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Deterioration of Frozen Semen of Bali Cattle after Cooling at 5°C

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ABSTRACT

Frozen semen is produced through several stages, which deteriorate spermatozoa. This research aimed to evaluate the deterioration degree of frozen semen after 5 °C cooling and freezing of Bali cattle. The samples included 10 male Bali cattle with a body weight of 542-668 kg, from which semen was collected once a week for five weeks. The deterioration of each individual's sperm was determined by observing two distinct straws. The parameters observed included viability, abnormalities, intact plasma membrane, and intact acrosome cap. Initial observations of the parameters were conducted following the addition of semen to diluent A1 (AD) as much as the volume of fresh semen. The semen in the AD group was not cooled and frozen. The A1 semen was then divided into two, namely, those with cooling at 5 °C for 4 hours (PT1) and at 5°C for 22 hours (PT2). The results showed that individual variations in Bali cattle caused significant differences in viability and intact plasma membrane of AD and PT1 groups, while PT2 did not differ in viability and intact plasma membrane spermatozoa. Abnormalities were significantly different between AD and PT2 groups, however PT1 did not differ in abnormalities spermatozoa. Intact acrosomal cap was significantly different in the AD, PT1, and PT2 groups. In conclusion, individual variations, including viability, abnormalities, intact plasma membrane, and acrosome cap of spermatozoa, were better at 4 hours compared to cooling at 5°C for 22 hours. A Cooling time of 4 hours at 5°C can be recommended for frozen semen processing.

Keywords: Abnormalities, Bali cattle, Intact acrosome cap, Intact plasma membrane, Viability

INTRODUCTION

Bali cattle are one of the original beef cattle in Indonesia. They should be developed, utilized, and preserved as native livestock resources with certain characteristics, and the ability to thrive in diverse environments. Bali cattle also have quite varied production performance and high reproductive capacity (Saili, 2020), which makes them suitable for producing frozen semen. Artificial insemination (AI) is one of the reproductive techniques used to exploit superior males. Therefore, the genetic dissemination of superior males is carried out as an effort to improve their performance. Implementing AI in cattle depends on the quality and quantity of semen ejaculated by a male, as well as the ability to maintain the quality and increase the volume of semen for a longer time. Hence, more female acceptors will be inseminated (Nyuwita et al., 2015). The use of frozen semen is one of the success factors for the AI program, which optimizes the functioning of the males and saves the cost of raising male cattle. The reason is that, frozen semen is more durable and can be used after several years, which necessitates the quality evaluation of semen (Zuidema et al., 2021).

Frozen semen is made using certain stages. The steps include a dilution process at 37 °C, cooling at 5 °C, adding cryoprotectants and equilibration, freezing in liquid nitrogen at -196 °C, and thawing (Zampini et al., 2020). Semen is stored for a certain time during the cooling process until it reaches a temperature of 5 °C. Long cold storage time increases lactic acid (Kowalczyk et al., 2020) and forms reactive oxygen species (Silvestre et al., 2021). Reactive oxygen species cause damage to polyunsaturated fatty acids in spermatozoa membranes (Aitken, 2017). Spermatozoa pass through various extreme changes in temperature and osmolarity, hence triggering the production of reactive oxygen species (Aitken and Drevet, 2020). High concentrations of reactive oxygen species in cells produce the oxidation of lipids and proteins (Lundgren et al., 2018). This process that sperm undergo can lead to cold shock, osmotic stress, and the formation of ice crystals, thereby reducing their quality in motility, permeability changes, and lipid components of the membrane (Peris-Frau et al., 2020). Furthermore, the ability of sperm to fertilize an egg is affected by the sperm cell membrane (Anifandis et al., 2014). Changes in the lipid components of the spermatozoa membrane can disrupt the stability and cause acrosomal damage (Nofa et al., 2018).

Individual variation is a genetic factor in the ability of males. Each cattle has a different genetic potential, which affects its semen quality. According to Fazrien et al. (2020), the quality of frozen semen produced by each cow is different. Bali cattle have different characteristics of viability values, intact plasma membranes, and abnormalities

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(Indriastuti et al., 2020). Furthermore, the value of viability decreases, when the abnormality increases after freezing (Indriastuti et al., 2020). Spermatozoa produced by each cattle have a different membrane composition and resistance, affecting their ability to withstand freezing and heat shock during thawing (Zamuna et al., 2016). The plasma membrane is needed as a protective organelle in the cell and a filter for exchanging intracellular and extracellular substances. The differences in the components of spermatozoa and the character of each individual affect the quality of produced frozen semen (Fazrien et al., 2020).

Frozen semen deterioration is often evaluated based on motility, but it is important to consider other factors, including viability, abnormality, intact plasma membrane, and intact acrosome cap (Santoso et al., 2021). The determinant of fertilization success depends not only on sperm motility, but also on the condition of an intact plasma membrane and sperm acrosome cap. Therefore, the current research aimed to evaluate the deterioration degree of frozen semen as an effect of the 5°C cooling and freezing process from different individual Bali cattle.

MATERIALS AND METHODS

Ethical approval

The study was conducted at the Singosari AI Center, Indonesia, according to the standard procedure SNI ISO 9001:2015 NO.G.01-ID0139-VIII-2019 and supervised by a veterinarian. The ethics committee of the Singosari AI Center provides ethical guidance and approval for responsible behavior when using bulls for semen collection. This research was approved by the Brawijaya University Ethical Committee number 121-KEP-UB-2022.

Experimental animal

The cattle used were male Bali cattle from the Singosari AI center, Malang, East Java, Indonesia. Semen was collected once a week for 5 weeks using an artificial vagina (Minitube, Germany). Semen was collected using a teaser to increase libido. Furthermore, the semen was obtained from 10 male cattle. The cattle samples were within the age range of 7-12 years with a body weight of 542-668 kg. The deterioration of each individual's sperm was determined by observing two distinct straws. Males were housed under the care of a veterinarian and provided with grass (22 kg), silage (3 kg), concentrates (4 kg), hay (1 kg), and minerals, including calcium and phosphor (0.06 kg).

Diluent preparation

The diluent used was tris-egg yolk consisting of 17.25 g tris aminomethane (Merck, Germany), 9.65 g citric acid (Serva, USA), 15.55 g lactose (Serva, USA), 27.95 g raffinose (Serva, USA), egg yolk, streptomycin (Meiji, Indonesia) 1 g/liter, penicillin (Meiji, Indonesia) 1,000,000 IU/liter, distilled water, and 13% glycerol (Merck, Germany) of the total required diluent. In the preparation procedure, tris aminomethane, citric acid, lactose, and raffinose were put into an erlenmeyer containing distilled water, then homogenized and heated to a temperature of 40°C for 10 minutes. Egg yolks 20% were added after the solution was cooled, then streptomycin and penicillin were added. Diluent was stored in the refrigerator for three days, after which the supernatant and pellet were separated. Approximately 13% glycerol was added according to the total required diluent (Tethool et al., 2021).

Research procedure

The fresh semen produced was observed macroscopically (color, consistency, pH, volume) and microscopically (concentration, abnormality, and motility) using a microscope (Olympus CX-23, Japan) to determine the feasibility. Diluent was added three times to each sample, namely A1, A2, and B. The following formulas 1-4 indicate the measurement of the diluent amount at the Singosari AI center following a study by Arif et al. (2020):

$$\text{Total volume} = \frac{\text{semen volume x concentration}}{25 \text{ million}} \quad \text{Formula 1}$$

$$\text{A1 volume} = \text{semen volume} \quad \text{Formula 2}$$

$$\text{A2 volume} = \frac{\text{Total volume} - (\text{A1 volume} + \text{semen volume})}{2} \quad \text{Formula 3}$$

$$\text{B volume} = \frac{\text{Total volume}}{2} \quad \text{Formula 4}$$

The deterioration was initially observed after semen was added to A1 diluent (AD). It was then divided into semen with cooling at 5°C for 4 hours (PT1) and 22 hours (PT2). Diluent A2 was added before storage for 4 and 22 hours. Meanwhile, diluent B was added after cooling for 4 and 22 hours, and equilibration was performed for 2 hours. Using 0.25 ml straws, sperm was filled and sealed into each straw before packaging and freezing in liquid nitrogen at -196°C. Finally, the frozen semen deterioration was observed after thawing at 37°C for 30 seconds (Santoso et al., 2021).

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Observation of semen deterioration

Spermatozoa viability was assessed by placing one drop (5 µl) of semen on the edge of the object glass and one drop of eosin-nigrosin (20 µl) on the semen in a ratio of 1:4, and then homogenized the mixture (Santoso et al., 2021). Furthermore, the mixture was prepared for analysis. The viability percentage was determined by dividing the number of live spermatozoa by the total sperm cells and multiplying by 100. Live and dead spermatozoa were indicated by transparent (colorless) and red heads, respectively (Mohamed et al., 2015).

Spermatozoa abnormalities were assessed using eosin-nigrosin staining. The semen was dripped onto the object glass and stained with eosin-nigrosin, and then thin smear preparations were made (Suhardi et al., 2020). Using a microscope (Olympus CX-23, Japan) with 400x magnification, 200 spermatozoa were counted in each of the five fields of view to determine the sperm count. The percentage of abnormality was obtained by counting the abnormal spermatozoa divided by the total number of sperm cells multiplied by 100 (Suhardi et al., 2020).

The acrosome cap was observed by making a thin slide of semen on the object glass, after which the preparations were dried and put into a 5% formalin solution at 37°C for 30 minutes. The slide was removed, washed using distilled water, and dried. Giemsa staining solution was prepared by adding 3 ml of Giemsa solution (Merck, Germany) drop by drop into 2 ml of standard pH 7 phosphate buffer solution, then adding 35 ml of aquabidest (Jayamas Medica, Indonesia). Subsequently, this solution was mixed until evenly distributed. The slide fixed in 5% formaldehyde (Merck, Germany) was put into the ready Giemsa staining solution for 4 hours in a water bath at 37°C. The stained slide was washed with distilled water and observed using a microscope (Olympus CX23, Japan - Optilab advanced V2, Indonesia) by counting 200 spermatozoa. Acrosomes of intact sperm were indicated by the purple top of the head, while those with light color imply deterioration (Chowdhury et al., 2014; Prihantoko et al., 2020).

The integrity of the spermatozoa membranes was tested using a hypoosmotic swelling test solution, consisting of 0.31 g of sodium citrate (Merck, Germany) and 0.565 g of fructose (Merck, Germany) dissolved in 50 mL of aquadest (Purwoistri et al., 2013). The test was conducted using 1 ml of the hypoosmotic solution from 150 ml osmol added to 0.1 ml of spermatozoa and incubated at 37°C for 30 minutes. In the next step, observation was performed with a microscope (Olympus CX23, Japan) with 400x magnification (Yendraliza et al., 2019). A circular tail characterized sperm cells with intact membranes at the end. Meanwhile, those with incomplete membranes were characterized by straight-tail conditions (Rajashri et al., 2017).

Statistical analysis

Statistical analysis was performed using SPSS software (version 25 IBM). Individual differences among Balinese cattle were obtained by one-way ANOVA and Duncan's multiple-range test. P value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Semen quality

Table 1 shows the characteristics of fresh semen produced by each Bali cattle. The fresh semen in the current research had the lowest and highest motility values of 73.24 ± 2.28% (Individual H) and 84.88 ± 1.17% (Individual C), respectively. The lowest and highest concentrations were 1226.8 ± 279.5 million/ml (Individual J) and 1982.2 ± 227.4 million/ml (Individual D), respectively. These differed from previous research, reporting the lowest and highest motility rates as 79.7 ± 0.09% and 85.6 ± 0.08%, respectively (Tethool et al., 2021). Moreover, in a study conducted by Tethool et al. (2021), the lowest and highest concentrations were 876.2 ± 225.3 million/ml and 1459.6 ± 294.1 million/ml, respectively. According to National Standard number 4869-1:2021, the suitable semen to be processed as frozen should have a motility value of ≥70% (Santoso et al., 2021). Therefore, the means values produced by each individual meet the criteria, indicating a suitable condition for processing frozen semen.

Table 1. Fresh semen quality of Bali cattle aged 7-12 years

Individuals	Color	Consistency	pH	Volume (ml)	Concentration (million/ml)	Abnormality (%)	Motility (%)
A	Milky white	Thick	6.40 ± 0.001	3.96 ± 0.78	1421.8 ± 240.7	4.80 ± 2.52	75.40 ± 7.46
B	Milky white	Thick	6.56 ± 0.09	2.10 ± 1.81	1453.0 ± 208.6	5.28 ± 3.02	77.40 ± 9.22
C	Milky white	Thick	6.56 ± 0.17	7.12 ± 1.78	1606.0 ± 95.7	4.68 ± 1.87	84.88 ± 1.17
D	Milky white	Thick	6.44 ± 0.17	3.88 ± 0.77	1982.2 ± 227.4	3.92 ± 1.47	80.96 ± 4.87
E	Milky white	Medium	6.36 ± 0.09	5.20 ± 2.12	1409.8 ± 255.9	3.68 ± 1.51	79.72 ± 4.03
F	Milky white	Medium	6.52 ± 0.11	5.20 ± 1.11	1323.4 ± 336.9	2.74 ± 1.33	81.48 ± 4.55
G	Milky white	Thick	6.40 ± 0.14	4.88 ± 1.03	1782.8 ± 153.9	3.66 ± 1.14	80.52 ± 6.42
H	Milky white	Thick	6.52 ± 0.11	2.72 ± 1.11	1758.4 ± 309.4	6.24 ± 1.70	73.24 ± 2.28
I	Milky white	Medium	6.56 ± 0.17	4.72 ± 2.67	1411.2 ± 218.4	6.48 ± 3.74	74.38 ± 6.95
J	Milky white	Medium	6.60 ± 0.14	7.00 ± 4.79	1226.8 ± 279.5	3.30 ± 0.60	79.34 ± 11.06

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Viability and abnormalities

As can be seen in Table 2, individual variations caused significant differences in the viability of spermatozoa in AD and PT1 groups ($p < 0.05$), while in the PT2 group there was no significant in the viability of spermatozoa. The highest values of individual variations in AD, PT1, and PT2 sperm viability were 92.43 ± 3.12% (Individual C), 72.90 ± 6.69% (Individual F), and 67.52 ± 5.14% (Individual B), respectively. Meanwhile, the lowest values were recorded for Individual F (84.84 ± 5.58%), followed by Individual G (65.74 ± 4.89%), and H (62.32 ± 6.36%). The viability of spermatozoa before freezing was higher than 71.82 ± 7.38% - 72.08 ± 6.63%, as reported by Hapsari et al. (2018). However, after freezing, it was lower than the amount of 75.79 ± 0.84% - 79.9 ± 20.84% in a study by Indriastuti et al. (2020).

Table 2. Viability and abnormalities value of spermatozoa in different Bali cattle individuals aged 7-12 years

Individuals	Viability (%)			Abnormality (%)		
	AD	PT1	PT2	AD	PT1	PT2
A	86.69 ± 5.26^{ab}	70.63 ± 5.49^{abc}	64. 85 ± 7.26	5.57 ± 1.11^{abc}	8.97 ± 1.29	13.58 ± 4.66^{b}
B	91.24 ± 3.93^{c}	72.65 ± 5.90^{c}	67.52 ± 5.14	6.46 ± 2.33^{c}	9.32 ± 1.37	10.93 ± 1.39^{a}
C	92.43 ± 3.12^{c}	67.23 ± 4.13^{ab}	63.69 ± 3.88	4.64 ± 2.61^{ab}	9.66 ± 1.04	10.46 ± 0.78^{a}
D	90.54 ± 3.42^{bc}	70.09 ± 6.65^{abc}	64.74 ± 4.01	5.45 ± 0.97^{abc}	9.49 ± 0.97	10.27 ± 1.25^{a}
E	88.48 ± 3.97^{abc}	70.81 ± 4.57^{abc}	65.77 ± 4.18	6.33 ± 1.76^{bc}	9.56 ± 1.57	10.59 ± 1.34^{a}
F	84.84 ± 5.58^{a}	72.90 ± 6.69^{c}	63.26 ± 7.14	4.91 ± 1.56^{abc}	9.48 ± 0.47	10.92 ± 1.30^{a}
G	90.66 ± 5.44^{bc}	65.74 ± 4.89^{a}	63.16 ± 5.01	4.46 ± 1.27^{a}	9.60 ± 1.37	10.45 ± 0.70^{a}
H	90.29 ± 2.34^{bc}	68.49 ± 6.89^{abc}	62.32 ± 6.32	4.83 ± 1.13^{abc}	9.65 ± 1.50	10.38 ± 1.45^{a}
I	88.62 ± 4.09^{abc}	71.66 ± 5.65^{bc}	64.43 ± 4.29	6.50 ± 2.99^{c}	9.25 ± 1.00	10.32 ± 1.36^{a}
J	91.49 ± 3.24^{c}	71.28 ± 4.73^{bc}	62.65 ± 4.95	4.97 ± 1.47^{abc}	9.25 ± 0.80	10.12 ± 1.24^{a}

^{a,b,c}Different superscripts in the same column were significantly different ($p < 0.05$). AD: After adding A1 diluent; PT1: Post-thawing cooling time of 4 hours; PT2: Post-thawing cooling time of 22 hours.

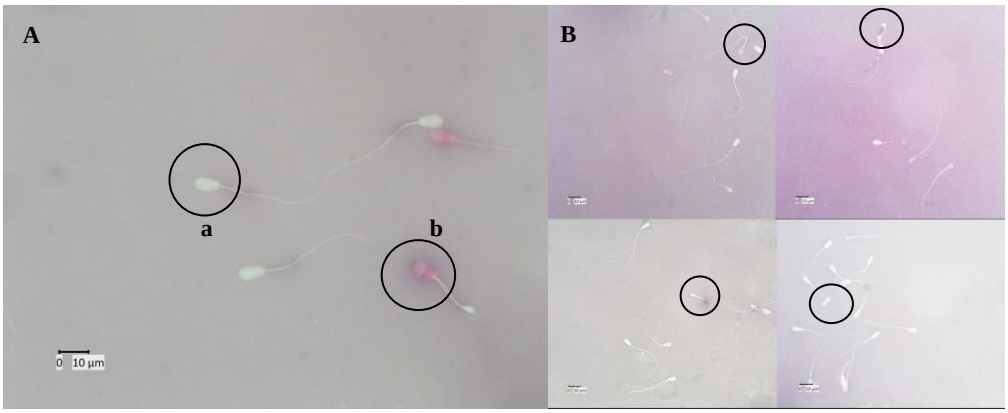

Figure 1. Identification of viability and abnormalities of spermatozoa Bali cattle aged 7-12 years. **A:** (**a:** viable [the head does not absorb color], **b:** nonviable), **B:** Abnormalities of sperm

Spermatozoa viability is one of the primary requirements and factors used for quality testing that shows fertilizing ability (Tanga et al., 2021) based on the number of sperm cells that can survive. It is assessed by exposing spermatozoa to eosin-nigrosine staining. Live spermatozoa cells are characterized by colorless spermatozoa heads (Figure 1A) since the function of protecting cell organelles and regulating the entry, and discarding of the required substances cannot occur properly. Damage to the plasma membrane of spermatozoa results in the disruption of metabolic processes, and consequently its death. The dead sperm cells cannot filter the staining liquid, leading to a colored head for the dead cells (Tanga et al., 2021). According to the results of the present study, individual variation in Bali cattle caused differences in the viability of the spermatozoa AD groups and PT1 groups because individuals in these groups had different cholesterol characteristics in their membranes. As reported in previous studies, individual variations affect spermatozoa viability due to the cholesterol characteristics that form condensation and plasma membrane integrity in each individual (Saez and Drevet, 2019; Indriastuti et al., 2020). Therefore, individuals with the highest viability values before freezing (AD) differed from those after freezing (PT1 and PT2). The low cholesterol content in the membrane results in the low resistance of spermatozoa to the freezing process, so the spermatozoa membrane will lose its function (Cornelius et al., 2015). Ice crystals are formed due to extreme temperature changes, and the freezing process in spermatozoa in this context causes deterioration of structure and plasma membrane (Sharma et al., 2015). Variations in male cattle affect spermatozoa viability (Mohammed et al. 2015). The ability of sperm cells to survive freezing varies between species and individuals of the same species (Yánez-Ortiz et al., 2021). This difference results from variations in each individual's biochemical properties and cell metabolism (Ali et al., 2022).

The value of spermatozoa viability is closely related to the integrity of the plasma membrane (Palacin et al, 2020). It was reported that the loss of spermatozoa viability was due to membrane damage induced by lipid peroxidation in the

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plasma membrane (Alahmar, 2019). The mechanism of membrane deterioration is initiated through the transition phase at the beginning of cooling. Furthermore, the cooling process changes the molecular structure of lipids, proteins, and nucleic acids, leading to a decrease in membrane fluidity (Sieme et al., 2015). During the freezing process, extracellular ice crystals are formed from diluent medium spermatozoa and an increase in the concentration of electrolytes in the spermatozoa cells (Öztürk et al., 2020). The formation of extracellular ice crystals increases the concentration of solutes contained. This osmotic gradient causes the water in the spermatozoa to diffuse out of the head through the plasma membrane, dehydrating the sperm cells. This condition causes the plasma membrane to be susceptible to lipid peroxidation by the activity of reactive oxygen species (Sobeh et al., 2020). According to Ramírez-Reveco et al. (2016), the percentage of post-thawing viability of sperm cells declared as the best value for artificial insemination is 64-80.

Individual variations of Bali cattle caused significant differences ($p < 0.05$) in the abnormalities values of AD and PT2 groups, while the PT1 group did not differ significantly ($p > 0.05$, Table 2). The highest values of the average abnormality obtained in AD, PT1, and PT2 were 6.50 ± 2.99% (Individual I), 9.66 ± 1.04% (Individual C), and 13.58 ± 4.66% (Individual A), respectively, while the lowest were 4.46 ± 1.27% (Individual G), 8.97 ± 1.29% (Individual A), and 10.12 ± 1.24% (Individual J), respectively. The results obtained were higher than that of Indriastuti et al. (2020), which were 3.45 ± 0.79 - 5.00 ± 0.37% and 4.15 ± 0.93 - 7.80 ± 1.29% before and after freezing. However, it was lower than the study by Surahman et al. (2021), which was 23.2% in semen after freezing.

The abnormality value indicates the percentage of spermatozoa with abnormal morphology throughout spermatogenesis or in the reproductive tract till ejaculation. Individual variations in the AD and PT2 groups led to significant differences in the abnormalities (Figure 1B), while the PT1 group did not differ in the abnormalities (Table 2). The differences in each of these individuals can be caused by the ability of each to maintain the stability of their cell membranes differently, hence, giving a different response to the abnormality of the spermatozoa produced. According to Parameswari and Sridharan (2019), the deterioration of the morphology can be avoided when the stability of the spermatozoa cell membrane is maintained. Furthermore, long cold storage time causes pressure changes and the production of free radicals (Bustani and Baiee, 2021). Extreme changes in osmotic pressure and ice crystals during cooling and freezing experienced by spermatozoa result in deterioration and abnormalities (Upadhyay et al., 2021). As a result of the freezing and thawing process, abnormalities in the midpiece of spermatozoa are acceptable (Ghirardosi et al., 2018). According to the 2021 Indonesian National Standard, a maximum of 20% spermatozoa abnormality is a requirement for frozen semen (Nugraha et al., 2021). Perry (2021) stated that the quality of semen could be doubted when it has a spermatozoa abnormality value of more than 20%. A high value of sperm abnormality could affect fertilization ability (Perry, 2021). According to a study of Firhamsah et al. (2022), Bali cattle with low spermatozoa abnormality values had a higher chance of successful insemination due to the number of cattle that do not return to heat for 60-90 days after mating or insemination.

Plasma membrane and intact acrosome

Individual variations caused significant differences in the intact plasma membrane of spermatozoa in AD and PT1 ($p < 0.05$), such a difference was not observed in the PT2 group ($p > 0.05$). Individual D had the highest intact plasma membrane values in the AD and PT1 groups, with 84.88 ± 6.07% and 67.8 ± 33.29%, while Individual B had the lowest intact plasma membrane values in the PT2 group, with 61.4 ± 51.95%, as shown in Table 3. The results obtained were in the same range as those by Indriastuti et al. (2020), which were 77.88 ± 1.64% - 86.22 ± 0.82% (before freezing) and 68.58 ± 0.86% - 77.09 ± 0.58%. However, it was higher than other research, which reported that Bali cattle's intact plasma membrane values after freezing were 43% (Diansyah et al., 2021) and 44.60 ± 0.6% - 48.21 ± 0.9% (Yendraliza et al., 2019).

The plasma membrane protects the spermatozoa against external conditions, such as entering certain substances (Diansyah et al., 2021). Sperm cells exposed to a solution with a higher osmotic pressure caused the tail to swell and coil, as shown in Figure 2A. Deterioration of the plasma membrane results in disruption of the metabolic process of spermatozoa cells, decreasing viability and increasing abnormalities (Ugur et al., 2019). Intact plasma membranes are also needed to perform normal functions and metabolism and induce capacitation and acrosome reactions (Cunha et al., 2017), enabling sperm cells to interact with oocytes (Tulake et al., 2015). The best value of intact plasma membrane was obtained in individual C. According to Indriastuti et al. (2020), variations of Bali cattle affect the proportion of intact plasma membranes. The proportion of the plasma membrane is influenced by the components of the membrane consisting of phospholipids, proteins, and carbohydrates (Öztürk et al., 2020).

Cell membranes containing cholesterol become sensitive to changes in temperature (De Toni et al., 2021). The condition of the spermatozoa cells membrane is influenced by the fatty acid composition and lipid ratio (Mandal et al., 2014). During freezing, extreme temperatures and osmotic pressure alter the structure and lipid composition of the plasma membrane in each part of the sperm cells (Cheng et al., 2022), which causes functional deterioration, resulting in decreased motility, circular movement, and premature death (Reis et al., 2016; Shan et al., 2021). The condition of the plasma membrane is related to the intact acrosome cap. This is because the deterioration of the plasma membrane is usually accompanied by that of the organelles of the intact acrosome cap cells, causing the release of enzymes needed during the fertilization process (Arvioges et al., 2021).

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The highest mean values of intact acrosomes of Bali cattle semen in AD, PT1, and PT2 groups were 88.58 ± 3.57% (Individual J), 74.04 ± 4.76% (Individual E), and 71.28 ± 9.99% (Individual B), respectively. Individual variations of Bali cattle caused significant differences in the intact acrosome cap values of AD, PT1, and PT2 groups ($p < 0.05$, Table 3). Before freezing, Bali cattle's average intact acrosome cap was 91.06% (Damayanti et al., 2021), while frozen semen was 64.12 ± 1.21-76.82 ± 1.55% (Prihantoko et al., 2020).

Table 3. Plasma membrane and intact acrosome value in different Bali cattle individuals aged 7-12 years

Individuals	Intact plasma membrane (%)			Intact acrosome (%)		
	AD	PT1	PT2	AD	PT1	PT2
A	79.05 ± 2.92^{ab}	57.17 ± 5.36^{a}	60.86 ± 2.13	81.14 ± 6.79^{a}	68.85 ± 6.49^{a}	65.61 ± 7.51^{abc}
B	84.10 ± 4.75^{cd}	66.33 ± 3.28^{cd}	61.45 ± 1.95	83.43 ± 2.63^{abc}	70.85 ± 4.96^{ab}	71.28 ± 9.99^{c}
C	84.88 ± 6.07^{d}	67.83 ± 3.29^{d}	60.23 ± 3.34	83.62 ± 6.45^{abc}	67.88 ± 4.08^{a}	60.91 ± 5.26^{a}
D	80.23 ± 6.19^{abc}	65.09 ± 4.09^{bcd}	60.97 ± 3.02	84.96 ± 5.41^{abc}	68.62 ± 5.68^{a}	62.74 ± 8.52^{ab}
E	82.88 ± 5.64^{bcd}	65.19 ± 4.51^{bcd}	60.37 ± 2.78	87.09 ± 6.12^{bc}	74.04 ± 4.76^{b}	65.56 ± 7.51^{abc}
F	81.80 ± 4.12^{abcd}	65.09 ± 4.53^{b}	60.30 ± 2.78	86.97 ± 6.55^{bc}	72.27 ± 6.27^{ab}	66.67 ± 8.34$_{abc}$
G	79.73 ± 5.07^{abc}	67.53 ± 3.24^{d}	60.23 ± 2.63	87.09 ± 5.29^{bc}	69.25 ± 4.98^{ab}	60.55 ± 2.67^{a}
H	82.18 ± 4.05^{bcd}	66.94 ± 3.25^{d}	60.05 ± 3.01	82.49 ± 7.39^{ab}	67.25 ± 5.67^{a}	63.34 ± 6.03^{ab}
I	77.61 ± 4.79^{a}	62.96 ± 2.97^{bc}	58.72 ± 3.54	84.27 ± 6.86^{abc}	68.88 ± 3.07^{a}	69.77 ± 9.74^{bc}
J	83.14 ± 6.62^{bcd}	66.93 ± 4.23^{d}	61.01 ± 2.50	88.58 ± 3.57^{c}	73.89 ± 5.10^{b}	59.55 ± 7.81^{a}

^{a,b,c}Different superscripts in the same column were significantly different ($p < 0.05$). AD: After adding A1 diluent; PT1: Post-thawing cooling time of 4 hours; PT2: Post-thawing cooling time of 22 hours.

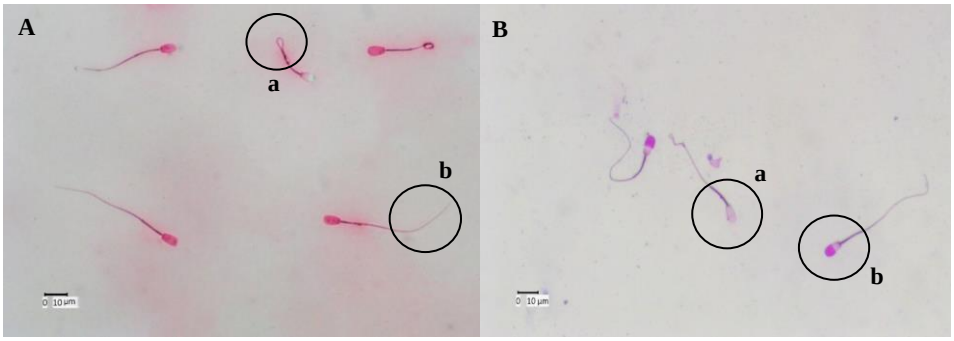

Figure 2. Identification of plasma membrane integrity using HOS test solution and acrosome cap using Giemsa staining in Bali cattle sperm aged 7-12 years. **A:** Intact plasma membrane (**a**), Deterioration plasma membrane (**b**), **B**: Intact acrosome cap (**a**), Deterioration acrosome cap (**b**)

The best percentage of intact acrosome cap in the PT1 group was for Individual E, with a value of 74.04%, but it was not different from individual J with a value of 73.89%. Before freezing, individual J had the highest intact acrosome cap value of 88.58%; hence, it maintained the condition of the acrosome cap after freezing. The differences obtained can result from variations in the protein profile of each individual. The distribution of tyrosine-phosphorylated acrosome protein from each male is different, resulting in individual differences in maintaining acrosome stability after the freezing process (Arai et al., 2017). Due to its small molecular weight, Giemsa staining can bind to proteins on the membrane and can pass through cell membranes that protect the acrosomes (Nofa et al., 2018; Prihantoko et al., 2020). Furthermore, the integrity acrosome is needed to ensure the success of spermatozoa in fertilizing the egg because the cap protects the enzymes contained (Sun et al., 2020).

The male sperm cells with a fertility rate of more than 53% have a high percentage of acrosome integrity (Yániz et al., 2021). Acrosome integrity of Bali cattle spermatozoa was observed using Giemsa staining. The dark purple color of the head indicated an intact acrosome cap, while the light purple or even colorless showed spermatozoa with incomplete acrosome caps (Figure 2B). The acrosome is a structure located in the apical part of the head, which plays a vital role in fertilization. Therefore, its deterioration results in the release of enzymes from the interior and directly causes the spermatozoa to lose their fertilizing ability (Hirose et al., 2020). The acrosome contains glycohydrolases and acrosins responsible for binding and penetrating the zona pellucida (Nagdas et al., 2016; Adrian et al., 2019). The binding of spermatozoa to the zona pellucida results in acrosome reactions and the release and activation of its enzymes, which allows penetration (Sawada and Saito, 2022). The percentage of intact acrosome caps in each individual is influenced by the ability of the membrane function to protect the sperm acrosome (Sitepu and Marisa, 2019).

CONCLUSION

The individual variations in semen before freezing can affect the parameters of spermatozoa deterioration, including viability, abnormalities, intact plasma membrane, and intact acrosome. Individual variations at 5°C in the cooling time of

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4 hours had better viability, abnormalities, intact plasma membrane, and acrosome caps of spermatozoa, compared to cooling at 5°C for 22 hours.

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Authors' contribution

Angelina Novita Tethool, Gatot Ciptadi, Sri Wahjuningsih, and Trinil Susilawati contributed to the study design, data analysis, and manuscript writing. Angelina Novita Tethool collected samples from the field and performed laboratory analysis. All authors reviewed the data from this study, performed statistical analyses, and approved the final draft of the manuscript. The authors reviewed and approved the final manuscript prior to submission to the journal.

Competing interests

The authors declare that there are no competing financial, and personal interests that might influence the research presented here.

Ethical considerations

The authors declare that this manuscript is original, has been checked by all the authors, and is not currently being considered for publication elsewhere.

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