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FROZEN SEMEN CHARACTERISTICS OF BALI BULL IN DIFFERENT AGE GROUPS

Karakteristik Semen Beku Sapi Bali pada Kelompok Umur Berbeda

Brilla Widya Witri[1], R Iis Arifiantini[2], Wahono Esthi Prasetyaningtyas[3], Ni Wayan Kurniani Karja[2]*

[1]Veterinary Biomedical Science Study Program, School of Veterinary Medicine and Biomedical Science, IPB University

[2]Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University

[3]Division of Anatomy, Histology and Embryology, School of Veterinary Medicine and Biomedical Sciences, IPB University

*Email: Karja_nwk@apps.ipb.ac.id

ABSTRACT

One factor that affects frozen semen characteristics is bull age. Increasing age induces changes in sperm that reduce frozen semen quality. This study aimed to evaluate the frozen semen characteristics of Bali bulls aged 3, 8, and 13 years. Frozen semen was derived from fresh semen with sperm motility > 70%. The frozen semen characteristics tested included motility, viability, plasma membrane integrity, acrosome integrity, and sperm abnormalities. Sperm motility was analyzed by computer-assisted sperm analysis (CASA). Viability was assessed using eosin-nigrosin staining; plasma membrane integrity was assessed using the hypoosmotic swelling test (HOST); acrosome integrity was assessed using Giemsa staining; and sperm abnormalities were assessed using Williams staining. The plasma membrane integrity of the 13-year-old group was significantly lower ($P < 0.05$) than that of the younger age groups. However, there were no significant differences ($P > 0.05$) in the other parameters. We conclude that age affects the integrity of the sperm plasma membrane in frozen semen from Bali bulls.

Keywords: *Age, Bali bulls, Frozen semen, Frozen semen characteristics*

ABSTRAK

Salah satu faktor yang mempengaruhi karakteristik semen beku adalah usia sapi jantan. Bertambahnya umur menyebabkan terjadinya perubahan pada sperma yang akan menurunkan kualitas semen beku. Penelitian ini bertujuan untuk mengevaluasi karakteristik semen beku sapi Bali jantan pada umur 3, 8 dan 13 tahun. Semen beku berasal dari semen segar dengan motilitas >70%. Karakteristik semen beku yang diuji adalah motilitas, viabilitas, integritas membran plasma, integritas akrosom, dan abnormalitas sperma. Motilitas sperma dianalisis dengan menggunakan *Computer-assisted sperm analysis* (CASA). Viabilitas diamati dengan pewarnaan eosin-nigrosin, integritas membran plasma diamati dengan *hypoosmotic swelling test* (HOST), integritas akrosom diamati dengan pewarnaan Giemsa, dan abnormalitas sperma diamati dengan pewarnaan *Williams*. Hasil penelitian menunjukkan bahwa nilai integritas membran plasma pada kelompok usia 13 tahun secara signifikan lebih rendah ($P < 0,05$) dibandingkan dengan kelompok usia lainnya, tetapi tidak ada perbedaan yang signifikan ($P > 0,05$) pada parameter lainnya. Disimpulkan bahwa umur memengaruhi integritas membran plasma sperma semen beku sapi bali.

Kata Kunci: *Karakteristik semen beku, Sapi Bali, Semen beku, Umur*

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INTRODUCTION

Age is one factor affecting the quality of frozen semen (Abah et al. 2023). Aging causes a decrease in sperm production due to anatomical changes in reproductive organs, abnormalities, and degeneration of germ cells in seminiferous tubules (Endo et al. 2024). Aging in males is associated with changes at all levels of reproduction, affecting steroidogenesis and gametogenesis (Bhasin et al. 2000). Aging in germ cells irreversibly disrupts spermatogenesis, increases apoptosis, decreases the capacity of cells to resist oxidative damage, and increases sperm abnormalities. All these disorders affect the characteristics of fresh and frozen semen produced and reduce the success of sperm fertilization (Dong et al. 2022).

Increased age correlates with increased oxidative stress in the semen. High oxidative stress reduces the success of the cryopreservation process, leading to a decrease in the quality of frozen semen produced (Castleton et al. 2022). Aging causes a decrease in the secretion of accessory glands. The liquid and protein contents of older individuals differ from those of younger individuals, which can affect semen characteristics, particularly sperm motility (Halvaei et al. 2020). Research in 3–9 years old Holstein cattle (Argiris et al. 2018) and Jersey cattle age group 2,5–3 and 6–8 years old (Bhatt et al. 2016) has shown that aging does not affect frozen semen quality. A previous study by Satrio et al. (2022) showed that increasing age reduced the quality of frozen semen in Simmental cattle age groups 2, 4, and 10 years old. Some parameters of Limousin cattle semen characteristics change with age, but it can still be used as frozen semen up to 12 years of age (Baharun et al., 2023).

The quality of frozen semen is also determined by the cryopreservation process of sperm. Cryopreservation is an assisted reproductive technology that improves genetics, controls venereal diseases, and optimizes livestock fertility (Khalil et al. 2018). Sperm is the most resistant cell to damage from cryopreservation compared to other body cells due to its low intracellular water content. However, cryopreservation is still detrimental to sperm (Ugur et al. 2019). The plasma membrane is most susceptible to cryo-damage, followed by mitochondria, acrosomes, and DNA damage (Khalil et al. 2018). Cryopreservation causes changes in metabolism, cell ultrastructure, cryocapacitation, Reactive Oxygen Species (ROS) production, chromatin damage, mitochondrial damage, RNA, and protein changes (Layek et al. 2022). All changes that occur will interfere with the transportation and survival of sperm in the female reproductive tract, thereby reducing sperm fertility (Sathe 2021).

Cryopreservation aims to maintain sperm with biological characteristics and functions that are still competent for fertilization (Nagata et al. 2019). Various types of damage that may occur in sperm cells prevent not all semen from being able to achieve this goal, so there are requirements that must be met by fresh semen so that it can be produced into frozen semen. In Indonesia, the requirements that must be met are stated in SNI Frozen Semen 4869:2021, Part 1; namely, semen to be frozen must come from fresh semen with a minimum sperm motility of 70% and a maximum abnormality rate of 20%. One of the adverse effects of cryopreservation is decreased sperm motility; thus, fresh sperm motility is selected to enhance post-thaw motility. Abnormal sperm causes toxic effects on healthy sperm through ROS production (Nagata et al. 2019). This study aimed to analyze the effect of the age of Bali bull with fresh sperm motility >70% on the characteristics of frozen semen produced.

MATERIALS AND METHODS

Place and time of research

The research was conducted at one of the Regional Artificial Insemination Centers (RAICs) in Indonesia from June to July 2024.

Semen sample

The semen utilized in this study was cryopreserved semen obtained from nine Bali bulls, categorized into three distinct age groups: 3, 8, and 13 years old, with three specimens in each group. The cryopreserved semen was derived from fresh

semen exhibiting progressive motility exceeding 70%, which was subsequently frozen in an egg yolk tris diluent (Arifiantini et al. 2024). Prior to analysis, the cryopreserved semen underwent thawing. The thawing process was conducted by immersing the straw in a 37°C water bath for 30 seconds (Arifiantini et al. 2024). Following thawing, the semen was analyzed for progressive motility, viability, plasma membrane integrity, acrosomal status, and sperm morphological abnormalities.

Progressive motility and sperm viability evaluation

Sperm motility was analyzed using computer-assisted sperm analysis (CASA; Sperm Vision Minitube version 3.5.6.2, Germany). A 3 µL sample was loaded into a 37 °C warmed *Leja* counting chamber. The software program was run following the guidelines. Observations were made from 5 fields of view (Indriastuti et al. 2020). Sperm viability was evaluated using eosin-nigrosine staining. Semen was mixed with eosin-nigrosine (1:2) in a glass slide and homogenized. Smear preparations were prepared from the mixtures. The preparations were dried on a heating table and observed under a microscope with 200 sperm (Pardede et al. 2020).

Sperm plasma membrane integrity and acrosome integrity evaluation

Sperm plasma membrane integrity was evaluated using the hypoosmotic swelling test (HOST). A total of 490 µL of HOST solution (1.351 g fructose and 0.735 g sodium citrate in 100 ml distilled water) was mixed with 10 µL of semen (1:50). The mixture was then incubated at 37°C for 30 minutes. A 5 µL sample of the solution mixture was placed on a warmed object glass and covered with a cover glass. The preparations were observed under a 400x magnification microscope using 200 sperm cells (Putri et al. 2023). Acrosome integrity was evaluated by Giemsa staining. Frozen semen was diluted 1:2 with NaCl, and then smear preparations were made and dried. The preparations were fixed using methanol for 10 minutes at room temperature and then rinsed with running water. The preparations were stained with Giemsa dye for 3 hours in a staining jar. The preparations were then rinsed with running water and dried. The preparations were then observed under a light microscope at 400x magnification. The evaluation was conducted using 200 sperm (Prihantoko et al. 2020).

Sperm abnormalities evaluation

Sperm abnormalities were evaluated using calbolfuchsin-eosine staining (*Williams* staining). Frozen semen was diluted 1:2 with physiological NaCl and a smear preparation was made and dried. The preparations were washed with absolute alcohol for 4 minutes and dried. Next, the preparations were rinsed with 2% chloramine solution for 2 minutes until the mucus disappeared and the preparations looked clean. The preparations were then washed with distilled water and 95% alcohol. The preparations were stained with *Williams* solution for 8-10 minutes, rinsed with running water, and dried. The preparations were observed under a microscope at 400x magnification. Abnormalities were observed in 500 sperm cells (Baharun et al. 2021).

Statistical analysis

Frozen semen characteristics data in each age group were analyzed using analysis of variance (ANOVA). The differences in group means were analyzed using Duncan's multiple range test (DMRT) with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Sperm cryopreservation has negative effects, including ice crystal formation, hyperosmolarity, changes in cell volume, and protein denaturation. Stressors that affect sperm during cryopreservation include ion imbalance, protease activation, energy deficiency, membrane phase transition, cytoskeleton destabilization, cellular acidosis, and free radical production (Ugur et al. 2019). These negative effects manifest as the oxidation of cellular compounds and damage to structures, including DNA, the acrosome, and the plasma membrane (Hezavehei et al. 2018). Plasma membrane destabilization at ≤ 5°C will affect Ca^{2+} homeostasis, acrosome integrity, and the changes in membrane composition.

Cryopreservation results in a decreased antioxidant capacity and increased lipid peroxidation in sperm membranes (Sharafi et al. 2022). Cryotolerance is highly dependent on the lipid composition of the sperm plasma membrane; differences in fatty acid profiles and omega-3/omega-6 ratios in sperm result in varying levels of cryotolerance (Hezavehei et al. 2018). The concentration of cholesterol in seminal plasma is also important because it replaces cholesterol in the sperm plasma membrane, thereby maintaining membrane fluidity (Beer-Ljubić et al. 2009).

Age-dependent lipid and cholesterol composition is related to the activity of accessory glands that regulate the components and their concentration in the seminal plasma (Badi et al. 2018). Omran et al. (2013) stated that antioxidant capacity decreases with age, making it unable to handle oxidative stress during cryopreservation. This study shows that frozen Balinese semen derived from fresh semen samples with motility > 70% exhibits characteristics, including progressive motility, viability, acrosome integrity, and abnormalities, that are not significantly different (P > 0.05) among the age groups of 3, 8, and 13 years (Figure 1–3), while plasma membrane integrity is statistically lower (P < 0.05) in the age group of 13 years (Figure 2). This result aligns with Kudratullah et al. (2024), who state that frozen semen with motility > 70% produces frozen semen quality that is not statistically different, except for plasma membrane integrity.

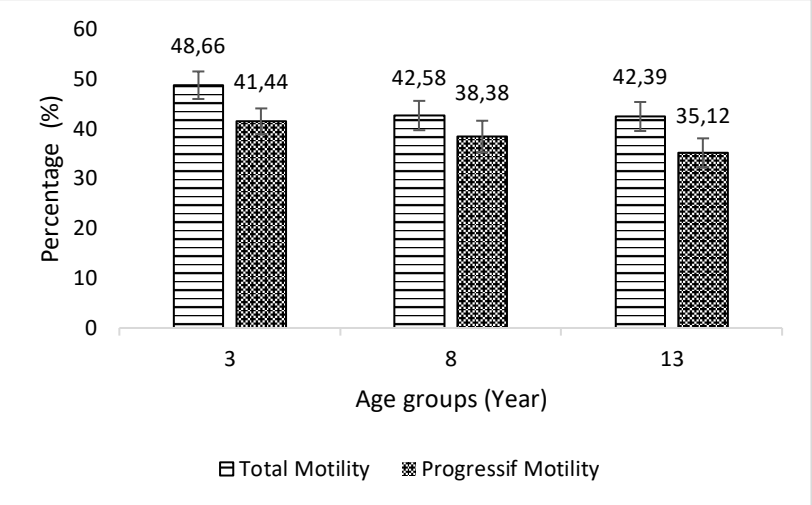

Figure 1. Total and progressive motility of frozen semen Bali bulls in different ages groups

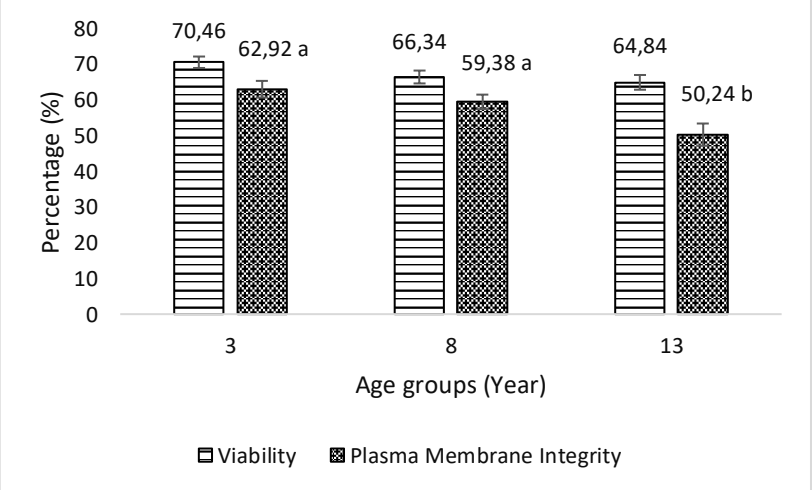

Figure 2. Sperm viability and plasma membrane integrity of frozen semen Bali bulls in different age groups
Different letter labels indicate significant differences (P < 0.05)

Sperm motility of frozen semen of Bali bull

Total and progressive motility were not significantly different (P > 0.05) among the age groups. The total motility of the 3-, 8-, and 13-year-old groups ranged from 42.58 ± 2.94% to 48.66 ± 2.76%. The progressive motility of the 3-, 8-, and 13-year-old groups ranged from 35.12 ± 2.89% to 41.44 ± 2.57%. This result aligns with findings from Murphy et al. (2018) and Baharun et al. (2023), which reported that the motility of frozen semen from bulls older than 1 year is not significantly different. This is partly because all frozen semen comes from fresh semen with > 70% motility. This finding supports Kudratullah et al. (2024), who stated that bulls with fresh semen motility > 70% produce frozen semen motility that is not significantly different. Sperm motility is a crucial parameter in assessing semen quality, as sperm must reach the ampulla of the fallopian tube for successful fertilization (Contri et al. 2013).

Sperm motility depends on the amount of energy available. Adenosine triphosphate (ATP) is used by the flagellar dynein-ATPase localized in the axoneme along the flagellum. Sperm produce ATP via glycolysis in the sperm head and flagellum and also through oxidative phosphorylation mechanisms in the mitochondria (Gallo et al. 2021). The primary effect of cryopreservation on post-thawed sperm motility is mitochondrial damage, which leads to a decrease in ATP production and sperm motility (Prihantoko et al. 2022). Damage to mitochondria triggers apoptotic mechanisms, which are also associated with decreased viability. Tail abnormalities also contribute to the decrease in progressive motility values; during cryopreservation, the tail may undergo irreversible flagellum looping, preventing sperm from moving forward (O'Connell et al. 2002)

Viability and plasma membrane integrity of frozen semen of Bali bull

Figure 2 presents the viability and plasma membrane integrity of frozen semen from Bali bulls aged 3, 8, and 13 years. The viability of the 3-, 8-, and 13-year-old groups ranged from 64.84 ± 1.6% to 70.46 ± 2.06%. Viability did not differ significantly (P > 0.05) among any of the age groups. These results are consistent with those of Baharun et al. (2023) on Limousin cattle, Melita et al. (2014), and Budiyanto et al. (2021) on Aceh cattle. The plasma membrane integrity of frozen semen of 3-, 8-, and 13-year-old groups was 62.92a ± 2.33%, 59.38a ± 2.03%, and 50.24b ± 3.09%, respectively. The plasma membrane integrity of the 13-year-old group was significantly lower (P < 0.05) than that of other groups. The results of this study align with those of Kudratullah et al. (2024), who found that frozen semen derived from fresh semen with motility > 70% has a post-thawing viability value that is not different, while plasma membrane integrity is significantly different. Sperm viability is the condition of the sperm cells in a state of life or death (Purnawan et al. 2023). Plasma membrane integrity is a condition in which the plasma membrane can still carry out its function as an ion transport control so that fluids outside the cell cannot enter the cell. Only sperm with an intact plasma membrane can survive complex changes in the female reproductive tract (Pardede et al. 2020).

The primary injury resulting from cryopreservation is due to membrane damage. The decrease in temperature during the cooling process causes a liquid-to-gel phase change, making the membrane more rigid and fragile. Phase change causes lipid phase separation, resulting in irreversible protein aggregation (Ugur et al. 2019). Plasma membrane damage is also caused by changes in osmotic pressure during the equilibration and freezing process—damage in the form of plasma membrane swelling (Khalil et al. 2018). Plasma membrane damage is also caused by increased ROS during cryopreservation, especially during the equilibration process (Prihantoko et al. 2022). Additionally, aging also increases ROS levels. High levels of ROS cause an imbalance of oxidation and antioxidation, leading to plasma membrane lipid peroxidation that alters the fluidity and integrity of the plasma membrane (Carreira et al. 2017). Sperm are susceptible to peroxidation because the sperm membrane comprises polyunsaturated fatty acids (Kogan et al. 2021). Sperm have minimal antioxidant capacity according to their cell size (Nago et al.

2021). Aging also causes a decrease in antioxidants in semen, especially antioxidant enzymes such as glutathione peroxidase and superoxide dismutase; therefore, excess ROS cannot be neutralized (Carreira et al. 2017). In addition to the disruption of ROS-antioxidant balance, the fatty acid composition of membrane lipids also changes during aging. The fatty acid composition shifts towards an increase in polyunsaturated fatty acids (PUFAs) compared to monounsaturated fatty acids (MUFAs) and an increase in triglycerides. These compositional changes decrease the fluidity and flexibility of the plasma membrane (Papsdorf and Brunet 2019). The combination of changes in plasma membrane lipid composition, along with decreased antioxidant capacity in the semen of older individuals, resulted in a reduced ability of 13-year-old sperm to withstand osmotic stress, physical effects, and oxidative stress during cryopreservation and thawing. Lipid peroxidation positively correlated with membrane damage (Catalá and Díaz 2016).

A decrease in the plasma membrane integrity decreases sperm viability. The integrity of the sperm plasma membrane strongly influences both its viability and overall membrane integrity. Decreased plasma membrane integrity can lead to sperm death. Dead sperm have higher membrane permeability; thus, dyes penetrate them more easily (Purnawan et al. 2023). Rapid changes in osmolarity and intracellular ice crystal formation during cryopreservation alter the surface protein and carbohydrate composition, thereby reducing sperm viability. The production of ROS during the cryopreservation process, combined with low antioxidant capacity, induces apoptotic pathways that ultimately reduce sperm viability (Hezavehei et al. 2018).

Acrosome integrity and sperm abnormality of frozen semen of Bali bull

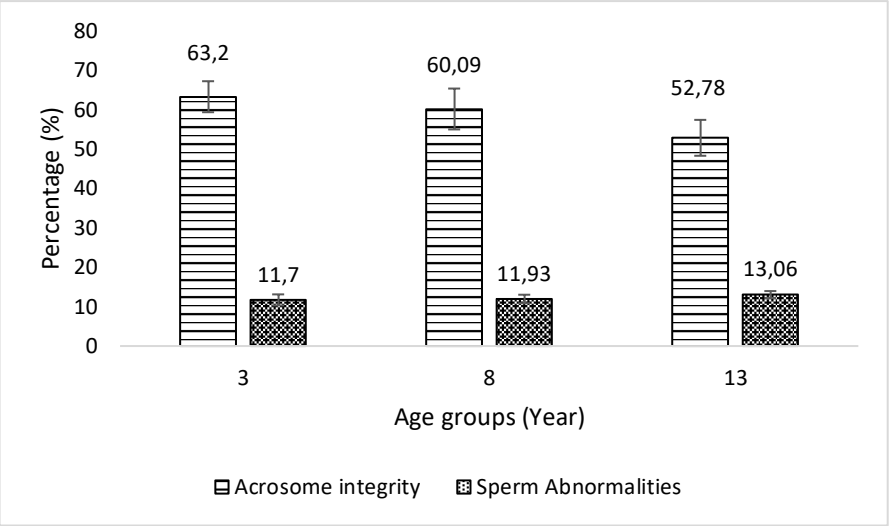

Figure 3. Sperm acrosome integrity and abnormalities of frozen semen Bali bulls in different age groups

Figure 3 presents the acrosome integrity of frozen semen from Bali bulls aged 3, 8, and 13 years. Acrosome integrity was not significantly different ($P > 0.05$) between the age groups. The results are supported by the research of Bhatt et al. (2016), which indicates that acrosome integrity is not affected by age. Acrosome integrity ranged from 47.78 ± 3.93% and 63.2 ± 4.55%. The acrosome is a membrane-bound organelle located in the anterior part of the sperm nucleus (Fatmila et al. 2024). Acrosome integrity of post-thawing semen must remain intact so that enzymes, including hyaluronidase and acrosin, can reach the female reproductive tract. These enzymes play a crucial for lysing the zona pellucida during fertilization. Sperm membrane and acrosome integrity are positively associated with field fertility (Yániz et al. 2021). Sperm must undergo capacitation and the acrosome reaction to fertilize the ovum. Capacitation and the acrosome reactions are vital for successful fertilization. The acrosome reaction

is triggered when the sperm attaches to the zona pellucida of the ovum. This reaction causes the release and activation of acrosome enzymes, allowing the sperm to penetrate the zona pellucida (Prihantoko et al. 2020). Therefore, the acrosome must remain intact until the acrosome reaction occurs.

The decrease in acrosome integrity can be attributed to reduced antioxidant protection activity and heat shock proteins due to cryopreservation process (Fatmila et al. 2024). Cold shock can cause acrosome vesiculation or a false acrosome reaction. Reactive oxygen species (ROS) from cryopreservation can damage the acrosome by oxidizing the acrosomal membrane lipids. Damage to the acrosome due to cryopreservation can be as high as 20% (Kumar et al. 2017). Changes during cryopreservation, particularly in the sperm plasma membrane and head ultrastructure, increase acrosome damage and result in the loss of enzymes in the acrosome. Similar to plasma membrane integrity, the acrosome integrity during cryopreservation is influenced by the lipid composition of the the acrosome membrane (Sun et al. 2020). Damage to the plasma and acrosome membranes can trigger a premature acrosome reaction, decreasing sperm fertilization capacity (Khalil et al. 2018).

Sperm abnormalities in this study were not significantly different ($P > 0.05$) across all age groups. This aligns with Baharun et al. (2023) in Limousin cattle and Menon et al. (2011) across several breeds, including Angus, Simmental, Charolais, Limousin, and Hereford. Abnormality values ranged from 11.7 ± 1.4 and 13.06 ± 0.88% (Figure 3). Observed sperm abnormalities included double head, narrow, microcephalus, undeveloped, macrocephalus, narrow at the base, pear-shaped, round head, abaxial, detached head, bent tail, cytoplasmic droplet, abnormal contour, and coil. Abnormalities in all groups were relatively low, at less than 20%. An abnormality rate exceeding 20% reduces fertilization success (Indriastuti et al. 2020). Assessing sperm abnormalities is crucial for evaluating bull fertility, as it relates to the presence or absence of deviations in spermatogenesis and sperm maturation (Felton-Taylor et al. 2020). Spermatogenesis disorder can lead to abnormalities in the sperm head, while issues in the maturation process can result in cytoplasmic droplets or tail abnormalities (Indriastuti et al. 2020).

Sperm abnormalities are influenced by testosterone concentration. Testosterone is needed during the spermatogenesis process, so the testosterone concentration in the testes determines the level of sperm abnormalities produced at that time (Baharun et al. 2021). Conditions that alter the profile of reproductive hormones, including testosterone–such as stress due to feeding and environmental factors, as well as disease–affect the percentage of sperm abnormalities. Feeding high-energy diets, grains, and excess concentrates will also increase the incidence of sperm abnormalities. Breed and environmental conditions influence sperm abnormality. Tropical areas exhibit higher rates of sperm abnormalities (Felton-Taylor et al. 2020). The quality of frozen semen is influenced by the quality of fresh semen used, the type of extender, the cryopreservation process, and the storage conditions of the frozen semen (Baharun et al. 2023). Cryopreservation increases the incidence of sperm abnormalities, particularly twisted tails and detached heads, due to cold shock (Khalil et al. 2018).

CONCLUSION

Age did not impact motility, viability, acrosome integrity, or abnormalities. However, it did influence the plasma membrane integrity of frozen semen from the Bali bull.

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