Influence of Sperm Number and Antioxidant Melatonin in Extender on the Quality of Post-Thawing Sheep Spermatozoa

Putri F[1], Karja NWK[2], Setiadi MA[2], Kaiin EM[3]

[1]*Animal Biomedical Science Program, School of Veterinary Medicine and Biomedical Sciences, IPB University, Jl Agatis, Kampus Dramaga, Bogor 16680, Indonesia*
[2]*Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedical Sciences, IPB University, Jl. Agatis, Kampus Dramaga, Bogor 16680 Indonesia*
[3]*Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong*
Email: karja_nwk@apps.ipb.ac.id

(received 11-08-2022; revised 21-10-2022; accepted 31-10-2022)

ABSTRAK

Putri F, Karja NWK, Setiadi MA, Kaiin EM. 2023. Pengaruh konsentrasi spermatozoa dan penambahan antioksidan melatonin pada pengencer terhadap kualitas spermatozoa domba post-thawing. JITV 28(1):1-10. DOI: http//dx.doi.org/10.14334 /jitv.v28.i1.3069.

Penelitian ini bertujuan untuk melihat pengaruh konsentrasi spermatozoa dan efektifitas suplementasi melatonin pada pengencer terhadap kualitas semen post-thawing. Semen ejakulat dikoleksi dengan menggunakan metode vagina buatan (MVB). Penelitian dilakukan dalam dua tahap, pertama semen dibekukan dalam pengencer andromed dengan konsentrasi yang berbeda dalam satu straw (50, 100, 200 juta perstraw), yang kedua semen dibekukan dalam pengencer yang disuplementasi melatonin dengan dosis berbeda (0, 0,5, 1,0 dan 1,5 mM melatonin). Parameter yang diamati adalah pergerakan spermatozoa dengan menggunakan Computer Assisted Sperm Analysis, keutuhan membran dan keutuhan akrosom. Data dianalisis menggunakan Anova dan di uji lanjut menggunakan uji Duncan. Hasil penelitian menunjukkan bahwa kualitas dan pola pergerakan semen domba tidak terdapat perbedaan signifikan saat dibekukan dengan konsentrasi 50, 100, maupun 200 juta perstraw (P>0,05). Penambahan melatonin dengan konsentrasi 1,0 mM pada pengencer memberikan pengaruh terhadap motilitas total dan motilitas progresif spermatozoa (P<0,05) tetapi tidak memberikan pengaruh yang signifikan terhadap persentase karakteristik pola gerak spermatozoa. Dari hasil penelitian dapat disimpulkan bahwa konsentrasi spermatozoa perstraw tidak memberikan pengaruh terhadap kualitas semen domba dan suplementasi melatonin dalam pengencer dapat mengurangi efek negatif selama proses frozen-thawed terhadap motilitas, tudung akrosom dan integritas membran plasma pada semen domba. Suplementasi melatonin dengan konsentrasi 1 mM pada bahan pengencer merupakan konsentrasi dengan kualitas terbaik pada penelitian ini.

Kata Kunci: Kriopreservasi, Konsentrasi Semen, Melatonin, Kualitas Post-thawing

ABSTRACT

Putri F, Karja NWK, Setiadi MA, Kaiin EM. 2023. Influence of sperm number and antioxidant melatonin addition in extender on the quality of post-thawing sheep spermatozoa. JITV 28(1): 1-10. DOI: http//dx.doi.org/10.14334/jitv.v28.i1.3069.

This study aimed to examine the effect of spermatozoa concentration and the effectiveness of melatonin supplementation in diluent on the quality of post-thawing semen. Ejaculated semen was collected using the artificial vaginal method (MVB). The study was carried out in two stages, firstly semen was frozen in andromed diluent with different concentrations in one straw (50, 100, 200 million per straw), and the second was frozen semen in diluent supplemented with melatonin with different doses (0, 0.5, 1, 0 and 1.5 mM melatonin). Parameters observed were the movement of spermatozoa using Computer Assisted Sperm Analysis, membrane integrity, and acrosome integrity. Data were analyzed using ANOVA and further tested using Duncan's test. The results showed no significant difference in the quality and movement pattern of sheep semen when frozen at concentrations of 50, 100, or 200 million per straw (P>0.05). Furthermore, adding melatonin to the diluent in this study affected spermatozoa's total motility and progressive motility at a concentration of 1.0 mM (P<0.05) but did not significantly affect the percentage of spermatozoa motility pattern characteristics. From the results, it can be concluded that the concentration of per straw spermatozoa does not affect the quality of sheep semen, and melatonin supplementation in diluent can reduce the effects of the frozen-thawed process on motility, acrosome cap, and plasma membrane integrity in sheep semen. Melatonin supplementation with a concentration of 1 mM in the extender was the highest quality concentration in this study.

Key Words: Cryopreservation, Melatonin, Post-thawing Quality, Sperm Number

INTRODUCTION

Cryopreservation of semen is an efficient technique to save sperm cells, allowing the preservation of gene pools and the expansion of desired reproductive merits (Salmani et al. 2013). However, the cooling, freezing, and thawing processes cause physical and chemical stress on the spermatozoa membranes, reducing their

viability and fertility (Chatterjee et al. 2005). One cause of this decline is due to the action of the reactive oxygen species (ROS) generated by the cellular components of semen, abnormal spermatozoa, and neutrophils, namely, a superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) (Perumal, 2014). Spermatozoa are susceptible to OS because their plasma membrane contains an unusually high percentage of polyunsaturated fatty acids (PUFAs) exposed to a damaging process called lipid peroxidation. Lipid peroxidation occurs as electrons from plasma membrane lipids are stripped away by ROS, which propagates a chain of redox reactions that eventually generates highly mutagenic and genotoxic electrophilic aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal (4- HNE), and acrolein (Bui et al. 2018). 4- HNE is derived from lipid hydroperoxides of ω- 6 fatty acids such as linoleic acid, and arachidonic DNA damage observed in spermatozoa results from OS. It is an essential characteristic of semen quality and, therefore, a useful marker in diagnosing male infertility (Bui et al. 2018).

The semen sample must be appropriately diluted to allow enough per straw sperm for artificial fertilization (Kalita et al. 2019). The combination of different storage temperatures and reduced sperm concentration at refrigerated insemination doses has recently been studied in cattle (Murphy et al. 2013; Murphy et al. 2016; Murphy et al. 2018). In rabbits, after 48 hours of refrigerated storage, higher sperm concentrations (30 and 60 x 10^6 spermatozoa/mL) were associated with higher overall motility (Johinke et al. 2015). Another study stated, In bovine, no significant reduction in fertility of liquid semen when sperm concentration was reduced from 10 to 1.5 x 10^6 sperm per insemination dose (Shanon et al. 1984). The suggested dose of semen is 0.1 to 0.2 ml with about 50 to 100 million active spermatozoa. About 50 to 100 million spermatozoa are needed in one dose for vaginal insemination, but 25 to 50 million are suggested for cervical insemination, and 15 to 20 million are enough for laparoscopic AI (Yusuf et al. 2005; Gergátz 2007). However, several scientists have used much higher numbers: 100 (Fernandez-Abella et al. 2003), 50 to 300 (Ehling et al. 2003), and 400 (King et al. 2004) million spermatozoa in one dose of semen. Because there is no straightforward enterprise standard, it is necessary to research whether there is an effect of concentration differences on the quality of post-thawing sheep spermatozoa.

Several studies were carried out to improve semen quality by adding antioxidants such as vitamin C (Trilaksana et al. 2016), glutathione (Salmani et al. 2013), and melatonin (Succu et al. 2011). In recent years, melatonin has been widely used as an additional antioxidant in cryopreservation. Ashrafi et al. (2013) reported that supplementation of melatonin in the freezing medium could counteract the adverse effects of the freeze-thawing process on the motility, viability, normal morphology, and plasma membrane integrity in bull spermatozoa with a concentration of 2 mM. At the same time, Succu (2011) reported that doses of one mM of melatonin led to higher viability rates and higher percentages of total motile and progressive motility. These results align with Pang et al. (2016), that reported melatonin can improve the quality of bovine frozen-thawed semen. The pineal gland releases melatonin (N-acetyl-5-methoxy tryptamine) in the brain; it is involved in many physiological functions, including regulation of seasonal reproduction, and influences the immune system and circadian rhythms (Awad et al. 2006; Jang et al. 2010). On the other hand, the antioxidant property of melatonin has been demonstrated in vivo. Melatonin can also maintain the semen characteristics of goats (Ramadan et al. 2009), rats (Sönmez et al. 2007), wild boars (Jang et al. 2010), and humans (Espino et al. 2010; Ortiz et al. 2011).

Differences in concentrations in extenders and melatonin supplementation as an antioxidant in extenders still need to be explored; because there is a lack of information regarding the effect of sperm concentration on freezing without the reduction in their post-thaw survival of spermatozoa and melatonin supplementation as an antioxidant in extenders on sheep. Hence the present study was taken up.

MATERIALS AND METHOD

Ethical approval

This research was approved by The Institution of Research and Community Services, Animal Care Use Committee Bogor Agricultural University, 231 – 2021 IPB.

Research animal

Two sheep from the Rehabilitation and Reproduction Unit of IPB were used in this study, aged 18-24 months and weighing 30-40 kg. Feed was given in the morning and evening as forage as much as 3 kg/head/day and concentrate with 11% protein as much as 0.5 kg/head/day. Drinking water is provided ad libitum.

Semen collection and cryopreservation

Spermatozoa collection from ejaculate was carried out once a week for two months with the artificial vaginal method (MVB) performed six times as the number of replications. The research was conducted in two steps. In the first step, the semen was diluted with an Andromed diluent at three different concentrations of 50, 100, and 200 million spermatozoa. Semen samples were aspirated into 0.25 ml straws. The second stage of semen was diluted with Andromed with the best concentration in the previous step and supplemented with antioxidant

melatonin at different doses (0, 0.5, 1.0, and 1.5 mM melatonin). Diluted semen samples were evaluated for quality; only semen with more than 70% motility was used in this study.

Furthermore, the packaged semen was equilibrated in a refrigerator at 5°C for 4 hours. After equilibration, each semen sample of each treatment was evaluated for quality. Freezing of semen was done by placing straws 10 cm above the surface of liquid nitrogen in tightly closed styrofoam for 20 minutes. Furthermore, the straws were put into liquid nitrogen (-196°C) and stored in a liquid nitrogen container for quality evaluation.

Semen evaluation

Spermatozoa Movement Assessment. The movement of spermatozoa was observed using Computer Assisted Sperm Analysis (CASA, Sperm Vision Minitube) at the Animal Reproductive Biotechnology Laboratory, Research Center for Biotechnology, BRIN. Observing the motility of spermatozoa refers to the modified Hariono (2021), in which the straws for each treatment were thawed by immersing them in water at 37°C in a water bath for 30 seconds. First, an evaluation was carried out by dripping 10 L of cement on an object glass and covered with a cover glass. Then, the sample was placed on a microscope (Zeiss Axio Scope A1). The following parameters were evaluated for post-thawed semen, namely: Motility: the percentage of motile sperm; Progressive motility: the percentage of progressively motile sperm; Curvilinear velocity (VCL: µm/second); Straight line velocity (VSL: µm/second); Average Path Velocity (VAP: µm/second), Amplitude of Lateral Head Displacement (ALH: µm) and Straightness (STR: %).

Membrane Integrity. Examine spermatozoa plasma membrane integrity (PMI) using the Hypoosmotic Swelling Test (HOS-Test). The composition of the HOS solution was 0.135 g fructose and 0.0737 g trisodium citrate $2H_2O$ in 10 mL milli-Q water. A sample of 20 L of cement was added to 80 L of HOS solution and left for 30 minutes in a water bath (37°C). After incubation, 10 µL of cement was placed on a glass object, covered with a cover glass, then observed under a light microscope (Nikon FDX-35) with a magnification of 400x. Straight tails characterize spermatozoa with plasma membrane damage. In contrast, circular or bulging tails characterize spermatozoa with plasma membrane integrity. The number of spermatozoa observed was at least 200 cells in 10 fields.

Acrosome Integrity**.** TBG staining begins with dripping cement and trypan blue solution, dissolved using 0.81% NaCl simultaneously and then slowly homogenized, made into smear preparations, and dried vertically. Next, the preparations were stained using a neutral red solution consisting of 86 ml 1 N HCL and 14 ml 37% formaldehyde solution, and 0.2 g Neutral Red (Sigma N-2880). The preparations were stained for 3 minutes, then dried, rinsed using running water, and dried again. In the next staining stage, the preparations were immersed in a jar containing 5% Giemsa solution, left at room temperature for three days, and then rinsed again by dipping into a container filled with water for 2 minutes. After drying, it was warmed using a heating table (40°C) (Nofa et al. 2018). An evaluation was conducted on 200 cells using a light microscope with an objective lens of 400x magnification. Spermatozoa with acrosome integrity (AIn) are purple on the head, while spermatozoa with incomplete acrosomes will have a pale lavender or faded color. Acrosome status was calculated by counting the number of spermatozoa with AIn divided by the total number of spermatozoa multiplied by 100%.

Statistical analysis

The data of spermatozoa assessment were shown as the mean± SD. Each experiment was repeated five times. The data were analyzed using One-Way Analysis of Variance (ANOVA), and Duncan's Multiple Range Test (DMRT) was performed to determine if there was any significant difference among the treatments at $P<0.05$. Statistical analysis was conducted using IBM SPSS® Statistics version 22.0 (IBM Corp., Armonk, NY, US).

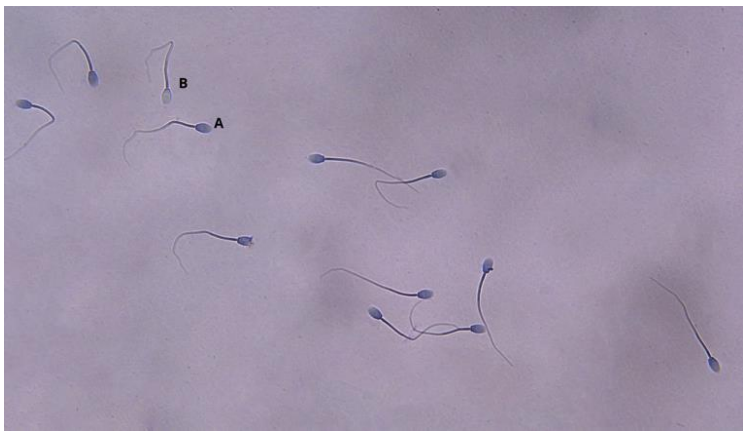

Figure 1. Acrosome staining using trypan blue-Giemsa examined at 400x: (A) acrosome integrity, (B) damaged acrosome

RESULTS AND DISCUSSION

Quality of frozen-thawed sheep spermatozoa with different concentrations per straw

Motility is an essential indicator of successful fertilization. Prior to cryopreservation, the quality of the fresh semen will determine whether the semen is suitable for processing. Toelihere (1985) stated that semen could be continued for processing: if it has a high mass movement value and motility, a concentration of 1500 million to 3000 million, and an abnormality value below 15 percent. Based on observation, high fresh semen motility (80.00±0) indicates that spermatozoa will survive through cooling or cryopreservation. The sperm motility percentage of frozen semen after thawing was >40%. In general, freezing and thawing procedures significantly reduced the quality of sheep semen. The percentages of total motility, progressive motility, plasma membrane integrity, and acrosome integrity of spermatozoa immediately after thawing are presented in Table 1.

The quality of frozen semen is influenced by the freezing process but is also closely related to the quality of fresh cement produced before the freezing process is carried out (Awad et al. 2006; Salmani et al. 2013; Nofa et al. 2018). The results showed that the quality after thawing of ejaculated spermatozoa met the requirements for artificial insemination based on the Indonesian National Standard number SNI 4869.3-2014 part 3: Goats and Sheep with the condition that the percentage of frozen semen motility was at least 40% or mass movement ++ (Badan Standardisasi Nasional 2014). The percentage of total motility was higher at a concentration of 100 million spermatozoa per straw, 45.74 ± 3.70, and did not show significant differences between treatment groups. These findings align with Holt (2000) and Lemma (2011) that the freezing and thawing process causes damage to spermatozoa, resulting in a decrease in post-thawing quality. It is well known that sperm can be affected by several factors during the freeze-thaw process, leading to loss of plasma membrane integrity and damage to the acrosome, mitochondrial dysfunction leading to irreversible loss of motility and fertility (Karja et al. 2013). The success of cryopreservation was also assessed by recovery rate (RR), the percentage of spermatozoa that recovered from the freezing process, which was calculated by comparing motile spermatozoa in fresh semen and after thawing (Arifiantini et al. 2007).

Table 1 shows a higher percentage of total motility at a concentration of 100 million per straw spermatozoa and does not show a significant effect between treatments (P>0,05). De Graft (2007) reported successful in vivo fertilization at 100 million spermatozoa per dose. In addition, this fertility rate is comparable to a dose of 50 million sperm; this supports the results of this study that lowering or increasing the sperm concentration per dose will not harm the fertility rate in vivo. Progressive motility percentage (Table 1) did not show a significant effect between the treatment groups. Progressive motility is one of the essential criteria for spermatozoa to fertilize oocytes (Kato et al. 2011). In in vivo fertilization, the spermatozoa ejaculated in the vagina and moved forward in the oviduct via the cervical canal and uterus (Hyakutake et al. 2018). Furthermore, spermatozoa change movement patterns to become non-progressive, and changes in movement patterns are required for spermatozoa to pass through the utero-tubal junction and enter the oviduct (Muiño et al. 2008). Progressive motility may decrease rapidly due to the depletion of glycolytic substrates and decreased energy (Ahmad et al. 2015).

Based on table 1, the value of acrosome integrity of sheep spermatozoa did not show any significant difference. The results showed that the acrosome integrity had different concentrations per straw spermatozoa, 45.00±5.00; 46.36±3.34; 46.17±5.39, respectively. The low percentage of acrosome integrity is associated with a low percentage of intact plasma membrane and motility (Sitepu et al. 2018). Based on table 1, sheep spermatozoa's plasma membrane integrity values showed no significant difference. The results showed that the acrosome integrity had different concentrations per straw spermatozoa, 46.50±3.50; 48.33±4.80; 48.00±4.27, respectively. Changes in the plasma membrane play an essential role in the capacitation process (Dapino et al. 2009) and cause a decrease in spermatozoa fertility (Thomas et al. 2006). Damaged spermatozoa and physiological changes in the membrane can be described in the motility of the spermatozoa (Gervasi & Visconti, 2017). In addition, an intact plasma membrane can reduce the lipid phase transition, protect the membrane's ion-exchange function, and decrease intracellular [Ca^{2+}] (Muiño et al. 2008). Changes in membrane permeability during cooling can cause an increase in intracellular [Ca2+] (Fuller & Whittingham, 1996). Peroxidation of fatty acids of spermatozoa due to damage to the plasma membrane can cause loss of motility (Sundararaman et al. 2012). The integrity of the plasma membrane is related to the metabolic ability of spermatozoa in energy production and ATP synthesis, not generally if there is damage to the membrane, which results in decreased motility and survival of spermatozoa (Nofa et al. 2018).

The movement patterns of post-thawing sheep spermatozoa with different concentrations are shown in table 2. The movement patterns evaluated were VCL, LIN, and ALH. Based on these results, it was found that the VCL value did not differ between all treatment groups, the lower VCL value was found in the group with a concentration of 50 million, and the highest VCL value

Table 1. Characteristics of sheep spermatozoa after freezing with different concentrations

Parameter (%)	Concentration (10^6/straw)		
	50	100	200
Total Motility	44.10±1.68[a]	46.36±3.34[a]	45.74±3.70[a]
Progressive Motility	29.36±6.94[a]	33.43±9.26[a]	32.08±4.23[a]
Plasma Membrane Integrity	46.50±3.50[a]	48.33±4.80[a]	48.00±4.27[a]
Acrosome integrity	45.00±5.00[a]	46.36±3.34[a]	46.17±5.39[a]

The same superscript letters following numbers in the same column indicate a non-significant difference (P<0.05)

Table 2. The movement pattern of sheep spermatozoa after being frozen at different concentration

Parameter	Concentration (10^6/straw)		
	50	100	200
VCL (μL/s)	92.02±8.71[a]	98.94±29.02[a]	95.48±25.19[a]
VSL (μL/s)	46.50 ±2.33[a]	48.33±3.20[a]	48.00±2.85[a]
VAP (μL/s)	65.28±3.86[a]	68.50±14.70[a]	66.79±12.72[a]
LIN (%)	0.49±0.30[a]	0.49±0.04[a]	0.51±0.05[a]
STR (%)	0.69±0.01[a]	0.69±0.02[a]	0.73±0.02[a]
ALH (μm/s)	5.94±0.74[a]	5.81±0.35[a]	6.03±0.56[a]

VC= Curvilinear Velocity, VSL= Straight Line Velocity, VAP= Average Path Velocity, LIN= Linearity, STR= Straightness, ALH= Lateral Amplitude of Head Displacement. The same superscript letters following numbers in the same column indicate a non-significant difference (P<0.05)

was found in the group with a concentration of 100 million. At the same concentration, there was no difference in the percentage of LIN between the 50 million, 100 million, and 200 million concentration groups. Meanwhile, the percentage of LIN in the 50 million and 100 million groups was lower than in the 200 million groups. The percentage of spermatozoa LIN at different periods did not differ between the treatments. The results showed no effect of spermatozoa concentration on changes in the percentage of LIN. The values for the amplitude of lateral head displacement (ALH) are presented in Table 2. The ALH values of spermatozoa in the 200 million group were higher than the 50 million and 100 million groups (P<0.05). Hyperactivation is part of the capacitation process (Colás et al. 2009). Spermatozoa undergo motility hyperactivation due to molecular changes in the flagellum (Abou-hailed & Tulsiani, 2009). Spermatozoa of various species have altered patterns of flagellar motion during the final stages of capacitation. This hyperactivated motility is characterized by large-amplitude solid flagellar beats and erratic swimming trajectories; the most common expression of hyperactivation appears to involve a decrease in the progressive movement and an increased bending of the flagellar midpiece while maintaining vigorous motility (Taitzoglou et al. 2004). Hyperactivation motility can be calculated using CASA by looking at the VCL, ALH, and LIN. (Hinrichs and Loux 2012). Based on Table 2, it can be seen that the spermatozoa did not show any hyperactivity, as Shojaei et al. (2012) explained that the determination of spermatozoa undergoes a change in movement to hyperactivation when the ALH value is >7 m.

Quality of frozen-thawed sheep spermatozoa with different doses of melatonin

Cryopreservation is a method that can support the conservation of animal genetic materials by freezing semen. The use of the conserved semen and spermatozoa is not limited by geographical distance and time (Pamungkas et al. 2014). The use of cement cryopreservation technology is quite often used for fish cement (Martínez-Páramo et al. 2017; Magnotti et al. 2018), livestock (Lv et al. 2019; Salinas et al. 2021; Yánez-Ortiz et al. 2021), and human (Tao et al. 2020). It is well known that the cryopreservation process of semen can lead to the formation of reactive oxygen species (ROS). ROS are formed due to an imbalance between the antioxidant content and free oxides, resulting in oxidative stress. Oxidative stress damages the plasma

membrane because phospholipids' membrane structure is sensitive to lipid peroxidation (Bucak et al. 2010). Total motility, progressive motility, plasma membrane integrity, and acrosome integrity of spermatozoa incubated with melatonin are presented in Table 3. Percentages of total motility and progressive motility in this study were assessed using CASA, and significant differences were found between treatments at different doses of melatonin. The percentages of total motility and progressive motility were higher (P<0.05) in the diluents supplemented with 1.0 and 1.5 mM melatonin (62.68±2.72 and 38.91±2.68; 58.11±2.40 and 35.05±1.68, respectively). The results showed a decrease in the integrity of the plasma membrane after the cryopreservation process, but the diluent supplemented with melatonin showed a higher value than the control. Melatonin protects mouse spermatozoa from the side effects of the peroxidative agent homocysteine reported by Sönmez et al. (2007). Melatonin has also been shown to protect spermatozoa from mercury oxidative damage (Rao & Gangadharan, 2008) and increase the hyperactivation of hamster spermatozoa (Fujinoki, 2008). Furthermore, Ramadan et al. (2009) reported that melatonin maintains the sperm quality of Damascus goats.

The percentage of progressive motility showed no significant difference (P>0.05) between the control group (45.74±3.70) and the diluent supplemented with 0.5 mM melatonin (48.63±3.01). Melatonin supplementation of 1 mM in the diluent increased the percentage of PMI and AIn (P<0.05) (62.22±0.30 and 67.23±2.6) compared to other groups. Several studies have confirmed that melatonin can reduce oxidative stress (Reiter, 1998; Jang et al. 2010). Pang et al. (2016) reported that melatonin could improve plasma membrane and acrosome integrity in frozen-thawed bovine semen. The beneficial effect of melatonin on plasma membranes is that melatonin stimulates the activity of enzymes involved in ROS metabolism and maintains cell membrane integrity (Izadpanah et al. 2015). In addition, melatonin can be an effective anti-apoptotic agent (Tsantarliotou et al. 2008). Other studies have shown that melatonin can prevent apoptosis by controlling Bcl-2 expression and ameliorating oxidative stress in the testes (Othman et al. 2016). Supplementation with one mM of melatonin was reported to prevent capacitation and apoptosis of mouse spermatozoa (Casao et al. 2010). This anti-apoptotic ability may cause increased motility and plasma membrane integrity. In addition, our study also found that melatonin supplementation at a dose of 1.5 mM can reduce the motility determined; this decrease in quality is thought to be due to melatonin at high concentrations can have a negative effect. Erez et al. (1992) found that high doses of melatonin in

Table 3. Characteristics of sheep spermatozoa after freezing with different doses of melatonin

Parameter (%)	Doses (mM)			
	0	0.5	1.0	1.5
Total Motility	45.74±3.70^{a}	48.63±3.01^{a}	62.68±2.72^{b}	58.11±2.40^{b}
Progressive Motility	32.08±4.23^{ab}	28.02±1.13^{a}	38.91±2.68^{c}	35.05±1.68^{bc}
Plasma Membrane Integrity	48.00±4.27^{a}	59.24±2.67^{b}	62.22±0.30^{b}	61.16±1.22^{b}
Acrosome integrity	46.17±5.39^{a}	59.88±1.70^{b}	67.23±2.61^{c}	66.61±1.52^{c}

Different letters at different doses show significant differences (P<0.05)

Table 4. The movement pattern of sheep spermatozoa after being frozen with different doses of melatonin

Parameter	Doses (mM)			
	0	0.5	1.0	1.5
VCL (μL/s)	95.48±25.19	78.14±1.52	86.75±3.51	90.19±4.75
VSL (μL/s)	48.97±8.29	41.33±0.84	42.90±1.14	43.33±2.23
VAP (μL/s)	66.79±12.72	60.90±1.71	60.79±2.85	61.45±3.88
LIN (%)	0.51±0.05	0.52±0.02	0.49±0.01	0.47±0.03
STR (%)	0.73±0.26^{b}	0.68±0.20^{a}	0.70±0.02^{ab}	0.69±0.01^{ab}
ALH (μm/s)	5.96±0.72	6.16±0.50	6.24±0.07	6.42±0.26

VCL= Curvilinear Velocity. VSL= Straight Line Velocity. VAP= Average Path Velocity. LIN = linearity. STR= Straightness. ALH= Lateral Amplitude of Head Displacement. Different letters at different doses show significant differences (P<0.05)

reproductive fluids can decrease sperm motility. Excessive levels of melatonin in semen affect the physiology of the human spermatozoa (Yie et al. 1991). Incubation of bull semen with melatonin between 0.5 and 4 mM for 60 min significantly reduced spermatozoa motility (Tanyildizi et al. 2006). This may be due to different cement processing procedures, different proportions of components in the plasma membrane, and different species (Zhang et al. 2015). However, these results do not fully explain the negative effect of high concentrations of melatonin on semen, and therefore further research should be carried out. In some literature, different doses are optimal for semen cryoprotection. For example, one showed that 1 and 2 mM were optimal concentrations, while another study found that the best concentration was 0.25 mg/mL in various species of fields (Appiah et al. 2019).

Total motility, progressive motility, PMI, and AIn of spermatozoa incubated with melatonin are presented in Table 3. Percentages of total motility and progressive motility in this study were assessed using CASA, and significant differences were found between treatments at different doses of melatonin. The percentages of total motility and progressive motility were higher ($P<0.05$) in the diluents supplemented with 1.0 and 1.5 mM melatonin (62.68±2.72 and 38.91±2.68; 58.11± 2.40 and 35.05±1.68, respectively). The percentage of progressive motility showed no significant difference ($p > 0.05$) between the control group (45.74±3.70) and the diluent supplemented with 0.5 mM melatonin (48.63±3.01). Melatonin supplementation of 1 mM in extender increased the percentage of PMI and AIn (62.22±0.30 and 67.23±2.6, respectively) compared to other groups.

The description of the acrosome status of the spermatozoa in Figure 1 uses TBG staining: (A) spermatozoa with AIn absorb purple color (B) spermatozoa with damaged acrosomes will have a pale lavender/faded color. Thus, if part of the spermatozoa acrosome is damaged or incomplete, the cells cannot absorb color correctly, and the staining results will look faded. Irregularity in the head can also indicate that the plasma membrane has been damaged and has the potential for acrosomal damage. The results showed that the acrosome status of spermatozoa supplemented with melatonin after thawing showed a reasonably high percentage ranging from 59-67%. The acrosome integrity percentage indicated no damage to the spermatozoa that could reduce its fertilization ability. Spermatozoa cannot fertilize if acrosomal damage exceeds 50% (Hernández et al. 2012).

The movement pattern of post-thawed spermatozoa with melatonin supplementation is shown in Table 4. There was no significant difference between VCL, ALH, and LIN values. Velocity and linearity contribute to essential characteristics of the spermatozoa function (Shibahara et al. 2003). By definition, linearity is linearly moving in a straight line, affecting progressive motility (Sarastina et al. 2007). Sarastina *et al.* (2007) state that if LIN>35%, the average spermatozoa move linearly straight ahead. The results of this study describe the LIN value ranging from 47 to 52%; this indicates that the spermatozoa move linearly straight ahead. The ALH value in this study showed no difference ($P>0.05$) with a value of 5 to 6 m. All frozen semen had ALH values <5µm, meaning the spermatozoa were not hyperactive (Sarastina et al. 2007; Kathiravan et al. 2011).

The relationship between the CASA variable and the fertilization ability indicates that metabolically active spermatozoa have high penetration potential. Good motility is needed for the ability of spermatozoa to penetrate the cumulus and reach the zona pellucida. Furthermore, the intact membrane will bind to the zona pellucida (Ducha et al. 2012; Okabe 2013). Conversely, Spermatozoa with damaged plasma membranes cannot bind to ZP (binding zone) because the membrane that binds the zona pellucida has lost its receptors (Ducha et al. 2012).

CONCLUSION

In conclusion, differences in spermatozoa concentration did not significantly affect the quality of sheep semen. They demonstrated that adding one mM of melatonin increased the effect of the frozen-thawed process on motility, acrosome integrity, and plasma membrane integrity in sheep semen.

ACKNOWLEDGMENT

Thank the Indonesian Endowment Fund for Education (LPDP) from the Ministry of Finance Republic Indonesia for granting the scholarship and supporting this research.

REFERENCES

Abou-haila A. Tulsiani DRP. 2009. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. Arch Biochem Biophys. 485:72–81. DOI:10.1016/j.abb.2009.02.003.

Ahmad M. Ahmad N. Riaz A. Anzar M. 2015. Sperm survival kinetics in different types of bull semen: Progressive motility. plasma membrane integrity. acrosomal status and reactive oxygen species generation. Reprod Fertil Dev. 27:784–793. DOI:10.1071/RD13400.

Appiah MO. He B. Lu W. Wang J. 2019. Antioxidative effect of melatonin on cryopreserved chicken semen. Cryobiology. 89:90–95. DOI:10.1016/j.cryobiol.20 19 .05.001.

Arifiantini I. Supriatna I. Aminah. 2007. Kualitas Semen Beku Kuda dalam Pengencer Susu Skim dan Dimitropoulos dengan Dimetilformamida Sebagai Krioprotektan. Media Peternak. 30:100–105.

Ashrafi I. Kohram H. Ardabili FF. 2013. Antioxidative effects of melatonin on kinetics. microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci. 139:25–30. DOI:10.1016/j.anireprosci.2013. 03.016.

Awad H. Halawa F. Mostafa T. Atta H. 2006. Melatonin hormone profile in infertile males. Int J Androl. 29:409–413. DOI:10.1111/j.1365-2605.2005.00624.x.

Badan Standardisasi Nasional. 2014. SNI 4869.3: 2014 Semen beku–Bagian 3 : Kambing dan domba. Jakarta (Indones): Badan Standardisasi Nasional.

Bucak MN. Tuncer PB. Sariözkan S. Başpinar N. Taşpinar M. Çoyan K. Bilgili A. Akalin PP. Büyükleblebici S. Aydos S. et al. 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: Antioxidants protect DNA integrity against cryodamage. Cryobiology. 61:248–253. DOI:10.1016/j.cryobiol.2010. 09.001.

Bui AD. Sharma R. Henkel R. Agarwal A. 2018. Reactive oxygen species impact on sperm DNA and its role in male infertility. Andrologia. 50:1–10. DOI:10.1111 /and.13012.

Casao A. Vega S. Palacín I. Pérez-Pe R. Laviña A. Quintín FJ. Sevilla E. Abecia JA. Cebrián-Pérez JA. Forcada F. Muiño-Blanco T. 2010. Effects of melatonin implants during non-breeding season on sperm motility and reproductive parameters in rasa aragonesa rams. Reprod Domest Anim. 45:425–432. DOI:10.1111/j.1439-053 1.2008.01215.x.

Chatterjee I. Richmond A. Putiri E. Shakes DC. Singson A. 2005. The Caenorhabditis elegans spe-38 gene encodes a novel four-pass integral membrane protein required for sperm function at fertilization. Development. 132:2795–2808. DOI:10.1242/dev.01868.

Colás C. Grasa P. Casao A. Gallego M. Abecia JA. Forcada F. Cebrián-Pérez JA. Muiño-Blanco T. 2009. Changes in calmodulin immunocytochemical localization associated with capacitation and acrosomal exocytosis of ram spermatozoa. Theriogenology. 71:789–800. DOI:10.101 6/j.theriogenology.2008.10.003

Dapino DG. Teijeiro JM. Cabada MO. Marini PE. 2009. Dynamics of heparin-binding proteins on boar sperm. Anim Reprod Sci. 116:308–317. DOI:10.1016/j.anire prosci.2009.02.018.

Ducha N. Susilawati T. Aulanni'am. Wahyuningsih S. Pangestu M. 2012. Ultrastructure and fertilizing ability of Limousin bull sperm after storage in CEP-2 extender with and without egg yolk. Pakistan J Biol Sci. 15:979–985. DOI:10.3923/pjbs.2012.979.985.

Ehling C. Wirth P. Schindler L. Hadeler KG. Döpke HH. Lemme E. Herrmann D. Niemann H. 2003. Laparoscopical intrauterine insemination with different doses of fresh. conserved. and frozen semen for the production of ovine zygotes. Theriogenology. 60:777–787. DOI:10.1016/S0093-691X(03)00049-9.

Espino J. Bejarano I. Ortiz Á. Lozano GM. García JF. Pariente JA. Rodríguez AB. 2010. Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa. Fertil Steril. 94:1915–1917. DOI:10.1016/j.fertnstert.2009.12.082.

Fernandez-Abella D. Preve MO. Villegas N. 2003. Insemination time and dilution rate of cooled and chilled ram semen affects fertility. Theriogenology. 60:21–26. DOI:10.1016/S0093-691X(02)01041-5.

Fujinoki M. 2008. Melatonin-enhanced hyperactivation of hamster sperm. Reproduction. 136:533–541. DOI:10. 1530/REP-08-0202.

Fuller S.. Whittingham D. 1996. Effect of cooling mouse spermatozoa to 4C on fertilization and embryonic development. J Reprod Fertil. (March):139–145. DOI:10.1007/978-94-017-4380-8_20.

Gervasi MG. Visconti PE. 2017. Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. Andrology. 5:204–218. DOI: 10.1111/andr.12320.

Gergátz E. 2007 A juhok mesterséges termékenyítése; in: Házi emlősállatok mesterséges termékenyítése. (The artificial insemination in sheep; in Artificial insemination in domestic mammals); Szerk. (Edited) Tamás Pécsi; Pécsi Tamás. Mezőgazda Kiadó.Bp. 978-9-63286-237-8

Hernández PJ.. Fernandez R.. Rodriguez S j. .. Soto MY.. Verona JE.. Garcia RA. 2012. Post-thaw acrosomal viability and reaction in sperm obtained from equine epididymis tail. Rev Salud Anim. 34:84–88.

Hinrichs K. Loux SC. 2012. Hyperactivated Sperm Motility: Are Equine Sperm Different? J Equine Vet Sci. 32:441–444. DOI:10.1016/j.jevs.2012.05.070.

Holt W V. 2000. Basic aspects of frozen storage of semen. Anim Reprod Sci. 62:3–22. DOI:10.1016/S0378-4320 (00)00152-4.

Hyakutake T. Mori K. Sato K. 2018. Effects of surrounding fluid on motility of hyperactivated bovine sperm. J Biomech. 71:183–189. DOI:10.1016/j.jbiomech.2018. 02.009.

Irez TO. Senol H. Alagoz M. Basmaciogullari C. Turan F. Kuru D. Ertiingealp E. 1992. Effects of indoleamines on sperm motility in vitro. Hum Reprod. 7:987–990.

Izadpanah G. Zare-Shahneh A. Zhandi M. Yousefian I. Emamverdi M. 2015. Melatonin Has a Beneficial Effect on Stallion Sperm Quality in Cool Condition. J Equine Vet Sci. 35:555–559. DOI:10.1016/j.jevs.2015.02.007.

Jang HY. Kim YH. Kim BW. Park IC. Cheong HT. Kim JT. Park CK. Kong HS. Lee HK. Yang BK. 2010. Ameliorative effects of melatonin against hydrogen peroxide-induced oxidative stress on boar sperm

characteristics and subsequent in vitro embryo development. Reprod Domest Anim. 45:943–950. DOI:10.1111/j.1439-0531.2009.01466.x.

Johinke D. de Graaf SP. Bathgate R. 2015. The effect of sperm concentration and storage vessel on quercetin-supplemented rabbit semen during chilled storage. Reprod Domest Anim. 50:567–573. DOI:10.1111 /rda.12525.

Kalita MK, Sinha S, Biswas RK, Deka BC, Bhuyan M, Ahmed K. 2019. Effect of sperm number of per dose on quality and fertility of frozen buck semen. J Entomol Zool Stud. 7:614–617.

Karja NWK. Fahrudin M. Setiadi MA. 2013. Vitro fertility of post-thawed epididymal ram spermatozoa after storage at 5° C before cryopreservation. Media Peternak. 36:26–31. DOI:10.5398/medpet.2013.36.1.26.

Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. 2011. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system - A review. Reprod Domest Anim. 46:165–172. DOI:10.1111/j.1439-0531.2010.01603.x.

Kato Y, Shoei S, Nagao Y, 2011. Capacitation status of activated bovine sperm cultured in media containing methyl-β-cyclodextrin affects the acrosome reaction and fertility. Zygote. 19:21–30. DOI:10.1017/S09671994 10000304.

King ME. McKelvey WAC. Dingwall WS. Matthews KP. Gebbie FE. Mylne MJA. Stewart E. Robinson JJ. 2004. Lambing rates and litter sizes following intrauterine or cervical insemination of frozen/thawed semen with or without oxytocin administration. Theriogenology. 62:1236–1244. DOI:10.1016/j.theriogenology.2004.01. 009.

Lemma A. 2011. Effect of cryopreservation on sperm quality and fertility. Artif Insemin Farm Anim. DOI:10.5772/16563.

Lv C. Wu G. Hong Q. Quan G. 2019. Spermatozoa cryopreservation: state of art and future in small ruminants. Biopreserv Biobank. 17:171–182. DOI:10.1089/bio.2018.0113.

Magnotti C. Cerqueira V. Lee-Estevez M. Farias JG. Valdebenito I. Figueroa E. 2018. Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Rev Aquac. 10:15–25. DOI:10.1111/raq.12145.

Martínez-Páramo S. Horváth Á. Labbé C. Zhang T. Robles V. Herráez P. Suquet M. Adams S. Viveiros A. Tiersch TR. Cabrita E. 2017. Cryobanking of aquatic species. Aquaculture. 472:156–177. DOI:10.1016/j.aquaculture. 2016.05.042.

Muiño R. Tamargo C. Hidalgo CO. Peña AI. 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. Anim Reprod Sci. 109:27–39. DOI:10.1016/j.anirepr osci.2007.10.007.

Murphy C. Fahey A.. Shafat A. Fair S. 2013. Reducing sperm concentration is critical to limiting the oxidative stress challenge in liquid bull semen. J Dairy Sci. 96:4634–4637. DOI:10.3168/jds.2012-6484.

Murphy C. Holden SA. Murphy EM. Cromie AR. Lonergan P. Fair S. 2016. The impact of storage temperature and sperm number on the fertility of liquid-stored bull semen. Reprod Fertil Dev. 28:1349–1359. DOI:10.1071/RD 14369.

Murphy EM. Eivers B. O'Meara CM. Lonergan P. Fair S. 2018. Effect of storage temperature. nitrogen gassing and sperm concentration on the in vitro semen quality and in vivo fertility of liquid bull semen stored in INRA96. Theriogenology. 108:223–228. DOI:10.1016/j.therio genology.2017.12.012.

Nofa Y. Karja NWK. Arifiantini RI. 2018. Status akrosom dan kualitas post-thawed spermatozoa pada beberapa rumpun sapi dari dua balai inseminasi buatan. Acta Vet Indones. 5:81–88. DOI:10.29244/avi.5.2.81-88.

Okabe M. 2013. The cell biology of mammalian fertilization. Dev. 140:4471–4479. DOI:10.1242/dev.090613.

Ortiz A. Espino J. Bejarano I. Lozano GM. Monllor F. García JF. Pariente JA. Rodríguez AB. 2011. High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J Pineal Res. 50:132–139. DOI:10.1111/j.1600-079X.2010.00822.x.

Othman AI. Edrees GM. El-Missiry MA. Ali DA. Aboel-Nour M. Dabdoub BR. 2016. Melatonin controlled apoptosis and protected the testes and sperm quality against bisphenol A-induced oxidative toxicity. Toxicol Ind Health. 32:1537–1549. DOI:10.1177/07482337145612 86.

Pamungkas FA. Batubara A. Anwar A. 2014. Kriopreservasi spermatozoa kambing Boer: Perbandingan dua bahan pengencer terhadap kualitas post-thawing dan kemampuan fertilisasinya. JITV. 19:130–137. DOI:10.14334/jitv.v19i2.1041.

Pang YW. Sun YQ. Jiang XL. Huang ZQ. Zhao SJ. Du WH. Hao HS. Zhao XM. Zhu H Bin. 2016. Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. Mol Reprod Dev. 83:993–1002. DOI:10.1002/mrd.22742.

Perumal P. 2014. Effect of superoxide dismutase on semen parameters and antioxidant enzyme activities of liquid stored (5°C) mithun (bos frontalis) semen. J Anim. 2014:1–9. DOI:10.1155/2014/821954.

Ramadan TA. Taha TA. Samak MA. Hassan A. 2009. Effectiveness of exposure to longday followed by melatonin treatment on semen characteristics of Damascus male goats during breeding and non-breeding seasons. Theriogenology. 71:458–468. DOI:10.1016 /j.theriogenology.2008.07.029.

Rao M V.. Gangadharan B. 2008. Antioxidative potential of melatonin against mercury induced intoxication in spermatozoa in vitro. Toxicol Vitr. 22:935–942. DOI:10.1016/j.tiv.2008.01.014.

Reiter RJ. 1998. Oxidative damage in the central nervous system: Protection by melatonin. Prog Neurobiol. 56:359–384. DOI:10.1016/S0301-0082(98)00052-5.

Salinas MB. Chuammitri P. Sringarm K. Boonyayatra S. Sathanawongs A. 2021. Current perspectives on ruminant sperm freezability: Harnessing molecular changes related to semen quality through omics technologies. Vet Integr Sci. 19:487–511. DOI:10. 12982/vis.2021.039.

Salmani H. Nabi MM. Vaseghi-Dodaran H. Rahman MB. Mohammadi-Sangcheshmeh A. Shakeri M. Towhidi A. Shahneh AZ. Zhandi M. 2013. Effect of glutathione in soybean lecithin-based semen extender on goat semen quality after freeze-thawing. Small Rumin Res. 112:123–127. DOI:10.1016/j.smallrumres.2012.12.015.

Sarastina. Susilawati T. Ciptadi G. 2007. Analisa beberapa parameter motilitas spermatozoa pada berbagai bangsa sapi menggunakan computer assisted semen analysis (casa). J Ternak Trop. 6:1–12.

Shannon P. Curson B. Rhodes A. Relationship between total spermatozoa per insemination and fertility of bovine semen stored in Caprogen at ambient temperature. New Zeal J Agr Res 1984;27:35-41.

Shibahara H. Obara H. Kikuchi K. Yamanaka S. Hirano Y. Suzuki T. Takamizawa S. Suzuki M. 2003. Prediction of Human Sperm Fertilizing Ability by the Hyperactivated Motility Patterns. J Mamm Ova Res. 20:29–33. DOI:0.1274/jmor.20.29.

Sönmez M. Yüce A. Türk G. 2007. The protective effects of melatonin and Vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats. Reprod Toxicol. 23:226–231. DOI:10.1016/j.reprotox.2006.11.003.

Succu S. Berlinguer F. Pasciu V. Satta V. Leoni GG. Naitana S. 2011. Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. J Pineal Res. 50:310–318. DOI:10.1111/j.1600-079X.2010.00843.x.

Sundararaman MN. Kalatharan J. Jawahar KTP. 2012. Computer assisted semen analysis for quantification of motion characteristics of bull sperm during cryopreservation cycle. Vet World. 5:723–726. DOI:10.5455/vetworld. 2012.723-726.

Taitzoglou IA. Chapman DA. Zervos IA. Killian GJ. 2004. Effect of plasmin on movement characteristics of ejaculated bull spermatozoa. Theriogenology. 62:553–561. DOI:10.1016/j.theriogenology.2003.11.016.

Tanyildizi S. Bozkurt T. Çiftçi O. Sakin F. 2006. In vitro effects of melatonin on hyaluronidase activity and sperm motility in bull semen. Turkish J Vet Anim Sci. 30:89–93.

Tao Y. Sanger E. Saewu A. Leveille MC. 2020. Human sperm vitrification: The state of the art. Reprod Biol Endocrinol. 18:1–10. DOI:10.1186/s12958-020-00580-5.

Thomas AD. Meyers SA. Ball BA. 2006. Capacitation-like changes in equine spermatozoa following cryopreservation. Theriogenology. 65:1531–1550. DOI: 10.1016/j.theriogenology.2005.08.022.

Toelihere MR. 1985. Inseminasi Buatan pada Ternak. Bandung (Indones): Angkasa Triwulanningsih.

Trilaksana I. Ndun R. Bebas W. 2016. Penambahan vitamin C pada pengencer fosfat kuning telur semen kalkun yang disimpan pada suhu 5°C. Bul Vet Udayana. 7:186–193.

Tsantarliotou MP. Kokolis NA. Smokovitis A. 2008. Melatonin administration increased plasminogen activator activity in ram spermatozoa. Theriogenology. 69:458–465. DOI:10.1016/j.theriogenology.2007.10.015.

Yánez-Ortiz I. Catalán J. Rodríguez-Gil JE. Miró J. Yeste M. 2021. Advances in sperm cryopreservation in farm animals: Cattle. horse. pig and sheep. Anim Reprod Sci. DOI:10.1016/j.anireprosci.2021.106904.

Yie S -M. Daya S. Brown GM. Deys L. YoungLai E V. 1991. Melatonin and aromatase stimulating activity of human seminal plasma. Andrologia. 23:227–231. DOI:10. 1111/j.1439-0272.1991.tb02543.x.

Yusuf TL. Arifiantini RI. Rshmiwati N. 2005. Daya tahan semen cair kambing peranakan etawah dalam pengencer kuning telur dengan kemasan dan konsentrasi spermatozoa yang berbeda. J Indones Trop Anim Agric. 4:217–223.

Zhang XG. Yan GJ. Hong JY. Su ZZ. Yang GS. Li QW. Hu JH. 2015. Effects of Bovine Serum Albumin on Boar Sperm Quality During Liquid Storage at 17°C. Reprod Domest Anim. 50:263–269. DOI:10.1111/rda.12481.