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The Quality of Frozen Semen of Limousin Bull in Various Semen Diluents

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ABSTRACT

Diluents are substances added to the semen to increase the semen volume, reduce sperm density, and maintain sperm viability. This study aimed to compare the effects of various semen diluents on the quality of frozen semen of Limousin bull. Three mature Limousin bulls were used as semen sources. The semen was collected using an artificial vagina and then evaluated for its quality. Sperms with motility less than 70% and abnormality greater than 20% were excluded from this study. After semen evaluation, each ejaculate was individually divided into four equal tubes and diluted with skimmed milk-egg yolk (SMEY), Tris–egg yolk (TEY), Tris–egg yolk–skimmed milk (TEYSM), or Andromed®. Diluted semen was loaded into a mini straw and then equilibrated at 5 °C for 4 h. Following equilibration, the straws were frozen. The percentages of sperm motility, viability, intact membrane, and sperm DNA integrity were evaluated after freezing. Furthermore, malondialdehyde (MDA) and aspartate aminotransferase (AST) enzyme concentrations were assessed after thawing. The sperm motility with TEY, TEYSM, and Andromed® did not differ significantly but was higher than with SMEY. Sperm viability, membrane intactness, and sperm DNA integrity did not differ among treatments. By contrast, the MDA levels and concentration of AST in the Andromed® diluent semen showed the lowest values compared with SMEY semen ($p<0.05$) but did not differ from those of TEY and TEYSM semen. To conclude, TEY, TEYSM, and Andromed® diluents performed equally well, and although they showed better results than SMEY, sperm diluted in SMEY are still suitable for artificial insemination.

Keywords: AST; frozen semen of Limousin; MDA; semen diluent

INTRODUCTION

Indonesia has several artificial insemination centers (AICs) spreading over different islands (Jakaria *et al.*, 2018). AICs produce frozen semen from superior males, used in artificial insemination (AI) technology. Artificial insemination in cattle is an efficient reproductive biotechnology (Choudhary *et al.*, 2016), easy to apply (Durrant, 2009), and useful for increasing livestock population and genetic quality (Morrell, 2011). Artificial insemination in Indonesia primarily uses frozen semen. However, the semen-freezing process can cause physical damage in the form of the plasma membrane and mitochondrial damage. Freezing can also result in DNA fragmentation, mRNA degradation, and chromatin changes (Ugur *et al.*, 2019).

Furthermore, according to Ugur *et al.* (2019), freezing affects reactive oxygen species (ROS) formation. The presence of malondialdehyde (MDA) and aspartate aminotransferase (AST) enzymes in post-thaw semen are indicators of high levels of ROS, which can cause a decline in semen quality (Waheed *et al.*, 2013; Ugur *et al.*, 2019). The success of the freezing process is influenced by the dilution and freezing techniques (Pena & Forsberg, 2000) and the type of diluent used (Jha *et al.*, 2019).

Diluents are substances added to the semen to increase the semen volume, reduce sperm density, and maintain sperm viability; thus, the process of frozen semen production requires a diluent to maintain sperm quality. Various types of diluents are currently used for both homemade and commercial uses, including skimmed milk-egg yolk (SMEY), Tris–egg yolk (TEY), and Andromed® (Susilawati, 2013; Baharun *et al.*, 2017). Some AICs have their own diluent compositions, including Tris–skimmed egg yolk. Skimmed milk-egg yolk diluent is a medium containing lipoprotein and lecithin to maintain sperm viability (Feradis, 2010) and protect sperm from cold shock. Milk also contains the enzyme lactenin, which is destroyed during heating. Heating milk to more than 80 °C, releases sulfhydryl groups (–SH), which function as reductive substances that regulate the oxidative metabolism of sperm (Widjaya, 2011).

Tris-egg yolk (TEY) contains substances needed by sperm, such as fructose, amino acids, and vitamins, from which sperm obtain sufficient energy to move progressively (Susilawati, 2013). Ax *et al.* (2000) stated that Tris buffer has several advantages, including maintaining pH, osmotic pressure, and electrolyte balance. According to Baharun *et al.* (2017), TEY has a complete composition, including Tris (hydroxymethyl) aminomethane, citric acid, fructose, aqua, egg yolk, penicillin, and streptomycin. Tris-skimmed milk-egg yolk diluent is a combination of Tris diluent with egg yolk-skimmed milk. This diluent has not been widely studied. The addition of 15% skimmed milk to the Tris-egg yolk diluent was able to suppress the decrease in the sperm viability of Simmental bulls stored for 2 days at a temperature of 5 °C (Widjaya, 2011).

The process of adding diluents to semen differs among AICs, including one- or two-step dilutions, but some use three- to four-step dilutions. One- and two-step dilution techniques are considered better than three- to four-step dilutions (Arief *et al.*, 2020). Frozen semen of Limousin bull is in great demand by farmers. Cows inseminated with Limousin frozen semen produce a large, fast-growing calf. Tris-skimmed milk-egg yolk diluent has not been studied scientifically. Thus, this study aims to comprehensively evaluate different diluents and find the most appropriate diluent to improve the quality of frozen semen of Limousin bull in Indonesia.

MATERIALS AND METHODS

Ethical Approval

This study was conducted following standard operational procedure SNI ISO 9001:2015 No. 824 100 15084 at the Ungaran AI Center in Central Java. A veterinarian supervised all methods in this study. The Ethical Committee of Ungaran AI Center in Central Java provided ethical guidelines and approval on the responsible conduct for bull semen collection.

Semen Collection and Evaluation

Semen collection was performed by a qualified bull master from the AIC. Three mature, healthy 3-year-old Limousin bulls were used as sources of semen. This study kept all bulls in individual cages equipped with feed and water. Feed was forage and concentrates, comprising up to 10% and 1% of body weight, respectively. The feed was given in the morning and afternoon, and drinking water was provided *ad libitum*. Semen was collected using an artificial vagina twice a week, in the morning, according to the standard protocol of the AIC. Immediately after collection, fresh semen was delivered to the laboratory for evaluation. The evaluations of the collected semen consisted of both macroscopic and microscopic assessments. The macroscopic evaluation included volume, color, consistency, and pH. The microscopic evaluation included mass movement, sperm motility, viability,

concentration, and sperm morphology (Arifiantini, 2012).

Diluent Preparation

The types of diluents used in this study were skimmed milk-egg yolk (SMEY), Tris-egg yolk (TEY), Tris-egg yolk-skimmed milk (TEYSM), and Andromed® (Table 1).

Semen Processing and Frozen Semen Quality Test

The standard for semen quality to be processed into frozen semen, according to the Indonesian Nasional Standard for frozen bull semen (Badan Standardisasi Nasional, 2017), is ≥70% sperm motility. The semen was divided into four tubes; each was diluted using SMEY, TEY, TEYSM, or Andromed® diluents, with a final dose of $100 × 10^6$ mL^{-1} or $25 × 10^6$ straw^{-1}. After dilution, the semen was packed in 0.25 mL mini straws (IVM, France) and equilibrated in a cooling cabinet for 4 h. Freezing of semen was performed using an automatic freezing machine (Minitube, Germany).

The frozen semen was then stored in a liquid nitrogen container for further testing. Before evaluation, the semen was thawed at 37 °C for 30 s. The semen was then transferred to microtubes and stored at 37 °C during evaluation. After thawing, sperm motility, viability, plasma membrane intactness, and DNA integrity were assessed. Additionally, the levels of MDA and AST enzymes were also evaluated.

Sperm Motility Evaluation

Evaluation of sperm motility was performed by dripping 10 μL of semen onto a warm glass slide and covering it with a cover glass. Sperm motility was observed using computer-assisted sperm analysis (CASA; Sperm Vision TM 3.7 Minitube, Germany), based on the analysis of digitized images from a computer connected to a microscope at 200× magnification. The test was automatically conducted at four fields of view (Michos *et al.*, 2013).

Table 1. Composition of various diluents used in the research

Ingredients	SMEY	TEY	TEYSM	Andromed®
Tris hydroxymethyl aminomethane (g)	–	3.03	1.6	–
Skimmed (g)	10	–	4	–
Citric acid (g)	–	1.78	0.9	–
Fructose (g)	2	1.25	–	–
Andromed® (mL)	–	–	–	20
Glycerol (%)	8	6	5	–
Egg yolk (%)	10	20	20	–
Pure water (mL) ad	100	100	75	80
Antibiotic				
Penicillin (IU/mL)	1000	1000	1000	–
Streptomycin (mg/mL)	1	1	1	–

Note: SMEY= skimmed milk–egg yolk, TEY= Tris–egg yolk, TEYSM= Tris–egg yolk–skimmed milk.

Sperm Viability and Sperm Plasma-Membrane Integrity Assessments

Sperm viability was assessed by mixing 50 µL of semen with 100 µL of eosin nigrosine stain, smeared, and dried at 37 °C. Examination of the integrity of the sperm plasma membrane was performed by mixing 50 µL of semen with 950 µL of HOS solution (Arif *et al.*, 2020).

Malondialdehyde (MDA) Levels Evaluation

MDA levels were tested using the thiobarbituric acid (TBA) sperm method (Anghel & Stela, 2010; Sukmawati *et al.*, 2014). One milliliter of thawed semen was centrifuged at 1000 ×g for 10 min (Kitman-T24, Tommy, Japan). The supernatant was discarded, and the sperm pellet was then washed twice with Tris HCl pH 7. Next, 1 mL of pure water was added to the pellet and 0.5 mL TBA (0.67 g 2-thiobarbituric acid, 100 mL distilled water, 0.5 g NaOH, and 100 mL glacial acetic acid). The sample was heated (90 °C) for 1 h and centrifuged at 4000 × g (10 min). The resulting supernatant was pink, and its absorbance was measured using a spectrophotometer at a wavelength of 532 nm. MDA coefficient: 1.56×105 M cm^{-1}. Results are expressed in MDA/108 nmol of sperm.

AST Enzyme Concentration Analysis

Concentrations of AST enzyme were analyzed using a commercial kit (Glory® Diagnostics, Spain). A total of 1 mL of thawed semen was centrifuged at 2500 × g for 10 min. Then, 50 µL of supernatant was added to 1 mL of commercial kit reagent, homogenized, and incubated at 37 °C for 1 min. The absorbance at 1, 2, and 3 min was calculated at a wavelength of 340 nm. AST concentration was calculated as U/L= ΔA/min × 3333 (Glory® Diagnostics).

Sperm DNA Evaluation

Testing of Sperm DNA damage was conducted using acridine orange (AO) staining. Twenty microliters of thawed semen were smeared on a clean glass slide. The smear was then air-dried and fixed in Carnoy's solution (a mixture of acetic acid and methanol, 1:3) for 4 h. After fixation, the slide was rinsed using pure water, air-dried, and then immersed in AO solution in the dark (12-15 h). The slide was air-dried at room temperature in the dark. The slide was observed using a fluorescence microscope at a magnification of 400× and excitation light of 450-490 nm in the dark (Said *et al.*, 2015; Santoso *et al.*, 2021). Sperm with intact DNA emits a green color, whereas fragmented sperm emits a yellow to orange color. Five hundred sperm cells were examined for each sample. The DNA damage was calculated by dividing DNA damage by the total number of sperm, multiplied by 100%.

Data Analysis

This study used a completely randomized design with 4 replications. The data obtained were analyzed using analysis of variance at a 95% significance level and Duncan's multiple range test. The data were processed using SPSS version 24.0. Data are presented as means ± standard error.

RESULTS

The Quality of Fresh Semen of Limousin Bull

Fresh semen from Limousin bulls is milky white to cream in color, with a medium-to-thick consistency. Semen volume and pH were 6.23±0.26 mL and 6.43±0.01, respectively. Microscopic evaluation showed good mass movements (+++), with sperm motility of 70%. Sperm concentration was 1128.17±46.85, with sperm viability of 93.84%±0.93%. Sperm membranes were mostly intact (93.28%±0.90%) with low sperm abnormality (1.44%±0.07%). In summary, the fresh semen of Limousin showed good quality and was suitable for semen freezing.

Quality of Frozen Semen of Limousin Bull in Various Diluents

Table 2 presents the quality of frozen semen of Limousin bull in various diluents. After thawing, the quality of frozen semen showed differences in total and progressive motility between types of diluents ($p<0.05$). The sperm total motility was higher ($p<0.05$) in the Andromed® diluent than those in SMEY, TEY, and TEYSM diluents. Sperm progressive motilities in Andromed®, TEY, and TEYSM were not significantly different ($p>0.05$), but all three values were higher ($p<0.05$) than those for SMEY. However, SMEY was still suitable for AI, according to Indonesian National Standards for bovine frozen semen quality, as it showed >40% sperm motility.

Sperm-DNA Damage in Frozen Semen of Limousin Bull

The results showed that frozen semen of Limousin bull diluted in various diluents had intact sperm DNA between 76.43%–80.21%, with no difference in DNA damage between treatments.

MDA and AST Levels in the Frozen Semen of Limousin Bull

Figure 1 shows the MDA and AST enzyme levels of frozen semen of Limousin bull in various diluents. MDA levels in frozen-thawed sperm with SMEY, TEY, TEYSM, and Andromed® diluents were 0.26±0.02, 0.07±0.01, 0.06±0.01, and 0.04±0.01 nmol/10^8, respectively. Sperm frozen in Andromed® diluent had lower MDA levels than those in SMEY ($p<0.05$), but there was no difference among the values for Andromed®, TEY, and TEYSM diluents. Concentrations of AST enzyme in frozen semen were 14.99±1.42, 7.77±1.85, 7.77±1.11, and 5.55±1.11 U L^{-1} for SMEY, TEY, TEYSM, and Andromed®, respectively (Figure 1).

Table 2. Quality of frozen semen of Limousin bull in various diluents

Variables	Diluent			
	SMEY	TEY	TEYSM	Andromed®
Fresh Semen				
Sperm motility (%)	--70--			
Sperm viability sperm (%)	------------------------------------93.84 ± 0.93------------------------------------			
Sperm IM (%)	------------------------------------93.28 ± 0.90------------------------------------			
After thawed semen				
TMOT (%)	55.70 ± 5.70[b]	61.08 ± 4.20[b]	58.99 ± 1.39[b]	76.98 ± 3.58[a]
PMOT (%)	41.60 ± 2.50[b]	53.20 ± 3.96[ab]	53.44 ± 1.24[ab]	66.34 ± 8.33[a]
Sperm viability (%)	77.46 ± 2.40[a]	80.56 ± 0.46[a]	80.51 ± 0.88[a]	78.50 ± 1.17[a]
Sperm IM (%)	76.43 ± 2.69[a]	79.58 ± 0.55[a]	80.21 ± 0.89[a]	77.35 ± 1.88[a]

Note: Means in the same row with different superscripts differ significantly ($p < 0.05$). SMEY= skimmed milk–egg yolk; TEY= Tris–egg yolk; TEYSM= Tris–egg yolk–skimmed milk; TMOT= total motility; PMOT= progressive motility; IM= intact membrane.
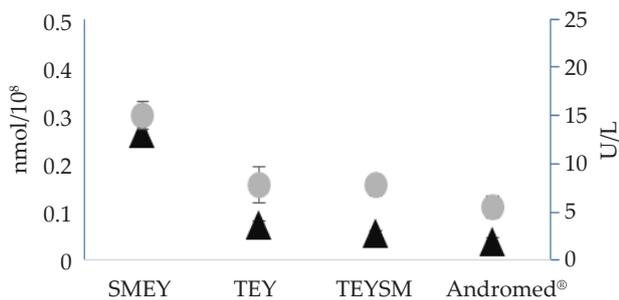

Figure 1. Malondialdehyde (▲) and aspartate aminotransferase enzyme (●) contents of frozen semen of Limousin bull in various semen diluents. SMEY= skimmed milk-egg yolk; TEY= Tris-egg yolk; TEYSM= Tris-egg yolk-skimmed milk.

DISCUSSION

The percentages of progressive sperm motility in the four diluents were under the requirements of SNI 4869-1:2017 for frozen bovine semen, with sperm motility of at least 40% (Badan Standardisasi Nasional, 2017). Khalil *et al.* (2018) reported that freezing and thawing processes decreased bull-sperm motility by 26.2%–60%. This study showed that the decreases in sperm motilities were between 3.6% and 28.4%. This decrease could be caused by oxidative stress, changes in osmotic pressure, or sperm temperature during the cryopreservation process (Sharafi *et al.*, 2015).

Semen diluents play a role in maintaining sperm quality during the cooling process. Andromed® contains soya lecithin, Tris, citric acid, fructose, antioxidants, glycerol, pure water, and additional antibiotics (Tylosin, Gentamicin, Spectinomycin, and Lincomycin) (Minitub, 2001). The antioxidant content of Andromed® is thought to be more optimally associated with the protein plasma membrane, which can prevent mechanical damage during semen processing. TEY contains substances needed by sperm as a source of nutrients. Tris contains citric acid and fructose, which act as a buffer to prevent changes in pH due to lactic acid formation during sperm metabolism. Tris also maintains osmotic pressure and electrolyte balance is a source of energy and protects sperm from cold shock (Raheja *et al.*, 2018). Skimmed

milk diluents contain lactose, which removes water from the cells, reducing the formation of ice crystals. Additionally, skimmed milk diluent acts as an osmotic-pressure buffer to avoid cell swelling and stabilize cell membranes (Best, 2015). Lactose in milk prevents intra-cellular crystallization by suppressing the osmotic pressure outside the cells. Ten percent whole milk or skim milk and 7% glycerol with antibiotics are commonly used for frozen bull semen. Skim milk contains nutrients that can be used by sperm as an energy source.

The addition of egg yolk to the diluent protects sperm from the influence of cold shock, and milk also contains lactenin, which is toxic to sperm. Lactenin is destroyed during heating, and heating milk at a temperature of more than 80 °C will release sulfhydryl groups (–SH), which function as reductive agents that regulate the oxidative metabolism of sperm (Raheja *et al.*, 2018). Successful use of SMEY, TEY, and Andromed® diluents has been widely reported, but studies of the TEYSM combination are lacking. The TEYSM diluent in this study maintained the quality of frozen semen of Limousin bull and could, therefore, be used as an alternative diluent. The use of a TEYSM combination diluent was previously reported by Widjaya (2011), who reported an increase in the motility of Simmental bull semen that was stored for 2 days at 5 °C with a motility percentage of 60.7%.

Using CASA facilitated the objective study of motility (Michos *et al.*, 2013). A high progressive motility percentage can improve sperm fertilization ability, which can be assessed using sperm kinematics parameters (Table 3). According to Kathiravan *et al.* (2011), curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) parameters are used to predict *in vivo* fertility. Moreover, Inanc *et al.* (2018) added that beat cross-frequency (BCF) and the amplitude of lateral head displacement (ALH) could be used as indicators of sperm fertility.

This study showed no differences among diluents in their effects on sperm kinematics [VCL (119.70–143.10 µm s⁻¹), VAP (74.23–85.01 µm s⁻¹), and VSL (58.43–70.11 µm s⁻¹) in all diluents]. According to Nagi *et al.* (2015), there was a close correlation, with a high significance, between each of the velocities (VCL, VSL, VAP) and non-return rate (NRR) at day 30 and pregnancy rate

Table 3. Sperm kinematic variables of frozen semen of Limousin bull in various semen diluents

Variables	Diluent			
	SMEY	TEY	TEYSM	Andromed®
TMOT(%)	55.70 ± 5.70[b]	61.08 ± 4.20[b]	58.99 ± 1.39[b]	76.98 ± 3.58[a]
PMOT (%)	41.60 ± 2.50[b]	53.20 ± 3.96[ab]	53.44 ± 1.24[ab]	66.34 ± 8.33[a]
VCL(μm/s)	135.35 ± 4.71[a]	143.10 ± 5.28[a]	126.98 ± 5.91[a]	119.70 ± 12.59[a]
VAP (μm/s)	74.23 ± 3.21[a]	85.01 ± 1.12[a]	78.02 ± 3.37[a]	75.57 ± 7.03[a]
VSL(μm/s)	58.42 ± 4.16[a]	70.11 ± 2.16[a]	64.848 ± 3.49[a]	63.36 ± 6.78[a]
BCF (Hz)	23.92 ± 1.59[a]	28.19 ± 2.08[a]	26.83 ± 1.06[a]	25.19 ± 1.82[a]
ALH (μm)	5.21 ± 0.31[a]	5.25 ± 0.41[a]	4.39 ± 0.10[a]	5.31 ± 0.27[a]

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). SMEY= skimmed milk–egg yolk; TEY= Tris–egg yolk; TEYSM= Tris–egg yolk–skimmed milk; TMOT= total motility; PMOT= progressive motility; VCL= curvilinear velocity; VAP= average path velocity; VSL= straight-line velocity; BCF= beat cross-frequency; ALH= amplitude of lateral head displacement.

Table 4. Decrease in sperm viability of frozen semen of Limousin bull in various diluents

Sperm viability	Diluent			
	SMEY	TEY	TEYSM	Andromed®
Fresh semen (%)	--------------------------93.84 ± 0.93 ------------------------------			
After thawed semen (%)	77.46 ± 2.4[a]	80.56 ± 0.46[a]	80.51 ± 0.88[a]	78.50 ±1.17[a]
Decreased sperm viability (%)	16.38	13.28	13.33	15.34

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). SMEY= skimmed milk–egg yolk; TEY= Tris–egg yolk; TEYSM= Tris–egg yolk–skimmed milk.

Table 5. Decrease in the integrity of sperm plasma membrane in frozen semen of Limousin bull in various semen diluents

Sperm intact membrane	Diluent			
	SMEY	TEY	TEYSM	Andromed®
Fresh semen (%)	-----------------------------93.28±0.90---------------------------			
After thawed semen (%)	76.43 ± 2.69[a]	79.58 ± 0.55[a]	80.21 ± 0.89[a]	77.35 ± 1.88[a]
Decreased of intact membrane (%)	16.85	13.7	13.07	15.93

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). SMEY= skimmed milk–egg yolk; TEY= Tris–egg yolk; TEYSM= Tris–egg yolk–skimmed milk.

(PR) at day 75. Further analysis of VAP helps predict the fertilization rates of frozen-thawed bull semen; sperm are demonstrating VAP values of >45 μm s⁻¹ show a high NRR and PR (Nagy *et al.*, 2015). The sperm VAP values for the four diluents tested here ranged from 74.23 to 85.01 μm s⁻¹. Therefore, sperm from all diluents can fertilize an egg.

The viability of post-thaw sperm of frozen semen of Limousin bull showed no difference between diluents. The sperm viabilities of frozen bovine semen in this study ranged from 77.46% to 80.56%, with the decrease in sperm viability ranging from 13% to 16% (Table 4). The decrease in sperm viability of frozen semen of Limousin bull was due to an increase in the number of damaged and dead sperm during the cryopreservation process and the formation of lactic acid in the diluent medium. The decreased sperm viability in egg yolk-based diluents can also be attributed to the higher calcium ions in the egg yolks, which can cause acrosomal damage (Coppock, 2019). Nagata *et al.* (2019) reported that a satisfactory value for post-thaw sperm viability for the success of AI is >55%. This study showed that sperm viability values in all four diluents were >55% and were satisfactory for AI, according to Nagara *et al.* (2019).

There was no difference in the sperm-membrane integrity of frozen semen of Limousin bull between

diluents, which ranged 76%-80%, with a decrease of 13%-16.85% (Table 5). Reductions in sperm membrane integrity can result in low potential for sperm fertilization (Mughal *et al.*, 2018). In this study, the membrane integrity values were still within the normal range.

Sperm DNA damage can be evaluated using AO staining (Kucuk, 2018). This study indicated that all frozen semen in the various diluents had low sperm-DNA damage. DNA damage can occur during dilution, cooling, freezing, and thawing in the presence of ROS (Bailee *et al.*, 2018; Al-Badry *et al.*, 2018). This study showed that the type of diluent and freezing process did not cause DNA damage. Low DNA damage may also be influenced by the superior bulls used in this study; all bulls in the AI center show superior quality and, thus, have good sperm quality.

Low sperm-MDA levels indicate minor damage to sperm cells. High sperm MDA levels are caused by ROS activity, which attacks lipids on the sperm membranes during semen processing and thawing. Leakage of the membrane will cause the release of cellular components such as lipids, proteins, and other ions. This loss, in turn, affects sperm susceptibility to oxidative stress because of the large number of saturated fatty acid components present in the sperm membrane (Andersen *et al.*, 2016). Sorongbe *et al.* (2019) reported that the MDA content of frozen buck semen with TEY diluent

was 1.25 ± 0.17 nmol/10^8. Frozen semen of Limousin bull diluted using SMEY also showed low MDA levels (Sukmawati *et al.*, 2014). MDA levels in the frozen semen of FH bull diluted using TEY were higher than those in this study (Asadpour *et al.*, 2012). In this study, MDA levels of frozen semen of Limousin bull were relatively low. Therefore, we conclude that sperm cell damage from lipid peroxidation was limited.

The concentration of AST enzyme in the semen diluted in Andromed® was lower than that in SMEY ($p<0.05$). There was no difference in the concentrations of AST enzyme between Andromed®, TEY, and TEYSM diluents. Borah *et al.* (2015) reported that the concentration of AST in frozen yak cattle semen in TEY diluent was 11.95 ± 1.02 U L^{-1}. The concentration of AST enzyme of frozen bull semen in TEY diluent was 13.17 ± 0.17 IU mL^{-1} and lower than that with Biochiphos plus diluent (14.67 ± 0.17 IU mL^{-1}) (Veerabramhaiah *et al.*, 2011). Moreover, El-Nagar (2017) reported that the concentration of AST enzyme in frozen semen of Friesian bull using TEY diluent was 40.25 ± 0.94 U L^{-1}. Frozen semen of buffalo in TEY with 7% glycerol had an AST enzyme concentration of 98.75 ± 1.49 U L^{-1} (Abdel-Khalek *et al.*, 2016). The concentration of the AST enzyme in this study was relatively low, so it can be concluded that there was limited cell damage.

Good quality semen is characterized by the lower activity of AST enzyme (Ehala-Aleksejev & Punab, 2016). The release of enzymes from the sperm occurs if there is damage to the sperm membrane due to cold shock or poor-quality diluent. Low release of AST enzyme from the sperm reflects the effectiveness of the diluent in maintaining the integrity of the sperm membrane. Generally, the best semen diluents can block the release of enzymes and other electrolytes from the sperm cells (El-Harairy *et al.*, 2018).

A suitable diluent can provide an energy source for sperm metabolic processes. It can maintain osmotic pressure and pH and suppress the decline in sperm quality during the freezing and thawing process (Zega *et al.*, 2015). Skimmed milk is suitable for freezing bull semen (Vishwanath & Shannon, 2000). However, this study showed that the progressive motility of sperm in the skimmed milk diluent was lower than those with the other diluents, and the MDA levels and AST enzyme concentrations were higher. Mammalian semen shows different tolerances to each type of diluent (Herrera *et al.*, 2017), and sperm sensitivity to peroxidation damage can differ among individuals. These differences result from variations in the composition of the sperm plasma membrane, although each bull is reared with the same system and feed management (Sukmawati *et al.*, 2014).

CONCLUSION

The use of SMEY, TEY, TEYSM, and Andromed® diluents can provide a source of nutrition for the viability of sperm of Limousin bull. Based on the results of this study, AICs in Indonesia can safely use all these diluents. TEY, TEYSM, and Andromed® diluents maintained the sperm quality of Limousin bull semen during the freezing process better than SMEY. However, sperm in SMEY are still suitable for AI.

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