Assessment of Sperm Acrosome Status, Malondialdehyde and Aspartate Aminotransferase Enzyme Concentration of Frozen Semen from Limousin and Simmental Bulls in Different Commercial Diluents

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ABSTRAK

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Kriopreservasi semen adalah proses pengawetan sel sperma pada suhu rendah, agar semen bekunya dapat digunakan di masa yang akan datang. Kualitas semen beku dipengaruhi oleh bahan pengencer. Tujuan dari penelitian ini adalah untuk membandingkan pengaruh pengencer komersial terhadap status akrosom, konsentrasi malondialdehid (MDA) dan enzim aspartat aminotransferase (AspAT) dari semen beku sapi Limousin dan Simmental. Semen yang digunakan dalam penelitian ini berasal dari sapi Limousin dan Simmental dengan 5 x penampungan (Faktor pertama). Prosedur satu langkah digunakan untuk metode pengenceran. Andromed®, Optixcell® dan Steridyl® digunakan sebagai pengencer (Faktor kedua). Data dianalisis dengan analisis varians (ANOVA) dilanjutkan dengan Tukey HSD dengan selang kepercayaan 5%. Hasil penelitian menunjukkan tidak terdapat interaksi (P>0,05) antara dua faktor terhadap status akrosom, kerusakan akrosom sperma Simmental dalam Steridyl® secara signifikan lebih rendah daripada yang lain (P<0,05), namun semua pengencer menunjukkan kerusakan akrosom sperma yang rendah. Penelitian juga menunjukkan tidak ada interaksi antara jenis pengencer dan rumpun pada konsentrasi MDA dan AspAT (P>0,05). Ketiga pengencer komersial yang digunakan memiliki kemampuan yang sama dalam melindungi status akrosom dan mempertahankan konsentrasi MDA dan AspAT pada pembekuan semen sapi Limousin dan Simmental. Ketiga pengencer komersial dalam penelitian ini dapat menjadi alternatif bahan pengencer semen beku sapi Limousin dan Simmental.

Kata Kunci: Status Akrosom, AspAT, MDA, Sapi Limousin dan Simmental

ABSTRACT

Tahar MA, Komariah, Nuraini H, Maulana T, Gunawan M, Arifiantini RI. 2022. Assesment of sperm acrosome status, malondialdehyde and aspartate aminotransferase enzym concentration of frozen semen from Limousin and Simmental bulls in different commercial diluents. JITV 27(3):122-129. DOI: http://dx.doi.org/10.14334/jitv.v27.i3.3049.

Sperm cryopreservation is the process of preserving sperm cells at low temperatures, so that its frozen semen can be used in the future. The quality of the frozen sperm is affected by the diluent. The objective of this study was to compare the effects of commercial diluents on acrosome status, malondialdehyde (MDA) and aspartate aminotransferase (AspAT) enzyme concentration of thawed Limousin and Simmental bull semen. Semen was collected twice weekly using an artificial vagina. The fresh semen processed into frozen semen had sperm motility of >70%. The one-step procedure was used for the dilution methods. Andromed®, Optixcell® and Steridyl® were used as diluents. Data were analyzed by analysis of variance (ANOVA) followed by Tukey HSD 5% confidence interval. The result showed no interaction (P>0.05) between two factors on acrosome status. The sperm acrosome damage of Simmental in Steridyl® was significantly lower than others (P<0.05), although all diluents showed low sperm acrosome damage. Also, no interaction between the type of diluent and breed on MDA and AspAT enzyme concentrations was detected (P>0.05). The results suggest that three commercial diluents have equal efficacy in protecting acrosome status and maintaining MDA and AspAT enzyme concentrations of frozen Limousin and Simmental bull semen. Therefore, all commercial diluents can be an alternative for Limousin and Simmental frozen semen.

Key Words: Acrosome Status, AspAT, MDA, Limousin and Simmental Bull

INTRODUCTION

Frozen semen of Limousin and Simmental is in great demand among breeders. Frozen semen of the two cattle in Indonesia is produced by the National Artificial Insemination Center (AIC) and the Regional AIC (RAIC). The quality of the frozen semen is influenced by the type of diluent (Zamuna et al. 2015). Homemade diluents are used in the AIC, while homemade and commercial diluents are used in the RAIC. Commercial diluents are more convenient to prepare, less time-consuming, and contain antibiotics according to international standards. Semen diluents for international trade must include a combination of the antibiotics gentamicin, tylosin, lincomycin, and spectinomycin (GTLS). These antibiotic combinations have been developed and are commonly used in the Americas and other European countries (Morrell & Wallgren 2014). The commercial diluent commonly used in RAIC is Andromed®, which contains soy lecithin. There are now several commercial diluents with different sources of lecithin; one of these is Steridyl®, has contains sterile egg yolk. The newest commercial diluent is Optixcell®, which contains liposomes.

Cryopreservation impairs lipid composition and sperm plasma membrane organisation, leading to the leakage of valuable intracellular enzymes, such as aspartate aminotransferase (AspAT) (Fraser et al. 2018), or energy substrates, such as adenosine triphosphate (ATP) (Fraser et al. 2007), ultimately resulting in cell death. Sperm undergo cold damage and cold shock at low temperatures above those that cause freezing, which reduces their viability (Gączarzewicz et al. 2010). In general, cold damage refers to damage caused in cells maintained at critical temperatures below those at which cells normally function. In contrast, cold shock refers to reduced viability caused by either a rapid drop in temperature or a sharp drop in temperature. There is some overlap between the effects of cold shock and cold damage on cell organelles, particularly cell membranes. After freezing and thawing the most commonly studied quality tests were sperm motility, viability, and plasma membrane integrity (Hidayati et al. 2018).

Assessment of acrosome status is still rarely performed, although it is important because it is related to sperm fertility. The sperm acrosome contains several enzymes that play an important role in penetrating the zona pellucida during fertilization (Prihantoko et al. 2020). Besides that Malondialdehyde (MDA) and AspAT concentrations of frozen sperm are important indicators of plasma membrane damage (Gączarzewicz et al. 2010). MDA concentration was caused by lipid peroxidation of sperm plasma membrane due to high reactive oxygen species (ROS) and decreased sperm quality (Subramanian et al. 2018). The objective of this study was to compare the effects of acrosome status, and concentration of MDA and AspAT enzymes in sperm from Limousin and Simmental bull semen, frozen in different commercial diluents. The finding of this study can be used as a consideration for the selection of alternative commercial diluents to improve the quality of frozen bull semen.

MATERIALS AND METHODS

This study was conducted between November 2021 and February 2022 in Ungaran AIC, Central Java. Laboratory of Biotechnology Research Center, National Agency for Research and Innovation, Cibinong. Reproductive Rehabilitation Laboratory, Department of Reproduction and Obstetrics, and Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University.

This study was performed according to the standard operating procedure (SOP) 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 ethics committee of Ungaran AI Center in Central Java provided ethical guidelines and approval for the reliable performance of bull semen collection.

Research design

This study was conducted in a factorial randomised block design with three types of commercial diluents (Andromed®, Optixcell®, and Steridyl®), two groups of cattle breeds (Limousin and Simmental), and five replicates. This study was an experimental laboratory. Samples in the form of fresh semen are processed into frozen semen. Limousin and Simmental use in this study was in the productive age (3-5 years old, weight around 800 kg), each consisting of three bulls. The bulls were kept intensively according to the RAIC SOPs. Semen collection was performed twice weekly using an artificial vaginal method by a bull master according to RAIC SOPs. The criteria for fresh semen used for this study is motility of 70%.

Preparation of the diluent

Each commercial diluent was mixed with aquabidest (according to the diluent brochure) in a ratio of 1:4 (Andromed®), 1:2 (Optixcell®) and 1:1.5 (Steridyl®). The diluent is then homogenised and placed in a water bath (35°C).

Macroscopic and microscopic evaluation of the fresh semen

The evaluation of the semen was performed according to Arifiantini (2012). Macroscopic evaluation

includes volume, the colour of semen, acidity (pH), and semen consistency. Microscopic evaluation is performed using a binocular microscope (Olympus CX23) and includes observation of sperm movement, sperm motility, sperm viability, sperm abnormalities, sperm concentration, and sperm plasma membrane integrity.

Sperm mass movement is observed by placing a drop of semen on a microscope slide and viewing it under a microscope at 100X magnification. Sperm motility was performed by dropping 10µl semen onto a microscope slide, diluting with saline in a 1:4 ratio, homogenising, and removing a drop, which was then covered with a coverslip. The sample was viewed under a microscope with 400x magnification. The assessment of progressive sperm motility is determined subjectively. The reported value ranges from 0-100% with a 5% scale (Susilawati 2013). Sperm viability and abnormalities were assessed using the eosin-nigrosin staining method. Furthermore, sperm plasma membrane integrity was assessed using a hypoosmotic swelling (HOS) solution. Finally, sperm concentration was measured using an SDM 6 photometer (Minitube, Germany).

Preparation of the semen cryopreservation

The dilution method used in this study was a one-step dilution, according to Arif et al. (2020), semen was diluted with the diluent at room temperature. Immediately after dilution, the semen was filled into a 0.25-mL mini-straw (Minitube, Germany) using an automated filling and sealing machine (automated Combisystem and Minijet printer; Minitube, Germany). After filling into the mini-straws, they were arranged on a freezing rack and equilibrated in a refrigerated chamber (4-5°C) for four hours. Freezing was performed in an automatic freezing machine (Minitube, Germany). The frozen semen is stored in a tank with liquid nitrogen for further storage time before evaluation.

Evaluation of sperm acrosome status in frozen-thawed semen

The acrosome status of sperm was assessed by the fluorescein isothiocyanate-peanut agglutinin (FITC-PNA) staining method according to Rajabi-Toustani et al. (2019) with slight modifications.

Evaluation of malondialdehyde and aspartate aminotransferase enzyme concentration in frozen-thawed semen

The evaluation of MDA concentration was performed using the thiobarbituric acid method. The MDA coefficient used was $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, which is expressed in nmol MDA/ 10^8 of sperm (Sukmawati et al. 2015). AspAT enzyme concentration was evaluated using an automated chemical analyzer (VetScan®, Abaxis Inc, Union City) and a commercial kit (VetScan®, Abaxis Inc, Union City).

Data analysis

The data obtained were tabulated using the Microsoft Excel 2010 program and then processed using the Statistical Package for the Social Sciences (IBM SPSS® 25). The normality of the data of each assessment was tested using the Kolmogorov-Smirnov test method. The normally distributed data were then analysed using analysis of variance (ANOVA) with a significance level of 95%. If there was a significant difference, Tukey HSD was performed, and data were presented as means ± standard errors (SEM).

RESULTS AND DISCUSSION

The fresh semen from Limousin and Simmental cattle in this study had a volume of 7.83±0.63 mL and 7.49±0.37 mL, with a pH of 6.45±0.03 and 6.45± 0.04, respectively. The consistency of the semen was light to medium, and the colour was creamy to yellowish. Mass motility showed +++, and sperm motility was 80-90%. Sperm viability was 90.29±0.20% and 91.88±0.21% with membrane integrity of 89.40 ± 0.20% and 90.72 ± 0.21%, respectively. Sperm concentration was 1465.73±74.71 x 10^6/ml and 1775.67±83.68 x 10^6/ml, respectively. All bulls showed minor sperm abnormalities, 5.78±0.14% and 5.12±0.19%, respectively.

The quality of fresh semen from the two groups of bulls used in this study was very good and met the requirements for freezing fresh semen. Indonesian National Standard 4869-1:2017 states that frozen semen consists of fresh semen with minimum motility of 70% (BSN 2017). The semen quality of these bulls is traceable because the animals used are selected bulls kept in a bull rearing system according to the AIC SOP.

Assessment of sperm acrosome status of frozen limousin and simmental semen in different commercial diluents

Intact acrosome status is essential as it is closely associated with sperm fertility. The acrosome contains proteolytic enzymes that play a crucial role in the fertilization process but can be damaged during the freezing process (Lopes et al. 2021). The enzymes included acrosine, hyaluronidase, and corona-penetrating enzymes required for penetration into the cumulus oophorus and pellucid zone (Zaenuri et al. 2018). Detachment of the sperm acrosome marks the,

Table 1. Acrosome status of Limousin and Simmental sperm are frozen in different commercial diluents.

Cattle breed	Diluents		
	Andromed®	Optixcell®	Steridyl®
Limousin (%)	93.84±0.60	95.65±0.40	97.22±0.52
Simmental (%)	95.88±0.31	96.40±0.46	97.63±0.36

Table 2.Acrosome status of sperm after frozen-thawed based on diluent or breed factor

Treatment		Acrosome status (%)
Diluent	Andromed®	94.86±0.45^{b}
	Optixcell®	96.03±0.31^{ab}
	Steridyl®	97.43±0.31^{a}
Cattle breed	Limousin	95.57±0.44^{b}
	Simmental	96.64±0.27^{a}

Different superscript letters following numbers in the same column indicate significant differences (P<0.05).

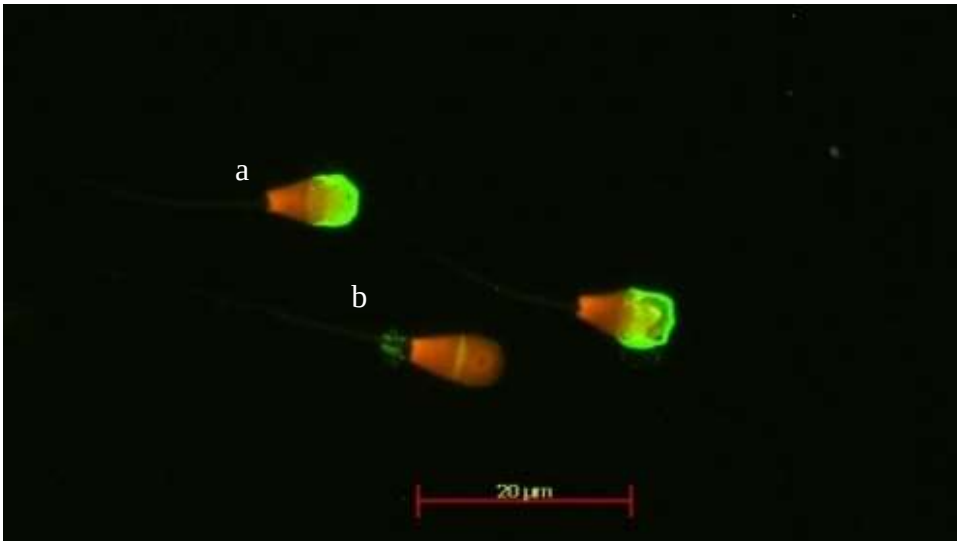

Figure 1. Sperm acrosome status using FITC-PNA staining. a. sperm with intact acrosome, b sperm with damaged acrosome

acrosomal damage to the sperm head by the acrosomal reaction. A green glow on the sperm head indicated the intact acrosome status, while the incomplete acrosome status emitted a red colour (Figure 1). The results showed that there was no interaction (P>0.05) between the types of diluents and breed on the acrosome status of semen after thawing (Table 1).

The sperm acrosome status in this study was influenced by the type of diluent and the breed of cattle. Sperm frozen in Steridyl® diluent with lecithin from sterile egg yolk showed less acrosome damage than Andromed® (Table 2). This study showed that the degree of acrosome damage was influenced by the breed of cattle. Simmental had less sperm acrosome damage than Limousin (Table 2). In addition, the acrosome is located inside the spermatocyte and is protected by the plasma membrane. According to Sukmawati et al. (2015) and Indriastuti et al. (2020), breed and individual cattle have different sperm plasma membrane compositions. The cholesterol/phospholipid ratio and the degree of saturation of carbon chains differ among cattle breeds. Some of these differences cause cells to be sensitive to other cryopreservation methods, as evidenced by the decreased sperm quality after freezing, including damage to the acrosome. Furthermore, the freezing rate may also influence semen freezing success.

Previous studies have shown that sperm acrosome status after thawing in Limousin and Simmental cattle ranged from 88.40-97.09% to 86.60-97.91% but using different semen diluents (Nofa et al. 2018). The difference in semen acrosome status among cattle breeds in this study follows the statement of Üstüner et al. (2015).

Assessment of malondialdehyde concentration of frozen Limousin and Simmental semen in different commercial diluents

Evaluation of MDA concentration was essential to determine the level of lipid peroxidation in semen. Malondialdehyde is an aldehyde compound produced by the peroxidation of unsaturated fatty acids. MDA production results from increased reactive oxygen species (ROS) in cells (Tsikas 2017). The higher the MDA concentration, the greater the damage to sperm, resulting in low sperm quality (Sukmawati et al. 2015). The MDA concentrations of frozen-thawed Limousin and Simmental semen in different commercial diluents are shown in Table 3. The results showed no interaction (P>0.05) between the types of commercial diluents and cattle breeds in the concentration of MDA in frozen-thawed semen

The MDA concentration of thawed semen in the three commercial diluents was relatively low. The MDA concentrations of some crossbred cattle in Bioxcell® diluent, Tris egg yolk, and citrate egg yolk were 1.8±0.1 nmol/10^8, 2.5±0.1 nmol/10^8, and 2.6±0.0 nmol/10^8, respectively (Khumran et al. 2015) According to Dwinofanto et al. (2019), the MDA concentration of frozen-thawed Bali cattle semen in Tris egg yolk dilution was 6.8±0.3 nmol/10^8. MDA is the most commonly measured biomarker of oxidative stress, i.e. lipid peroxidation. The number of PUFAs that can contribute to MDA is much higher, and the amount of MDA in semen samples is an index of lipid peroxidation (Sharafi et al. 2015). In this study, the MDA concentration was very low in all cattle breeds and all diluents, ranging from 0.022 to 0.151 nmol/108; therefore, we can assume that the degree of lipid peroxidation in semen was also low. This result might be related to the number of PUPAs in the plasma membrane of both cattle breeds.

The assesment of aspat concentration of frozen Limousin and Simmental semen in different commercial diluents

The plasma membrane surrounds the entire cell and is directly affected by environmental changes. Another parameter related to the cell membrane, especially its integrity, was the activity of AspAT in seminal plasma. The concentration of enzyme activity in seminal plasma describes the status of sperm damage, especially of the midpiece (Tejaswi et al. 2016). The concentration of AspAT enzyme in frozen-thawed semen of Limousin and Simmental cattle in different commercial diluents are shown in Table 4. Analysis of variance showed that the type of commercial diluent and the breed of cattle and the interaction between the two factors has not affect the AspAT enzyme concentration in semen (P>0.05).

AspAT is an intracellular enzyme located mainly in the mid-piece of sperm tail (Soeparna & Arifiantini, 2013). Membrane damage in the tail, particularly in the midpiece, results in the release of the AspAT enzyme from the cell. The release of AspAT affects mitochondria; as a result, adenosine triphosphate (ATP) the production is halted, and spermatozoa stop moving (du Plessis et al. 2015). The release of AspAT from sperm into the seminal plasma is associated with increased sperm plasma membrane permeability. This condition leads to a decrease in the biological value of sperm (Frydrychová et al. 2010).

AspAT concentration in this study ranged from 75.00 to 90.33 U/L, and there was no interaction between cattle breed and diluents. The result of this study was almost the same as that reported by Hammad et al. (2019) from frozen Friesian-Holstein bull semen in Citrate-yolk diluents and was 75.20±1.11 U/L. In addition, Arif et al. (2020) reported that the AspAT concentration in frozen-thawed Limousin semen diluted

Table 3. Malondialdehyde concentration of frozen semen from Limousin and Simmental cattle frozen in different commercial diluents

Cattle breed	Diluents		
	Andromed®	Optixcell®	Steridyl®
Limousin (nmol/10^8)	0.040±0.010	0.039±0.011	0.025±0.007
Simmental (nmol/10^8)	0.151±0.115	0,043±0.013	0,022±0.005

Table 4. Concentration of AspAT enzyme in frozen-thawed semen of Limousin and Simmental cattle in different commercial diluents

Cattle breed	Diluents		
	Andromed®	Optixcell®	Steridyl®
Limousin (U/L)	86.67±8.25	90.33±7.54	75.00±20.31
Simmental (U/L)	80.33±4.33	90.33±5.84	76.67±2.91

in skim milk diluent was 8.33±1.14 U/L. The concentration of AspAT enzyme in fresh buffalo semen was also reported by El-Sharawy et al. (2017) and ranged from 57.7±0.82 U/L to 64.5±0.75 U/L. The three commercial diluents used in this study were able to maintain the normal configuration of the sperm plasma membrane; therefore, the release of the AspAT enzyme was still at a normal concentration.

Cryo-induced oxidative stress is associated with excess production of ROS resulting in biochemical and physical damage to the sperm membrane structures and subsequently leading to the reduced fertilising ability of sperm (Asadpour et al. 2021). All three commercial diluents contain antioxidants. The antioxidant content of the three diluents used in the study counteracted the effects of oxidative stress exceptionally well. This result is reflected in the low MDA and AspAT concentrations, so it was suspected that oxidative stress damage to the sperm plasma membrane was also low. This result follows the opinion of Hezavehei et al. (2018) that adding antioxidants to the diluent during the cryopreservation process can neutralise ROS and maintain the quality of thawed sperm. The function of antioxidants in the diluents was to add or remove an electron to neutralise ROS, stabilise free radicals, and inhibit oxidation (Ahmadi et al. 2016). Isnaini et al. (2019) confirmed that antioxidants must be used appropriately to maintain sperm quality during cryopreservation; high antioxidants may cause toxic effects in sperm cells.

A semen diluent contains an energy source and provides components to protect the sperm plasma membrane (Rizal & Riyadhi 2016). When sperm plasma membranes are not maximally protected, the absence of lecithin leads to sperm death to reduce cold shock (Nguyen et al. 2019). A drop in temperature from 37 ^{o}C to -60 ^{o}C can lead to cold shock, osmotic stress, and ice crystal formation. These factors can reduce the permeability of the sperm plasma membrane, potentially causing acrosomal damage (Ugur et al. 2019). The three commercial diluents in this study contain different sources of lecithin, and all have components sperm need to protect during freezing. Therefore, acrosome damage, MDA, and AspAT concentrations were low.

CONCLUSION

Commercial diluents with different lecithin-based ingredients showed equal efficacy in maintaining intact acrosome status and supported MDA and AspAT concentrations of thawed Limousin and Simmental semen. All commercially available diluents can be alternatives for Limousin and Simmental frozen semen.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests with respect to the material covered in the manuscript.

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