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Research Article

EFFECT OF *MORINGA OLEIFERA* SEED EXTRACT SUPPLEMENTATION ON POST-THAW QUALITY OF SIMMENTAL BULL SEMEN DILUTED IN CEP EXTENDER

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Abstract
The success of Artificial Insemination (AI) programs in cattle largely depends on the quality of semen after freezing. This study aimed to evaluate the effect of supplementing cauda epididymis plasma (CEP) diluent by *Moringa oleifera* seed extract (MSE) on the post-thaw quality of Simmental bull semen. Semen samples were collected by bulls with individual motility ≥70% and processed at the Singosari Artificial Insemination Center between October and December 2024. The study employed a Completely Randomized Design (CRD) with four MSE concentrations: 0, 1, 5, and 10 mg/mL. Each test was replicated six times for motility, viability, and membrane integrity. Observations were conducted utilizing microscopy and evaluated statistically by ANOVA and Duncan's test. The results indicated that 5 mg/mL of MSE yielded the highest semen quality post-thaw, by motility of 43.33±3.73%, viability of 83.17±1.07%, and membrane integrity of 84.08±1.06% ($P<0.05$). However the other treatments showed lower semen quality post-thaw of motility, viability, and membrane integrity. These outcome demonstrate that MSE supplementation enhances the antioxidant addition of the CEP diluent, effectively reducing oxidative damage and preserving semen quality during cryopreservation. This suggests that *Moringa oleifera* seed extract represents a promising natural additive for improving the efficacy of frozen semen used in artificial insemination programs.
Keywords: antioxidant, semen, cryopreservation, extender, cep

INTRODUCTION

Indonesia's rising population has pushed up the demand for beef, but domestic production has yet to keep pace, leaving the country dependent on imports to fill the gap (Kusumaningrum et al., 2021). In 2024, Indonesia had over 18.6 million beef cattle (BPS, 2024), but this still fell short of market needs. Simmental cattle, a *Bos taurus* breed from Switzerland, are favored in Indonesia for their rapid growth and superior carcass traits (Hasnudi et al., 2019). Artificial insemination (AI) has become a central strategy for growing the Simmental population, enabling farmers to improve genetics from utilizing semen by high-quality, superior bulls. Artificial insemination also reduces inbreeding and lowers the risk of disease transmission (Baruselli et al., 2017). However, its effectiveness depends heavily on the quality of semen after it has been frozen and thawed. Before AI can be performed, the semen must be diluted in an appropriate medium and stored at low temperatures to maintain its quality.

To have semen quality after ejaculation, several preservation techniques have been done, all of which involve storing semen in an appropriate diluting medium. These preservation

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methods have kept sperm viable, and diluents comprising Cauda Epididymis Plasma (CEP) have been essential, providing nutrients, stabilizing pH, and maintaining osmotic balance (Ducha et al., 2020). According to Ducha et al. (2021), two common storage methods are refrigeration at 4–5 °C and freezing in liquid nitrogen at –196 °C. Storing semen at –196 °C is intended for long-term preservation; at this ultra-low temperature, spermatozoa can remain viable for more than 25 years, provided the freezing and storage procedures are performed correctly.

The cryopreservation process can still result in sperm cells damage, often due to ice crystal formation, osmotic stress, and oxidative damage (Ezzati et al., 2020). Excess reactive oxygen species (ROS) impair motility, viability, and membrane integrity (Ugur et al., 2019). The damaging effects of free radicals that occur during storage can be reduced by adding antioxidants to the diluent medium. These compounds protect sperm cells by preventing free radicals from damaging crucial components including lipids, proteins, and carbohydrates (Silvestre et al., 2021).

Moringa oleifera contains strong antioxidants comprising flavonoids and ascorbic acid (Arshad et al., 2025). Moringa seed extract (MSE), which contains a high level of flavonoids (around 47 mg/g), can have neutralize reactive oxygen species (ROS) and inhibit lipid peroxidation (Bupu et al., 2022; Carrera-Chávez et al., 2020). Most existing studies on *Moringa* seed extract have focused on goats or sheep; therefore, the present study utilized Simmental bull semen to extend and complement previous findings. This study examines how the addition of MSE to CEP diluents affects post-thaw motility, viability, and membrane integrity of Simmental bull semen, contributing to advancements in sustainable livestock biotechnology.

MATERIALS AND METHODS

Research Design

The number of bulls used in this experiment was 1 with 2 ejaculations, semen from the ejaculated bull was pooled into 6 replicates. The main manipulation involved varying the concentration of moringa seed extract (0 mg/mL, 1 mg/mL, 5 mg/mL, and 10 mg/mL) and 6 samples each treatments combined with a two-day frozen storage method. The samples were evaluated for spermatozoa motility, viability, and membrane integrity before freezing and after thawing.

Preparation of Moringa Seed Extract

The preparation of Moringa seed extract (MSE) starts by drying the seeds in an oven at 60°C for eight hours (Carrera-Chávez et al., 2020). The dried moringa seeds are ground into a powder and macerated using 96% ethanol as a solvent for two 18-hour periods (Ertürk et al., 2018) at ratios of 1:10 and 1:5 (Yasacaxena et al., 2023). After 36 hours of homogenizing Moringa seed powder with ethanol, the mixture is filtered to separate the solvent from the solid residue. The resulting filtrate is then evaporated using a rotary evaporator at 50°C to obtain a concentrated extract. The four concentrations 0, 1, 5, and 10 mg/mL of MSE are based on the previous study of Carrera-Chávez et al. (2020).

Preparation of CEP Dilution

The CEP diluent was prepared following Ducha (2018). Its per-liter composition have included: 15 mmol NaCl, 7.0 mmol KCl, 3.0 mmol $CaCl_2 \cdot 2H_2O$, 3.0 mmol $NaHCO_3$, 8.0 mmol $MgCl_2 \cdot 6H_2O$, 11.9 mmol $NaHCO_3$, 8.0 mmol NaH_2PO_4, 20.0 mmol KH_2PO_4, 55 mmol fructose, 1.0 g sorbitol, 2.0 g BSA, 133.7 mmol Tris, 1000 IU penicillin, 1 g streptomycin, and 42.6 mmol citric acid. All components were kept in sterile Otsuka water utilizing the aliquot technique, then sterilized by a 0.2 µm Millipore membrane. Afterward, 20% egg yolk was added and homogenized. The mixture was stored in a refrigerator for 2–4 days until it had formed into two layers, and the supernatant was collected for use as the diluent. All diluents were stored at 4–5 °C.

Freezing Process

Fresh semen by Simmental bulls was collected at BBIB Singosari, Malang, utilizing an artificial vagina (AV). Macroscopic evaluations included assessing the volume, color, pH, odor, and consistency, while microscopic evaluations included sperm concentration, mass and individual motility, morphological abnormalities, and membrane integrity.

According to Rahayu and Ducha (2022), the dilution process consist of three stages: A1, A2, and B. The first stage, A1, is to have out after quality control by mixing fresh semen that has the required standards with the diluent. In this step, the diluent is added in the same volume as the fresh semen (1:1). The A2 dilution is then prepared by having the remaining amount of diluent needed, calculated utilizing the following formula:

$$V.A2 = V.total - (V.semen + V.A1 + V.B)$$

The diluted semen is then stored in a refrigerator at 4–5 °C for about 18 hours. After this cooling period, dilution B is carried out by having the final volume of diluent calculated as follows:

$$\text{V.B} = \frac{V.A1 \times sperm\ concentration \times V.semen}{2}$$

The pre-freezing stage gradually spans a temperature range from 5 °C to –140 °C over a 7-minute period using a DigitCool machine. After this step, the semen is fully frozen in liquid nitrogen at –196 °C.

Sperm Motility Observation

Sperm motility was assessed by placing 10 µL of semen on a glass slide and observing the movement patterns under a microscope at 100× magnification (Rosnizar et al., 2021). Individual sperm motility data were based on the progressive movement of the spermatozoa (Setiadi et al., 2019). The requirement for good sperm motility percentage was ≥ 40% (SNI, 2024).

Sperm Viability Observation

Eosin–nigrosin staining was used on semen samples placed on a glass slide to assess sperm viability. The prepared smears were examined under a microscope at 100× magnification. Viability was determined by calculating the percentage of live spermatozoa, with unstained heads, relative to the total number observed (Malinda et al., 2021).

Sperm Membrane Integrity Observation

Membrane integrity was assessed utilizing the hypoosmotic swelling test. In this procedure, 100 µL of semen was incubated with 1 mL of a 125 mOsm/L hypoosmotic solution at 37 °C for 30 minutes. A 0.2 mL portion of the mixture was then smeared onto a microscope slide, and 200 spermatozoa were examined under a ×400 microscope. The percentage of membrane integrity was based on the proportion of spermatozoa showing characteristic swelling compared to the total number observed (Fuadatin & Hariani, 2024).

Statistic Analysis

The data were first tested for normality using the Kolmogorov–Smirnov test. An ANOVA was then performed to assess the effect of Moringa seed extract dosage on spermatozoa viability, motility, and membrane integrity, followed by Duncan's test to examine homogenity. The statistical software used in this study was IBM SPSS Statistics 25 with a significance level (α=0.05). In this study, variation in the concentration of moringa seed extract is the main manipulation factor and spermatozoa quality evaluations are the response variable.

RESULTS AND DISCUSSION

Fresh Semen Quality Evaluation

The fresh semen collected by Simmental bulls showed good quality before freezing (Table 1). The ejaculate had a milky white color, dense consistency and an average volume of 11.8 ± 1.7 mL, which falls within the normal range for this breed. The sperm concentration reached 1,670 × 10^6 cells/mL, a value higher than those reported in some earlier studies. Differences among individual bulls are likely due to several factors, including collection frequency and nutritional status, which can affect accessory gland secretions and mantain pH stability (Komariah et al., 2020).

Motility averaged 73.2 ± 1.8% by abnormalities ≤ 20%, which meets the (SNI, 2024) standards for AI-quality semen. Progressive motility has the sperm's ability to move forward efficiently—an essential fertility indicator that is affected by genetics, management practices, and environmental conditions. Viability (95.75 ± 0.3%) and membrane integrity (91.5 ± 0.7%) were also high, confirming that the semen was of good quality and suitable for the freezing process (Mohanty et al., 2018).

Sperm Motility Evaluation

Adding Moringa Seed Extract (MSE) to have the CEP diluent had a significant effect on sperm motility both before freezing and after thawing ($P < 0.05$) (Table 2). Before freezing, the highest motility was observed at the 1 mg/mL dose (55.00 ± 6.45%), whereas after thawing, the best outcome was achieved by 5 mg/mL (43.33 ± 3.73%). Despite a general have after thawing, post-thaw motility at 5 mg/mL remained above 40%, meeting (SNI, 2024) standards.

The decrease in motility after freezing is largely due to stress and membrane damage caused by reactive oxygen species (ROS). The phenolic and flavonoid compounds in MSE likely have counteracted these effects by acting as antioxidants, neutralizing ROS, and reducing lipid peroxidation (Castaldo et al., 2019; Olszowy, 2019). These outcomes are consistent with previous studies showing that a 5 mg/mL dose of MSE maintains sperm motility more effectively than either higher or lower concentrations.

Sperm Viability Evaluation

Moringa Seed Extract (MSE) supplementation also had a significant effect on sperm viability ($P < 0.05$) (Table 3). Before freezing, the 1 mg/mL dose had slightly higher viability (88.42 ± 1.06%), but after thawing, the best outcome was observed at 5 mg/mL (83.17 ± 1.07%). Microscopic observations (Figure 1) have a greater number of live spermatozoa—identified by their unstained heads—in the 5 mg/mL treatment group.

The improved viability with MSE supplementation is likely due to its antioxidant properties, which reduce ROS buildup and stabilize the sperm plasma membrane. The flavonoids and phenolics in MSE act as free-radical scavengers, protecting sperm cells from oxidative damage (Iqbal et al., 2022). Thus, adding 5 mg/mL of MSE to the CEP diluent appears to have been the most effective dose for preserving sperm vitality after cryopreservation.

Sperm Membrane Integrity Evaluation

Membrane integrity has a similar trend (Figure 2). The 5 mg/mL treatment has the highest integrity both before has (88.42 ± 1.57%) and after thawing (84.08 ± 1.06%), whereas the 10 mg/mL dose resulted in the lowest values. Microscopic observations (Figure 2) indicated that spermatozoa with curved tails, reflecting intact membrane integrity, were most prevalent in the 5 mg/mL treatment group.

The sperm plasma membrane is highly vulnerable to oxidative stress because it contains large amounts of polyunsaturated fatty acids (PUFAs). The antioxidant components of MSE likely mitigated ROS-induced damage to membrane lipids and mitochondria, thereby reducing cold-shock effects during cryopreservation (Albertini et al., 2022). Mantaining membrane integrity is vital for sperm function and fertilizing ability (Bezerra et al., 2023).

Efforts to improve the effectiveness of the Artificial Insemination (AI) program require maintaining the quality of the spermatozoa used. In this study, spermatozoa were preserved using a CEP diluent and subsequently subjected to a freezing process. Fructose in the CEP diluent serves as a primary energy source for spermatozoa during the cooling phase (Bustani and Baiee, 2021). However, exposure to ultra-low temperatures (−196 °C) during cryopreservation can induce cold shock, leading to structural and functional damage to spermatozoa. To reduce these effects, 13% glycerol is incorporated into the CEP diluent prior to freezing, where it functions as an intracellular cryoprotectant. Glycerol helps maintain cellular water balance by modulating ice crystal formation and reducing membrane damage (Rozi and Ducha, 2020).

The bioactive compounds in Moringa Seed Extract (MSE) trigger various protective mechanisms that support the maintenance of essential cellular functions and preserve membrane integrity during cryopreservation (Khalil et al., 2020). In addition, spermatozoa treated with MSE exhibit enhanced mitochondrial membrane potential, underscoring its role in reducing apoptotic events during freezing (Kumar et al., 2023; Mohlala et al., 2023). In addition, natural enzymatic antioxidants comprising glutathione peroxidase and glutathione reductase work alongside MSE, creating a synergistic defense against oxidative stress during the freezing and thawing processes (Adeoye et al., 2018; Matsuzaki & Sasanami, 2017).

Moringa Seed Extract (MSE) significantly affect post-thaw sperm motility, viability, and membrane integrity, with 5 mg/mL emerging as the most effective concentration. These outcomes reinforce the notion that oxidative stress is a key factor in compromising semen quality during cryopreservation, as excessive ROS can damage the lipids and proteins that form sperm membranes. The antioxidant components in MSE comprising flavonoids, phenolics, vitamins C and E, β-carotene, and trace minerals like zinc and selenium help neutralize ROS and stabilize membrane lipids, thereby reducing cryo-induced injury (Jayawardana et al., 2015). Several studies indicate that supra-optimal doses may diminish efficacy or even cause adverse effects, suggesting that higher concentrations do not necessarily yield better outcomes. For instance, Wahjuningsih et al. (2019) reported that a 7% MO concentration in goat semen led to poorer post-thaw sperm quality compared with a 5% concentration, possibly due to pro-oxidant activity or the presence of antinutritional compounds such as tannins.

In the present study, a moderate concentration of *M. oleifera* seed extract (5 mg/mL) was found to preserve sperm motility, viability, and membrane integrity, likely due to its antioxidant activity. However a study involving cryopreserved ram sperm used for fertilization of bovine oocytes showed that a moderate concentration of MO seed extract (10 mg/mL) sustained a high cleavage rate of approximately 83%, whereas higher concentrations did not provide additional benefits, indicating the presence of an optimal antioxidant dose (Guedea-Betancourt et al., 2022).

Moreover, the fertility assessment of these compounds and their interactions with conventional semen extenders for Simmental bull semen remain insufficiently understood. Variations in the measure of any cryopreservation supplement's effect on fertility and reproductive outcomes substantially influence antioxidant effectiveness and sperm protection.

Table 1. Quality evaluation of fresh Simmental semen

Macroscopies parameter	Averages and standard deviation
Color	White milk
pH	6.4±0.28
Consistency	Dense
Volume (mL)	11,8±1.7
Concentration	1670.0±160.0
Individual Motility	73.2±1.8
Abnormality	17.05±1.9
Viability	95.75±0.3
Membrane Integrity	91.5±0.7

Table 2. Motility of Simmental spermatozoa

Doses (mg/mL)	Motility Percentage Average (%) ± Deviation Standard		Reduction (%)
	Before Freezing	Post-Thawing	
0	55.83±5.34[a]	34.17±3.44[a]	21.67
1	55.00±6.45[b]	40.00±4.08[b]	15.00
5	54.17±6.72[b]	43.33±3.73[b]	10.83
10	55.00±6.45[a]	29.17±3.44[a]	25.83

Table 3. Viability of Simmental spermatozoa

Doses (mg/mL)	Viability Percentage Average (%) ± Deviation Standard		Reduction (%)
	Before Freezing	Post-Thawing	
0	87.58±1.10[ab]	74.29±1.89[a]	12.67
1	88.42±1.06[b]	80.75±0.95[b]	7.67
5	88.25±1.22[b]	83.17±1.07[c]	5.08
10	87.67±1.31[a]	71.50±1.04[a]	16.17

Table 4. Membrane integrity of Simmental spermatozoa

Doses (mg/mL)	Membrane Integrity Percentage Average (%) ± Deviation Standard		Reduction (%)
	Before Freezing	Post-Thawing	
0	83.42±1.13[ab]	71.58±2.15[a]	11.83
1	87.75±1.38[ab]	79.92±1.06[b]	7.83
5	88.42±1.57[b]	84.08±1.06[b]	4.33
10	83.17±0.85[a]	68.75±1.70[a]	14.42

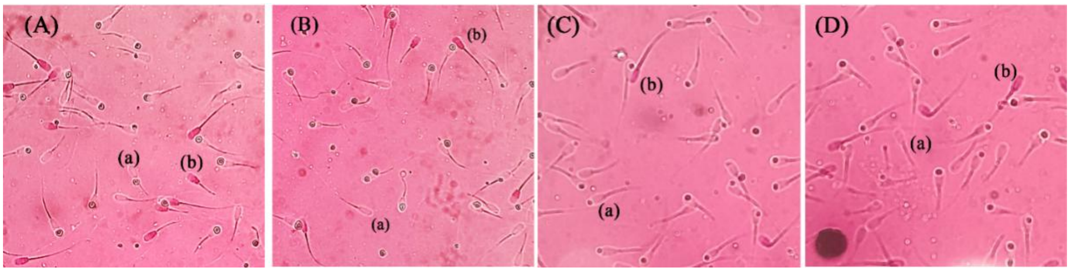

Figure 1. Sperm viability of Simmental cattle post-thawing (400×): (A) 0 mg/mL; (B) 1 mg/mL; (C) 5 mg/mL; (D) 10 mg/mL. Transparent heads = live sperm; red = dead sperm

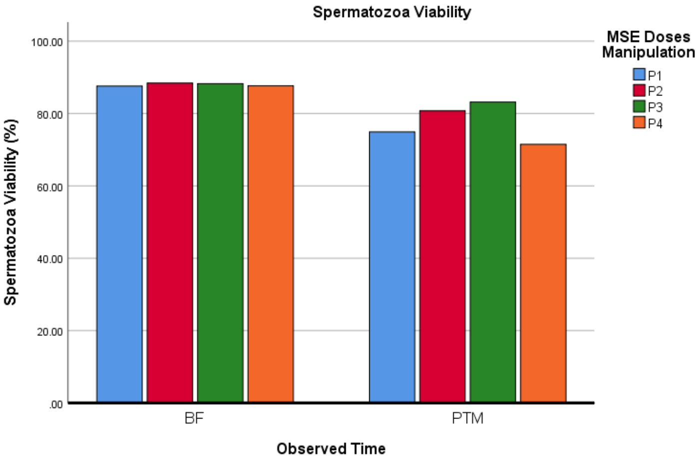

Figure 2. Scale bar of sperm viability percentage

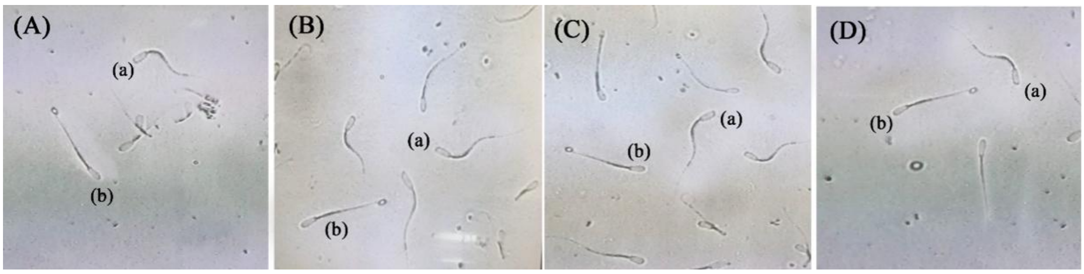

Figure 3. Sperm membrane integrity of Simmental cattle post-thawing (400×): (A) 0 mg/mL; (B) 1 mg/mL; (C) 5 mg/mL; (D) 10 mg/mL Curved tails = intact membranes; straight = damaged

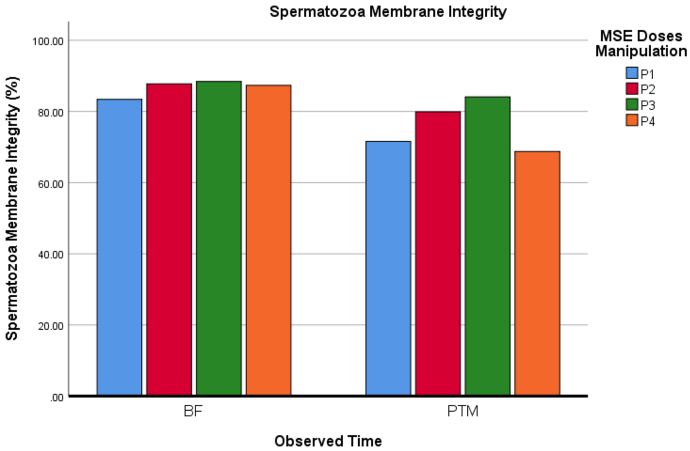

Figure 4. Scale bar of sperm membrane integrity percentage

CONCLUSIONS

In summary, supplementing with the CEP diluent for Simmental bull semen by Moringa Seed Extract (MSE) enhanced to produce antioxidant activity, preserved sperm viability and motility, and offered protection against membrane damage. The optimal concentration was 5 mg/mL, to have yielding motility of 43.33 ± 3.73%, viability of 83.17 ± 1.07%, and membrane integrity of 84.08 ± 1.06%. Overall, these in vitro outcomes highlight MSE's potential as a promising antioxidant additive for improving frozen semen preservation. Artificial insemination (AI) centers play a practical role in improving sperm quality and enhancing fertilization potential, thereby serving as key platforms for advancing reproductive biotechnologies in livestock. However, further research is needed to have better understand the mechanisms behind MSE's effects and to conduct an in vivo fertility trial for enhancing antioxidant capacity and sperm quality during cryostorage in cattle.

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