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Characteristics and Potential Production of Frozen Semen of Pasundan Bull

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ABSTRACT

Pasundan cattle are one of the Indonesian domestic resources of animal-genetic materials that need to be developed and conserved. The aims of this study were to evaluate the characteristics of sperm and the potential production of frozen semen of Pasundan bulls. Ten Pasundan bulls were divided into two groups based on their fresh sperm motilities. Pasundan bulls were grouped based on their sperm motilities into group A (70-79%) and group B (80-89%). Secondary data were collected and confirmed with the primary data. The semen volume, pH, color, consistency, mass movement, sperm concentration, motility, viability, membrane integrity, abnormality, and DNA integrity of fresh semen were evaluated. The semen was then diluted, frozen, and stored at -196°C. The results showed that the pH of the fresh semen in group B was lower ($p<0.05$) than group A. Sperm concentration per ejaculate showed no difference with a range of $4312.36x10^6$ to $6303.52x10^6$. The viability and DNA integrity of fresh semen were not different between group A (84.41±0.99%; 91.19±0.79%) and group B (86.35±2.16%; 92.58±0.35%). DNA integrity of frozen semen of group B (89.81±1.18%) was higher ($p<0.05$) than that of group A (86.83±0.60%). The sperm motility of frozen semen of group A (<40%) was lower compared to SNI number 4869-1:2017. The frozen semen production of Pasundan bulls was between 144.18 to 191.29 straws/ejaculate. In conclusion, only bulls from fresh-semen sperm motility 80%-89% (group B) are eligible to be used as a semen source for artificial insemination.

Keywords: Pasundan cattle; characteristics of sperm; frozen semen

INTRODUCTION

Indonesia is a country that rich in genetic resources (GRs), including GRs for cattle. The Global Data Bank of Animal Genetic Resources states that cattle are categorized in a "risk" state of decline (FAO, 2015). Pasundan cattle is the result of the adaptation of more than 10 generations of crossing between Sundaicus/ Banteng/Bali cattle, Javanese, Ongole, and Madura cattle (Kementan, 2014). Pasundan cattle is a domestic breed having advantages in maintenance efficiency, resistance to drought, tropical diseases, and stress due to changes in the weather (Dwitresnadi *et al.,* 2015). Legalization to protect the genetic resources of Pasundan cattle of Indonesian livestock was stipulated by Decree of the Minister of Agriculture No. 1051/Kpts/SR.120/10/2014 (Kementan, 2014).

A report from the West Java Central Statistics Agency in 2015 showed a decline in Pasundan cattle population from 50,000 heads in 2013 to 40,000 heads in 2015 (Dwitresnadi *et al.,* 2015). On the other hand, increasing breeding and improving the genetic quality of cattle are required to maintain the population. Artificial Insemination (AI) using frozen semen is one of the efforts to increase the population and genetic quality of Pasundan cattle. Regulation of the Minister of Agriculture No. 10/Permentan/PK210/2016 requires that 60% of AI should be made from native and/or local breed (Kementan, 2016).

The selection of superior Pasundan bull is carried out through individual selection methods and pedigree. Bull selection is made by breeding soundness examination (BSE). A bull BSE includes the following three components; must be physically sound (includes scrotal circumference (SC)), have good sexual behavior, and be able to deliver good quality semen to the females they serve. Age of puberty and sexual maturity are the most important factors in BSE assessment due to their effects on the quality of semen (Barth, 2018). Semen analysis commonly included in BSE are sperm concentration, sperm motility, and sperm morphology (Stowe *et al.,* 2013), as well as mass activity (Palmer, 2016).

The quality of frozen semen is determined to support the success of the AI program (Herbowo *et al.,*

2019). Sperm motility is an important factor in the quality of frozen semen. The capability of sperm to survive during the freezing process (freezing capability) is an important aspect to select the bull in AI center. Sperm motility decreases about 40% during freezing (Sorenson, 1979). One of the causes of damage to the head membrane of the sperm during storage is the presence of Reactive Oxygen Species (ROS) (Len *et al.*, 2019). During the freezing process (cryopreservation), the viability of sperm will be reduced due to hyperosmotic diluents and temperature changes (Len *et al.*, 2019; Indriastuti *et al.*, 2020). Individual variation in frozen semen can affect sperm motility, viability, membrane integrity, abnormalities, and DNA fragmentation of frozen semen. Freezing capability is influenced by the individual aspect of animals (Indriastuti *et al.*, 2020). Freezing capability and the characteristic of semen (macroscopic evaluation and sperm motility) has been investigated on the sperms of Pasundan bull. Sperms of Pasundan bull were able to recover after the freezing process by 59.62±5.57% (Baharun *et al.*, 2017).

Indonesian National Standardization (SNI) number 4869.1: 2017 requires the motility of fresh semen at least 70% and frozen semen at least 40% with a minimum of two individual movements (BSN, 2017). Spermatozoa motility and bull's capabilities to produce frozen semen are very important. Therefore, more comprehensive studies are required to evaluate the semen of Pasundan bull. This study was aimed to evaluate the characteristics of spermatozoa and the potential production for frozen semen of superior Pasundan bulls from two different spermatozoa motility groups.

MATERIALS AND METHODS

Animals

All procedures performed in this study were approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, IPB University (Ethics Approval No: 161/KEH/SKE/VII/2019). A total of 10 Pasundan bulls aged 3-6 years with body weights (BW) range of 380-430 kg were used in this study. Fresh forage (10% BW per head per day) and concentrate (1% BW per head per day) were given in the morning and evening. Drinking water was provided ad libitum. Moreover, Pasundan bulls with fresh-semen sperm motility ≥ 70% (SNI number 4869-1: 2017) were divided into two groups. Group A (sperm motility 70-79%) and group B (sperm motility 80%-89%).

Semen Collection and Evaluation

Semen was collected twice a week every morning using an artificial vagina as a Standard Operating Procedures (SOP) of the Ciamis-West Java Regional Artificial Insemination Center (RAIC). Semen was taken to the laboratory for macroscopic and microscopic evaluations (Arifiantini, 2012). The macroscopic evaluation includes volume, color, consistency, and pH. The microscopic evaluation includes mass movement, sperm motility, sperm concentration, sperm viability, and sperm abnormalities. Secondary data were obtained from laboratory records, and primary data were collected during the experimental period.

The mass motion was evaluated by dripping one drop of semen on the slide glass then observed under a microscope (Olympus CX 23, Japan) at 100x magnification. Sperm motility was evaluated at different stages; fresh semen and after being frozen and thawed using the Computer Assisted Semen Analyzer (CASA; Andro Vision, Minitub Germany). The characteristics of various motility movements were divided into total motility, progressive motility, progressive fast motility, progressive slow motility, circular motility, vibrating, and immotile. A total of 25 µL semen was diluted with 725 µL of 0.9% NaCl, homogenized, and then dropped on a glass slide and cover with a cover glass. Observations were made under a microscope at 400x magnification. Sperm concentration (per mL) was calculated using the Photometer SDM 6 (Minitub, Tiefenbach, Germany).

The viability of sperm was evaluated by mixing 20 µL of semen and 80 µL of eosin-nigrosin dyes (1:4) on the slide glass, then observed under a microscope at 400x magnification. Intact plasma membrane (IPM) was evaluated using the hypoosmotic swelling test (HOS Test) by mixing 20 µL of semen in 1 mL of hypoosmotic medium (0.3 g fructose and 0.7 g sodium Citrate into 100 mL distilled water then incubated in a water bath (37^{o}C) for 30 minutes (Arifiantini, 2012). Evaluation was carried out under a microscope with 400x magnification.

DNA integrity was tested by staining with Acridine Orange (AO) and observed using a fluorescence microscope (Yusrina *et al.*, 2018). Fresh semen preparations were conducted by fixing with Carnoy solution of acetic acid and methanol (1:3). Observations were then made under a microscope with 400x magnification with an excitation light of 450-490 nm and a barrier filter of 530 nm in dark conditions.

Estimation of Semen Production

The number of motile sperms per ejaculate was calculated by multiplying the volume of semen by motility, and the concentration of sperm per ejaculate. The amount of frozen semen straw produced was calculated by dividing the number of motile sperm to the insemination dose ($25x10^6$ sperm in 0.25 mL or $100x10^6$ sperm mL^{-1}).

Semen was frozen using Tris Egg Yolk (TEY) extender with reference to the SOP of the AI center for the process of frozen-semen production. Semen was equilibrated in a cool top (5°C) for four hours and then packed using the MPP Uno, automatic filling and sealing machine (Minitub, Tiefenbach, Germany). The freezing process was carried out in a liquid nitrogen vapor above using a 60 x 40 x 30 cm^3 Styrofoam box for ten minutes. Frozen semen was then stored in a liquid nitrogen container (-196° C) for further evaluation (sperm motility, viability, membrane integrity, and DNA integrity).

Statistical Analysis

Data were analyzed using the student T test and were processed using SPSS version 26. Data were presented with the mean ± standard error of the mean (SEM). P values less than 0.05 were considered statistically significant.

RESULTS

The results showed that the sperm motility of group A (75.8±1.57%) was significantly lower than group B (83.57±1.12%) (Table 2). These results were then used as the basis for grouping Pasundan bulls to determine the quality of semen, sperm motility characteristics, and potential production of frozen semen.

Macroscopic examination (Table 1) showed that the semen volume, consistency, and color were not different with a semen range of 5.14-8.20 mL. The color of the semen was milky white with medium consistency. The pH of the semen showed that group B (6.30) was lower ($p<0.05$) than that of group A (6.46). Microscopic characteristics of semen (Table 1) showed mass movements in both groups A and B, which were positive 2 (++). Sperm concentration per mL (725.20×10^6 mL^{-1}; 856.60×10^6 mL^{-1}) and sperm abnormalities (9.41%; 10.22%) in the 2 groups

Table 1. Characteristic of semen of Pasundan bulls in two different sperm motility groups

Variables	Sperm motility groups	
	A	B
Number ejaculates	80	80
Volume ejaculate (mL)	8.20 ± 1.32	5.14 ± 0.80
pH	6.46 ± 0.02^a	6.30 ± 0.00^b
Color	Milk white	Milk white
Consistency	Medium	Medium
Mass movement	2.60 ± 0.24	2.60 ± 0.24
Sperm concentration (10^6/mL)	725.20 ± 107.65	856.60 ± 75.10
Sperm concentration per ejaculate (10^6)	6303.52 ± 1714.12	4312.36 ± 667.39
Sperm abnormality (%)	9.41 ± 1.21	10.22 ± 0.66

Note: A= the sperm motility group of fresh semen 70%-79%; B= the sperm motility group of fresh semen 80%-89%. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 2. Characteristics of fresh semen and frozen semen of Pasundan bulls in two different sperm motility groups

Variables	Sperm motility groups	
	A	B
Fresh semen		
Total motility (%)	91.12 ± 0.76^a	94.76 ± 0.94^b
Progressive motility (%)	75.87 ± 1.57^a	83.57 ± 1.12^b
Progressive fast motility (%)	25.74 ± 3.11	28.20 ± 3.17
Progressive slow motility (%)	30.95 ± 5.52	41.86 ± 7.08
Circular motility (%)	19.18 ± 3.53	13.51 ± 5.75
Vibrating (%)	15.25 ± 0.96^a	11.19 ± 1.17^b
Immotile (%)	8.88 ± 0.76^a	5.24 ± 0.94^b
Sperm viability (%)	84.41 ± 0.99	86.35 ± 2.16
Sperm membrane integrity (%)	72.96 ± 4.25	79.07 ± 2.52
Sperm DNA integrity (%)	91.19 ± 0.79	92.58 ± 0.35
Frozen semen		
Total motility (%)	84.36 ± 1.62	87.41 ± 2.65
Progressive motility (%)	32.93 ± 4.06	46.52 ± 6.99
Progressive fast motility (%)	9.67 ± 7.50	1.32 ± 0.47
Progressive slow motility (%)	21.62 ± 9.31	43.08 ± 12.51
Circular motility (%)	1.64 ± 0.28	2.12 ± 1.02
Vibrating (%)	51.43 ± 3.21	40.89 ± 5.08
Immotile (%)	15.64 ± 1.62	12.59 ± 2.65
Sperm viability (%)	43.13 ± 5.92	59.46 ± 5.41
Sperm membrane integrity (%)	51.27 ± 2.44	59.02 ± 2.38
Sperm DNA integrity (%)	86.83 ± 0.60^a	89.81 ± 1.18^b

Note: A= the sperm motility group of fresh semen 70%-79%; B= the sperm motility group of fresh semen 80%-89%. Means in the same row with different superscripts differ significantly ($p<0.05$).

were not different. Sperm concentrations per ejaculate in the two groups also showed no difference with a range of 4312.36x10^6 to 6303.52 x10^6 of sperm.

The sperm motility after freezing was not different which were 32.93% and 46.52%, respectively, in group A and B. Viability of sperm in fresh and frozen semen sperm showed the same value (p>0.05) between the two groups of bulls, 84.41% and 86.35%, and 43.13% and 59.46%. The IPM of fresh semen or after being frozen was also not different from the values of 72.96% to 79.07% and 51.27% to 59.02%. Sperm with intact DNA (not fragmented) of fresh semen did not differ (91.19% to 92.58%) however, DNA integrity of frozen thawed in group B (89.81%) were higher (p<0.05) than in group A (86.83%) (Table 2).

The results showed that total motility, progressive motility, vibrating, and immotile fresh semen differ among groups A and B (Table 2). Total motility and progressive motility in group B were higher (p<0.05; 94.76% and 83.57%) than in group A, whereas vibrating in group A was higher than in group B. Movement of sperm motility in semen freezing did not differ between groups.

The potential for frozen semen production per ejaculate did not different between groups A and B, with the potential for frozen semen production were 191.29 and 144.18 straws per ejaculate, respectively (Table 3).

Table 3. Potential of frozen semen production on Pasundan bulls in two different sperm motility groups

Variables	Sperm motility groups	
	A	B
Total sperm motile (10^6)	4782.23 ± 1283.01	3604.41 ± 560.96
Straw production per collection (pieces)	191.29 ± 51.32	144.18 ± 22.44

Note: A= the sperm motility group of fresh semen 70%-79%; B= the motility group of fresh semen sperm 80%-89%.

DISCUSSION

Pasundan cattle as genetic resources of Indonesian domestic livestock have several advantages in the quality of semen as well as other native Indonesian cattle, such as Bali and Madura cattle. Semen consists of sperm and plasma in the ratio of 10% sperm and 90% plasma (Indriastuti *et al.,* 2020). The average ejaculate volume in this study was higher compared to those reported by Baharun *et al.* (2017), i.e., 6.7 mL and 3.80 mL, respectively. The ejaculate volume of Pasundan bulls was almost the same as the other native Indonesian cattle, such as Bali cattle, which is between 6.32 mL (Indriastuti *et al.,* 2020) and 6.44 mL (Nabilla *et al.,* 2018). The volume of ejaculate is also related to the libido which is influenced by testosterone (Herdis, 2017).

The color and consistency of Pasundan bull's semen were the same in two different groups. The color, consistency, mass movement, and concentration of sperm are interrelated parameters because the color of semen is determined by the density (concentration) of sperm and manifested in semen consistency and sperm mass movement. The same results were obtained in Sumba Ongole bull's semen which showed a moderate consistency with creamy in semen color (Maulana *et al.,* 2019).

The pH of the semen in group B was lower (6.30). Semen from normal bull has pH range of 6.4 to 7.8 (Garner & Hafez, 2000). Zhou *et al.* (2015) reported that the spermatozoa could be influenced directly by the pH of semen. Acidic environmental conditions had a greater influence than the alkaline environment on sperm viability. Therefore, decreasing the pH of bull semen increased viability and longevity (Contri *et al.*, 2013). In this study, the viability of fresh semen in group B was higher (p>0.05) than in group A.

The pH level of semen also has implications for physiological conditions and some diseases. The characteristics of seminal plasma such as pH, metabolic products, or free radicals can be changed by bacteria (Okazaki *et al.*, 2010). Moreover, bacteria could also affect the function of sperm (Okazaki *et al.,* 2010; Bussalleu *et al.*, 2011). On the other hand, the presence of inflammatory cells and the results of bacterial metabolism affected the sperm environment, such as pH conditions (Sarkar *et al.*, 2011). Therefore, the acidic environment, due to a pathological condition, can reduce sperm function (Contri *et al.*, 2013).

Sperm concentration is the number of sperm cells per mL of semen. Sperm concentrations in this study per mL or per ejaculate were not different. Some bulls have a low volume but have a high concentration. On the other hand, some bulls have a high volume of semen but with a low concentration. The sperm concentration of Pasundan bulls was quite low compared with the normal range of sperm concentration of adult bull, which is 800 to 1200 x10^6 mL^{-1} semen (Campbell *et al.* 2003). Sperm concentration of Pasundan bulls in this study was also lower than Bali bulls (1164.81x10^6 mL^{-1}) (Indriastuti *et al.,* 2020), SO bulls (1256.42x10^6 mL^{-1}) (Maulana *et al.,* 2019), PO bulls (1286.0x10^6 mL^{-1}) (Ratnawati *et al.,* 2018), Madura bulls (1076.0x10^6 mL^{-1}) (Ratnawati *et al.,* 2018), and Aceh bulls (1194.00x10^6 mL^{-1}) (Zulyazaini *et al.,* 2016). The concentration of sperm in the bull semen is influenced by the testicle size and the frequency of semen collection. The scrotal circumference had a positive correlation with semen volume, sperm concentration, and motility in the Bali bulls (Saputra *et al.*, 2017).

The viability of sperm in fresh semen of Pasundan bulls in this study was almost the same as reported by Baharun *et al.* (2017) (84.37%). The viability of sperm in Bali bulls was 85.0% (Matahine *et al.,* 2014), and in Madura bulls were 85.0% (Ratnawati *et al.,* 2018). Moreover, the viability of sperm is also influenced by the breed of cattle. Some reports showed the sperm viability of Limousin and FH bulls were different (95.12% VS 85%) (Diliyana *et al.,* 2014; Samik *et al.,* 2014).

Plasma membrane integrity and sperm abnormalities of Pasundan bull in this study were not significantly different between groups. The integrity of the plasma membrane of the sperm after freezing decreased between 19.98% (B group) to 21.5% (A group). The result in this study was similar with the report of Casas &

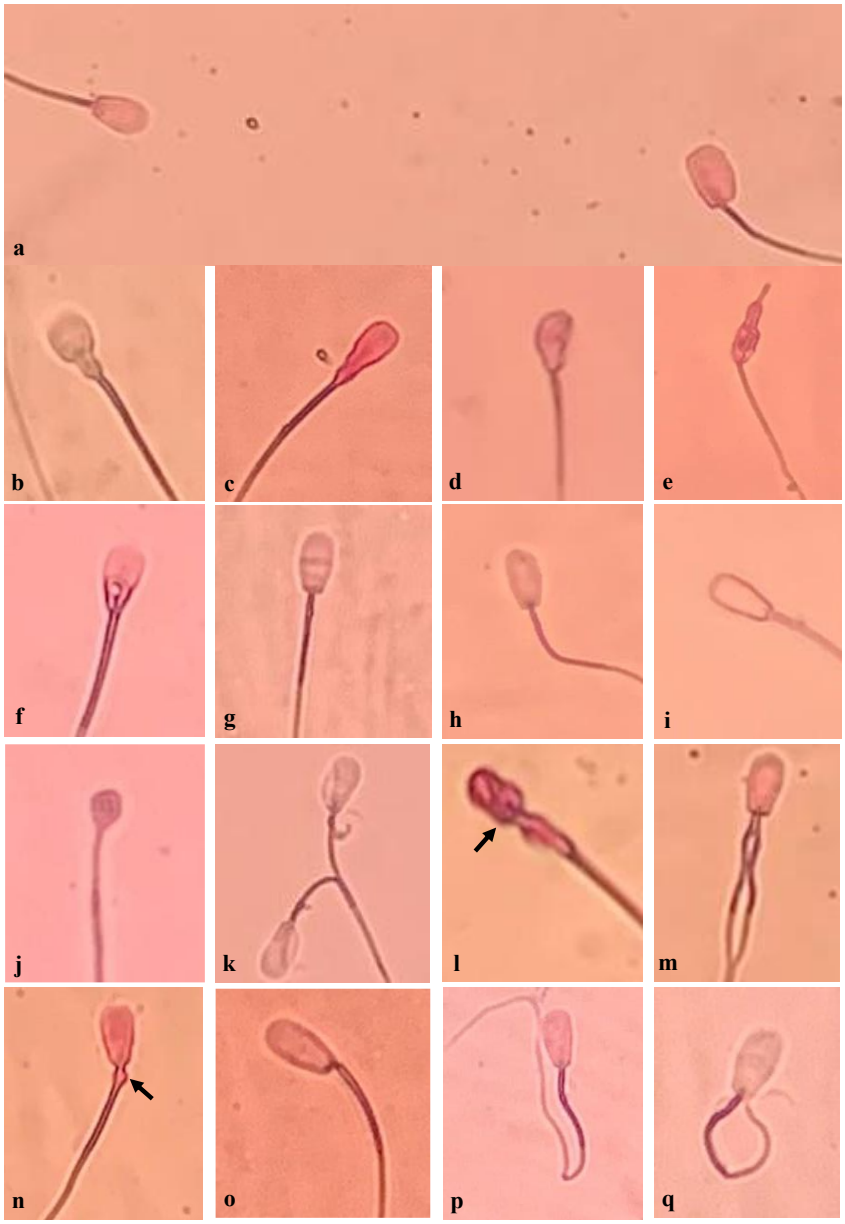

Figure 1. Sperm head morphology; normal and macrocephalus (a), pear shape (b), narrow at the base (c), abnormal contour (d), undeveloped (e), narrow (f), nuclear pouches (g), knobbed acrosome (h), flat acrosome (i), microcephalus (j), double head (k). Sperm tail morphology; defective midpiece (l), double tail (m), accessory vestigial midpiece (n), abaxial (o), coiled tails simple bent (p), coiled tails double folded (q).

Althouse (2009) that cooling and warming could damage the lipoproteins in the membrane of spermatozoa. The plasma membrane is responsible for regulating sodium, potassium, and calcium ions concentrations which are needed for mitochondrial activity and sperm motility (Pereira *et al.,* 2017). Another study by Sukmawati *et al.* (2014) showed that motility occurs if sperm have a membrane that works well to produce energy.

The structure and function of mitochondria are affected by the morphological changes of the sperm mitochondria and midpiece defect (Figure 1-l,1-n) during cryopreservation (Agarwal *et al*., 2014; Meyers *et al*., 2019; Indriastuti *et al*., 2020). The structural changes that occurred in post-thawing spermatozoa cells related with cells adaptation to the change of energy sources. These changes reduced Adenosine Tri Phosphate (ATP) production by the mitochondria, which essential for sperm motility (Meyers *et al*., 2019). In addition, the changes also affected the cellular connectivity of sperm (Agarwal *et al*., 2014). Mitochondria changed its structure and decreased the function by 15% after freezing (Khalil *et al*., 2018). Mid piece defect affects metabolism in the mitochondria, which is responsible for the conversion of ATP and ADP for the sperm movement energy (Meyers *et al*., 2019).

DNA integrity is an indicator of sperm fertility (Ajina *et al.,* 2017). Sperm DNA is packaged in prot-

amine so that they can maintain the quality of DNA by protecting from Reactive Oxygen Species/ROS attacks (Gonzalez-Rojo *et al.,* 2018). DNA damage can be caused by various factors such as heat stress, genetics, spermatogenesis, and inappropriate chromatin structure. In this study, DNA integrity after freezing was lower for both groups, 86.83% (group A) and 89.81% (group B), compared with fresh semen 91.19% (group A) and 92.58% (group B). An average decrease in intact DNA or DNA fragmentation across groups in this study was 3.56%. The result indicated that DNA fragmentation of sperm in Pasundan bulls during cryopreservation was higher than that reported for Limousin, Ongole, Brahman, and Simmental bulls, which was around 1.84%. However, DNA fragmentation of sperm in Pasundan bull observed in this study was lower than that reported by Priyanto *et al.* (2015) using the same breed of cattle and Sperm-Bos-Halomax® (Halotechdna, Spain) test which around 14.56%. Another report showed the level of damage after freezing in Bali bulls using Sperm – Bos – Halomax® was only 3.00% (Indriastuti *et al*., 2020).

Sperm motility is one of the parameters that is often used to evaluate sperm fertility. The results showed a decrease in sperm motility after changes in temperature (Tabel 2). The extreme osmolality occurred during freezing will damage the composition of the lipid plasma membrane that eventually causes a decrease in sperm motility. Sperm motilities during post freezing in both groups were varying (Table 2). Semen storage at low temperatures resulted in structural damage to sperm due to cold shocks and the formation of ROS (Fattah *et al.,* 2017). The study showed that the total motility (Table 2) of post thawing sperm in both groups was not significantly different.

Progressive motility consists of fast, slow, and circular motilities. Other sperm movements besides progressive are vibrating, and the rest is immotile. The result showed that progressive motility after thawing in group A sperm did not meet the standard of frozen semen criteria based on SNI number 4869-1: 2017 (BSN, 2017). The study also showed that the progressive motility of the sperm after thawing in Pasundan bulls was lower than in Bali bulls. Indriastuti *et al.* (2020) reported the progressive motilities of sperm after thawing in Bali bulls were between 64.69% and 71.23%.

The potential for the production of frozen semen is the ability of bulls to produce frozen semen. The potential can be calculated in each ejaculate or production in one year with the assumption of 40 weeks of production or a total of 80 times with the assumption of twice a week semen collections (Ditjennak, 2018). The production of frozen semen produced by each individual bull depends on the number of motile sperm present in an ejaculate. The results of this study showed that there was a lot of frozen semen from bulls in group A that did not meet the standard, while frozen semen from group B, most of them meet the criteria of Indonesian National Standard of frozen semen. Results showed that the potential production of frozen semen from all groups was not different (Table 3).

The local breed of cattle at least, can produce 7,500 straws per year (Ditjennak, 2018). The potential production of frozen semen by Pasundan bull in this study was average 167 straws per ejaculate, which was equal to 13,400 straws for one year of 40 weeks. This amount was high enough for local breed bull, although it was seen that there are quite high individual variations with high standard error values. Another local breed cattle, i.e., Madura cattle, have the potential to produce frozen semen around 79 and to 99.14 straws per ejaculate or around 6,627 to 7,980 straws a year (Komariah *et al.,* 2020; Aisah *et al.,* 2017). In contrast, Bali cattle can produce frozen semen as many as 11,843.17 straws per year (Indriastuti *et al.,* 2020). The potential for production of frozen semen is influenced by the volume and concentration of semen, having a positive correlation (Aisah *et al.,* 2017).

CONCLUSION

Characteristics and potency of frozen semen of Pasundan bulls were not different between group A (the motility of fresh semen 70%-79%) and group B (the motility of fresh semen 80%-89%). However, the pH of the semen and DNA integrity of semen post thawing were different. The sperm motility after freezing of group A did not meet SNI's standard. The group B of Pasundan bulls in Ciamis RAIC eligible as sources of semen in order to maintain the genetic resources of local Indonesian cattle by increasing the population of Pasundan cattle through AI technology.

CONFLICT OF INTEREST

Asep Gunawan and Cece Sumantri serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. Authors declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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