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Quality of Sexed Sperm of Bali Bull in Regional Artificial Insemination Center of Riau Province

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ABSTRACT

This study aimed to evaluate the quality of frozen semen of Bali bull resulted from sexing procedure on calf or offspring production with desired sex. The tested sperm of Bali bull were collected from Bali bull raised at Regional Artificial Insemination Center of Riau Province (BIBD Riau). The study was carried out in 2 stages. The first stage was X and Y chromosome separation by albumin method. The extender used in the sexing procedure is trice citrate fructose and egg yolk. The second stage was mainly testing the sexed sperm collected in 60 Bali cow in Langkat Village, Bengkalis Regency. To determine the quality of post thawing frozen semen collected from the sexing procedure, the study evaluated motility, viability, mortality, abnormality and plasma membrane integrity of the spermatozoa. The pregnancy rate, calving rate, and birth accuracy of inseminated sexed sperm to offspring' sex were also evaluated. The evaluation resulted in motility (66.3-75.3%), viability (70-78.5%), plasma membrane integrity (60-65.8%), abnormality (6.05-8.05%), mortality (20.05-30.05%), and pregnancy rate (83.33-90%). The calving rate on this study was 100% with the birth accuracy of 81.8% for male offspring and 40% for female offspring. As conclusion, the sexed sperm evaluated on this study have fairly good fertility.

Keywords: Bali bull, Calving rate, Pregnancy rate, Sexed sperm

10

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Introduction

Artificial insemination is one of technological means used to buttress in increasing livestock population. The added value of artificial insemination can be obtained by controlling the offspring' sex that is determined by X and Y chromosome within spermatozoa (Hafez and Hafez, 2016). The approach, so called sperm sexing, is intended to facilitate conservation of endangered species, supplying offspring for fattening, and other purposes (Prasad *et al.*, 2010).

There are numerous sperm sexing methods available, such as sephadex using-filtration (Dowson *et al.* 19 1986), Percoll gradient (Lizuka *et al.*, 1987), electrophoresis (Blottner *et al.*, 1994; Airsworth *et al.*, 2007), swim-up (Garner and Hafez, 200), and albumin method (Hafez and Hafez, 2016). The success rate of using the sexed-spermatozoa is 8-18.5% (Garner and Seidel, 2008), meanwhile the ratio of X and Y chromosome is 50%:50% (Hunter, 1982). Factors affecting the success rate of sperm sexing are *Bovine Serum Albumin* (BSA) concentration, retention time of spermatozoa in passing the BSA,

and spermatozoa concentration (Maxwell *et al.*, 2004).

The quality of sexed spermatozoa can be assessed based on its post thawing motility, not less than 40% (SNI, 2017). The pregnancy rate and the sex accuracy of the intended offspring from artificial insemination using sexed spermatozoa can be used as feasibility indicators for spermatozoa freezing. The pregnancy test of sexed spermatozoa on *Peranakan Ongole* (PO, Ongole Cross Breed) showed 40% of pregnancy (Susilawati, 2003). Aini *et al.* (2016) reported that X chromosome-containing sperm is obtained from 49.17% of upper fraction, while the Y chromosome-containing sperm found in the 51.40% lower fraction. Sitmorang *et al.* (2013) artificial insemination using the upper fraction semen result in the increment of female offspring produced, up to 65%. The sperm sexing on this research was carried out at *Balai Inseminasi Buatan Daerah* (BIBD; Regional Artificial Insemination Center) of Tenayan Raya, Riau Province. The sexed sperm were then tested on Bali cow in Langkat Village, Bengkalis Regency. This research aimed to evaluate the quality of frozen sexed semen of Bali bull in Riau province in producing sex-desired offspring.

Materials and Methods

Materials used on this research are Bali bull in *Balai Inseminasi Buatan Daerah* (BIBD; Regional Artificial Insemination Center) of Tenayan Raya, Riau Province; and 60 Bali cow in Langkat Village, Siak Kecil. The semen dilution was performed by using tris citrate fructose buffer and 20% of egg yolk as control (the common dilution performed in BIBD Tenayan Raya). Tris citrate fructose buffer added with 10-30% of egg albumin was used as separator media of X and Y chromosome-containing semen. The quality of semen was assessed by using Hypoosmotic Swelling Test (35 gram of fructose + 0.73 gram of sodium citrate), distilled water, and eosin-nigrosine. The freezing process was performed by using -196°C of liquid N_2 . All chemical compounds used on this research were purchased from Sigma-Aldrich (St. Louis, MO, USA). The estrous cycle synchronization of Bali cow was carried out by using GnRH (Fertagyl[®], Intervet International) and PGF_{2α} (Dinoprost tromethamine). Laboratory instruments used during the research are microscope (Olympus BX20, x400, Tokyo, Japan), 1 cm-diameter syringe, straw, cover glass, and object glass.

The research consisted of 2 stages. **Stage 1**; separating male and female semen. The semen of Bali bull used on this research showed 70% of motility and less than 10% of abnormality. The assessment for motility (% M) concentration, % of viability (% H), and plasma membrane integrity (% MPU) were performed at Laboratory of Reproduction Biology Faculty of Agriculture and Animal Science, UIN Suska Riau. The spermatozoa separation was done by creating 2 columns of albumin (@ 3 ml) that consist of 10 and 30% of albumin with extender composition shown on Table 1 (Sitomorang *et al.*, 2013). The sperm sexing followed this procedure: 3 ml of extender media containing 30% of egg albumin is transferred into 1 cm-diameter syringe, followed by another 3 ml of extender media containing 10% of egg albumin. Hence, there are 2 columns with 3 cm of height each. Semen that has been evaluated and show significant feasibility for sexing is then diluted with control media of tris citrate (1:1), and then each 1 ml is transferred to the upper column (10% of egg albumin layer).

The separation time is 10 minutes, 30 times repeated of collection process. Semen is then collected through drainage system, with condition that 3 ml of first semen coming out is

categorized as lower layer sperm that contains Y chromosome, while the next 3 ml semen contains X chromosome (upper layer semen). Each collected semen is then diluted with 6 ml of control tris citrate and centrifuged (2,500 rpm; 5 minutes) to wash the sperm from the solution. The precipitate is then diluted with control tris citrate to acquire semen with concentration of 100 million sperm/ml, stored in 5°C for 45 minutes, equilibrated for 3 hours. Semen is then frozen by placing 8 cm straw on the surface of liquid nitrogen for 10 minutes, then slowly dipped into liquid nitrogen, and stored in the -196°C container. The frozen semen is then thawed to be evaluated. The assessed parameters are sperm motility (%), sperm viability (%), mortality, and plasma membrane integrity. The percentage of spermatozoa motility represents spermatozoa that moves progressively (moving forward). It is evaluated subjectively on the 8 areas under microscope with 400x of magnification (Rasul *et al.*, 2001). The given number is within 0-100% range with 5% scale. Percentage of spermatozoa viability is performed by forming semen specimen stained with eosin-nigrosine. A drop of fresh semen is placed on the object glass, added with 1 drop of 0.2% of eosin-nigrosine solution. They are then mixed and covered with object glass, forming 45° of angle. The specimen is observed under 400x magnified microscope. Viable spermatozoa has clear head, while the spermatozoa with red head shows its non-viability. The viability is calculated by dividing the total viable spermatozoa by total counted spermatozoa, then multiplied by 100%.

The plasma membrane integrity is tested by using Hypoosmotic Swelling Test solution. The solution comprises 1.35 g of fructose + 0.73 sodium nitrate, solved in distilled water up to 100 ml. 200 μl of hypoosmotic solution is added with 20 μl semen, mixed until homogenous, and then incubated in 37°C temperature for 45 minutes. A thin spread specimen of it is evaluated under microscope with 400x magnification, with minimum number of 200 spermatozoa. The round or swelled tail of spermatozoa indicates the spermatozoa with solid plasma membrane integrity. Meanwhile, spermatozoa with disturbed plasma membrane integrity is represented with straight tail or without any swelling (Jeyendran *et al.*, 1992).

Stage 2. Sperm collected from the sexing process were tested to 60 Bali cow in Desa Langkat, Kecamatan Siak Kecil, Bengkalis

Table 1. The composition of tris-citrate extender containing 10% and 30% egg albumin

Extender	10% albumin	30% albumin	Extender in Tenayan Raya AIS
Tris hydroxil methylamine (g)	2.422	2.422	3.028
Citrate acid (g)	1.340	1.340	1.7
Fructose (g)	1.000	1.000	1.25
Egg yolk (ml)	20	20	20
Streptomycin (mg)	100	100	100
Penicillin (IU)	100.000	100.000	100.000
Albumin ml	10	30	-
Distilled water (ml)	70	50	75.1
Glycerol	-	-	6

Regency, with these following conditions: has been produced offspring and has 3 to 3.5 of Body Condition Score (BCS). The Bali cow were rectally palpated by local veterinarian/authorization to ensure that they were not currently pregnant. Bali cows were divided into 2 groups (@ 30 heads). The first group were inseminated with X sperm, while the second were inseminated with Y sperm. All Bali cow were synchronized with GnRH hormone (3 ml) on the first day and PGF_{2α} on day 7 after GnRH injection. Bali cow that show estrus symptoms were inseminated with the sexed sperm resulted from stage 1. The pregnancy test was conducted 2 months after insemination by rectal palpation. Offspring sexes were recorded to determine the accuracy of sexed-sperm that had been inseminated to offspring's sex. Pregnancy rate and calving rate value were also recorded.

Data analysis

Data from both first and second stage are shown in average, standard of deviation, and percentage (Steel and Torrie, 1991). The difference of median between treatments on second stage were statistically analyzed by using T-test.

3 Result and Discussion

Fresh semen quality

The fresh semen quality on this research (shown on table 1) demonstrate mass movement ++ and 70% of individual movement. The individual movement on this research does not contradict with the study on Bali bull that conducted by Rahmawati *et al.* (2008) in UPTD Bali. The viability of fresh semen on this research is 95.20±0.96%. This finding is different compared to previous studies by Pratiwi *et al.* (2008) that found the viability of spermatozoa from Ongole-Cross bull is 93.5±2.1% and Sunarti *et al.* (2016) who stated that the viability of spermatozoa from Bali bull in South East Sulawesi is 94.71±1.38%. However, the fresh semen of Bali bull on this research is still feasible to be diluted. It is supported by Hafez (2016) who stated the minimum viability for semen to be processed is 80%.

The semen collected on this research has low spermatozoa abnormality (6.61±0.19%) since it is less than 20% (Hafez and Hafez, 2016). Ax *et al.* (2008) stated that spermatozoa that will undergo further processing (dilution and freezing)

must comply the minimum value for abnormality not more than 15%, since it will be affecting the fertility. The spermatozoa concentration of the collected fresh semen is 1,432.50±450.50x10⁶/ml and categorized as normal. This is based on Hafez and Hafez (2016), spermatozoa concentration of bull ranges from 8 to 20 x10⁸/ml, with volume 5 to 15 x10⁹ per ejaculation. The spermatozoa number per ejaculation is ranging from 1,000 to 2,000 million/ml (Vishawanath and Shann (4) 2000).

The plasma membrane integrity of spermatozoa on this research is 89.06±0.40%. Spermatozoa whose plasma membrane integrity less than 60% is infertile spermatozoa (Revell and Mrode, 1994). Spermatozoa that has high motility, and ++ or +++ of mass movement will has high membrane integrity as well (Saili *et al.*, 2000).

Quality of sexed spermatozoa

The spermatozoa quality that has been undergone sexing treatment by albumin method are shown on Table 2. Motility percentage (% M), viability percentage (% H), and plasma membrane integrity (% MPU) of X and Y sperms have no significant difference. The motility of X and Y sperms on this research are 75.3±6.4% and 66.3±6.5% respectively, demonstrating no significant difference between the upper and lower column sperms. According to *Badan Standardisasi Nasional* (SNI, 2017; National Standardization Organization of Republic of Indonesia), the motility of post thawing semen in 37°C for 30 minutes should not less than 40%. Thus, the motility percentage of collected semen on this study complies that standard.

The X sperm shows greater motility than Y sperm (75.3% vs 66.3%). It might be possible due to the energy consumption used by sperm to passage the albumin media from lower to upper fraction. The concentration of lower fraction is greater than the upper fraction. So, the energy usage is greater to pass the lower fraction and it corresponds for reduced motility, or even weak and immotile. Hafez (2016) stated that the number of sperm entering fraction will fall off along with the increasing concentration of the extender media that responsible for high media's viscosity, enabling only motile spermatozoa to pass through.

The motility of sexed sperm on this study is different compared to the value reported by Gunawan *et al.* (2017) who evaluated the sexed sperm of Bali bull at Regional Artificial

1
Table 2. The quality of fresh semen of Bali Bull in Regional Artificial Insemination Center of Tenayan Raya, Pekanbaru

Parameter	Quality of fresh semen
Volume (liter)	7.2
Colour	yellowish white or cream
pH	7.00± 0.00
Viscosity	viscous
Viability (%)	95.20± 0.96
Mass movement	++
Individual movement (%)	70.00 ± 0.00
Motility (%)	73.33±2.88
Concentration (10 ⁹ /ml)	1,744.57± 1.55
Abnormality (%)	6.61 ± 0.19
Membrane integrity (%)	89.06 ± 0.40

Insemination of Banyumulek (75.3% vs 42.15% for X sperm and 66.3% vs 39.52 for Y sperm). Putri *et al.* (2015) reported that the post thawing motility of Friesian Holstein is 52.37% for X sperm and 48% for Y sperm. Breed, thinner media, fresh sperm volume might be factors of this difference.

The plasma membrane integrity of sexed sperm on this study are 65.8±6.5% for X sperm and 60±0.6% for Y sperm. The values do not show significant different that might be caused by the same extender media used (tris citrate fructose and egg yolk). It shows that during sexing procedure, the phospholipid of sperm could preserve and generate dynamic intracellular surface to protect the sperm from the environment condition. Ducha *et al.* (2012) stated that egg yolk on the extender solution can scavenge ROS to keep spermatozoa for having solid plasma membrane integrity-maintaining the ultrastructure of the spermatozoa itself. Sariozkan *et al.* (2010) stated that Low Density Lipoprotein (LDL) of egg yolk acts as protector for phospholipid membrane integrity. The sperm membrane plays role as media for energy (ATP) transport produced by mitochondrial Krebs-Cycle. The progressively motile sperm has to possess solid membrane integrity. Moreover, intracellular fluid of the sperm membrane will be required for fertilization (Ax *et al.*, 2008).

The abnormality values of sexed sperm on this study have no significant difference. The respective abnormality for X and Y sperms are 6.05±2.59 and 8.05±2.59 (shown on table 2). These abnormalities found on this study might resulted from the dilution and freezing procedures. According to the standard prescribed by National Standardization Agency of Republic of Indonesia (SNI, 2017), the abnormality of bull should not more than 20%. Thus, the abnormality values of sexed sperm on this study have corroborated the good quality of sperms.

The viability percentage of sexed sperm on this study are 78.5±7.8% (X sperm) and 70±0.5% (Y sperm) (shown on table 2) – with no significant difference. The mortality of sexed sperm is declining for lower column sperm or Y sperm. The liquid fraction used on this study might responsible for this finding. The lower fraction is more viscous than the upper fraction – sperm requires more energy to pass the lower fraction. This condition will macerate the sperm and eventually kill it. Noakes *et al.* (2017) confirmed that the viscosity differences in dilution affects the viability of sperm.

Pregnancy rate

The pregnancy rate of Bali cow inseminated with X semen (female) is 90±6% and 83.33±5.1% for cow inseminated with Y semen (male) (shown on Table 3). Those value do not demonstrate any significance difference. It might be resulted from the optimal reproductive condition of cow used in this study, thus the hormonal-synchronization could work effectively. Estrus-synchronized cow on this study show 100% estrus symptoms. Irikura *et al.* (2003) stated that the same dosage of GnRH affects the working length of PGF_{2α} on melisis luteum corpus.

Furthermore, the insignificant difference on the pregnancy rate could be a result of the semen deposition that performed at the 4th cervical ring (Jainudeen *et al.*, 2000). It is supported by Irikura *et al.* (2003) who stated that the pregnancy rate is affected by semen deposition during artificial insemination.

The pregnancy rate on this study is greater compared to previous studies by Sitomorang *et al.* (2013) on dairy cow (83.33-90% vs 53-6-%), Sailli *et al.* (2017) on Bali cow in South Konawe, South East Sulawesi (83.33-90% vs 64.52-81.25%), and Rosita *et al.* (2014) on the artificial insemination using frozen sexed semen on Ongole Cross cow. Breed, age, and rearing management might be responsible for these differences (Hafez and Hafez, 2016).

Calving rate

The calving rate of Bali cow inseminated by using X sperm (female) on this study is 81.8% for female offspring and 18% for male offspring. Meanwhile, the Y sperm-inseminated cow produced 40% male offspring and 60% female offspring (shown on Table 3). It shows that the female offspring birth is more dominant than male offspring birth on the Y sperm-inseminated cow. Sex ratio of calf is 50:50 for female and male offspring (Hafez, 2016). It might be caused by the greater number of X sperm compared to Y sperm – proving that the difference ratio of X and Y sperm on each column in which greater number of spermatozoa in upper column is correlated with the greater percentage of X sperm (Sitomorang *et al.*, 2013). The similar result was reported by previous study that artificial insemination that use sperm from upper fraction will increase the female offspring birth, while the use of lower fraction sperm will escalate the male offspring birth (Afati, 2004; Sitomorang *et al.*, 2013).

Table 3. The quality of sexed sperm

Sexed sperm	Sperm quality				
	% Motility	% Viability	% Plasma membrane integrity	% Abnormality	% Mortality
X Sperm / female	75.3 ± 6.4	78.5 ± 7.8	65.8 ± 6.5	6.05 ± 2.59	20.05 ± 1.77
Y sperm / male	66.3 ± 6.5	70 ± 0.5	60 ± 0.6	8.05 ± 2.59	30.05 ± 1.5

Table 4. The pregnancy rate, calving rate and accuracy of inseminated sexed sperm to offspring's sex

Inseminated Sperm	Total of Bali cow (head)	Pregnancy rate (%)	Accuracy of seed sperm to calf's sex	
			Male	Female
X Sperm (female)	30	90 ± 6 (27/30)	18.2 % (5/27)	81.8% (22/27)
Y sperm (male)	30	83.33±5.1 (25/30)	40% (10/25)	60% (15/25)

Finding on this study is different compared to Said and Afiati (2012) who reported that spermatozoa sexing on Bali bull by using albumin column method will produce 80.77% male offspring on cow that inseminated with sperm that predicted contain Y chromosome. Gunawan *et al.* (2017) stated that the calving rate of male offspring in West Nusa Tenggara can increase up to 78.26% and 89.5% in smallholder farmer (Gunawan *et al.* 2015) on the cow inseminated with sexed sperm that separated by using albumin column method. This difference can be resulted from 11 season, age, the parity of the cow, feed and nutrition (Rosenfeld and Roberts, 2004; Green *et al.*, 2008). Rorie (1999) reported that artificial insemination procedure also affects the sex ratio of offspring produced.

Conclusions

The sexing procedure for sperm from Bali bull produces same quality of X and Y sperm on their motility, viability, plasma membrane integrity, abnormality, and motility – respectively as much as 75.4%:66.3%, 78.5%:70.1, 65.8%:60%, 6.05%:8.05%, 20.05%:30.05%. The sexed sperm on this study have good fertility – 90% 1 X sperm and 83.33% for Y sperm; with 100% calving rate and accuracy of sexed sperm to offspring's sex as much as 81.8% for female and 40% for male.

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