Research Article

Sungkai Leaf (*Peronema canescens* Jack) Extract as a Natural Antioxidant for Mitigating Cryo-Injury in Bali Bull Spermatozoa

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Abstract | Conservation of the genetically valuable Bali bull necessitates high-quality frozen semen for successful artificial insemination (AI) This programs can increase livestock population growth through increasing pregnancy rates, shortening breeding intervals, and utilizing semen from superior bulls. This study examines the effectiveness of Sungkai leaf (*Peronema canescens* Jack) extract as a cryoprotectant due to its antioxidant properties. Fresh semen were collected at the Artificial Insemination Center (BBIB) Singosari, Jawa Timur. Samples were diluted in an extender consist of Tris-egg yolk, then divided into four treatment groups: without extract (control/T0), 0.10 mg/100 mL diluent (T1), 0.15 mg/100 mL (T2), and 0.20 mg/100 mL (T3) of the extract. Pre-freeze and post-thaw sperm motility, viability, abnormalities, and sperm membrane integrity were assessed following standard protocols. The results showed that the addition of Sungkai leaf extract on Tris-egg yolk extender significantly impacted the cellular quality of Bali bull sperm. Specifically, the addition of 0.15 mg/100 mL Sungkai leaf extract (T2) significantly improved both pre-freezing and post-thawing sperm motility (83.40±1.44 and 73.23±1.66%), viability (83.95±1.08 and 78.80±1.23%), and sperm membrane integrity (87.50±1.07 and 82.60±1.85%) respectively, in comparison to T0 ($p<0.05$). Notably, Sungkai leaf extract did not significantly affect sperm abnormalities pre-freezing or post-thaw ($p>0.05$), with all groups (T0, T1, T2, and T3) remaining below the maximum standard limit (20%). These findings suggest that Sungkai leaf extract, particularly at 0.15 mg/100 mL, can effectively enhance the cellular quality of Bali bull sperm during cryopreservation and post-thawed.

Keywords | Antioxidant, Bali bull, Cellular quality, Post-thaw, Semen cryopreservation, Sungkai leaf extract

Received | July 25, 2025; **Accepted** | September 21, 2025; **Published** | October 13, 2025
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Citation | Novita R, Diatmono DFF, Cahyono HP, Padmawati FG, Suhartanto B, Widayati DT (2025). Sungkai leaf (*Peronema canescens* Jack) extract as a natural antioxidant for mitigating cryo-injury in bali bull spermatozoa. Adv. Anim. Vet. Sci., 13(10):2277-2286.
DOI | https://dx.doi.org/10.17582/journal.aavs/2025/13.10.2277.2286
ISSN (Online) | 2307-8316

INTRODUCTION

The preservation of semen quality is a critical factor in the success of artificial insemination programs aimed at improving the reproductive efficiency and genetic quality of cattle. In tropical countries such as Indonesia, Bali bulls (*Bos javanicus*) are one of the most important local genetic resources due to their adaptability, feed efficiency, and resistance to harsh environmental conditions. However, their reproductive performance is often limited by a decline in spermatozoa quality during cryopreservation, which significantly affects fertility outcomes. This breed

represents a domesticated variant of the wild Banteng (Warman *et al*., 2025). Bali cattle are crucial to Indonesia's livestock sector due to their strong adaptability to new environtments, notable tolerance to tropical environments, local flora, and high parasite resistance (Suprapto *et al*., 2021). Bali cattle also exhibit high fertility and low calf mortality rates (Warman *et al*., 2023; Baliarti *et al*., 2020). Furthermore, Bali cattle are widely recognized as a potential source of meat, carcass production, and known for their high growth rates (Nugraha *et al*., 2022). Although the Bali cattle population is currently abundant and distributed across various regions in Indonesia, their utilization as meat-producing livestock must be balanced with efforts to increase their population to ensure sustained conservation (Warman *et al*., 2023). Accelerating the population growth of Bali cattle in Indonesia can be achieved through AI programs, implemented at both commercial and smallholder farm levels (Warman *et al*., 2025). The application of AI promises improved genetic quality of calves, increased productivity, reduced operational costs, and a lower risk of disease transmission (Warmadewi and Bidura, 2021). The success of AI programs, particularly for Bali cattle, is largely hinges on the quality of its semen (Warman *et al*., 2025; Warmadewi and Bidura, 2021).

Semen quality, a crucial determinant of Bali bull fertility, is determined by numerous factors such as genetics, diet, and environmental parameters like season, temperature, and humidity index (Warman *et al*., 2025). Ongoing studies into advanced semen preservation techniques, alongside improved extenders and cryoprotectants, demonstrates potential for extending the motility and viability of frozen semen. This, in turn, promises to enhance the efficiency of AI implementation nationwide (Sahiruddin *et al*., 2021). Various semen extenders, derived from both animal and plant protein sources, are available for cattle breeding (Suprapto *et al*., 2021). The majority of bull semen extenders typically incorporate 20% egg yolk or an equivalent substitute, along with antioxidants that are essential to enhance post-thaw semen quality by neutralizing damaging reactive oxygen species (ROS). Furthermore, antioxidants can reduce lipid peroxidation and stabilizing cell membranes (Prastiya *et al*., 2023; Su *et al*., 2019). Nevertheless, issues such as toxicity, biocompatibility, and cost associated with synthetic antioxidants have spurred a shift in research interest toward plant-derived natural antioxidants (Authaida *et al*., 2025). Several studies have investigated the supplementation of plant-based antioxidants in semen extenders for various species, including bulls, bucks, rams, and boars. Examples include extracts from green tea (Wijayanti *et al*., 2023), Javanese turmeric (Bintara *et al*., 2025), cactus (Ramírez-Chequer *et al*., 2025), orange, pineapple, and beet (Bozzi *et al*., 2022).

Despite extensive research into semen preservation, the potential of Sungkai leaf extract as an antioxidant for semen cryopreservation remains largely unexplored. Natural plant extracts rich in bioactive compounds have gained increasing attention as alternative antioxidants due to their safety, accessibility, and multifunctional properties. Sungkai (*Peronema canescens* Jack), is a traditional medicinal plant in Southeast Asia that contains secondary metabolites such as flavonoids (17.60±0.50 mg GaE/g extract) and phenolic (40.03±0.60 mg GaE/g extract) with strong antioxidant and anti-inflammatory activities (Novita *et al*., 2025). Previous studies have reported its potential in modulating immune responses, protecting against oxidative damage, and improving physiological performance in animals (Diniyati *et al*., 2025; Dillasamola *et al*., 2021). However, its application in reproductive biotechnology, particularly in semen preservation, remains poorly explored. This substance may help preserve spermatozoa cellular quality throughout the pre-freezing, freezing, and cryopreservation stages. These flavonoids directly preventing the generation of ROS during semen processing and preservation (Nurfauziyah *et al*., 2024; Montano *et al*., 2022). While various plant extracts have been investigated, a significant gap remains in understanding the specific benefits of Sungkai leaf extract for enhancing semen preservation. Investigating the addition of Sungkai leaf extract as a potential plant-based antioxidant in Tris-egg yolk extenders in this study could lead to advancements in reproductive technology for cattle breeding, particularly by enhancing AI success. Key aspects warranting evaluation include its antioxidant capacity and overall impact on sperm cellular quality parameters during both pre-freezing and post-thawing of Bali bull sperm. Furthermore, this study also determining the optimal concentration of Sungkai leaf extract in semen extenders is crucial to maximize its protective benefits while minimizing potential toxicity.

MATERIALS AND METHODS

Animals used, locations, and study period

Fresh semen were collected from five 7-years old Bali bulls, each weighing approximately 588 kg. Clinically, the bulls were in good health, exhibited high libido, and had normal reproductive systems. The bulls were housed individually in temperature-controlled enclosures, maintained between 22–27°C, at the Artificial Insemination Center (BBIB) Singosari, Jawa Timur. A distinct preparation of Sungkai leaf extract was performed at Laboratory of Tropical Animal Research Center (TARC) and post-thawed semen analysis was performed at Laboratory of Animal Physiology and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta. This study was carried out during the period of October to December 2023.

Experimental design

A completely randomized design (CRD) was implemented

to minimize experimental error and sure statistical validity. Four treatments groups included: A Tris-egg yolk extender was prepared without Sungkai leaf extract and designated as the control (T0), and Tris-egg yolk extender enriched with 0.10 mg/100 mL (T1), 0.15 mg/100 mL (T2), and 0.20 mg/100 mL (T3) Sungkai leaf extract, each treatment replicated 10 times. Sperm quality parameters were assessed both pre- and post-cryopreservation.

Preparation of sungkai leaf extract

Sungkai leaves were collected from the Musi Rawas Utara District in Sumatra Selatan Province. Upon collection, leaves were separated from twigs, and a 500 g sample was dried, powdered, and repeatedly sieved to achieve a fine powder. The dried Sungkai leaf powder was prepared at a concentration of 200 g per liter in 70% ethanol. The mixture was then subjected to maceration for three days to perform the extraction. To ensure optimal extraction efficiency and minimize evaporation, the maceration container was sealed with aluminum foil. Following maceration, the saturated leaves were pressed and filtered through filter paper to collect the crude Sungkai leaf extract. The solvent was subsequently removed from the extract using an evaporator at 50°C and 45 rpm, yielding a concentrated Sungkai leaf extract. The extraction procedure for sangkai leaf extract yielded a product with antioxidant properties, specifically a composition rich in flavonoids and phenols (Novita *et al.*, 2025).

Praparation of extender

The extender was prepared according to established protocols from BBIB Singosari. The process, which included Tris-aminomethane, raffinose pentahydrate, citric acid, and lactose, involved their dilution in 80 mL of distilled water. The solution was subseqeuntly homogenized by stirring at 100°C for 10 minutes. After cooling to 37°C, penicillin and streptomycin were added, followed by an additional 10–15 minutes of homogenization. Subsequently, the solution (20 mL) was homogenized with isolated egg yolk for 30 minutes. The resulting extender was brought to 4°C and stored for 24 hours under cold conditions before use.

Semen collection and evaluation

Two ejaculates were collected from each bull using a sterilized artificial vagina (AV). Collected samples were transported directly to the laboratory. Initial fresh semen analysis involved macroscopic examination of volume, color, pH value, smell, and consistency. This was followed by microscopic evaluation of semen concentration, motilities (individual and mass motility), viability, and morphological abnormalities (primary and secondary abnormality). Semen samples were evaluated according to the guidelines of the National Standardization Agency of Indonesia (4869-1:2024) and BBIB Singosari, which is certified by ISO 17025:2017 for laboratory competence and ISO 9001:2015 for quality management systems. Samples showing >70% motility and <20% abnormalities were considered suitable for subsequent processing (BSN, 2024). The starting quality of fresh semen, as used in this study, is detailed in Table 1.

Table 1: Characteristics of fresh Bali bull semen.

Parameters	Bali bull fresh semen evaluation
Volume (mL)	7.36±1.48
Color	Milky white
pH value	6.60±0.00
Smell	Distinctive
Consistency	Moderate
Concentration (x 10^7/mL)	1,119.66±113.85
Mass motility	+++
Individual motility (%)	83.33±1.52
Viability (%)	84.26±5.11
Abnormalities (%)	7.37±1.59

Semen diluent and extract addition

Three distinct extenders, designated extender A1, A2, and B, were formulated for this study. Extender A1 and A2 was an Tris-egg yolk extender supplemented with Sungkai leaf extract. Extender B shared a similar Tris-egg yolk formulation but incorporated 13% glycerol in place of the Sungkai leaf extract. Glycerol was added as a standard method by BBIB Singosari to prevent cold shock during freezing. The addition of glycerol was incorporated into the extenders for all treatment groups (T0, T1, T2, and T3). Semen was initially diluted at a 1:1 ratio with extender A1 and subsequently equilibrated at 4–5°C. After being equilibrated for three hours, extender A2 was gradually added to the mixture. The amount of extender A2 added was calculated based on semen concentration. During this process, the temperature was maintained at 4–5°C, and the mixture was allowed to stand for approximately 18 hours. After a total equilibration period of approximately 24 hours, extender B was added just before the mixture was filled, sealed, and frozen in straws. The calculation for the amount of extender A2 added is expressed by the following formula:

$$Vt = \frac{Va \times M \times K}{D} \times 100$$

Vt: total semen volume (mL), Va: diluted semen volume (mL), M: spermatozoa motility (%), K: spermatozoa concentration (x10^7/mL), and D: desired dose (10^9/mL).

Meanwhile, the calculation for the amount of extender B to be added is expressed in the following formula:

$$Vb = \frac{Vt}{2}$$

Vb: volume of extender B added (mL) and Vt: total semen volume (mL).

Pre-freezing and post-thawing evaluation

Observations conducted both pre-freeze and post-thaw encompassed cellular quality parameters. Motile activity of the sperm was assessed by combining 10 µL of semen with 40 µL of saline on a microscope slide, covering with a coverslip, and examining seven distinct fields of view at 400x magnification. Sperm viability was determined using eosin-nigrosine staining. A 5 µL semen sample was carefully mixed with 20 µL of eosin-nigrosine stain on a microscope slide. The air-dried smear was then examined at 400x magnification. Live spermatozoa exhibited white heads, while dead spermatozoa displayed reddish heads. The abnormalities of spermatozoa including: broken tails, broken heads, coiled tails, folded tails, small heads, and large heads, were assessed using viability preparations. Sperm membrane integrity was assessed using the hypo-osmotic swelling (HOS)-test, a 10 µL of semen was mixed with 1000 µL of HOS solution and incubated at 37°C for 30 minutes. After incubation, a 10 µL mixture was transferred to a microscope slide and observed at 400x magnification. spermatozoa with preserved membrane integrity showed a coiled tail feature, spermatozoa with a damaged membrane, on the other hand, exhibited a straight tail (Fitriana *et al*., 2025; Baity *et al*., 2024; Prihantoko *et al*., 2020). Results from all observations are presented as percentages.

Statistical analysis

The data were statistically analyzed using One-Way ANOVA followed by Post Hoc Multiple Comparisons Tukey's-b to identify significant differences among treatment groups. All statistical analyses were performed using IBM SPSS version 26 (IBM, USA), with the level of significance was set at 5% ($p<0.05$). The results are expressed as Mean±Standard Error of the Mean (SEM).

RESULTS

Influence of sungkai leaf extract on bali bull sperm motility

The addition of various Sungkai leaf extracts significantly affected the pre-freezing spermatozoa motility of Bali bull semen ($p<0.05$). Specifically, treatment group T2 exhibited a significant difference from the T0 and T1 groups, while showing no significant difference when compared to T3 group. The results also indicated that T3 was not significantly different from T1 (Table 2). Post-thaw analysis (Table 3) revealed that group T2 exhibited superior total sperm motility, significantly differing ($p<0.05$) from groups T0, T1, and T3. There was no significant difference between T1 and T3. Overall, the administration of 0.15 mg/100 mL Sungkai leaf extract (T2) effectively maintained sperm cellular quality on pre-freeze and post-thaw conditions.

Table 2: Impact of Sungkai leaf extract on pre-freeze Bali bull sperm quality (Mean±SEM).

Treatments	Motility (%)	Viability (%)	Abnormalities (%)	Sperm membrane integrity (%)
T0	73.23±1.65^{a}	77.80±1.59^{a}	11.50±0.61	82.35±1.12^{a}
T1	77.33±1.04^{ab}	79.60±1.57^{ab}	11.22±0.38	82.55±1.11^{a}
T2	83.40±1.44^{c}	83.95±1.08^{b}	9.82±0.47	87.50±1.07^{b}
T3	79.00±3.26^{bc}	80.20±1.20^{ab}	11.47±0.73	84.90±1.35^{ab}
F values	10.178	3.491	1.975	4.241
p values	0.000	0.025	0.135	0.012

^{a,b,c} Different superscripts in the same column indicate a significant difference ($p<0.05$), F values: representing the ratio of between-group variance to within-group variance, p values: probability of observed sample, T0: without Sungkai leaf extract, T1: 0.10 mg Sungkai leaf extract per 100 mL diluent, T2: 0.15 mg Sungkai leaf extract per 100 mL diluent, and T3: 0.20 mg Sungkai leaf extract per 100 mL diluent.

Table 3: Impact of Sungkai leaf extract on post-thaw Bali bull sperm quality (Mean±SEM).

Treatments	Motility (%)	Viability (%)	Abnormalities (%)	Sperm membrane integrity (%)
T0	59.41±1.79^{a}	69.15±2.55^{a}	12.22±0.70	76.80±1.46^{a}
T1	66.35±1.80^{b}	73.60±2.54^{ab}	12.02±0.94	79.80±0.77^{ab}
T2	73.23±1.66^{c}	78.80±1.23^{b}	10.75±0.62	82.60±1.85^{b}
T3	60.40±2.11^{ab}	69.40±1.90^{a}	11.97±0.88	77.40±1.28^{a}
F values	11.830	4.524	0.701	3.564
p values	0.000	0.009	0.557	0.023

^{a,b,c} Different superscripts in the same column indicate a significant difference ($p<0.05$), F values: representing the ratio of between-group variance to within-group variance, p values: probability of observed sample, T0: without Sungkai leaf extract, T1: 0.10 mg Sungkai leaf extract per 100 mL diluent, T2: 0.15 mg Sungkai leaf extract per 100 mL diluent, and T3: 0.20 mg Sungkai leaf extract per 100 mL diluent.

Impact of sungkai leaf extract on bali bull sperm viability

The supplementation of Sungkai leaf extract significantly improved the pre-freezing viability of Bali bull sperm (Table 2). Specifically, group T2 showed a significant increase in viability compared to the T0 ($p<0.05$), but no significant difference was observed when compared to groups T1 and T3 ($p>0.05$). Post-thaw observations (Table 3) revealed that Sungkai leaf extract in group T2 resulted in a significant difference compared to groups T0 and T3 ($p<0.05$), although it did not significantly differ from group T1 ($p>0.05$). Based on these results (Tables 2 and 3), both pre-freezing and post-thawing viability demonstrated optimal percentages when Sungkai leaf extract was administered at 0.15 mg/100mL in the extender (T2). Viability observations are presented in Figure 1.

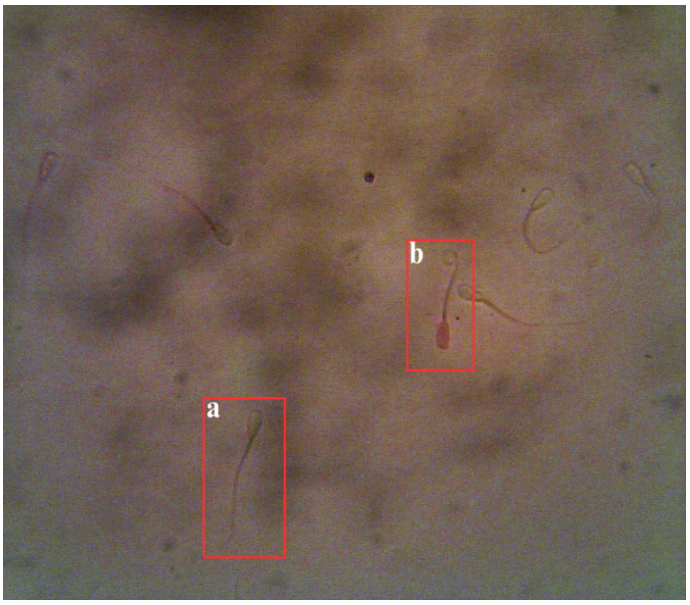

Figure 1: Viability of Bali bull spermatozoa at 400x magnification: (a) live spermatozoa, characterized by unstained (white) heads, and (b) dead spermatozoa, characterized by red heads due to the absorption of eosin-nigrosine stain.

Influence of sungkai leaf extract on bali bull sperm abnormalities

Pre-freezing abnormalities (Table 2) and post-thawing (Table 3) abnormalities observations showed that adding Sungkai leaf extract to the extender did not significantly affect the percentage of abnormalities across any treatment group ($p>0.05$). All treatment groups consistently maintained abnormality levels below the maximum standard limit (20%). Furthermore, post-thaw observations (Table 3) revealed no significant increase in abnormalities, indicating the extract did not negatively impact sperm morphology. Spermatozoa cells abnormalities are presented in Figure 2.

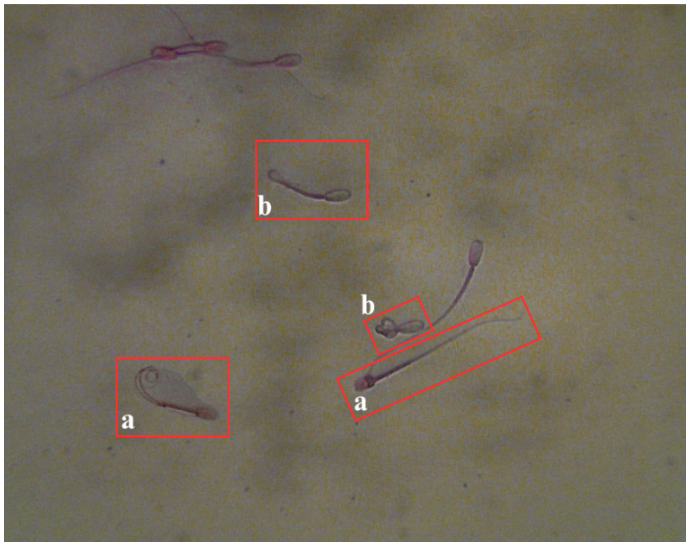

Figure 2: Abnormalities observed in Bali bull spermatozoa at 400x magnification. (a) A primary abnormality characterized by a double tail and a small head. (b) A secondary abnormality showing a bent tail.

Impact of sungkai leaf extract on bali bull sperm membrane integrity

Pre-freezing observations (Table 2) indicated that group T2 exhibited significantly different ($p<0.05$) sperm membrane integrity percentages compared to groups T0 and T1, yet revealed no significant differences from T3 ($p>0.05$). Furthermore, post-thawing observations (Table 3) revealed that the addition of Sungkai leaf extract at 0.15 mg/100 mL extender (T2) effectively maintained spermatozoa membrane integrity, with results significantly different from those of groups T0 and T3, while showing no significant difference when compared to group T1. The results related to sperm membrane integrity are ilustrated in Figure 3.

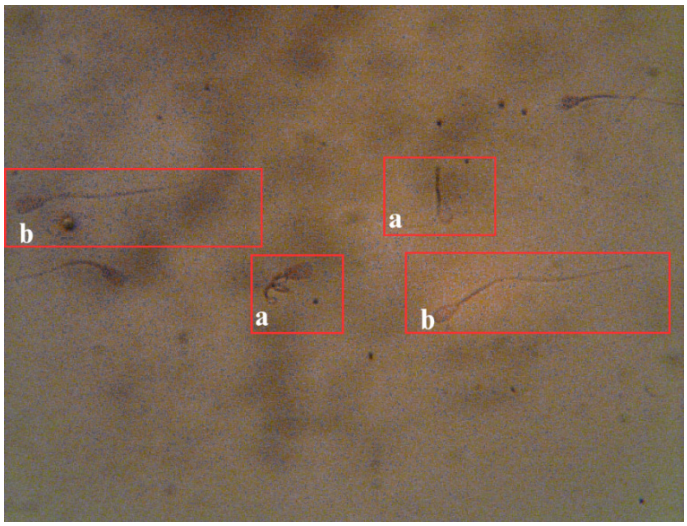

Figure 3: Membrane integrity of Bali cattle spermatozoa observed at 400x magnification: (a) Intact plasma membrane, characterized by a coiled tail, and (b) Damaged plasma membrane, characterized by a straight tail.

DISCUSSION

The findings presented in Tables 2 and 3 demonstrate the significant influence of Sungkai leaf extract addition in semen extender on the percentage of motility, viability, abnormalities, and sperm membrane integrity. Notably, the pre-freeze and post-thaw motility percentages were above the standard range, indicating their suitability for AI (BSN, 2024). Furthermore, the reduction in motility observed in semen supplemented with Sungkai leaf extract was comparatively low. Previous study, for instance, reported a broader range of 24–64% decrease in pre-freezing and post-thawing motility in human sperm (Ozkavukcu *et al*., 2008). The decline in semen motility during the pre-freezing stage is primarily attributed to temperature fluctuations during the equilibration process between semen and the extender (Bintara *et al*., 2025; Fitriana *et al*., 2025). Conversely, the reduction in semen motility during the freezing and post-thawing stages is a consequence of cold shock and lipid peroxidation induced by ROS (Fitriana *et al*., 2025; Bintara *et al*., 2023). Drastic temperature changes increase mitochondrial metabolism, and a byproduct of this metabolic process is the formation of ROS (Baity *et al*., 2024). An excessive amount of ROS binds to unsaturated fatty acids within the spermatozoa membrane, induced lipid peroxidation (Bintara *et al*., 2025; Fitriana *et al*., 2025). Lipid peroxidation and oxidative stress compromise the structural and functional integrity of the spermatozoal membrane. This damage disrupts the membrane's ability to maintain cellular homeostasis, which is crucial for sperm viability and function. This disruption results in the leakage of nutrients and ions, such as calcium (Ca^{2+}), potassium (K^{+}), sodium (Na^{+}), and the aspartate aminotransferase enzyme, which is crucial for adenosine triphosphate (ATP) production, from within the cell membrane. Consequently, this leads to a reduction in the frequency of spermatozoa movement (Prihantoko *et al*., 2020).

Sperm motility is a crucial factor in successful sperm transport during fertilization. Highly motile semen significantly determines the ability of spermatozoa to penetrate the cumulus oophorus and zona pellucida of the ovum (Soto-Heras *et al*., 2023). Consequently, good sperm motility directly influences fertilization rates and the conception rate in AI programs (Warman *et al*., 2025; Baity *et al*., 2024). The results demonstrate that adding Sungkai leaf extract can maintain spermatozoa motility during both pre-freezing and post-thawing processes (Tables 2 and 3). This suggests the presence of high antioxidant activity, which inhibits free radicals that can damage sperm membranes and reduce motility (Fitriana *et al*., 2025; Baity *et al*., 2024). Specifically, antioxidant components like flavonoids in Sungkai leaves can directly donate hydrogen atoms to free radical compounds, thereby stabilizing them. Furthermore, flavonoids are known to chelate metal ions, which slows and prevents the excessive formation of ROS (Novita *et al*., 2025; Montano *et al*., 2022). This inference is drawn from the research findings, where the T0 group, without the addition of Sungkai leaf extract, consistently demonstrated the lowest motilities during pre-freeze and post-thawing.

The percentages of pre-freezing (Table 2) and post-thawing sperm viability (Table 3) in Bali cattle semen were in accordance with findings from previous studies (Baity *et al*., 2024; Iskandar *et al*., 2022). Pre-freezing semen viability is influenced by the dilution and equilibration processes (Bintara *et al*., 2023, 2025). During these processes, spermatozoa continuously metabolize to produce ATP as an energy source. Excessive ATP production can lead to a decrease in semen pH (Rotimi *et al*., 2024). A decrease in semen pH, tending towards acidity, adversely affects sperm function and can result in spermatozoa death (Zhou *et al*., 2015). Furthermore, the observed decrease in post-thawing spermatozoa viability is attributed to oxidative stress, resulting from excessive ROS production (Fitriana *et al*., 2025; Prihantoko *et al*., 2020). Excessive oxidative stress during cryopreservation can induce cellular apoptosis due to mitochondrial damage and caspase activation (Fleming and Thomson, 2025; Shi *et al*., 2024), consequently, there is a decreased overall sperm viability, impaired motility, and reduced fertilization capacity (Shi *et al*., 2024).

Based on previous studies, excessive ROS production leads to deoxyribonucleic acid (DNA) fragmentation and can negatively impact sperm function. This is because peroxidases damage sperm mitochondria and plasma membranes (Kowalczyk, 2022; Dutta *et al*., 2019). Furthermore, ROS can induce oxidative modifications, leading to the accumulation of mutations within mitochondrial DNA, which impair sperm motility and viability by inhibiting energy production (Juan *et al*., 2021; Dutta *et al*., 2019). Sungkai leaves contain polyphenolic antioxidant compounds that exhibit several activities. These include the ability to scavenge and neutralize free radicals through electron or hydrogen atom donation, chelate transition metal ions such as Fe^{2+} and Cu^{2+}, which are involved in ROS formation via the Fenton reaction, and modulate gene expression and the activity of enzymes involved in endogenous antioxidant defense through modulation of signal transduction pathways like Keap1-Nrf2 (Kowalczyk, 2022). In addition to these direct mechanisms, polyphenols have been observed to impede the catalytic activity of xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Fraga *et al*., 2023). These enzymes are involved in ROS production in spermatozoa, and their inhibition can reduce the levels of oxidation that can damage spermatozoal cellular quality

(Fitriana *et al*., 2025; Nurfauziyah *et al*., 2024; Fraga *et al*., 2023; Zini *et al*., 2009).

The levels of semen morphological abnormalities (primary and secondary abnormalities) observed both pre-freezing (Table 2) and post-thawing (Table 3) were not significantly different ($p<0.05$). Based on these findings, post-thawing semen abnormalities was also determined to be low (<20%), indicating its suitability for AI programs (BSN, 2024). The absence of a significant effect of Sungkai leaf extract on sperm abnormalities may be attributed to the low baseline rate of abnormalities in the Bali bull semen used in this study, the consistent age range of the Bali bull sires, uniform management practices, and homogeneous environmental conditions which left little scope for further improvement (Warman *et al*., 2025). Similar findings have been reported in previous studies where antioxidant supplementation effectively improved motility and membrane integrity but showed limited impact on sperm morphology when initial abnormality levels were minimal (Bouhadana *et al*., 2025; Warman *et al*., 2025; Felton-Taylor *et al*., 2020). Therefore, the lack of a significant response in sperm abnormalities should not be interpreted as a lack of biological activity of Sungkai leaf extract, but rather as an indication that its protective effect is more pronounced in functional parameters susceptible to oxidative stress than in morphological traits that are largely predetermined (Bouhadana *et al*., 2025; Dimitriadis *et al*., 2023).

The abnormalities of spermatozoa cells are influenced by hormones during spermatogenesis and cryopreservation process (Dementieva *et al*., 2024; Baharun *et al*., 2021; Byrne *et al*., 2017). Several protein hormones, produced by the anterior pituitary such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), exert their effects on specific testicular cells to regulate and promote testosterone biosynthesis. Moreover, testosterone subsequently supports spermatogenesis and correlates with the semen quality produced (Byrne *et al*., 2017; Ardiyansyah and Utomo, 2014). Furthermore, previous studies have demonstrated that the cryopreservation process may impair both morphological integrity and functiononal capacity of sperm, leading to defects such as coiling, bending, tearing, or detachment of the tail from the sperm head (Dementieva *et al*., 2024; Raad *et al*., 2018). However, the observed low post-thawing abnormalities values (Table 3) are potentially attributable to the positive effect of antioxidant activity during the cryopreservation process (Bansal and Bilaspuri, 2010). High post-thawing sperm abnormalities can impede fertilization, inhibit embryonic development, and enhance the risk of transmitting genetic defects (Fitriana *et al*., 2025; Baity *et al*., 2024; Dementieva *et al*., 2024).

Observations of sperm membranes revealed that the plasma membrane integrity of Bali bull sperm was consistent with previous study (Indriastuti *et al*., 2020). Notably, treatment group T2 exhibited optimal results, differing significantly ($p<0.05$) from other treatment groups in both pre-freezing and post-thawing assessments (Tables 2 and 3). Conversely, the decline in post-thaw sperm motility, viability, and membrane integrity percentage observed in group T3 compared to T1 and T2 (Table 3). The results suggest that 0.15 mg/100 mL of Sungkai leaf extract (T2) may represent the maximum tolerable concentration for addition. As previously described, plant-derived and other antioxidants mitigate oxidative stress by directly scavenging ROS, thereby preserving sperm morphology and function (Authaida *et al*., 2025; Bintara *et al*., 2025; Ramírez-Chequer *et al*., 2025; Wijayanti *et al*., 2023). However, excessive amounts can exert a pro-oxidant effect, impairing ROS-scavenging mechanisms and resulting in excessive oxidative stress and compromised sperm quality. This condition is identified as the "antioxidant paradox" (Authaida *et al*., 2025; Speisky *et al*., 2022). The antioxidant paradox occurs when polyphenols, instead of solely scavenging free radicals, undergo autoxidation to generate hydrogen peroxide (H_2O_2). Further explanation, at controlled levels, H_2O_2 enters cells via aquaporin channels and activates the Keap1-Nrf2 pathway, thereby enhancing endogenous antioxidant defenses and stabilizing the spermatozoa membrane. However, excessive H_2O_2 surpasses the redox-buffering capacity, leading to lipid peroxidation, loss of membrane integrity, and impaired sperm function (Mu and Kitts, 2023). It has been further explained that the supplementation of excessive antioxidants in sperm extenders can be toxic to spermatozoa, resulting in reduced sperm viability and motility (Pimpa *et al*., 2024; Zini *et al*., 2009). This is a critical concern, as membrane integrity is essential for sperm to maintain their biological functions, metabolic processes, immune evasion, and molecular recognition and binding (Xue *et al*., 2025).

The plasma membrane of mammalian sperm contains high levels of polyunsaturated fatty acids (PUFAs), which are fatty acids possessing more than two carbon double bonds (Gautier and Aurich, 2022; Kowalczyk, 2022). The unconjugated double bonds within the methylene groups of PUFAs reduce the strength of the methylene's carbon-hydrogen bonds. This increased vulnerability of hydrogen makes it susceptible to oxidative damage (Dutta *et al*., 2019). Lipid peroxidation, caused by ROS, can lead to the loss of 60% of the fatty acids present in the plasma membrane. This negatively impacts membrane fluidity, increases ion permeability, inhibits enzyme and receptor activity, and ultimately disrupts sperm membrane integrity, leading to reduced motility and impaired sperm-

oocyte interaction (Rahma *et al*., 2024; Dutta *et al*., 2019). Moreover, the paucity of enzymatic antioxidants within the sperm cytoplasm renders these cells particularly vulnerable to ROS-induced damage (Qamar *et al*., 2023; Kowalczyk, 2022). Excessive ROS production overwhelms endogenous antioxidant defenses, necessitating the addition of exogenous antioxidants to restore normal oxidative processes and achieve homeostasis (Rahma *et al*., 2024). Consistent with this, the addition of exogenous antioxidants from Sungkai leaf extract can protect the plasma membrane from damage caused by excessive ROS production. Sungkai leaves contain polyphenolic compounds, which are characterized by the presence of at least one aromatic ring substituted with one or more hydroxyl groups. These polyphenols can donate hydrogen atoms from the hydroxyl groups of their phenolic rings to react with free radicals, producing less reactive flavonoid phenoxyl radicals (Novita *et al*., 2025; Salehimanesh *et al*., 2025; Speisky *et al*., 2022). Flavonoid phenoxyl radicals possess a stable resonance structure and conjugated double bonds, allowing for electron delocalization. This effectively reduces and eliminates the damaging effects of free radicals on sperm membranes (Salehimanesh *et al*., 2025; Speisky *et al*., 2022).

CONCLUSIONS AND RECOMMENDATIONS

This study demonstrates that the supplementation of Bali bull semen extender with Sungkai leaf extract significantly enhances sperm quality parameters during cryopreservation. Specifically, the optimal supplementation of 0.15 mg/100 mL effectively maintained several pre-freeze and post-thaw sperm cellular quality (motility, viability, and membrane integrity), while not adversely affecting sperm abnormalities. The observed beneficial effects are primarily attributed to the antioxidant properties of polyphenolic compounds, particularly flavonoids, present in Sungkai leaves. Future studies are required to explore the protective molecular interactions between Sungkai leaf polyphenols, lipid peroxidation, and sperm DNA integrity. However, to validate its practical application in Bali cattle AI programs, future *in vivo* studies focusing on conception rates are essential.

ACKNOWLEDGMENT

The authors are grateful to Balai Pembiayaan Pendidikan Tinggi (BPPT) and Lembaga Pengelola Dana Pendidikan (LPDP) for their support for this study through the Beasiswa Pendidikan Indonesia (BPI) program. Special appreciation is also given to the BBIB Singosari, Jawa Timur, TARC Laboratory, and Faculty of Animal Science, Universitas Gadjah Mada, for providing essential facilities for this study.

NOVELTY STATEMENT

Although the properties of Sungkai leaves (*Peronema canescens* Jack) have been extensively studied, their potential as an antioxidant supplement to enhance the quality of Bali bull spermatozoa during cryopreservation has, to the best of our knowledge, remained unexplored. This study, therefore, presents the first investigation into the efficacy of Sungkai leaf extract as a plant-based antioxidant additive for semen extenders used in Bali bull semen preservation. Our findings demonstrate that adding an optimal concentration of the extract significantly improves spermatozoa motility, viability, and membrane integrity both pre-freezing and post-thawing. This study offers a promising, natural alternative for improving AI programs aimed at conserving the genetically valuable Bali bull population.

AUTHOR'S CONTRIBUTION

RN, BS, and DTW designed the study.
RN performed the fieldwork experiments.
RN, DFFD, HPC, and FGP performed data analysis, conducted the literature search, and wrote the original manuscript.
RN, DFFD, BS, and DTW performed data interpretation, edited, and reviewed the manuscript.
BS and DTW supervised the study.

Generative AI and AI-assisted technology statement

This article utilized Generative Artificial Intelligence (AI) for grammar checking to enhance accuracy in the writing process.

Conflict of interest

The authors have declared no conflict interest.

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