Journal of Advanced Veterinary Research

https://advetresearch.com

Extract of Cincau (*Mesona palustris* B.) Supplementation in Semen Extender Improves Boer Goat Sperm Cryopreservation

Sri Wahjuningsih[1*], Muhammad Nur Ihsan[1], Doni Andri Siswoyo[1], Dian Tria Fatmila[1], Aulia Firmawati[2]

[1]*Department of Animal Production, Faculty of Animal Husbandry, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia.*
[2]*Department of Veterinary Public Health, Faculty of Veterinary Medicine, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia.*

ARTICLE INFO

Original Research

Received:
09 July 2021

Accepted:
02 October 2021

Keywords:

Antioxidant, Black *cincau*, Boer goat, Frozen semen, Semen quality

ABSTRACT

The spermatozoa freezing process of Boer goat sperm leads to cold shock and lipid peroxidation, which increases reactive oxygen species. To reduce oxidative stress, antioxidants are required as semen diluents, which can be supplemented with the extract of black *cincau* (*Mesona palustris* B.) leaves. This study aimed to analyze the effects of black *cincau* leaf extract supplementation at dosages of 0%, 1.5%, 2%, and 2.5% in a basic diluent of skim milk yolk on the quality of post-thawing frozen semen and to determine the in vivo fertile quality of frozen semen through artificial insemination. This study used a completely randomized design using 110 sperm samples. There were four dilution treatments: skim milk yolk supplemented with 0%, 1.5%, 2%, and 2.5% (v/v) black *cincau* leaf extract, and added to sperm samples labeled T0, T1, T2, and T3, respectively. The motility, viability, membrane integrity of spermatozoa, and malondialdehyde (MDA) levels were observed. The best quality of spermatozoa from this study was used for artificial insemination to determine the percentage of conception rate and observed until the female gave a birth. Treatment of T2 (2% black *cincau* leaf extract supplementation) was found to have the best record on motility, viability and integrity of plasma membranes, and lower level of MDA. The results of artificial insemination on 30 female goats using T0 (without black grass jelly extract supplementation) and 30 female goats using T2, each resulted in a conception rate of 63.33% vs 80%. It could be concluded that skim milk yolk base diluent supplemented with 2% black *cincau* leaf extract has the capability to protect the motility, viability, and plasma membrane integrity, decreased MDA level of sperm after freezing and increase conception rate.

J. Adv. Vet. Res. (2021), 11 (4), 247-253

Introduction

The wide use of artificial insemination is the key technology of livestock production for achieving genetic progress and maintenance of genetic diversity (Waberski, 2018). Artificial insemination is an alternative to increase the reproductive capacity of livestock and is expected to accelerate the breeding, productivity, and genetic quality of goats (Ren *et al*., 2019), cattle (Lamb and Mercadante, 2016), and pigs (Rodriguez *et al*., 2017). To achieve an excellent result and for the artificial insemination program to be carried out properly, best quality semen from superior males is needed to be stored properly for a long time, distributed to various regions and artificially inseminated into females (Sitepu and Marisa, 2018).

Indonesian local goats normally have a smaller body size and low production performance. However, they can adapt to unfavorable environments and low quality feed. Therefore, crossbreeding is performed to introduce genetic sources with high productivity performance. Boer goats have excellent body conformations, good carcass quality, fast growing, and have high adaptability (Casey and Van Niekerk, 1988); furthermore, Boer goats have high resistance against diseases and can increase the carcass percentage of crossbred goat (Malan, 2000). Based on those characteristics, Boer goats are potential genetic resources in crossbreeding programs for improving Indonesian local goat productivity [Duridic *et al*., 2012).

The quality of frozen semen is a major determinant to the success of the artificial insemination program. Semen cryopreservation extend the viability of spermatozoa, fertilization capacity, and increase the reproductive efficiency of males (Casali *et al*., 2017). The semen preservation method has been developed for the past few decades. However, the decline of sperm quality during this process has not been resolved (Olivera-Muzante *et al*., 2011; Paul *et al*., 2018). The sperm cell motility rates after cryopreservation have been usually lower than when fresh semen is used. The composition of the extender before the freezing process is one of the most important factors that affects cryopreservation.

The cold shock resistant and cryoprotectant are necessary to preserve sperm during freezing and thawing. Cryopreservation and thawing might disrupt the plasma membrane of spermatozoa and reduces their post-thawing motilities and viabilities (Wahjuningsih *et al*., 2019). During the freezing process, a cold shock occurs to frozen spermatozoa due to a decrease in temperature to −196 °C and lipid peroxidation

*Corresponding author: Sri Wahjuningsih
E-mail address: yuning@ub.ac.id

ISSN: 2090-6277/2090-6269/

(LPO) caused by an increase in reactive oxygen species (ROS) (Sariözkan *et al*., 2014). The production of ROS by sperms is a normal physiological process, but an imbalance between ROS generation and scavenging activity is detrimental to the sperms.

Antioxidants are required to prevent the damage to the spermatozoa plasma membrane caused by LPO. Previous studies showed that exogenous antioxidant treatments improved the semen performance by reducing the effects of oxidative stress (Fang *et al*., 2017; Syarifuddin *et al*., 2017; Eriani *et al*., 2018). Researchers have studied the potential of antioxidant supplementations to overcome this problem by reducing ROS accumulation during the preservation process (Len *et al*., 2019). Natural antioxidant supplementation from plant extracts to maintain the quality of semen has been studied, including Moringa oleifera leaf extract (Wahjuningsih *et al*., 2019), grape seed procyanidin extract (Nigella sativa Oil), and honey (Wen *et al*., 2019).

One of the plants that contain antioxidant property is the grass jelly plant (*cincau*). It has several types, namely green grass jelly (Cyclea barbata Miers), black *cincau* (*Mesona palustris* B.), and grass jelly shrub (Premna parasitica Blume). The antioxidant activity of black *cincau* leaves extracted using ethyl acetate and methanol yielded an inhibition concentration 50 (IC50) of <200 µg/mL. Black *cincau* extract contains flavonoids, phenols, and tannins, with an antioxidant amount of 1054.7600 mg/L and IC50 of 4.3670 mg/ml, which classifies it as very strong antioxidant (Tarnajaya *et al*., 2018). The effect of skim milk yolk base diluent supplemented with Cincau leaf extract on the quality of post-thawing goat semen and its fertility has not been studied. Therefore, this study aimed to analyze the effect of black *cincau* leaf extract supplementation at different levels in skim milk yolk base diluent on the frozen quality of post-thawing goat semen and to test the in vivo fertility of frozen semen through artificial insemination.

Materials and methods

Ethical approval

This research has obtained ethical approval from the Ethics Commission of Brawijaya University (058-KEP-UB-2020).

Samples collection

Semen were collected from four healthy male goats aged 3–3.5 years old, weighing 50–56 kg, which were farmed in the Sumbersekar Field Laboratory of the Faculty of Animal Husbandry, University of Brawijaya. Sampling was carried out twice a week using the artificial vaginal method, using a Doe in heat as a teaser.

Evaluation of semen quality

Semen quality was evaluated in the Sumbersekar Field Laboratory and Animal Reproduction Laboratory of the Faculty of Animal Husbandry, UB's Central Laboratory of Biological Sciences. The evaluation was based on standard criteria (Rather *et al*., 2016). If there were no abnormalities in color and odor, then microscopic examination was continued to determine the motility, viability, abnormalities, membrane integrity, and spermatozoa concentration.

A total of 128 semen ejaculates were collected from goats, out of which 110 ejaculates were found suitable for preservation. Only semen with a progressive motile spermatozoa quality of more than 75% and normal morphology of more than 85% were used in this study.

Microscopic analysis evaluation of semen includes progressive motility (Lukman *et al*., 2014), viability, and morphology of the abnormality, which can be observed using eosin–nigrosin staining (Ducha *et al*., 2012), and hypo osmotic swelling test (Karunakaran and Devanathan, 2016; Eriani *et al*., 2018).

Spermatozoa with intact plasma membrane are characterized by a coiled spermatozoa tail, whereas spermatozoa with damaged membranes are characterized by a straight tail (Ramu and Jeyendran, 2013). The sperm concentration was counted subjectively with a hemocytometer Neubauer Chamber. LPO levels of spermatozoa in the semen sample was estimated by measuring the malondialdehyde (MDA) production using thiobarbituric acid test (Morte *et al*., 2008; Kumaresan *et al*., 2009).

Preparation of Black cincau leaf extract and test for antioxidants and phytochemical content

The black *cincau* leaf extract was prepared based on Puspitasari and Wulandari (Puspitasari and Wulandari, 2017). Four hundred grams of dried black *cincau* leaf powder are macerated using 3 L of 96% ethanol for 3 days. The macerate was then concentrated using a rotary evaporator at 45 oC to obtain the extract. The extract was placed in a 1.5-mL Eppendorf tube and stored in the freezer at −20 °C. The antioxidant activity test was carried out using the 2,2 dipenyl-1-picrylhidrazyl method based on Maesaroh *et al*. (2018). The phytochemical content test was conducted based on Puspitasari and Wulandari (2017) and the vitamin content was determined based on the method by Techinamuti and Pratiwi (2018). A 0.1 gram of black *cincau* extract was weighed and dissolved in 100 mL aquabidest. Samples with a concentration of 1000 ppm were then taken 1 mL and diluted until the volume reached 10 mL. The diluted sample was then measured using a UV-Vis spectrophotometer at a wavelength of 265 nm.

Experimental design

This study used a completely randomized design, using four treatments with 110 sperm samples. The basic diluent was skim milk–egg yolk (Herbowo *et al*., 2019), which was prepared by dissolving 10 g of skim milk powder (Tropicana Slim) and 1 g of fructose (Merck, KgaA, Darmstadt Germany) in 100-mL distilled water. The mixture was then heated at 92 °C for 10 min and then cooled to 32 °C. Fresh yolk from <3 days old of chicken eggs at a concentration of 5%, 1000 IU/mL of penicillin (Meiji, Japan), 1 mg/mL of streptomycin (Meiji, Japan), and 7% glycerol (Merck, KgaA, Darmstadt Germany) were added to the egg milk scheme yolk extender. Four different treatment diluents were prepared by adding 0%, 1.5%, 2.0%, and 2.5% black *cincau* leaf extract to 100 ml basic diluent, and labeled as T0, T1, T2, and T3, respectively. After preparation, the diluent was kept in a water bath at 37 °C for semen dilution. The osmolarity values of freezing media used in each treatment were 270 mOsm/kg (T0), 310 mOsm/kg (T1), 352 mOsm/kg (T2), and 395 mOsm/kg (T3).

Semen processing

Filling and sealing of straws

Extended semen was filled in 0.25 ml French mini straws (IMV Technologies, France), and subsequently sealed using a filling-sealing machine (Minitube, Germany).

Equilibration, vapor exposure, freezing, and thawing

The straws were laid on a stainless steel rack and placed

in a cooling cabinet for 4 h to attain a temperature of 3º C–4 °C. After equilibration, rack of straws from the cooling cabinet was transferred into a Styrofoam box with LN2. The LN2 quantity in the Styrofoam box was so decided that its upper surface remained 4 cm below the rack. Straws were exposed to LN2 vapored for 10 min. Finally, the straws were immediately transferred into liquid nitrogen goblet of a LN2 container and stored at −196°C for 2 weeks prior to thawing. Thawing of semen straws was done at 37 °C for 30 s in a water bath.

Artificial insemination

Healthy fertile does (n = 68) aged 3–3.5 years old (already had at least one offspring) were selected for artificial insemination. Each doe was estrus-synchronized via a subcutaneous injection of 1.5 mL PGF2α (Lutalyse, Holland).

Does were observed twice daily for heat behavior. To accelerate and detect the heat condition, a mature goat was shown in front of them in a separate room for 30 min. A total of 60 does were detected on to be in heat; eight does were excluded from the study because they were not in heat. The does were equally divided into two groups (30 does each). The first group of does was inseminated with skim milk–egg yolk without black *cincau* leaves extract supplementation (T0), considered as a control group, while the rest of 30 does (second group) was inseminated with the best frozen semen treatment (2% black *cincau* leaves extract). Each doe was inseminated twice; initial intracervical insemination was performed at 12 h, after the first heat sign, a second insemination was performed 10 h later. All straws (150 x 106 sperm/straw) used in the experiment were thawed in warm water (37 °C) for 30 s and then loaded into the insemination gun (IMV Technologies). Success of pregnancy was observed until the female inseminated using T0 and T2 gave birth.

Statistics analysis

All data were expressed as the mean values ± SD. Data of the progressive motility, viability, plasma membrane integrity, and MDA were evaluated by analysis of variance. In order to know the relationship between parameter, the Pearson's correlation was performed. All statistical analysis were performed using SPSS 17.0 and the Duncan's Multiple Renge Test (DMRT) was used for further analysis if there was a significantly difference at α = 0.05.

Results

This research used fresh Boar goat semen, which was examined macroscopically to determine the quality of volume, color, and pH. The microscopic examination parameters are mass movement, individual movement, concentration, viability, abnormality, and integrity of the spermatozoa plasma membrane. The quality of fresh semen of Boer goat is presented in Table 1.

The results on motility, viability, abnormality, and membrane integrity of diluted, equilibrated, and post-thawed sperms of Boer goat treated with various concentrations of black *cincau* extract are presented in Tables 2. The black *cincau* extract in this study yielded total phenol = 39.79 ± 0.87

Table 1. Macroscopic and microscopic variables of fresh semen of Boer goat.

Variable	Mean ± SD
Volume (ml)	1.25 ± 0.29
pH	6.70 ± 0.27
Color	Milky white
Mass Motility	+++
Individual motility (%)	80.55 + 8.35
Viability (%)	87.64 ± 6.56
Abnormality (%)	5.23 ± 0.52
Plasma membrane integrity (%)	88.34 ± 6.58
Concentration(10^6/ml)	2945.25 ± 125.52
MDA (μM)	4.94 ± 0.15

Table 2. The motility, viability, plasma membrane integrity, and MDA concentration of sperms of Boer goats treated with various concentrations of black *cincau* extract in different phases of semen processing.

Parameters	Treatments*)	Phases of semen processing		
		Dilution	Equilibration	Post-thawing
Average motility (%)	T0	66.42 ± 5.63^{a}	51.21 ± 4.84^{a}	43.42 ± 3.67^{a}
	T1	67.35 ± 5.65^{b}	54.23 ± 5.18^{b}	45.95 ± 4.21^{b}
	T2	71.35 ± 6.35^{c}	63.28 ±5.32^{c}	50.40 ± 4.62^{c}
	T3	67.29 ± 6.19^{b}	55.22 ± 4.35^{b}	44.60 ± 3.90^{ab}
Average viability (%)	T0	75.25 ± 3.83^{a}	61.36 ± 4.25^{a}	51.48 ± 3.25^{ab}
	T1	76.35 ± 5.57^{b}	63.23 ± 3.57^{b}	52.24 ± 3.36^{b}
	T2	82.12 ± 6.26^{c}	78.12 ± 6.29^{c}	59.21 ± 4.55^{c}
	T3	76.29 ± 5.65^{ab}	61.28 ± 4.65^{a}	51,44 ± 4.12^{a}
Average plasma membrane integrity (%)	T0	76.12 ± 5.22 ^{a}	68.62 ± 3.36a	53.23 ± 2.80^{a}
	T1	77.15 ± 4.12^{b}	68.78 ± 3.27^{a}	54.29 ± 3.17^{b}
	T2	82.59 ± 6.76^{c}	75.41 ± 5.93^{b}	60.45 ± 3.75^{c}
	T3	7.22 ± 5.17^{b}	68.59 ± 5.59^{a}	54.24 ± 3.13^{b}
Average MDA (μM)	T0	4.95 ± 0.10^{a}	5.98 ± 0.18^{b}	7.47 ± 0.18^{c}
	T1	4.94 ± 0.20^{a}	5.92 ± 0.12^{a}	7.35 ± 0.15^{b}
	T2	4.98 ± 0.13^{a}	5.90 ± 0.08^{a}	6.25 ± 0.12^{a}
	T3	4.99 ± 0.11^{a}	5.96 ± 0.02^{ab}	7.38 ± 0.10^{bc}

Note: Means value in each parameter with different superscripts in the same column differ significantly (P < 0.05). *)Treatments means skim milk yolk supplemented with 0% (T0), 1.5% (T1), 2% (T2), and 2.5% (T3) (v/v) black *cincau* leaf extract.

mgGAE/gram, total flavonoids = 169.50 ± 1.77 mgQE/gram, vitamin C = 3.86 ppm, and vitamin E = 147.87 ppm.

Spermatozoa motility is an important parameter of semen quality that needs to be considered before artificial insemination. Motility strongly correlates with the ability of the sperm to fertilize the ovum. The evaluation of the motility of individual spermatozoa at several phases of semen processing is listed in Table 2. This study confirmed that the cryopreservation process significantly reduced Boer goat sperm motility indicated by the reduced post-thawing motility of sperms compared with the effects of dilution and equilibration of the black *cincau* extract supplementation (Table 2). Sperms diluted in skim milk yolk based diluents containing 2% (v/v) black *cincau* extract had better post-thawed motility than those diluted in T0, T1, and T3.

A supplementation of 2% black *cincau* extract can maintain the viability of frozen semen after thawing, higher than those found in the control group and other groups treated with different concentrations.

The spermatozoa plasma membrane integrity was also examined in this study. The addition of black *cincau* extract to skim milk was for improve membrane integrity of plasma membrane of sperms. The results showed that the addition of a proper concentration of black *cincau* extract could significantly ($P < 0.05$) maintain the integrity of the plasma membrane of spermatozoa. Sperms diluted in skim milk yolk diluents containing 2% (v/v) black *cincau* extract (T2) had better plasma membrane integrity than the control group or other treatments.

In all treatments, MDA levels of semen samples increased after freezing and thawing (post-thawing) compared with during dilution and equilibration. There was a significant difference ($P < 0.05$) in MDA levels between treatments. Table 2 shows that if the MDA concentration at post-thawing is compared, among all treatments, T2 had the lowest MDA concentration. The antioxidant components in black *cincau* extract may protect the viability and integrity of the plasma membrane through scavenging the LPO and prevent the formation of MDA.

The results of this study indicated that motility has a significantly positive correlation with viability ($r = 0.957$) and plasma membrane integrity ($r = 0.825$). Motility, viability, and plasma membrane integrity have significantly negative correlation with MDA concentrations, $r = -0.745$, $r = -0.728$, and $r = 0.715$, respectively (Table 3).

Frozen semen showing the best quality (T2) and control (T0) was used in the artificial insemination to find out the fertility quality in vivo. T0 and T2 were each inseminated into 30 does. Artificial insemination using frozen semen T0 contained 19 pregnant goats from 30 goats carried out by IB, while at T2 there were 24 pregnant goats from 30 goats carried out by IB. Conception rates on T0 and T2 were 63.33% vs 80%, respectively.

Discussion

The semen cryopreservation technology as an artificial insemination method has numerous advantages for animal husbandry. However, the implementation of artificial insemination with frozen–thawed semen to goat breeding is limited by the poor ability of frozen spermatozoa. Therefore, the recovery of good quality sperm cells, which is used for further cryopreservation, is necessary. In this study, the addition of black *cincau* as antioxidants was intended to obtain a better quality of Boer goat semen after the freezing process. Semen quality was assessed based on step of semen processing: dilution, equilibration, and post-thawing on the variables of motility, viability, plasma membrane integrity, and sperm abnormality.

The results showed that 2% black *cincau* extract significantly ($p<0.05$) produced a higher percentage of motility, viability, and plasma membrane integrity than the control and other treatments. The study indicated that the addition of black *cincau* extract with the proper concentration in skim milk could improve the quality of frozen semen of Boer goats. This research indicated that supplementation of black *cincau* extract could reduce LPO reactions that can damage the plasma membrane of spermatozoa. The presence of antioxidant activity in black grass jelly is influenced by the presence of phenolic compounds such as flavonoids and phenolic acids.

According to Wahjuningsih *et al*. (2019) and Layek *et al*. (2016), the addition of some herbal extracts, which contain antioxidants into semen diluents, can improve the frozen-semen quality. The best concentration of black *cincau* extract that could maintain the high post-thawing quality of Boer goat sperms was 2% (v/v). The results showed that 1.5% (T1) and 2.5% (T3) black *cincau* extract supplementation have lower semen quality results than 2% (T2). The results obtained indicated that the effects of the appropriate concentration of antioxidants for maintaining semen quality (Sariözkan *et al*., 2014). An addition of 1.5% black *cincau* extract (T1) did not provide optimal protection for spermatozoa, while 2.5% (T3) causes an increasing of osmotic pressure in the diluent, which had a negative impact on the spermatozoa metabolism process. A disrupted spermatozoa metabolic process will decrease energy production in the form of ATP, thereby reducing the motility and viability of spermatozoa.

An addition of 2% black *cincau* extract supplementation (T2) significantly ($p<0.05$) obtained a better result than T0, T1, and T3 (Table 2). The results of this study support the fact that black *cincau* extract supplementation that contains antioxidants at an optimum level can improve the quality of post-thawed semen based on its motility, viability, and plasma membrane integrity. Black *cincau* extract supplementation with the right concentration in the basic diluent of skim milk yolk has a positive effect on improving the quality of frozen goat semen.

A drastic increase in temperature during thawing leads to a high metabolic activity, which means increased production of free radicals. In addition, the plasma membrane of spermatozoa cells undergoes a process of degradation due to the bad effects of clotting so that it is very vulnerable to free radical attack. The spermatozoa did not undergo such unfavorable conditions at the dilution and equilibration phase. It is in unfavorable conditions like this that the black *cincau* extract prevents excessive LPO reactions in the plasma membrane of spermatozoa cells caused by free radicals and other oxidants.

Reaction Oxygen Species (ROS) generated by spermato-

Table 3. Pearson's correlation coefficient between sperm motility, vialbility, plasma membrane integrity, and MDA concentration.

	Sperm motility	Sperm viability	Plasm membrane integrity	MDA concentration
Sperm motility	1	0.957**	0.825**	-0.745**
Sperm viability		1	0.859**	-0.728**
Plasma membrane integrity			1	-0.715**
MDA concentration				1

**Correlations are significant at $p<0.01$

zoa is involved in normal physiological processes such as sperm capacitation, acrosome reaction, maintenance of fertilizing ability, and stabilization of the mitochondrial capsule in the midpiece in bovine (Awda *et al*, 2009; Goncalves *et al*., 2010). However, excess ROS production in semen will have a detrimental effect on spermatozoa (Takeshima *et al*, 2020). The lipid of the plasma membrane of goat sperm has a higher concentration of unsaturated fatty acids compared with other ruminants. Therefore, in the thawing process, the lipid of the plasma membrane can be damaged, resulting in LPO. Susceptibility to cold temperatures is associated with a higher ratio of unsaturated fatty acids than saturated fats, which may result in the formation of a high LPO (Bansal and Bilaspuri, 2011).

Mammalian spermatozoa membrane is rich in polyunsaturated fatty acids (PUFA), which make it very sensitive to ROS. Previous studies have shown that decreased motility of frozen spermatozoa after thawing is associated with membrane destabilization resulting in increased permeability to ions and production of ROS (Awda *et al*, 2009). The process of preservation results in over production of ROS, which is extremely detrimental to spermatozoa. ROS promotes the peroxidation of lipids, resulting in intracellular oxidative burden. The sequence of events involves LPO, loss of membrane integrity with increased permeability, reduced sperm motility, structural DNA damage, and apoptosis (Fang *et al*.,2017). In addition, cryopreservation can cause DNA damage (Eveson, 2016). Consequently, the fertility following artificial insemination using cryopreserved semen is lower than that of fresh semen in most species.

Based on the results of the analysis of black *cincau* extract, it yielded total phenol = 39.79 ± 0.87 mgGAE/gram, total flavonoids = 169.50 ± 1.77 mgQE/gram, vitamin C = 3.86 ppm, and vitamin E = 147.87 ppm. This shows that the black *cincau* extract has an antioxidant activity that can suppress free radicals due to phenolic compounds. The presence of phenolic compounds in black *cincau* can play a role in inhibiting free radicals by donating one electron to an unpaired electron in free radicals, so that it can reduce the number of free radicals. Vitamins C and E can inhibit free radical chain reactions. The collaboration between vitamins C and E can defend spermatozoa from damage by capturing free radicals (Maslukhah *et al*., 2016).

Radical metabolic wastes such as ROS and hydrogen peroxide are one of the byproducts of oxygen to provide energy (ATP). These reactive compounds can disrupt the membrane from LPO reactions (Tatone *et al*., 2010). Antioxidants block LPO chain reactions by transferring one electron to a radical compound or by accepting electrons to neutralize the metabolites. These processes will prevent the detrimental effects of free radicals on the spermatozoa plasma cell membrane.

The MDA level is one part of the semen quality assessment using biochemical methods (Kordan *et al*., 2013). The plasma membrane of spermatozoa is susceptible to LPO. This LPO process occurs when PUFA, the largest constituent of the plasma membrane that surrounds cells and cell organelles, are broken down. Lipid oxidation of sperm membranes results in an MDA compound, which is toxic to the cells. MDA is an aldehyde compound resulting from LPO, which is toxic to cells. The higher the level of LPO that occurs, the more fatty acid chains are broken into aldehyde compounds so that more MDA content is formed (Tatone *et al*., 2010).

According to Susilowati *et al*. (2019), plasma membrane integrity has a negative correlation with MDA and a positive correlation with viability and motility of spermatozoa. Previous research also found that there was a negative correlation between viability of spermatozoa and levels of MDA. The lower the spermatozoa viability is, the higher the MDA level (Guthrie and Welch, 2012; Febrianti *et al*., 2014). The greater the motility and viability of spermatozoa, the lesser LPO occurs (Tatone *et al*., 2010).

MDA compounds cause damage to the spermatozoa membrane and decrease the integrity of the spermatozoa membrane (Sanocka and Kurpisz, 2004). Supplementation with 2% black *cincau* extract (T2) obtained the lowest MDA compared with T0, T1, and T3. Antioxidant compounds will react with free radicals to minimize damage to the spermatozoa cell membrane. For all concentrations of black *cincau* extract supplementation, MDA levels during post-thawing had a higher value ($P<0.05$) than during dilution and equilibration.

The high MDA levels during post-thawing are caused by the activity of ROS, which disrupted lipids in the spermatozoa membrane. The higher the level of LPO that occurs, the more fatty acid chains are broken into aldehyde compounds so that more MDA content is formed. Sperm plasma membrane impairment during the freeze–thawing process can explain the reduced ratio of post-thawing sperm motility, the increased ratio of damaged acrosomes, and the higher MDA concentrations (Fathi *et al*., 2019).

At the time of frozen semen thawing process, there is contact between the semen and oxygen, which allows the formation of reactive oxygen compounds. At the same time, there was a drastic increase in the temperature of the semen, which spurred the metabolic rate of spermatozoa, increasing the concentration of free radicals as a product of metabolism.

Cold shock will cause damage to cell structure and function. Free radicals will disrupt and take electrons from unsaturated fatty acids that make up the phospholipids of the cell plasma membrane. The membrane will lose its permeability, leads the release of many cellular components, including lipids, proteins, and ions. Excessive production of ROS during cryopreservation has been associated with the reduced post-thawed motility, viability, membrane integrity, antioxidant status, and fertility and sperm functions (Bansal and Bilaspuri, 2011). LPO can cause changes in membrane function, which results in decreased metabolism, morphology, spermatozoa motility, and fertility (Gallo *et al*., 2021).

Artificial insemination (AI) technology is one of the proven reproductive technologies to improve the genetic quality and population of livestock (Bearden and Fuquay, 2004). The evaluation of the success of AI can be measured from the number of pregnant female goats, which indicates an increase in reproductive efficiency. Conception rate (CR) is one indicator of fertility that can be used to assess reproductive efficiency (Pardede *et al*., 2020), and Dogan *et al*. (2015) stated that the CR is cor¬related with the quality of semen. The quality of semen must be considered in order to obtain a good fertility rate for the artificial insemination of farm animal. The spermatozoa motility, viability, and plasma membrane integrity in the skim milk yolk diluent with 2% black *cincau* extract supplementation showed the highest percentage, while MDA concentration were the lowest. The results of artificial insemination on 30 female goats using T0 (without black grass jelly extract supplementation) and 30 female goats using T2, each yielding a conception rate of 63.33% vs 80%. The conception rate at T2 is higher than T0 because the quality of frozen semen at T2 is higher than at T0. The results of this study are in accordance with the results of research from previous researchers which proved that good semen quality will have a big influence on the success of artificial insemination and increase CR (Mekonnen *et al*., 2010; Kebede *et al*., 2018; Kumaresan *et al*., 2017; Sitali *et al*., 2017).

Conclusion

A supplementation of 2% black *cincau* extract in skim milk

yolk base diluent has the best protective effect on the motility, viability, plasma membrane integrity, decreased MDA level of frozen–thawed sperms and increase the conception rate.

Acknowledgement

This research was funded by the Research Grants for Professors and Doctors of the Brawijaya University, Indonesia (No. 726/UN10.F05/PN/2020). The authors would like to thank the Chancellor of Brawijaya University and the Dean of the Faculty of Animal Husbandry, Brawijaya University. We also thank Mr Mochammad Bahrudin and Mr. Rochim for the technical support.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Awda, B.J., Mackenzie-Bell, M., Buhr, M.M., 2009. Reactive oxygen species and boar sperm function. Biology of Reproduction 81, 553–561.

Bansal, A.K., Bilaspuri, G.S., 2011. Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International 2011, 1-7.

Bearden, H.J., Fuquay, J.W., 2004. Applied animal reproduction. Reston Publishing Company, Inc., Virginia.

Casali, R., Pinczak, A., Cuadro, F., Guillen-Munoz, J.M., Mezzalira, A., Menchaca, A., 2017. Semen deposition by cervical, transcervical and intrauterine route for fixed-time artificial insemination (FTAI) in the ewe. Theriogenology 103, 30–35.

Casey, N.H., Van Niekerk, W.A., 1988. The boer goat. I. Origin, adaptability, performance testing, reproduction and milk production. Small Ruminant Research 1, 291-302.

Dogan, S., Vargovic, P., Oliveira, R., Belser, L.E., Kaya, A., Moura, A., Sutovsky, P., Parrish, J., Topper, E., Memili, E., 2015. Sperm protamine-status correlates to the fertility of breeding bulls. Biology of Reproduction 92, 92.

Ducha, N., Susilawati, T., Aulanniam, A., 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 Journal of Biological Sciences 15, 979-985.

Duridic, D., Grizelj, J., Dobranic, T., Harapin, I., Vince, S., Kocila, P., Folnozic, I., Lipar, M., Gracner, G.G., Samardzija, M., 2012. Reproductive performance of boer goats in a moderate climate zone. Vet. Arh. 82, 351-358.

Eriani, K., Azhar, A., Ihdina, M., Rosadi, B., Rizal, M., Boediono, A., 2018. Quality enhancement of aceh swamp buffalo (Bubalus bubalis) frozen semen by supplementing β-carotene. Tropical Animal Science Journal 41, 1-7.

Eveson, D.P., 2016. The sperm chromatin structure assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Animal Reproduction Science 169, 56-75.

Fang, Q., Wang, J., Hao, Y.Y., Li, H., Hu, J.X., Yang, G.S., Hu, J.H., 2017. Effects of iodine methionine on boar sperm quality during liquid storage at 170C. Reprod. Domestic Animal Endocrinology 52, 1061–1066.

Fathi, M., Zaher, R., Ragab, D., Gamal, I., Mohamed, A., Abu-El Naga, E., Badr, M., 2019. Soybean lecithin-based extender improves Damascus goat sperm cryopreservation and fertilizing potential following artificial insemination. Asian Pacific Journal of Reproduction 8, 174-180.

Febrianti, K.I., Rahayu, S., Marhendra, A.P.W., Soewondo, A., 2014. Kadar MDA spermatozoa setelah proses pembekuan. Biotropika: Journal of Tropical Biology 2, 142-147.

Gallo, A., Esposito, M.C., Tosti, E., Boni, R., 2021. Sperm motility, oxidative status, and mitochondrial activity: exploring correlation in different species. Antioxidants 10, 1131.

Goncalves, F., Barretto, L.S.S., Arruda, R.P., Perri, S.H.V., Mingoti, G.Z., 2010. Effect of antioxidants during bovine in vitro fertilization procedures on spermatozoa and embryo development. Reproduction in Domestic Animals 45, 129–135.

Guthrie, H.D., Welch, G.R., 2012. Effects of reactive oxygen species on sperm function. Theriogenology 78, 1700-1708.

Herbowo, M.T., Arifiantini, R.I., Karja, N.W.K., Sianturi, R.G., 2019. Cryopreservation of Swamp Buffalo Semen in Skim Milk Yolk-based Diluent with Two Different Cryoprotectants. Tropical Animal Science Journal 42, 13-18.

Karunakaran, M., Devanathan, T.G., 2016. Evaluation of bull semen for fertility-associated protein, in vitro characters and fertility. Journal of Applied Animal Research 45, 136-144.

Kebede, A. 2018. Review on factors affecting success of artificial insemination. International Journal of Current Research and Academic Review 6, 42-49.

Kordan, W., Fraser, L., Wysocki, P., Strzezek, R., Lecewicz, M, Mogielnicka-Brzozowska, M., Dziekonska, A., Soliwoda, D., Kozioro wska-Gilun, M., 2013. Semen quality assessments and their significance in reproductive technology. Polish Journal of Veterinary Sciences 16, 823–833.

Kumaresan, A., Johannisson, A., Al-Essawe, E.M., Morrell, J.M., 2017. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can dis¬criminate between above-and below-average fertility bulls. Journal of Dairy Science 100, 5824-5836.

Kumaresan, A., Kadirvel, G., Bujarbarbaruah, K.M., Bardoloi, R.K., Das, A., Kumar, S., Naskar, S., 2009. Preservation of boar semen at 18 °C induced lipid perox¬idation and apoptosis-like changes in spermatozoa. Animal Reproduction Science 110, 162-171.

Lamb, G.C., Mercadante, V.R.G. 2016. Synchronization and artificial insemination strategies in beef cattle. Veterinary Clinics of North America Food Animal Practice 32, 335–334.

Layek, S.S., Mohanty, T.K., Kumaresan, A., Parks, J.E., 2016. Cryopreservation of bull semen: evolution from egg yolk based to soybean based extenders. Animal Reproduction Science 172, 1-9.

Len, J.S., Koh, W.S.D., Tan, S-X., 2019. The roles of reactive oxygen species and antioxidants in cryopreservation. Bioscience Reports 39, BSR20191601.

Lukman, H.Y., Busono, W., Wahjuningsih, S., Suyadi, S., 2014. Sperm motility and viability after α-tocopherol dilution in Tris aminomethane-base extender during cold storage in Bali bull. International Journal of ChemTech Research 6, 5726-5732

Maesaroh, K., Kurnia, D., Al Anshori, J., 2018. Perbandingan metode uji aktivitas antioksidan DPPH, FRAP dan FIC terhadap asam askorbat, asam galat dan kuersetin. Chim. Nat. Acta 6, 93-100.

Malan, S.W., 2000. The improved Boer goat. Small Ruminant Research 36, 165–170.

Maslukhah, Y.L., Widyaningsih, T.D., Waziiroh, E., Wijayanti, N., Sriherfyna, F.H., 2016. Faktor pengaruh ekstraksi *cincau* hitam (Mesona Palustris Bl) skala pilot plant: kajian pustaka. Jurnal Pangan dan Agroindustri 4, 245-252.

Mekonnen, T., Bekana, M., Abayneh, T., 2010. Reproductive performance and efficiency of artificial insemination smallholder dairy cows/heifers in and around Arsi-Negelle, Ethiopia. Livestock Research for Rural Development 22, 1-5.

Morte, M.I., Rodrigues, A.M., Soares, D., Rodrigues, A.S., Gamboa, S., Ramalho-Santos, J., 2008. The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: seasonal distinctions and correlations with DNA strand break. Animal Reproduction Science 106, 36–47.

Olivera-Muzante, J., Fierro, S., Gil, J., 2011. Conception rates in ewes after AI with ram semen preserved in milk-egg yolk extenders supplemented with glycerol. Reproduction in Domestic Animals 46, 508–512.

Pardede, B.P., Agil, M., Yudi, Y., Supriatna, I., 2020. Relationship of frozen-thawed semen quality with the fertility rate after being distributed in the Brahman Cross Breeding Program. Vet. World 13, 2649-2657.

Paul, R.K., Balaganur, K., Bahire, S.V., Kumar, D., Singh, R., 2018. Supplementation of cauda epididymal plasma improves sperm characteristics following liquid preservation of ram semen at 3–5 °C. Reproduction, Fertility and Development 30, 1389–1401.

Puspitasari, A.D., Wulandari, R.L., 2017. Antioxidant activity, determination of total phenolic and flavonoid content of Muntingia calabura L. Extracts. Pharmaciana 7, 147-158.

Ramu, S., Jeyendran, R.S., 2013. The hypo-osmotic swelling test for the evaluation of sperm membrane integ¬rity. Methods in Molecular Biology 927, 21-25.

Rather, H.A., Islam, R., Malik, A.A., Lone, F.A., 2016. Addition of antiox-

idants improves quality of ram spermatozoa during preservation at 4 ^{0}C. Small Ruminant Research 141, 24-28.

Ren, F., Fang, Q., Feng, T.Y., Li, Y., Wang, Y.H., Zhu, H.J., Hu, J.H., 2019. Lycium barbarum and Laminaria japonica polysaccharides improve Cashmere goat sperm quality and fertility rate after cryopreservation. Theriogenolog 129, 29–36.

Rodriguez, A.L., Soom, A.V., Arsenakis, I., Maes, D., 2017. Boar management and semen handling factors affect the quality of boar extended semen. Porcine Health Management 3, 1-12.

Sanocka, D., Kurpisz, M., 2004. Reactive oxygen species and sperm cells. Reproductive Biology and Endocrinology 2, 12-19.

Sariözkan, S., Bucak, M.N., Tuncer, P.B., Buyukleblebici, S., Canturk, F. 2014. Influence of various antioxidants added to TCM-199 on post-thaw bovine sperm parameters, DNA integrity and fertilizing ability. Cryobiology 68, 129-133.

Sitali, M.C., Mwanza, A.M., Mwaanga, E.S., Parsons, I.R., Parsons, N.J. 2017. Sperm morphology and sperm quality of bulls raised on commercial farms in Zambia. International Journal of Advanced Biological and Biomedical Research 7, 27-33.

Sitepu, S.A., Marisa, J., 2018. Utilization of frozen goat semen with addition sweet orange essential oil to improve genetic quality in Ujung Teran Village. Journal of Saintech Transfer 1, 170-174.

Susilowati, S., Triana, I.N., Wurlina, W., Arimbi, A., Srianto, P., Mustofa, I., 2019. Addition of L-arginine in skim milk extender maintains goat spermatozoa quality in chilled temperature for five days. Veterinary World 12, 1784-1789.

Syarifuddin, N.A., Toleng, A.L., Rahardja, D.P., Ismartoyo, Yusuf, M., 2017. Improving libido and sperm quality of bali bulls by supplementation of Moringa oleifera leaves. Media Peternakan 40, 88-93.

Takeshima, T., Usui, K., Mori, K., Asai, T., Yasuda, K., Kuroda, S., Yumura, Y., 2021. Oxidative stress and male infertility. Reproductive Medicine and Biology 20, 41-52.

Tarnajaya, K., Pangkahila, A., Pangkahila, W., Siswanto, F.M., 2018. Pemberian ekstrak daun Cincau (*Mesona Palustris* Bl) meningkatkan kadar superoksida dismutase (SOD) tikus wistar (*Rattus norvegicus*) jantan yang diinduksi latihan fisik berlebih. Jurnal Biomedik 10, 9-15.

Tatone, C., Emidio, G.D., Vento, M., Ciriminna, R., Artini, P.G., 2010. Cryopreservation and oxidative stress in reproductive cells. Gynecol. Endocrinology 26, 563-567.

Techinamuti, N., Pratiwi, R., 2018. Review: Metode analisis kadar vitamin C. Farmaka 16, 309-315.

Waberski, D., 2018. Artificial insemination in domestic and wild animal species. In: Niemann, H. and Wrenzycki, C (Eds). Animal Biotechnology, Springer, Cham, Berlin, Germany.

Wahjuningsih, S., Ciptadi, G., Ihsan, M.N., Isnaini, N., Rahayu, S., 2019. Supplementation of *Moringa oleifera* leaves' extract in Tris-egg yolk extender on the quality and fertility of cryopreserved Senduro goat sperm. Livestock Research for Rural Development 31, 1-8

Wen, F., Li, Y., Feng, T., Du, Y., Ren, F., Zhang, L., Ma, S., Li, F., Wang, P., Hu, J., 2019. Grape seed procyanidin extract (gspe) improves goat sperm quality when preserved at 4 °C. Animals 9, 810.