Research Article

Protection of Myrmecodia Extract in Tris Diluent on PO Bull Sperm Quality During Freezing

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Abstract | The cooling process causes oxidative stress and affects sperm motility. Antioxidants in the diluent can maintain the motility of bull sperm during the cooling process until after thawing. This research examines the effect of Myrmecodia extract (MYE) supplementation, which has high antioxidants, on semen diluent during the cooling to cryopreservation process. Specifically, five PO bulls aged four years were collected using an artificial vagina (AV) twice weekly at the Semangga District Animal Health Center from August to September 2023. Semen diluted to a minimum of 25 million/mL is packaged in 0.25 mL straws. Treat Andromed® as a comparison, P0 tris egg yolk (TYE), P1 TYE+MYE 1 mL, P2 TYE+MYE 2 mL. The parameters observed include fresh semen quality, sperm motility, sperm viability, sperm membrane integrity, and recovery rate. The results showed that MYE 2 mL supplementation ($P<0.05$) affected the quality of sperm motility during equilibration and post-thawing compared to P0 and P1. MYE 2mL supplementation in tris diluent has almost the same capabilities as Andromed ® commercial diluent. The ability to maintain cell membrane integrity is indicated by the RR value reaching 77.99%. In conclusion, Supplementation of myrmecodia extract in 2 mL of egg yolk Tris diluent affected sperm protection during the equilibration and cryopreservation processes, following Indonesian National Standard 4869-1:2021 frozen bovine semen.

Keywords | Cryopreservation, Myrmecodia extract, Tris egg yolk, PO bull, Tannin, Protection

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INTRODUCTION

Smallholder farms widely use artificial insemination (AI) techniques in cattle to increase their productivity. Research by Nurcholis *et al.* (2019) AI impacts increasing cattle productivity. The AI process is generally influenced by several factors, i.e., estrus detection, inseminator capability and semen quality. Before AI was implemented, making frozen semen until thawing was an important condition for the success of AI. The process of making frozen semen has several stages that must be prepared, namely material preparation, dilution process, equilibration and freezing. During the equilibration process, sperm will experience oxidative stress caused by cold stress. Bull sperm contains unsaturated fatty acids because cattle are mammals. According to Sanocka and Kurpisz (2004), the plasma membrane of mammalian spermatozoa contains many lipids, phospholipids, and PUFA, which are susceptible to lipid peroxidation when interacting with reactive oxygen species (ROS). High ROS levels can cause damage to sperm membranes and structures (Aitken, 2017). In the equilibration process, sperm will lose balance, which increases ROS. In addition, the freezing process can cause the formation of ice crystals (Nurcholis *et al.*, 2016) and

cause damage to the sperm plasma membrane. Sperm, under normal conditions, antioxidant defense mechanisms can fight the formation of ROS to reduce the negative effects they cause (Ighodaro and Akinloye, 2017).

Antioxidant supplementation in semen diluents is widely used as a low-cost and natural alternative to maintain sperm quality. According to Ros Santaella and Pintus (2021), using natural plant extracts as additional diluents can protect sperm during cooling. Several extracts have high antioxidant content, such as green tea extract (Prastiya *et al*., 2023), soybean extract (Ratnawati *et al*., 2023), Lycopene (Bintara *et al*., 2023), red fruit oil (Nurcholis *et al*., 2021), Eurycoma longifolia extract (Baiee *et al*., 2018). Myrmecodia contains secondary metabolite compounds in the form of flavonoids and tannins which can inhibit dangerous cell proliferation through its antioxidant activity (Imaniar *et al*., 2022). Myrmecodia has a high flavonoid content of 119.52 mg/g (Nofiyanti *et al*., 2019). Flavonoids are natural antioxidants that can act as reducers of hydroxyl radicals, superoxides and peroxyl radicals. In addition, Myrmocodia contains 313 ppm of tocopherol, which reduces 96% of free radicals at a concentration of 12 ppm (Subroto and Suprapto, 2006).

Based on the potential of myrmecodia which contains flavonoids, antioxidants and tannins, researchers suspect that myrmecodia extract supplemented in a diluent can maintain the PO of bovine sperm during the cooling and freezing process, like other plant extracts that contain tannins. Therefore, this study aims to determine the potential of myrmecodia extract supplemented with diluent on the quality of post-thawing sperm in PO cattle.

MATERIALS AND METHODS

Animals

Five PO bulls for four years were reared intensively by breeders and in individual cages kept in 2.7 × 2. m. Grass and bran feed is given to PO bull in the morning and evening. PO bull semen collection is carried out in the morning 06.30 – 07.00 am at the Semangga District Animal Health Center. Cattle semen was collected twice weekly using an artificial vagina (AV) of August to September 2023. The artificial vagina (AV) used during collection has a temperature of 50-55 °C. The body temperature of PO cattle is measured using an infrared thermometer, before semen collection. The research locations are in several places, such as the animal health center, Semangga District, Agricultural Quarantine, and Universitas Musamus. Processing of cattle semen refers to the Indonesian national standard SNI: 4869-1:2021 (BSN, 2021) for frozen cattle semen.

Semen evaluation

Currently semen evaluation is the only method that is effective and easy to apply to detect the fertility of bulls. The semen assessment process is macroscopic, including volume, consistency, color and pH (6.4-8 scale using paper indicators). All microscopic evaluations used a binocular microscope (Olympus CX43, Japan) integrated with a monitor. This evaluation includes sperm mass movement, sperm motility, sperm viability, sperm concentration, sperm morphology, and sperm membrane integrity.

Observation of sperm motility (%) using a microscope with a magnification of 200x and 400x. Mix five drops of 0.9% NaCl with one drop of fresh semen. Motility value determination using 0-100% .

Observation of sperm viability (%) and sperm abnormalities (%) were observed before making preparations for Eosin Nigrosin (EN). They are dripping 4-5 EN to 1 drop of fresh semen, homogenizing, and preparing a smear. Validity was evaluated with 200x magnification, and live sperm did not absorb color, while dead sperm did absorb the color. The minimum count is 200 sperm cells. Evaluate abnormalities in sperm by dividing normal and abnormal sperm multiplied by 100%. The post-sexing evaluation process also uses this method.

The sperm concentration x (106) was observed by looking at five boxes on a Neubauer slide chamber (0.100 mm and 0.0025 mm, German brand). One box in the middle, two other containers at the top left and right, and two at the bottom left and right. Observation of sperm concentration under a microscope with a magnification of 100 x or 200 x. Calculation of the concentration is done by looking at the head of the sperm that is in the box, which is counted as 1, while the head of the sperm that is on the boundary is counted as ½. The formula for counting sperm is the number of boxes 5 x 5 visual fields x 5 dilution factor (1:200) x 10,000

Sperm membrane integrity (%) can be observed by hypoosmotic swelling (HOS) (Ramu and Jeyendran, 2013). 50 µL semen was added to 1000 µL HOS solution (1.351 g fructose and 0.735 g sodium citrate in 100 mL distilled water with an osmolarity of 150 m). The mixture was homogenized and then incubated at 37°C for 30 minutes in a water bath. The evaluation was conducted randomly in 10 fields of view to observe a minimum of 250 sperm. Calculations were performed using a phase-contrast binocular microscope with a magnification of 400 times. The intact sperm plasma membrane is marked with a coiled or inflated tail, while damaged sperm is marked with a straight tail. The percentage of sperm membrane integrity was calculated by comparing the number of reacting sperm (positive HOS) divided by the number of counted sperm x 100%.

Preparing diluent and semen processing

This study used tris egg yolk and several diluents Table 1. Sperm evolved to a final concentration of 50 million/mL. The diluted semen is stored in 0.25 mL straws with a minimum concentration of 25 million/mL per straw. The regulation of the Director General of Livestock, Department of Agriculture No. 12207/Hk.060/F/12/2007 After evaluating sperm motility > 70%, the process of mixing diluent and semen with a ratio of 9:1. Homogenize the mixture, equilibrate at 5°C and incubate at 38°C after 4, 12, and 24 hours (Salman *et al.*, 2023), to observe sperm motility, viability, and membrane Integrity. The next process is freezing the semen at a temperature of -130°C for 10 minutes and storing it at a temperature of -196°C for 72 hours. A post-thawing evaluation was conducted to see the recovery rate at 37°C for 30 seconds. During the observation process, the semen was stored in a water bath.

Table 1: Egg yolk Tris buffer composition supplemented with Myrmecodia extract.

Composition	Andormed ®	P0	P1	P2
Buffer tris (g)	-	1.5	1.5	1.5
Egg yolk (%)	-	20	20	20
Glycerol (%)	-	6	6	6
Streptomycin (g/mL)	-	0.1	0.1	0.1
Penicillin (mg)	-	100.00	100.00	100.00
MYE (mL)	-	-	1	2
Distilled water (mL)	80	75	75	75
Andromed ® (mL)	20	-	-	-

Data analysis

Analysis of normality and variance is used to determine homogeneity with the Shapiro-Wilk test. A two-way analysis of variance with Duncan's multiple range test was performed if P<0.05 (SPSS version 22.0).

RESULTS AND DISCUSSION

Fresh semen quality of PO cattle

The study's findings showed that the quality of fresh semen of PO cattle in general can be seen in Table 2. The average fresh semen volume of five PO cattle was 4.7 ml to 5.4 ml, pH between 5.8 to 6.5, thick and medium consistency. Microscopic observations showed sperm mass movement (+++), sperm motility between 78.5% to 85%, sperm viability between 82.5 to 92.0%, sperm concentration 958 x (106) to 1027x(106)/mL, Normal sperm is 94.8 to 96.6%, and abnormal sperm is between 3.4 to 5.2%.

The fresh semen volume of PO cattle is an average of 4.7 ml – 5.4 ml. Nurcholis *et al.* (2021) volume semen sapi PO pada umur 3 tahun rata-rata 4.8 ml. Penelitian lain voleme semen sapi PO mencapai 6.1 ml (Zamuna *et al.*, 2016). This difference can occur due to differences in the type of livestock, feed, environment, and genetics. According to Bhave *et al.* (2020), Genetics and the environment affect the production and quality of cattle semen. The pH value of fresh semen is within normal limits, namely 5.8 – 6.5. The pH of fresh semen for cattle generally is 6.4 – 6.6 (Prastiya *et al.*, 2023). Semen pH is usually correlated with sperm motility, reinforced by Dhumal *et al.* (2021) that semen pH is closely related to sperm motility. The motility and viability of fresh semen sperm typically have normal values above 70% and 80% (Nurcholis *et al.*, 2021). The quality of fresh semen in this study complies with Indonesian national standards (SNI 4869-1:2021). In general, fresh semen from PO cattle is of suitable quality for further processing from dilution to freezing. Indicators that fresh semen can be frozen must have sperm motility >70%, sperm viability >75%, and abnormalities <20% (Iskandar *et al.*, 2022; Nurcholis *et al.*, 2021).

Table 2: Characteristics of fresh semen from PO bull.

Characteristics	PO1	PO2	PO3	PO4	PO5
Macroscopic					
Volume(ml)	5.4±0.69	4.7±0.35	5.0±0.55	5.2±0.50	5.4±0.69
pH	6.5±0.15	5.8±0.18	6.3±0.10	6.5±0.15	6.5±0.15
Color	Cream	Cream	Cream	Cream	Cream
Consistency	Moderate	Moderate	Moderate	Moderate	Moderate
Microscopy					
Movement of sperm	+++	+++	+++	+++	+++
Total sperm motility (%)	82.5±1.65	78.5±1.05	80.1±1.20	85.0±1.45	81.0±1.10
Sperm viability (%)	87.6±0.95	83.7±1.00	82.5±0.85	92.0±1.05	87.0±1.80
Sperm concentration x (10^6)	1027±80.95	958±78.50	995±90.00	1010±90.00	1022±82.10
Morphology					
Normal sperm (%)	96.4±0.97	94.8±0.70	95.0±0.82	95.2±0.25	96.6±0.30
Abnormal sperm (%)	3.6±0.15	5.2±0.31	5.0±0.27	4.8±0.70	3.4±0.65

Table 3: Effect of MYE on maintaining sperm after equilibration (Mean ±SE).

Tr	Motility (%)			Viability (%)			Membrane Integrity (%)		
	4h	12h	24h	4h	12h	24h	4h	12h	24h
Adr	63.0±1.00[a]	66.0±1.05[a]	75.0±0.05[a]	69.0±2.05[a]	75.5±1.02[a]	83.5±1.10[a]	56.5±0.05[a]	61.5±1.05[a]	67.0±1.00[a]
P0	62.5±2.15[a]	63.2±1.10[b]	70.8±1.20[b]	67.2±2.05[a]	72.5±1.45[b]	80.8±1.15[b]	52.8±1.15[b]	57.5±2.35[b]	62.2±2.22[b]
P1	63.8±2.07[a]	64.5±1.85[b]	71.4±1.05[b]	68.5±2.00[a]	73.0±1.25[b]	81.5±1.10[b]	53.0±1.00[b]	58.3±2.20[b]	63.0±2.28[b]
P2	63.7±2.15[a]	66.5±2.60[ac]	75.4±1.05[ac]	69.4±2.25[a]	76.0±2.65[ac]	84.0±1.05[ac]	56.0±1.05[ac]	61.0±2.75[ac]	66.8±2.95[ac]

Note: Tr (Treatment), Adr (Andromed ®), P0 (TYE without MYE), P1 (TYE + MYE 1 mL), P2 (TYE + MYE 2 mL), Different lowercase letters (a,b,c) on the same colomn ($P < 0.05$).

MYE ON MAINTAINING SPERM AFTER EQUILIBRATION

The research results are in Table 3. Treatment P2 ($P<0.05$) on sperm motility after equilibration for 24 hours (75.4 ± 1.05) was higher compared to P0 and P1. However, P2 has the same sperm motility percentage ($P>0.05$) as Andromed®. Sperm viability in treatment P2 showed an influence ($P<0.05$) from treatments P0 and P1. However, there was no difference with Andromed® comparator diluent. The best balance is based on sperm motility and sperm viability within 24 hours. The integrity of PO cattle sperm cell membranes after thawing showed the highest reaction to exposure to HOS solution in Andromed®, followed by P2, P1 and P0.

The relationship between diluent and equilibration time can be explained that the longer the equilibration time (24h) will increase the motility of P2 sperm, which is 75.4% compared to 4h and 12h. Another finding was that 24-hour equilibration resulted in motility of 53.8% (Salman *et al*., 2023). However, according to Fleisch *et al*. (2017), the 2h equilibration time gave the best results, namely 85.1%, compared to 24h, i.e., 83.5%. Reinforced by Nurcholis *et al*. (2021) 2h and 6h equilibration times, the percentage of sperm motility was 72.15% and 60.00%. This difference is understandable because the diluent and the equilibration temperature differ. The results of this study explain that balance time is very important in adapting the plasma membrane of sperm cells to new conditions. However, until now, there has been no agreement to determine the exact draw time. The difference in equilibration time only has a small impact on sperm quality after thawing but does not reduce the fertilization rate. Factors that influence the decline in sperm quality are cell membrane imbalances caused by phospholipid balance and imbalance processes. Shan *et al*. (2021) stated that male reproductive function has been proven depends on the homeostatic stability of sperm lipids. Antioxidants and egg yolk play an important role in this process. Egg yolk contains phospholipids, which play a role in protecting sperm when cooling and freezing. In addition, antioxidants can protect sperm from the toxic effects caused during this process (Zhang *et al*., 2012). Sun *et al*. (2019) stated that phospholipids can be a reservoir of cholesterol and help protect sperm cell membranes and the acrosome from cryogenic damage. If the sperm freezing process is correct, it can be maintained for over ten years. Semen stored for up to 25 years can still be used for AI (Bahmaid *et al*., 2023).

The viability of sperm diluted using MYE gave a difference ($P<0.05$), which was possible because of the levels of antioxidants and tannin content in MYE. Antioxidants play an important role in counteracting lipid peroxidation by maintaining the sperm cell membrane during equilibration. Green tea extract (GTE), which contains antioxidants, affects the motility and viability level before freezing (Prastiya *et al*., 2023). In addition, MYE contains flavonoids and tocopherol, which protect cell membranes.

The integrity of the cell membrane can indicate that the sperm is in an active state, which is indicated by the reaction of the sperm tail becoming coiled. The integrity of the sperm membrane can be maintained with the help of TYE and the antioxidants contained in MYE yaitu flavonoid and tocopherol. Tocopherol is a fat-soluble antioxidant localized in cell membranes and functions as a coolant for lipid peroxidation due to ROS (de Lamirande and Gagnon, 1992). In addition, the tannin content in myrmecodia is thought to increase the integrity of intact plasma membranes. According to Fitiyah *et al*. (2017), 5% tannin can increase sperm motility and can effect maintaining sperm motility with storage 14 days Bali bull. The amount of tannin given to P1 was not sufficient to help protect sperm, so the P0 and P1 treatments tended to have the same results.

PTM SPERMA DAN RECOVERY RATE

In Table 4, post-thawing sperm motility (PTM) using 2 mL extra myrmecodia influences sperm cell protection during freezing and storage. However, P2 treatment ($P<0.05$) compared to P1 and P0 was not different from the Andromed® comparison but tended to be higher than Andromed®. This is suspected because MYE contains tocopherol, flavonoids and tannin. Research on Entada abyssinica extract containing tannin can increase post-thawing progressive motility and maintain plasma membrane integrity (Sobeh *et al*., 2020). Adding 1.5mM tocopherol maintained increased NCDs and sperm viability (Ratnani *et al*., 2020). Apart from that, all

Table 4: Post thawing motility sperm PO bull.

Tr	Total motility (%)	Sperm viability (%)	Membrane integrity (%)	Abnormality (%)
Adr	63.0±1.00[a]	69.0±1.00[a]	65.0±0.05[a]	6.0±1.00[a]
P0	55.5±1.15[b]	61.0±1.50[b]	57.8±1.20[b]	7.0±1.15[a]
P1	56.0±1.00[b]	62.5±1.25[b]	58.4±1.05[b]	6.0±1.15[a]
P2	63.5±0.10[ac]	69.0±1.15[ac]	65.4±1.05[ac]	6.5±1.00[a]

Note: Tr (Treatment), Adr (Andromed ®), P0 (TYE without MYE), P1 (TYE + MYE 1 mL), P2 (TYE + MYE 2 mL), Different lowercase letters (a, b, c) on the same colomn ($P < 0.05$).

Table 5: PO bulls sperm motility recovery rate value.

Tr	Motility sperm (%)		
	Fresh semen	Post thawing	RR
Adr	81.42±1.25	63.0±1.00	77.37±1.95[a]
P0		55.5±1.15	68.16±1.60[b]
P1		56.0±1.00	68.77±1.50[b]
P2		63.5±0.10	77.99±1.85[ac]

Note: Tr (Treatment), Adr (Andromed ®), P0 (TYE without MYE), P1 (TYE + MYE 1 mL), P2 (TYE + MYE 2 mL), Different lowercase letters (a,b,c) on the same colomn ($P < 0.05$).

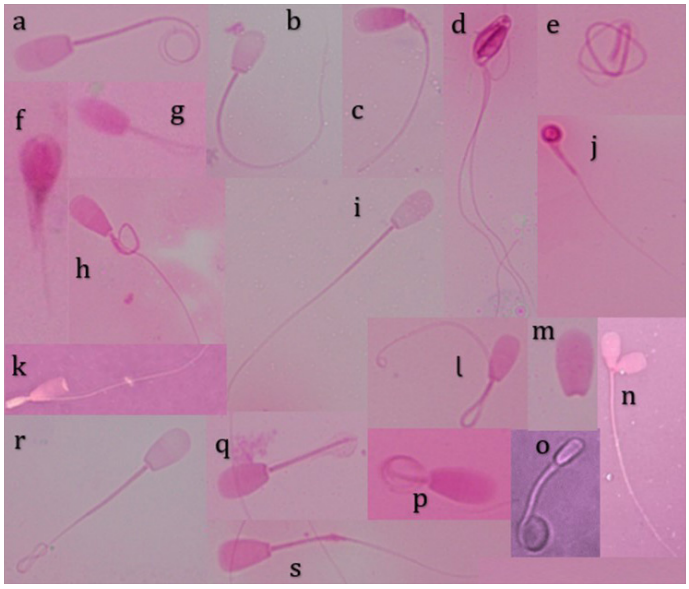

Figure 1: Morphology and sperm membrane reactions: (a) abnormal tail turning, (b) abnormal midpiece upwards, (c) broken neck, (d) abnormal free cell membrane on the head, small head and two tails, (e) no head, (f) pyriform head, (g) tail rotating to the head, (h) coiled principal piece, (i) normal sperm, (j) small round sperm head, (k) abnormal acrosome, (l) distal midpiece reflexes, (m) head free neck, (n) double head, (o) sperm reaction to HOS, indicated by coiled tail, (p) severely coiled midpiece, (q) midpiece interrupted in the middle, (r) tail twisting abnormality at the tip, (s) tail proximal cytoplasmic droplet.

treatments had no effect ($P>0.05$) on sperm abnormalities in PO bulls. Seventeen sperm abnormalities in PO bulls could be identified (Figure 1). Further research needs to

be carried out regarding the use of tannin in myrmecodia extract on sperm quality. The ability to survive sperm after freezing can be seen from the sperm motility recovery rate (RR) value in Table 5. The results of this study show that the RR in treatment P2 and Andromed ® as a comparison is not different (P>0.05). Decreased sperm motility based on the RR value between 25.92% - 17.92%. The sperm motility ability to return to life after freezing reached 77.99%. Indicate protection of tannin-containing buffers from potential and active MYE during freezing and storage processes. The results of a study by Liman *et al.* (2022) stated that tannins and their derivatives have natural protective properties that have the potential to improve the viability of sperm cells after freezing. Standar semen beku yang disyaratkan oleh SNI 4869-1:2021, motilitas sperma PTM > 40%. The results of this research are in accordance with the standards applied in Indonesia. Further research regarding the use of MYE more than 2 ml needs to be carried out to determine its effect on PTM and RR values. This is because in this study the P2 treatment was the same as P0.

CONCLUSIONS AND RECOMMENDATIONS

Supplementation of myrmecodia extract in 2 mL of egg yolk Tris diluent affected sperm protection during the equilibration and cryopreservation processes. PTM evaluation of PO cattle sperm has a percentage by the recommendations of SNI 4869-1:2021 for frozen cattle semen.

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NOVELTY STATEMENT

Prime myrmecodia extract supplementation is high in antioxidants in the form of flavonoids and tocopherols used in diluting and freezing PO cattle semen, and this research found the best concentration for its use.

AUTHOR'S CONTRIBUTION

NN conceptualization, drafting of the original manuscript, and validation. SMS collecting data. AB revising grammatically, creating illustrations, and validation.

ETHICAL APPROVAL

Ethical feasibility of animals The Faculty of Veterinary Medicine, Universitas Gadjah Mada (UGM) approved this research through decision No.23/EC-FKH-2022.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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