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# Progressive Motility, DNA Fragmentation, Intact Plasma Membrane, and Acrosome Status of Frozen Semen Bali and Simmental Bulls

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**ABSTRACT**

In increasing the population of bulls in Indonesia, it is necessary to utilize Artificial Insemination (AI) technology. AI reproductive technology aims to increase reproductive efficiency and use superior semen in livestock and can prevent the spread of reproductive diseases. Frozen semen is one of the factors that influence the success of AI. Thus, the quality of frozen semen must be maintained so that fertility remains good. This study aimed to determine the quality of frozen semen of Bali and Simmental bulls at the Artificial Insemination Center, South Sulawesi. This study used frozen semen from two Bali and Simmental bulls. Parameters observed were progressive motility, deoxyribonucleic acid fragmentation (DNA), acrosome status, and intact plasma membrane. The progressive motility was tested using Computer-assisted sperm analysis (CASA Sperm Vision Minitube), the DNA fragmentation using acridine orange dye, the acrosome status using peanut agglutinin and the intact plasma membrane staining using HOS solution. This study give result that the progressive motility, DNA fragmentation, and acrosome status of spermatozoa in frozen semen of Bali and Simmental bulls were not significantly different ($P>0.05$), while the whole plasma membrane of spermatozoa in Bali bulls frozen semen was significantly higher ($P<0.05$) than Simmental bulls. The results indicate that based on the parameters of progressive motility, DNA fragmentation, and acrosome status of spermatozoa, both bulls breeds had the same quality of frozen semen. Meanwhile, according to the spermatozoa intact plasma membrane, frozen semen of Bali bulls had better quality than that of Simmental bulls.

Keywords: Artificial Insemination, frozen semen quality, Bali and Simmental bulls

## INTRODUCTION

One of the existing first generation reproductive technologies which have proven its success in increasing the genetic quality improvement of livestock is artificial insemination (AI) technology. This technology can produce good quality of offspring in large numbers by utilizing superior males. Furthermore, (AI) is considered capable of producing genetically enhanced offspring [1], [2]. Generally, AI activities use frozen semen which is produced through several stages to be inseminated into the female reproductive tract.

Regulation of the Minister of Agriculture Number 10 of 2016 discusses frozen cement production at Artificial Insemination Centers (BBIB, BIB and BIBD) Provision and Distribution of Frozen Cement Ruminants and Indonesian National Standard (SNI) Frozen Cement Number 4869.1: 2017.The bulls used as source to produce frozen semen at BBIB, BIB and BIBD are superior bulls [3]. Frozen semen is utilized to obtain superior domestic and foreign males because it can be stored for a very long time and is free from infectious diseases caused by natural mating. The main purpose of making good frozen semen is to increase the success rate of pregnancy which is similar to natural mating with the application of biotechnology (IB) [4]. The Center for Artificial Insemination located in the Pucak Region of South Sulawesi is one of the institutions which provide the needs of frozen semen. The process of frozen semen production will lead to up to 50% of quality decrease due to the cryopreservation process [5], [6]. This is what causes the need for quality testing of frozen semen to determine the extent of quality degradation that occurs after the freezing process. Spermatozoa quality can be seen through several parameters including progressive motility, deoxyribonucleic acid (DNA) fragmentation, and intact plasma membrane. These parameters play a very important role in the process of fertilization and embryo development in female cattle. This study aims to determine the quality of frozen semen produced at the Pucak Regional Artificial Insemination Center because information concerning the quality of frozen semen evaluated on the parameters of progressive motility, deoxyribonucleic acid (DNA) fragmentation, acrosome status, and intact plasma membrane is still very limited in number.

## MATERIALS AND METHODS

### Research Materials

The research was conducted from November 2021 to February 2022 at the center of biotechnology, the National Research and Innovation Agency (BRIN). The frozen semen was obtained from two Bali and Simmental bulls maintained by the Regional Artificial Insemination Center (RAIC) of South Sulawesi, aged 6-7 years

### Evaluation of Frozen Semen Quality

The quality of frozen semen was evaluated at BRIN by observing the progressive motility parameters using Computer-assisted sperm analysis (CASA Sperm Vision Minitube, Germany).

DNA fragmentation was observed under a Z2 fluorescent imager microscope (Zeiss Company, Germany) using acridine orange (AO) staining (Sigma-Aldrich®, St. Louis MO). The test was carried out by reviewing the semen sample on a glass object. After that, the reviewed semen was fixed in Carnoy for 2 hours which was then rinsed with distilled water, soaked in AO overnight, rinsed with distilled water, and dried. Then, it was observed on a fluorescent microscope with a magnification of 40 × 10. The center part of the damaged head is green/dark yellow while the intact one is green.

$$\text{DNA Fragmentation Percentage} = \frac{\text{Number of Damaged Spermatozoa}}{\text{Total Number of Spermatozoa}} \text{ X } 100\%$$

Intact Plasma Membrane was using the Hypoosmotic Swelling method (HOS Test). Ten (10) µl of semen was added to 30 µl of HOS solution, after which it was homogenized so that the solution was evenly distributed and incubated at 37 °C for 30 minutes. Testing for an intact plasma membrane is carried out by dripping the mixed solution which has been incubated on a glass slide and evaluated using a microscope with a magnification of 40 x 10. Spermatozoa that have an intact plasma membrane are marked with a coiled or thick-looking tail while those that are damaged are marked with a straight tail. The percentage of spermatozoa that reacted to the HOS solution was calculated by the following formula:

$$\text{Intact Plasma Membrane Percentage} = \frac{\text{Number of Reacted Spermatozoa}}{\text{Total Number of Spermatozoa}} \text{ X } 100\%$$

Acrosome status was observed under a Z2 fluorescent imager microscope (Zeiss Company, Germany) using peanut agglutinin (PNA) staining (Sigma-Aldrich®, St. Louis MO). The test was performed by reviewing the semen sample on a glass object. The semen that had been reviewed in preparations was then fixed in 96% ethanol for 10 minutes and dried. Then, drip 20-30-micron PNA and incubate at $37^0$C for 30 minutes. Drip 5 microns of propidium iodine (Sigma-Aldrich®, St. Louis MO) and incubate for 10 minutes. The preparations were then washed using aquadest solution. After that, the object glass was covered using a cover glass and was observed under a fluorescent microscope at 40 × 10 magnification. The damaged spermatozoa in the the acrosome at the tip of the head will be green while the intact one is reddish orange.

$$\text{Percentage of Acrosome Status} = \frac{\text{Number of Damaged Spermatozoa}}{\text{Total Number of Spermatozoa}} \text{X } 100\%$$

## Analisa Data

The data obtained were analyzed using a comparative test, specifically statistical analysis of the T test (T-test Independent sample), to compare the samples of Bali and Simmental bulls.

## RESULTS AND DISCUSSIONS

Evaluation of semen quality is one of the approaches to determine the fertility of bulls, although there is no accurate test to measure the level of fertility. The main objective for evaluating frozen semen is to increase the success rate of pregnancy, which is similar to natural mating, with the application of artificial insemination (AI) biotechnology. The observations of progressive motility, DNA fragmentation, whole plasma membrane, and acrosome status are among the very important characteristics of spermatozoa associated with the ability of spermatozoa to fertilize oocytes. The percentage of progressive motility, DNA fragmentation, intact plasma membrane, and acrosome status can be seen in Figure 1.

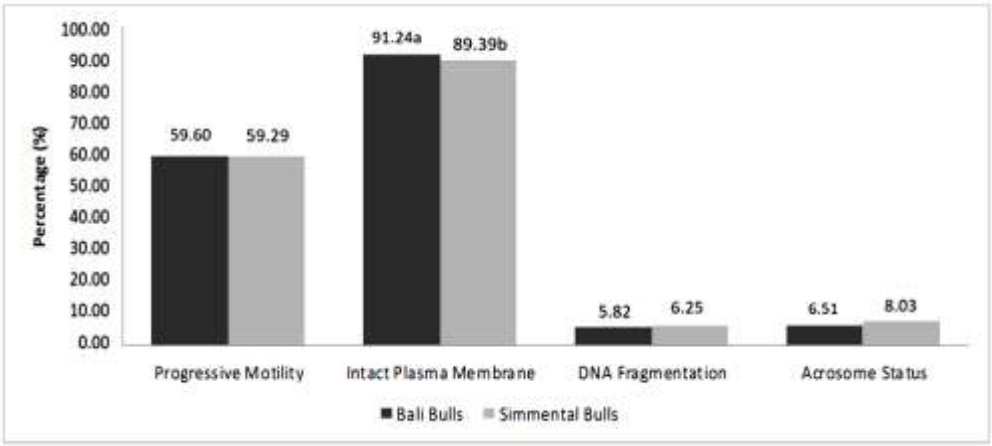


Figure 1. Percentage of progressive motility, DNA fragmentation, intact plasma membrane, and acrosome status. Note: Different superscripts in the line shows significant difference ($P<0.05$),

### Spermatozoa Progressive Motility

Progressive motility is that spermatozoa can swim or advance forward in a straight line or circle. Progressive movement of spermatozoa plays an important role for spermatozoa to enter the vaginal canal and achieve or fertilize the viable egg**.** The result of this study revealed that the percentage of sperm motility of Bali and Simmental bulls frozen semen (59.60±2.66% vs. 59.29±1.94%) was not significantly different ($P>0.05$) (Fig. 1). This showed that frozen semen of Bali and Simmental bulls produced at the Pucak Regional Artificial Insemination Center had

the same quality. According to Morell [7], progressive motility is required for spermatozoa to enter and move into the cumulus oophorus to reach the oocyte. In vivo, spermatozoa that can maintain longer total motility during incubation may allow spermatozoa to survive in the female reproductive tract, undergo capacitating, and carry out fertilization into oocytes [8]. The result of this study was higher than that previously reported by Jalius and Depison [9] on ongole bull which had a progressive motility percentage value of 38.14%.

Progressive motility assessment provides information on the fertility rate of the males used. The higher the progressive motility values of an animal, the higher the animal's fertility level. According to Lee *et al*. [10], the motility of spermatozoa is one of the most important criteria in determining the rate of fertilization, and the characteristics of normal spermatozoa motility determine the success of spermatozoa fertilization. In addition Evans and Maxwell [11] added that there are three types of spermatozoa motility, including progressive motility (forward movement), rotational motility (rotating movement), and oscillatory or convulsive motility without forward movement or positional movement.

**Spermatozoa Intact Plasma Membrane**

The intact plasma membrane test is one of the vital parameters to be observed because it is related to the permeability of the plasma membrane which plays a role in protecting spermatozoa. Damage which occurs to the head of spermatozoa membrane will affect the viability, whereas the damage which appears in the tail of the spermatozoa membrane will affect the motility [12]. The percentage of intact plasma membrane of spermatozoa in this study (91.24±1.28 % vs. 89.39±1.43) showed a significant difference between Bali and Simmental bulls ($P<0.05$). This shows that Bali bulls have a high percentage value compared to that of Simmental bulls. The difference is presumed due to the different types of bull breeds being utilized. This shows that spermatozoa in Bali cattle are more resistant to changes in temperature while in the frozen semen production process than Simmental semen. It may also be due to differences in livestock [13]. Likewise, Chandolia *et al.* [14] also argues that post-thawing heat shock after being frozen can affect the resistance of spermatozoa; one of the contributing factors is differences in livestock breeds.

The result of this study was higher than that of the previous study reported by Marawali [15] which stated that the percentage of intact plasma membranes in frozen semen of Bali bull was 39.08%. The integrity of the spermatozoa plasma membrane is an important factor in spermatozoa metabolism, capacitating, acrosomal reactions, and binding of spermatozoa to the egg surface. The plasma membrane of spermatozoa serves to protect spermatozoa organelles and to transport electrolytes for spermatozoa metabolism [16]. Butar [17] explained that a damaged plasma membrane can affect the physiological function and metabolism of spermatozoa, causing spermatozoa to die.

**Spermatozoa DNA Fragmentation**

Spermatozoa DNA fragmentation testing shows chromatin integrity as the main indicator for spermatozoa chromatin maturity and the spermatozoa DNA which acts as a carrier of genetic material can be found in both the nucleus and the mitochondria. The nuclear DNA compounds found in the head of the spermatozoa are crucial for the fertilization to take place.

The percentage of DNA fragmentation of Bali and Simmental bulls spermatozoa in Figure 1 were 5.82±2.06% vs. 6.25±1.31%, indicating no significant difference (P>0.05). The result of this study showed that there was less damage to spermatozoa DNA compared to that of previous studies. Priyanto *et al*. [18] reported that each breed has different level of DNA fragmentation. Brahman bull showed the lowest DNA damage (06.83±1.25%) and the highest was Simmental bulls (19.68±11.24 %). However, the result of the current study was higher than that reported by Indriastuti [19] which found that the percentage of DNA fragmentation that occurred in frozen semen of Bali bulls was 4.79±0.87. Although spermatozoa with chromatin abnormalities can access the ovum in vivo, their DNA cannot condense in the normal post-insemination period so that it will lead to abnormalities or delayed formation of the pronucleus, delayed division, impaired embryonic development, and the failure of maternal recognition and maintenance of early pregnancy [20]. Evaluation of DNA fragmentation in frozen semen of Bali and Simmental bulls using acridine orange (AO) dye can be seen in Figure 2.

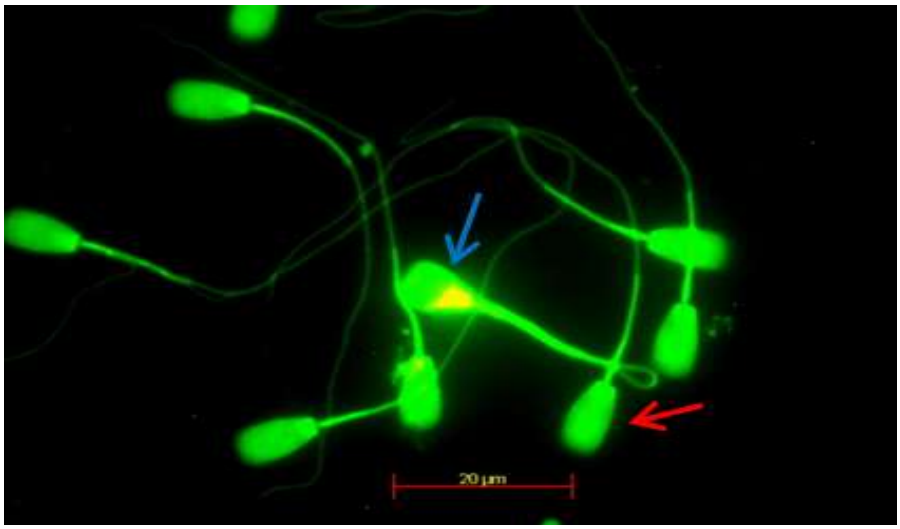


Figure 2. Observation of DNA fragmentation of Bali and Simmental bulls. Blue arrow: DNA-fragmented spermatozoa; red arrow: DNA-unfragmented spermatozoa.

DNA fragmentation in spermatozoa represents the integrity of spermatozoa chromatin which acts to maintain the stability of the paternal DNA packaging from the process of spermatogenesis to fertilization. The fertilization will run optimally if the chromatin is ripe. According to Rodriguez-Martinez [21], damage to DNA chromatin can occur due to changes in DNA polymers and continuously exposed to various physical and chemical environments that have the potential to change the natural structure of the DNA.

**Spermatozoa Acrosome Status**

Acrosome is a derivative of the Golgi apparatus formed in the early stages of spermiogenesis which mainly occur in the spermatid phase [22]. Acrosome can be found in the anterior of spermatozoa head and acrosomal damage can be employed as an indicator which causes the reduction of spermatozoa function due to the lack of cellular components and inactivation of the main protein, thereby reducing spermatozoa fertility [23]. The percentage of incomplete spermatozoa acrosome status in Bali and Simmental bulls were 6.51±2.66% and 8.05±1.91%, respectively (Figure 1), which demonstrated no significant difference (P>0.05). The acrosome sheath contains acrosomal materials, specifically enzymes (hyaluronidase, acrosin, etc.) that play a role in inducing acrosomal reactions and interactions with the zona pellucida during fertilization [24]. For this reason, the acrosome status must remain intact to protect the release of enzymes and genetic material before being released into the female reproductive organs [25]. The observation results the acrosome status of Bali and Simmental bulls can be seen in Figure 3.

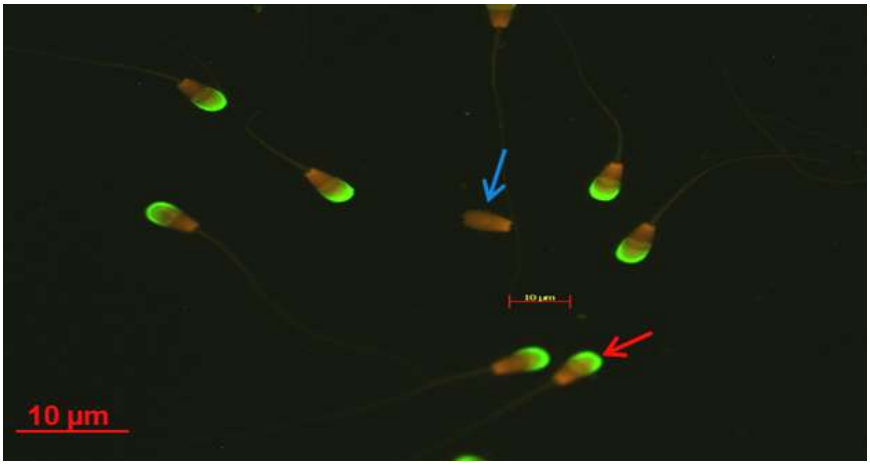


Figure 3. Observation of Bali and Simmental bulls acrosome status. Blue arrow: incomplete spermatozoa; red arrow: complete spermatozoa.

The percentage of incomplete acrosome status in this study was higher than that of Nofa *et al.* [26] which exhibited 2.09% and 5.0% of incomplete acrosomes in Simmental bulls, but were lower than that obtained by Ardhani *et al*. [25] with 24.52% of incomplete acrosomes in Bali bulls. To detect the spermatozoa acrosome membrane consisting of Inner Acrosome Membrane (IAM) and Outer Acrosome Membrane (OAM) using peanut agglutinin dye. There is a part of the acrosome that is damaged or cannot absorb the solution or a pale color is visible on the head, so the spermatozoa cells are damaged in the plasma membrane and have the potential for the acrosome to be incomplete or damaged. Spermatozoa must have an intact acrosome to be able to perform the function of acrosome reaction at the right time to release enzymes and facilitate spermatozoa in penetrating the zona pellucida [27]. Changes that occur

in the lipid components of the spermatozoa membrane will affect the stability of the membrane and can potentially cause damage to the acrosome [28], [29].

## CONCLUSION

Both Bali and Simmental bulls have same quality of frozen semen evaluated on the parameters of progressive motility, DNA fragmentation, and acrosome status. Meanwhile, according to the intact plasma membrane, Bali bulls had better quality of frozen semen than Simmental bulls.

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