rpm for five minutes yields a better outcome as compared with centrifugation for seven minutes.

Table 1: Proportions of X- and Y-bearing sperm in the non-sexed and sexed semen.

	Non-sexed semen	Sexed semen (Y-bearing sperm)
	Average (%)	Average (%)*
X-bearing sperm	47.23	19.21
Y-bearing sperm	52.77	80.79
Total	100	100

*Significant difference between Y- and X-bearing sperm ($p<0.05$).

Conception rate following AI using sexed and non-sexed semen

In this research, 64 cows inseminated with non-sexed semen, with a pregnancy success at first AI of 28 cows [conception rate (CR) = 40.62%], while 65 cows inseminated with single-dose sexed semen AI with a pregnancy success at first AI of 35 cows (CR= 61.54%) and 21 cows inseminated with double-dose sexed semen AI with a pregnancy success at first AI of 18 cows (CR= 56.25%). The results of this research indicate that our AI success rate with sexed semen was better than rates reported in previous research. According to Cooke *et al*. (2014), the success of pregnancy decreases when using sexed semen, while the research by Susilawati *et al*. (2015) reported a rate of about 44% success when performing AI with double-dose sexed semen obtained *via* the Percoll gradient density centrifugation method. Campanille *et al*. (2011) research also revealed that deposition of sexed semen into Mediteranian Italian buffalo heifers increased pregnancy rates when compared to those of non-sexed semen (38.8%, 37.7%, respectively). The low CR in cows inseminated by using non-sexed semen might be because there was injection of BIO ATP® neither additional high-protein feed given after AI. The provision of BIO ATP® and additional feed with a high protein content can prevent the early death of embryos, as most pregnancy failures are caused by such. Demiral *et al*. (2007) reported as well that insufficient progesterone hormone levels are another cause of pregnancy failure. Low CR can also be caused by low semen quality, which may result from Percoll gradient density centrifugation and freezing, causing sperm membrane damage.

The percentages of calf sex (male or female) born following AI can be seen in Table 2. Table 2 shows that AI with non-sexed semen led to just over half of the non-sexed semen calves being male (54.17%), while AI using single-dose sexed semen yielded a male calf of 42.11%, for a non-significant difference ($p>0.05$). However, according to Table 1, AI involving double-dose semen sexing led to a male calf of 78.95%. which has given the proportion of Y-bearing sperm found in the semen sample. Thus, the percentage of male calves born after a double dose of sexed semen led to a significant of more male calves.

The differences in male calf birth rates were caused by some factors, including the variable percentages of Y-bearing sperm in the semen samples. According to Xu *et al*. (2000), differences in male calf birth rates are influenced by the Y-bearing sperm proportion, including during freezing, fertilization and while in the straw. Based on Hafez and Hafez (2008), Y-bearing sperm boast faster motility than X-bearing sperm, while X-bearing sperm is slower but more likely to survive. Therefore, a larger number of Y-bearing sperm promotes an increased opportunity for male calves. Demiral *et al*. (2007) suggested that a difference between the estrous and AI time of more than six hours can lead to a higher pregnancy success rate, but does not influence the sex of the calf.

In this research, AI using double-dose sexed semen resulted in male calf about 78.95% of the time. This result was better than that achieved by Susilawati (2014), who reported a male calf percentage of about 75%, representing an outcome that was lower than the Y-bearing sperm proportion of about 87% in the initial semen sample. This discrepancy occurs because the number of doses used in AI can affect the number of Y-bearing sperm available while trying to fertilize the ovum. Although sexing was completed by increasing the percentage of Y-bearing sperm, the quality of the semen was decreased due to the sexing treatment carried out. According to Hayakawa *et al*. (2012), semen sexing performed using the flow cytometry method can result in physical/physiological damage to the sperm that has an impact on fertility.

The evaluation of the quality of sperm before AI showed that the percentages of sperm in the non-sexed and sexed semen were 36% and 31.4%, with concentrations of 31.67 and 16.12 million sperm per straw. According to Mohanty *et al*. (2018), the most important semen quality parameter is progressive concentration and motility (moving forward), because only progressive spermatozoa are capable of fertilizing an egg. The National Standardization Agency added that the quality of semen is very important for the success of AI. Suitable frozen semen for insemination must have a concentration of 25 million sperm/straw, with a spermatozoa motility rate of 40%. This is not consistent with the results of post thawing motility that we observed for the non-sexed and sexed frozen semen.

Low sperm motility and concentration in the straw causes the sperm to not be able to fertilize the egg. As X-bearing sperm may survive, they may thus have a greater

Table 2: Accuracy of calf sex following non-sexed, single-dose sexed and double-dose sexed semen.

Sex	Single dose of non-sexed semen		Single dose of sexed semen		Double dose of sexed semen	
	Total	Percentage (%)	Total	Percentage (%)	Total	Percentage (%)
Female	11	45.83	22	57.89	4	21.05
Male	13	54.17	16	42.11	15	78.95
Total	24	100	38	100	19	100

ability to perform fertilization. According to Rahman and Pang (2020), Y-bearing sperms live faster presumably due to increasing expression of certain proteins causing apoptotic sperm that leading to Y-to-X ratio shift under stressful condition. By increasing the number of doses or adding to the volume and concentration of Y-bearing sperm in AI, the opportunity for Y-bearing sperm to inseminate the ovum would be bigger. Thus, using a double dose of sexed semen would be more likely to produce a male calf, as was seen in this study. In addition, adding doses to AI will also increase the CR and pregnancy rate.

CONCLUSION

In conclusion, the percentage of Y-bearing sperm in the non-sexed semen was 52.77%, while that in sexed semen obtained using Percoll gradient density centrifugation was about 80.79%, The proportions of male calves born from crossbred Ongole cows inseminated by using a single dose of non-sexed semen, a single of dose sexed semen and a double dose of sexed semen were 54.17%, 42.11% and 78.95% respectively.

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