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Frozen Semen Characteristics of Limousin Bull at Different Ages

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ABSTRACT

Strategies to increase the population and productivity of beef cattle can be implemented through reproductive management, such as artificial insemination (AI) using frozen semen. This study aims to evaluate the characteristics of frozen semen of Limousin bulls of different ages, which can still be used for insemination programs up to 12 years of age. This study used frozen semen of Limousin bulls ages 3, 8, and 12 years produced in 2021 at the Artificial Insemination Center in Singosari, East Java Province, Indonesia. Five bulls from each different age group were used for replication. Computer-assisted sperm analysis was used to determine sperm motility, viability, and abnormalities using eosin–nigrosine staining. Plasma membrane integrity was analyzed using the hypoosmotic swelling test. The acrosomes' integrity was evaluated using FITC-PNA-PI, protamine deficiency using chromomycin A3, and DNA fragmentation testing using the acridine orange fluorescent technique. The results showed that the parameters of sperm motility, viability, abnormality, plasma membrane integrity, and protamine deficiency showed no significant differences in all age groups. The kinematic parameters (straightness and beat cross frequency) of the 3-year-old group were significantly higher ($p<0.05$) compared with those of the other groups. Parameters of acrosome integrity showed a higher prevalence in the 3-year-old group compared with those of the other groups. Furthermore, the DNA fragmentation of the 12-year-old group was significantly higher ($p<0.05$) compared with that of the other groups. The research concludes that increasing the age of Limousin bulls can reduce acrosome integrity and DNA fragmentation.

Keywords: acrosome; DNA fragmentation; frozen semen; Limousin; sperm quality

INTRODUCTION

Over the past century, the renowned Limousin beef breed originated in central France and has undergone selective breeding to enhance economically significant traits (Mariadassou *et al*., 2020). To enhance cattle population and productivity, advanced reproductive technologies are applied. One of such technique is artificial insemination (AI), which involves using superior-quality cattle, including Limousin cattle (Indriani *et al*., 2013). Determining the fertility of domestic animals is essential, particularly in males used for AI and breeding (Tanga *et al*., 2021; Iskandar *et al*., 2023).

Bull fertility is an essential factor in the success of AI-assisted breeding programs and is responsible for failures in animal breeding (Rahman *et al*., 2017). Baharun *et al*. (2021) reported that low fertility is associated with the increasing ages of bulls. Simmental bulls have a reproductive peak in the age range of 3–8 years. The highest quality of semen production in bulls occurs at the age of 5 years and will decrease slowly after passing the peak age (Satrio *et al*., 2022). An increasing male age correlates with the decreased reproductive function (Belloc *et al*., 2014), which is related to the hormonal function associated with the reduced testosterone concentration (Baharun *et al*., 2021). Furthermore, aging in cattle also causes degeneration of the seminiferous tubules and reduces the number of germ cells, Sertoli cells, and Leydig cells (Jiang *et al*., 2013), which can interfere with the process of spermatogenesis (Tesi *et al*., 2020) and eventually reduce semen quality (Satrio *et al*., 2022). The decreased quality of frozen semen, such as sperm motility (kinematic movement), capacitation status, and DNA integrity, is closely related to the increasing age of male animals (Satrio *et al*., 2022).

Sperm motility, viability, abnormality, and plasma membrane integrity are essential to the quality of frozen semen, which affects freezing capability (Baharun *et al*., 2017). The freezing ability of sperm to survive during the freezing process is a crucial aspect of selecting the bull in the AI Center. The AI Center conventionally (macro- and micro-) assesses semen quality, which allows older bulls to be used for frozen semen production. Using frozen semen from old bulls for the AI program can result in the decreased semen quality due to oxidative stress (Aitken, 2020), which can reduce sperm

fertility. Therefore, this study aims to comprehensively identify the effect of age on the sperm characteristics of Limousin bulls. The results are expected to be one of the primary references in determining bull selection and culling policies in the Indonesian AI Center in the future.

MATERIALS AND METHODS

The materials used in this study were frozen semen of Limousin bulls obtained from the AI Center at Singosari, East Java Province, Indonesia. The bulls at the Singosari AIC were managed following standard operational procedures (SNI ISO 9001:2015 No. G.01-ID0139-VIII-2019), supervised by a veterinarian, and fulfilled every principle of animal welfare. The research was conducted at the Genomic Laboratory, National Research and Innovation Agency, Bogor, Indonesia. The semen is distributed in liquid nitrogen containers with a capacity of 10 L. Before and after distribution, sperm motilities are checked in multiple samples using a light microscope and following the Indonesian National Standard for sperm motility (>40% motility).

Experimental Design

This study used frozen semen from Limousin bulls with a tris-egg yolk extender. Five bulls from each of the 2021-produced frozen semen were used for replication in various age groups. Based on the secondary data on semen production from January to December 2021, the bulls were divided into age groups for each ejaculate of an individual bull.

Frozen-Thawed Semen

Frozen semen from five bulls per different age groups produced in 2021 were used in this experiment. The parameters of this study included sperm motility, viability, abnormality, plasma membrane integrity, acrosome integrity, protamine deficiency, and DNA fragmentation. The frozen semen was thawed in a water bath at 37 °C for 30 s. Then, the straws were dried with a tissue, and the semen was removed and inserted into the Eppendorf tube by cutting the manufactory and laboratory plugs on both ends of the straw. Subsequently, the tube containing the semen was placed in a water bath at 37 °C for further evaluation.

Sperm Motility, Viability, and Abnormality Analysis

Sperm motility was measured by placing a drop of 5–10 μL thawed semen on a glass slide covered with a cover glass. Motility was evaluated using computer-assisted semen analysis (CASA) at a temperature of 38 °C using the Sperm Vision Program (Minitub, Tiefenbach, Germany). The parameters measured included sperm progression (%), distance curve path (DAP: μm/second), distance curve linear (DCL: μm/second), distance straight line (DSL: μm/second), average path velocity (VAP: μm/second), curvilinear velocity (VCL: μm/second), straight linear velocity (VSL: μm/second), straightness (STR: VSL/VAP, linearity of forward progression (LIN: VSL/VCL), wobble (WOB: VAP/VCL), and average lateral head displacement (ALH: μm). The beat cross frequency (BCF: Hz) is the frequency with which the sperm head crosses the middle path of the sperm, measured in Hertz. Observations of sperm motility were conducted under the microscope at a magnification of 200×, and as many as seven fields were examined with a value ranging from 0% to 100%.

Sperm viability was tested by placing a drop of 10–20 μL of semen on a glass slide. Eosin–nigrosine solution at a ratio of 1:2 was mixed with the semen, making a thin smear on a glass slide, and dried using a Bunsen burner. It was then examined under a microscope with 400× magnification for a minimum of 200 cells or 10 visual fields. Viable sperm showed a clear-colored head, whereas dead sperm showed a purplish-red head.

Sperm abnormalities were determined by placing a drop of 10–20 μL of semen on a glass slide, following the same procedure, and observed under the microscope (Olympus BX 51, Tokyo, Japan) at a 400× magnification for a minimum of 200 sperm cells in 10 visual fields.

Sperm Plasma Membrane Integrity, Acrosome Integrity, Protamine Deficiency, and DNA Fragmentation Analysis

Plasma membrane integrity was determined by placing a drop of 10–20 μL of semen into the HOST medium solution (30 μL) for 40 min at 37 °C. The mixture was then dropped on a glass slide, covered with a glass cover, and observed under a microscope at 400× magnification for a minimum of 200 cells or 10 visual fields. Sperm with plasma membrane integrity showcased a coiled tail reaction.

Acrosome integrity was assessed using the FITC-PNA-PI, carried out by assessing the semen on a glass slide and drying it over a Bunsen burner. The semen sections were then fixed in 200 mL of 96% ethanol solution for 10 min and then air-dried (Rajabi-Toustani *et al*., 2019); 20–30 μL of PNA solution was mixed with the sample and incubated for 30 min at 37 °C then mixed with PI (10 μL) and set for 5 min at 37 °C. Washing was carried out using a PBS solution and air-dried, after which the dry sample was covered with a cover glass, glued with clear nail polish, and examined under a fluorescent microscope (Figure 1).

Protamine deficiency was determined using the chromomycin A3 fluorescent technique by reviewing the semen on a glass slide and drying it over a Bunsen burner. The semen was then fixed in 200 mL of Carnoy's solution for 10 min at −4 °C, washed with PBS, and air-dried. A 20–50 μL of CMA3 solution was placed on a glass slide, incubated for 20–30 min at 37 °C, glued using clear nail polish, and examined under a fluorescent microscope (Figure 1).

DNA fragmentation testing was performed using the acridine orange (AO) fluorescent technique by Said *et al*. (2015). Semen was placed on a glass slide and subsequently dried using a Bunsen burner. Semen was fixed in 200 mL of Carnoy's solution for 2 h, rinsed with distilled water, and dried. After drying, the samples

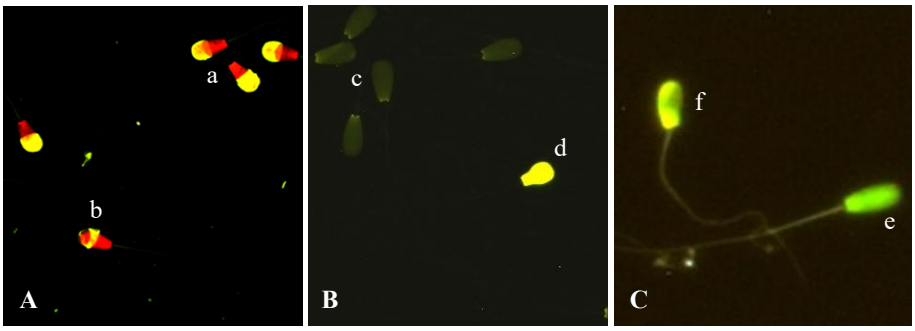

Figure 1. Fluorescence microscopy of Limousin bull spermatozoa: A. Acrosome integrity assessed by FITC-PNA staining: a. sperm with bright yellow over the acrosomal cap–sperm with intact acrosome; b. sperm no FITC-PNA staining–sperm with loss-acrosome. B. Sperm protamine deficiency assessed by chromomycin A3 (+CMA3) staining: c. sperm with yellowish green round-headed sperm cells show protamine deficiency; d. sperm with bright yellow round-headed sperm cells show normal protamine content. C. DNA fragmentation assessed by acridine orange staining: e. sperm with green fluorescence in the head–sperm with normal DNA; f. sperm with green to yellow fluorescence in the head–sperm with damaged DNA (DNA fragmentation).

were immersed overnight in 250 mL of AO solution for 5 min and rinsed with distilled water. The slide was covered with a cover glass, sealed with clear nail polish, and examined under a fluorescent microscope (Figure 1).

Statistical Analysis

The data obtained were presented as the mean ± standard deviation and analyzed using a two-way analysis of variance at $p<0.05$. Duncan's multiple-range test was carried out when significant differences existed among the ages. Data were estimated using SPSS version 26 (IBM® Corp., Armonk, NY, US).

RESULTS

Frozen Sperm Motility

In this study, there was no significant difference in the percentage of frozen semen motility, viability, abnormality, and plasma membrane integrity in Limousin bulls aged 3, 8, and 12 years (Table 1).

As shown in Table 2, the CASA movement patterns (Table 2) on the STR parameter between the semen of a 3- and 8-year-old bulls are significantly different ($p<0.05$). In contrast, the STR values of a 12-year-old bull were not entirely different from those aged 3 and 8 years. The WOB parameters of a 3-year-old bull were lower compared with those of bulls aged 8 and 12 years. The BCF parameters of a 3-year-old bull showed a significant difference ($p<0.05$) when compared with those aged 8 and 12 years. Furthermore, there were no significant differences in the kinematic parameters: sperm progression, DAP, DCL, DSL, VAP, VCL, VSL, LIN, and ALH.

Table 1. Motility, viability, abnormality, and plasma membrane integrity of frozen semen of Limousin bulls

Variables (%)	Age		
	3 years ± SD	8 years ± SD	12 years ± SD
Motility	64.37 ± 3.86	65.13 ± 3.86	62.74 ± 6.20
Viability	70.21 ± 5.14	74.11 ± 2.83	72.04 ± 4.12
Abnormaly	6.56 ± 5.11	7.60 ± 3.98	5.57 ± 2.029
Plasma membrane integrity	76.06 ± 5.81	74.82 ± 3.55	75.88 ± 1.56

Sperm Plasma Membrane Integrity, Acrosome Integrity, Protamine Deficiency, and DNA Fragmentation

The evaluation of frozen semen from Limousin bulls aged 3, 8, and 12 years were performed using the fluorescence analysis shown in Table 3. There was a significant difference in acrosome integrity at all ages ($p<0.05$). A 3-year-old Limousin bull had higher acrosome integrity than those aged 8 and 12. There was no significant difference in protamine deficiency at all ages. Meanwhile, DNA fragmentations in 3-year-old bulls were significantly higher ($p<0.05$) than those in 8 and 12 years-old bulls.

Table 2. Computer-assisted semen analysis (CASA) movement patterns in the sperm of Limousin bulls at different age

Variables	Age		
	3 years ± SD	8 years ± SD	12 years ± SD
Progressif	58.74 ± 3.58	58.56 ± 4.46	57.07 ± 5.57
DAP (μm/s)	35.20 ± 3.16	38.24 ± 1.83	35.46 ± 4.91
DCL (μm/s)	56.17 ± 5.52	61.02 ± 2.99	55.03 ± 9.14
DSL (μm/s)	21.90 ± 0.42	22.07 ± 0.83	20.83 ± 2.10
VAP (μm/s)	80.01 ± 8.08	89.05 ± 4.39	82.20 ± 12.43
VCL (μm/s)	127.31 ± 13.90	141.60 ± 6.83	127.10 ± 22.40
VSL (μm/s)	49.87 ± 1.42	51.77 ± 2.18	48.62 ± 5.61
STR (%)	0.62 ± 0.04^{a}	0.57 ± 0.01^{b}	0.58 ± 0.03^{ab}
LIN (%)	0.39 ± 0.033	0.36 ± 0.0083	0.38 ± 0.03
WOB (%)	0.62 ± 0.013^{b}	0.62 ± 0.0054^{ab}	0.64 ± 0.023^{a}
ALH (μm)	5.11 ± 0.81	6.11 ± 0.40	5.43 ± 1.08
BCF (Hz)	28.05 ± 1.03^{a}	25.97 ± 0.64^{b}	25.78 ± 0.56^{b}

Note: Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 3. Results of the average percentages of acrosomes, protamine deficiency, and DNA fragmentation of Limousin bulls at different age

Variables (%)	Age		
	3 years ± SD	8 years ± SD	12 years ± SD
Acrosome	96.50 ± 0.35[a]	95.90 ± 0.54[b]	94.90 ± 1.74[b]
Protamine deficiency	3.70 ± 3.21	2.44 ± 3.56	2.90 ± 1.98
DNA fragmentation	8.50 ± 3.74[a]	5.90 ± 1.98[ab]	3.20 ± 1.15[b]

Note: Means in the same row with different superscripts differ significantly ($p<0.05$).

DISCUSSION

The results showed that the percentages of frozen semen motility, viability, abnormality, and plasma membrane integrity were not significantly different in Limousin bulls aged 3, 8, and 12 years. Sperm motility is the most important index to evaluate sperm quality and fertilization ability. The average sperm motility results obtained in Limousin bulls aged 3, 8, and 12 years are all above 40%, which adheres to the motility quality requirements of the Indonesian National Standard (SNI) 4869-1:2021 (BSN, 2021). Increasing the size of the testes results in a greater number of seminiferous tubules, leading to an increase in the production of sperm cells. A high motility percentage is correlated with the high ability of mitochondria to produce ATP, which supports the movement of sperm flagella (Gallo *et al*., 2021). Sperm movement depends on the availability of sufficient energy for activity, which is obtained through the hydrolysis of dynein ATPase from ATP along the axoneme-microtubule pathway (Tourmente *et al*., 2015). Meanwhile, Magdanz *et al*. (2019) reported that the energy for sperm movement through the mechanisms of glycolysis and oxidative phosphorylation (OXPHOS) produces ATP in microtubules (Oberoi *et al*., 2014). However, these results differ from the research by Satrio *et al*. (2022), who reported that older bulls have reduced sperm motility. This is due to the decreased mitochondrial activity (Oberoi *et al*., 2014) due to the inactivation of the OXPHOS pathway (Amaral *et al*., 2013) to produce ATP (Ryu *et al*., 2019), thereby reducing sperm motility in old bulls.

According to Hidalgo *et al*. (2021), an excellent progressive percentage in bull fertility is 39.33%. The progressive of frozen semen for Limousin bulls aged 3, 8, and 12 years showed good results because it exceeded the standard value. According to Susilawati (2013), there are three patterns of sperm motility, namely, the hyperactive group (VCL value ≥ 100 μm/s, LIN <60%, and ALH ≥ 5 μm). In a study by Massanyi *et al*. (2008), the progressive parameters range from 46.14% to 68.57% (DAP: 19.23–24.44 (m/s), DCL: 37.43–47.20 (m/s), DSL: 14.27–18.92 (m/s), VAP: 45.26–57.31 (m/s), VCL: 87.45–110.37 (m/s), and VSL: 33.77–44.31 (m/s) (Hz)). The results of this study showed that several parameters that were not significantly different exceeded the normal values, indicating that the sperm exhibited a high quality. CASA is believed to be a more efficient method of assessing the quality of sperm because it can precisely and accurately measure various aspects of sperm fertility (Yata *et al*., 2020).

Sperm viability is one of the determining criteria for the quality of spermatozoa, as seen from the number of surviving spermatozoa, which was carried out using the eosin–nigrosine staining method. According to Reveco *et al*. (2016), a viability percentage of 64%–80% of frozen semen is suitable for AI. In this study, frozen semen of Limousin bulls aged 3, 8, and 12 years had viability >70%, indicating a good quality for AI. The results of this study are similar to those reported by Melita & Mulyadi (2014) and Budiyanto *et al*. (2021), who found that age does not affect the sperm viability of Aceh bulls.

In this study, the analysis of spermatozoa abnormalities showed no significant difference in spermatozoa abnormalities in bulls aged 3, 8, and 12 years. The observed abnormalities ranged from 5.57±2.02% to 7.60±3.98% (Table 1). The highest percentage of abnormal spermatozoa was found in Limousin bulls aged 8 years, with a value of 7.60%, while the lowest percentage was observed in Limousin bulls aged 12 years, with 5.57%. These abnormalities in spermatozoa can be attributed to disruptions either during the spermiogenesis stage or during the maturation process in the epididymis (Iskandar *et al.,* 2022). The abnormal morphology of spermatozoa is related to fertility. The high abnormality comes from the storage process and the physiological conditions of the diluent used. Abnormal spermatozoa will increase during the cooling and freezing processes due to cold shock and an imbalance in osmotic pressure resulting from metabolic processes that continue during storage (Solihati *et al*., 2008). Failures in the process of spermatogenesis or spermiogenesis, genetic factors, disease, and unsuitable environmental conditions cause primary abnormalities. The abnormal structure of any part or organelle of the sperm can affect sperm motility (Sun *et al*., 2020). Felton-Taylor *et al*. (2020) reported no association between age and other sperm traits across breeds, such as midpiece abnormalities. There was a slight increase in < 20-month-old bulls passing on vacuolation and knobbed acrosome thresholds compared with older age groups. Acrosome status might be an example of the advised practice of culling specific bulls when they consistently exhibit these abnormalities at a young age (Beggs *et al*., 2020). Young bulls (~11 months old) are culled in commercial AI facilities when their ejaculates contain <75% of normal spermatozoa (Rahman *et al*., 2018).

There were no variations in the integrity of the spermatozoa plasma membrane among Limousin bulls aged 3, 8, and 12 years. The integrity of the plasma membrane is crucial for the proper functioning of spermatozoa as it directly impacts metabolic processes and is closely associated with spermatozoa motility and viability (Iskandar *et al*., 2022). According to Loux *et al.* (2014), the intact plasma membrane value must be equal to or higher than the spermatozoa motility value to ensure optimal functionality. The plasma membrane integrity in spermatozoa is essential because it plays a vital role in the fertilization process, fertility, and sperm

function for the success of AI. The plasma membrane maintains homeostasis (Rahman *et al*., 2020), defends against foreign body agents, and interacts with the other cells, such as the oocytes and the epithelial lining of the female reproductive tract (Jha *et al*., 2020). Satrio *et al*. (2022) reported that the plasma membrane integrity value must equal or exceed the spermatozoa motility value. Damage to the spermatozoa membrane will cause loss of motility, rapid metabolic changes, morphological changes, the release of the acrosome cap, and the release of intracellular components. It has been found that after returning to isotonic conditions, the plasma membrane integrity decreases (Oldenhof *et al*., 2013). Plasma membrane integrity influences cell organelles, causing the spermatozoa to be able to move progressively (motile) and stay alive (Kaeoket *et al*., 2011).

The acrosome is located between the plasma membrane of the head and the nucleus (Iranpour, 2013). Sperm acrosome integrity in this study showed that 3-year-old Limousin bulls have higher acrosome integrity compared with those aged 8 and 12 years. One of the determinants of fertility is acrosome integrity because, for successful fertilization by AI, spermatozoa must have intact acrosomes when inseminated into the female reproductive tract and must react in a timely manner when they reach the site of fertilization (Rajabi-Toustani *et al*., 2019). According to Kumar *et al*. (2016), the freezing–thawing and equilibration processes significantly increased the damage percentage of acrosome integrity. Inappropriate cryopreservation and thawing procedures damage the acrosome, rendering the sperm cell impaired in fertilization (Demirhan *et al*., 2020).

Male infertility is correlated with abnormal protamine ratios (Francis *et al*., 2014). Abnormal sperm chromatin condensation and DNA damage cause infertility (Kumaresan *et al*., 2020). This study showed a protamine deficiency of <4% in Limousin bulls aged 3, 8, and 12 years, which is lower than the results of the study by Carreira *et al*. (2017), who found a 23.05% protamine deficiency in spermatozoa. Dehghanpour *et al*. (2017) said that using the CMA3 and TUNEL tests showed a significant increase in sperm DNA protamine deficiency.

Factors that cause damage to spermatozoa DNA due to internal and external factors can affect sperm quality. Internal factors are determined genetically and are susceptible to external factors, such as infection or exposure to other oxidative materials, which results in DNA damage. DNA fragmentation in this study showed significant differences in Limousin bulls at all ages, with the 3-year-old bull having the highest DNA fragmentation at 8.5±3.74 and the 12-year-old bull having the lowest DNA fragmentation at 3.2±1.15. The results of this study are the same as those of Fortes *et al*. (2012), who reported that younger male bulls had a higher percentage of DNA fragmentation index than adult male bulls. In Nellore males, it has been found that young (1.8–2 years) and old (8–14.3 years) bulls are more prone to DNA damage than adult (3–7 years) bulls, with young bulls exhibiting more impaired protamine than old bulls and old bulls showing more nuclear oxidative damage (Carreira *et al*., 2017). The result showed that the rates of sperm DNA fragmentation among 3, 8, and 12-year-old Limousin bulls were within the normal range. The established standard tolerance for DNA damage in the frozen semen of bulls is generally accepted to be between 10% and 20% (Everson, 2016). These findings indicate that the studied bulls across different age groups exhibited rates of DNA fragmentation that fell within the expected and acceptable levels, suggesting overall healthy sperm quality even after freezing. Oxidative stress causes extensive damage to the sperm DNA, sperm membrane damage, and decreased sperm motility (Ribas-Maynou & Benet, 2019). Moreover, an environment with high temperatures affects testicular temperatures and causes damage to the spermatozoa's DNA (Evenson, 2016).

CONCLUSION

The study showed that older Limousin bulls had reduced acrosome integrity and DNA fragmentation in the sperms of frozen semen. In addition, frozen semen from Limousin bulls up to 12 years of age can still be used for insemination programs.

CONFLICT OF INTEREST

The authors declared that there was no conflict of interest in the publication of this paper.

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