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RESEARCH ARTICLE

Potential of Brown Macroalgae Extract (*Sargassum ilicifolium* (*Turner) C. Agardh*) on the Quality and Protamine-1 (PRM1) Protein Levels of Frozen-thawed Semen in Simmental Bulls

C. Damaris[1,2], A. Budiyanto[2], P.I. Sitaresmi[3*], K.D. Prihantoko[3,5], M.F. Hudaya[3], D.A. Mahari[2,3], F.A. Pamungkas[3], H.P. Cahyono[4], E.M.N. Setyawan[2], N. Kusumorini[6], and A. Kusumawati[2*]

[1]Livestock Embryo Center, Kp. Pasir Pogor, Cipelang, Cijeruk Subdistrict, Bogor, West Java, Indonesia, 16740; [2]Department of Reproduction and Obstetrics, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Jalan Fauna No. 2, Karangmalang, Sleman, Yogyakarta, Indonesia, 55281; [3]Research Center for Animal Husbandry, National Research and Innovation Agency, Cibinong, Science Center, Jalan Raya Jakarta-Bogor, Bogor, Indonesia, 16915; [4]Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada, Jalan Fauna No. 2, Karangmalang, Sleman, Yogyakarta, Indonesia, 55281. [5]Post-Doctoral Program, Research Center for Animal Husbandry, National Research and Innovation Agency, Cibinong, Science Center, Jalan Raya Jakarta-Bogor, Bogor, Indonesia 16915. [6]Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia.
*Corresponding author: uma_vet@ugm.ac.id; pradita.iustitia.sitaresmi@brin.go.id

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ABSTRACT

Semen cryopreservation provides numerous advantages, but it also results in reduced semen quality following thawing. In order to mitigate these negative consequences, antioxidants are often added to semen extenders. Brown macroalgae has recently been shown to be a natural source of antioxidants. The purpose of this study was to investigate whether supplementation of extender with four concentrations of macroalgae extract (*Sargassum ilicifolium* (Turner) C. Agardh) would improve the quality of sperm post thawing. A total of 30 semen samples collected twice weekly over a period of four weeks from four Simmental bulls were used in this study. These samples were divided into five parts, diluted using extender supplemented with *S. ilicifolium* extract at 0, 250, 500, 1000 and 2000ppm, and cryopreserved in liquid nitrogen. Post-thaw sperm motility, viability, membrane integrity, DNA fragmentation, reactive oxygen species (ROS), malondialdehyde (MDA) levels, and protamin-1 (PRM-1) protein concentration were analyzed. Results demonstrated that semen samples frozen in extender supplemented with *Sargassum ilicifolium* extract exhibited reduced levels of spermatozoa damage, as indicated by significantly better sperm progressive motility, viability, membrane integrity, and reduced DNA fragmentation due to lower ROS production compared to the control group ($P<0.05$). The best results in terms of sperm motility, viability, membrane integrity, and lower DNA fragmentation were observed in the 1000ppm treatment group. Reduced ROS levels in the treatment groups were associated with lower MDA and increased PRM-1 levels. Moreover, DNA fragmentation was inversely related to PRM-1 levels. Overall, the best results across multiple parameters were observed in the 500–1000ppm treatment range. Further studies are needed to fully explore the potential of *S. ilicifolium* extract in maintaining semen quality during the freeze–thaw process.

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INTRODUCTION

Semen cryopreservation enables the long-term storage of sperm and aids the widespread dissemination of superior germ plasm to enhance genetic improvement in livestock kept at far-off places (Yánez-Ortiz *et al.*, 2022). This modern technique involves dilution of semen using extenders containing some cryoprotectant, slowly lowering the temperature, freezing in liquid nitrogen at -196°C, and then thawing the cells. It is considered the most effective method for preserving the long-term ability of sperm to fertilize eggs (Upadhyay *et al.*, 2021). Nevertheless,

cryopreservation causes structural harm to spermatozoa, such as disruption of the DNA, acrosome, and plasma membrane, which lowers the fertilizing ability of frozen sperm (Handayani *et al.*, 2021). Overactive mitochondrial activity produces reactive oxygen species (ROS), which damage cellular components and organelle function. Cold shock, osmotic and oxidative stress, and intracellular ice crystal formation are additional stressors that arise during freezing (Yoon *et al.*, 2015). Consequently, despite its advantages, cryopreservation negatively affects the structural integrity and function of sperm.

The reduced ability of post-thaw spermatozoa to fertilize the ovum is a result of molecular damage caused by cryopreservation (Yánez-Ortiz *et al.*, 2022). According to previous reports, cryopreserved semen shows decreased sperm motility, mitochondrial membrane potential, and acrosomal integrity, increased plasma membrane permeability, and increased ROS production (Peris-Frau *et al.*, 2020). Reactive oxygen species include hydroxyl radicals (OH-), superoxide anions (O_2-), and hydrogen peroxide (H_2O_2), which induce lipid peroxidation, mitochondrial disruption, DNA deterioration, and cell death (Hezavehei *et al.*, 2018). Antioxidant protection of semen is compromised as it contains excessive ROS without adequate endogenous antioxidants. For instance, superoxide dismutase (SOD) activity drops to 38-66% during freezing and thawing (Bilodeau *et al.*, 2000). These workers also found that the level of protamine-1 (PRM-1) protein, a major chromatin structural protein in bull spermatozoa, decreased in damaged sperm DNA. This protein was subsequently found to be associated with high fertility (Dogan *et al.*, 2015). Oxidative stress primarily affects membrane lipids, proteins, nuclear DNA, and protamines. Abnormal expression of PRM-1 protein is associated with sperm that are less viable, less motile, and have more chromatin damage than sperm with normal expression of PRM-1 protein (Kritaniya *et al.*, 2020).

Cryoprotective agents, such as glycerol, are added to the semen extender during cryopreservation to prevent cryoinjury; however, high concentrations of these agents are potentially toxic to sperm (Hezavehei *et al.*, 2018). It is generally agreed that adding antioxidants is necessary to prevent excessive ROS development and increase sperm viability after thawing (Peris-Frau *et al.*, 2020). Researchers have found that macroalgae, especially brown ones like *Sargassum ilicifolium* (Turner) C. Agardh, are abundant in bioactive antioxidant compounds, such as fucoxanthin, fucoidan, and phlorotannins. These compounds exhibit antioxidant, antibacterial, and anti-inflammatory effects (Susanto *et al.*, 2017; Lutfia *et al.*, 2020; Arguelles, 2021). Brown macroalgae have plenty of phenolic compounds, which exhibit protective effect against oxidation. This makes them good natural supplements for semen extender (Arguelles and Sapin, 2022). This study aimed to evaluate the effects of supplementation of bull semen extender with *S. ilicifolium* extract at concentrations of 250, 500, 1000 and 2000ppm on post-thaw sperm quality of Simmental bulls, prompted by the abundant macroalgae resources in Indonesia. The results of this study are expected to highlight the importance of *S. ilicifolium* in the local cattle market to improve post-thaw semen quality and optimize artificial insemination programs in the country.

MATERIALS AND METHODS

Ethical approval: The study was conducted in accordance with the guidelines of the Ethics Commission for Animal Care and Use of the National Research and Innovation Agency (BRIN) (073/KE.02/SK/05/2025), Indonesia.

Collection and preparation of *Sargassum ilicifolium* extract: Samples of the brown macroalgae *Sargassum ilicifolium* were collected from the coastal area of Sepanjang Beach, Gunungkidul Regency, Yogyakarta (-8.137133858422729, 110.568364949342), Indonesia, during July-August 2024. After three rounds of distilled water rinsing, the samples were freeze-dried for 48h at a condenser temperature of -49.4°C. To remove contaminants, the dried samples were crushed and sieved. Maceration was performed for 48h in 96% ethanol (1:10 w/v), followed by filtration using Whatman No. 1 filter paper. The extraction process was repeated twice for the remaining residue. The combined filtrates were concentrated using a rotary evaporator (BUCHI™ Rotavapor™ Scholar System) at 40°C to obtain a thick extract, which was subsequently stored at 4°C in sterile Falcon tubes until further use (Saberivand *et al.*, 2022). A thick extract was prepared at a stock concentration of 10,000ppm for supplementation to the semen extender by dissolving 0.05 g of thick extract in diluent A or B with a volume of 5 ml each, then diluted to concentrations of 2000, 1000, 500 and 250ppm.

Semen collection and cryopreservation: In this study, 3-year-old Simmental bulls (n=4), with an average body weight of 803.38±47.23 kg, raised at the National Artificial Inseminition Center in Central Java, were used for semen collection. The bulls were housed under naturally prevailing climatic conditions in semi-open individual bull pens. They were offered a diet comprising forage and concentrate (10.0 and 1.0% of body weight, respectively), with free access to clean drinking water. Management, such as weekly exercise, nail trimming, and deworming, was performed every two months. A total of 30 semen samples collected twice weekly over a period of four weeks from four Simmental bulls, using an artificial vagina were used in this study. The collected semen samples were immediately transported to the adjacent laboratory for initial evaluation of ejaculate volume, sperm concentration, and progressive motility in accordance with Indonesian National Standard (SNI) No. 4869-1-2017 “Frozen Bull Semen”. Ejaculates exhibiting more than 70% progressive motility, and sperm concentration exceeding $1x10^9$ sperm/mL were selected for dilution and freezing.

A skim milk-egg yolk extender was prepared by dissolving 100 g of skim milk in 960mL of distilled water previously heated to 50°C. The mixture was then heated to 92°C and subsequently cooled to 37°C. Antibiotics (1 g of penicillin (Meiji Seika Pharma, Japan) and 1 g of streptomycin (Thermo Fisher Scientific, Singapore]) were added and homogenized. The extender was divided into two equal parts (A and B). Part A was prepared by combining 445mL of skim-milk antibiotic buffer with 10% (v/v) egg yolk and 1% (v/v) fructose (Merck). Part B consisted of 365mL of skim-milk antibiotic buffer, 10% (v/v) egg yolk, 1% (v/v) fructose, and 16% (v/v) glycerol (Sigma-Aldrich), which was homogenized thoroughly.

Sargassum ilicifolium extract was added to parts A and B of the extender at concentrations of 250, 500, 1000 or 2000ppm. Semen aliquots were diluted at 29 °C with extender A, cooled to 5$^{5\ °C}$and diluted with extender B to a final sperm concentration of 2.5x10^6 spermatozoa/0.25mL. After 4h of equilibration, samples were filled in 2.5mL straws (IMV®Technologies, L' Aigle, France), which were then sealed. The straws were held in steel racks (Cooltop, Minitube, Germany) and subjected to controlled freezing using a programmable cell freezer (IMV® Technologies, France) at the following cooling rates: from 4°C to -10°C at 5°C/min, from -10°C to -100°C at 40°C/min, and from -100°C to -140°C at 20°C/min. The straws were frozen and then stored in liquid nitrogen for at least for 48h until they were examined further (Saberivand *et al.*, 2022).

Post-thaw sperm motility assessment: Post-thawing semen evaluation began with thawing the frozen semen by placing the straw in a water bath at 37ºC for 30sec, and transferring the liquid semen from the straw to a microtube. For sperm motility assessment, 20µL of semen was put on a glass slide, covered with a coverslip, and examined under a microscope at 37 °C (100x magnification). The percentage of motile spermatozoa was estimated by observing five different fields, and motility was scored from 0 to 100% based on the proportion of spermatozoa moving in a straight line (Goshme *et al.*, 2021).

Sperm viability assessment: Sperm viability was assessed by eosin staining (Ismaya, 2017). A 1:4 ratio of semen specimen to stain was prepared, applied to a glass slide, and examined under a microscope at 400x magnification. Viable sperm were identified by dye exclusion, with their heads appearing translucent, whereas non-viable sperm absorbed the eosin stain, and their heads appeared red (Fig. 1). At least 200 sperm cells were examined to determine the percentage of viable sperm (Goshme *et al.*, 2021).

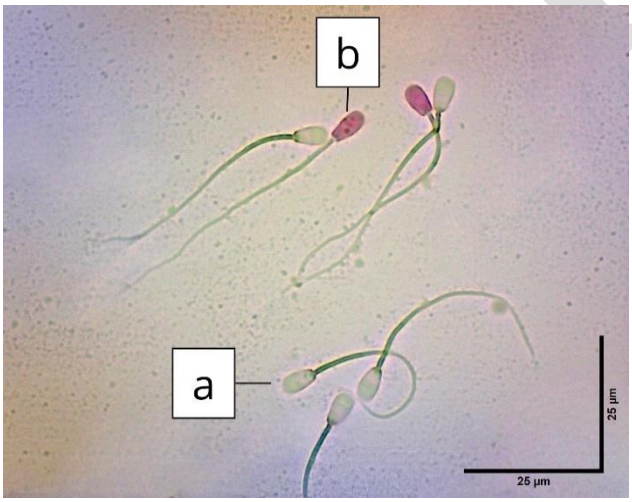

Fig. 1: Assessment of spermatozoa viability using eosin staining. (a) Viable spermatozoa appear translucent; (b) non-viable spermatozoa appear red (new figure that revised).

Plasma membrane integrity assessment: The hypo-osmotic swelling test (HOST) was used to assess the integrity of the sperm plasma membrane, as described by Gangwar *et al.* (2018), with slight modifications. A hypo-osmotic solution (0.9 g fructose+0.49g sodium citrate in 100mL distilled water) was used. After adding 200µL of HOS solution to 20µL of semen, the mixture was incubated for 45 min at 37°C. Thin smears were prepared, fixed in methanol for 10 min, rinsed, dried, and examined under 400× magnification. Sperm with intact plasma membranes were indicated by coiled tails, whereas those with damaged membranes showed straight tails (Fig. 2). A total of 200 sperm cells were examined for PMI assessment.

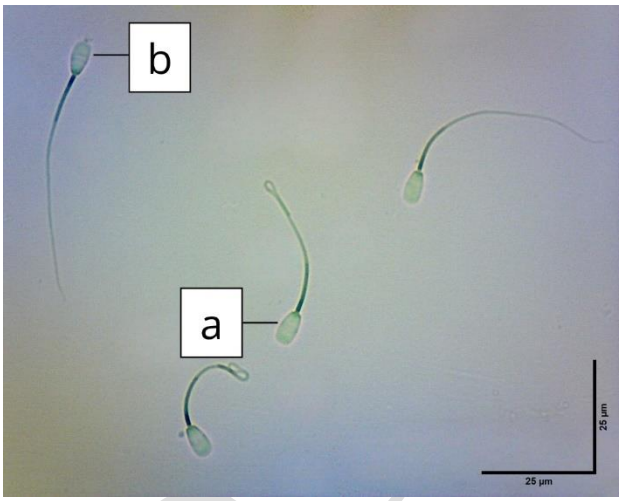

Fig. 2: Assessment of sperm plasma membrane integrity using the HOST method. (a) Intact sperm membrane indicated by a coiled tail; (b) damaged sperm membrane indicated by a straight tail.

Sperm DNA fragmentation analysis: Sperm DNA fragmentation was assessed using the TUNEL assay kit (Abbkine®, China) according to the manufacturer's instructions included with the kit. Following fixation with paraformaldehyde, permeabilization, and incubation with the TUNEL reaction mixture for 60 min at 37°C, sperm cells were evaluated using a fluorescence microscope at 520 nm. Intense green fluorescence indicated fragmented DNA, whereas dull green fluorescence indicated intact DNA (Prihantoko *et al.*, 2022). A total of 200 sperm cells were examined to determine the percentage of sperm DNA fragmentation.

Measurement of ROS activity: In this study, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen) was used to evaluate sperm ROS activity. Following centrifugation and PBS washing (1:9), the sperm cells were treated with 20µM DCFDA for 30 min at 37°C in the dark. After incubation, the cells were re-pelleted and resuspended in PBS. Under a fluorescence or confocal microscope (BioRad MRC-1024; 517–627 nm), ROS-positive cells were stained with brilliant green. At least 200 sperm cells were examined. The percentage ROS activity was calculated by dividing the total number of fluorescence-exhibiting sperm cells by the total number of sperm cells examined.

Sperm MDA levels were assessed using the Elabscience MDA (malondialdehyde) ELISA kit (Catalog E-EL-0060; Elabscience, USA), following the manufacturer's instructions. Semen and PBS (1:9) were centrifuged at 2–8°C to obtain a cell suspension. Then 50µL of sample or standard was added to each well, followed by 50µL of biotinylated detection antibody and

incubation at 37°C for 45 min. Wells were aspirated, washed thrice, and incubated with 100µL HRP conjugate for 30 min. After five additional washings, 90µL of substrate reagent was added and incubated for 15 min. Then, 50µL of stop solution was used to stop the reaction, which caused the solution to turn yellow. An ELISA reader was used to detect absorbance at 450 nm. The sensitivity of the MDA ELISA kit was 18.75ng/mL, while the inter-assay and intra-assay CV were <10%.

PRM-1 protein quantification: Semen and PBS (1:9) were centrifuged to collect the cell suspension. PRM-1 protein levels in sperm were quantified using a commercial ELISA kit (Cat. No. EIA06265Bo (Wuhan Enlibio Biotech Co., Ltd.), following the manufacturer's instructions. After preparing the reagents, standards, and samples, the wells were loaded with sperm pellets or PRM-1 standards and sealed. The plates were incubated for 90 min at 37°C. Then, 100µL biotinylated anti-PRM-1 antibody solution (prepared 30 min before) was added after two washes and incubated for 60 min. Afterwards, 100µL enzyme working solution (prepared 30 min earlier) was added and incubated for 30 min at 37°C. Plates were washed five times before adding 100µL color reagent and incubated in the dark for 30 min at 37°C. After adding 100µL of color reagent C, the absorbance was measured using an ELISA reader at 450 nm within 10 min. The sensitivity of the PRM-1 ELISA kit was 0.158ng/mL.

Statistical analysis: The Statistical Package for the Social Sciences (SPSS) version 27.0 for Windows and Microsoft Excel were used to analyze all the data. The Shapiro–Wilk test was employed to assess data normality, and Levene's test was used to evaluate homogeneity among groups. All parameters were normally distributed, except for ROS. Normally distributed data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's post hoc test and Pearson's correlation analysis. Non-normally distributed data were analyzed using the non-parametric Kruskal–Wallis test, followed by the Mann–Whitney post hoc test with Bonferroni correction and Spearman's rank correlation test. Results are presented as mean±SD, and statistical significance was considered at P<0.05.

RESULTS

Sperm motility, viability, and membrane integrity: The results presented in Table 1 indicate that the post-thaw semen exhibited significant differences in all microscopy parameters, including sperm motility, viability, and membrane integrity, between the control and treatment groups (P<0.05), with the extract-treated groups showing higher values than the control. Although sperm motility was significantly different between the control and treatment groups, post-hoc analysis revealed non-significant differences among the four extract concentrations (250 – 2000ppm). In contrast, sperm viability and membrane integrity differed significantly (P<0.05) across the various brown-algae supplementation levels. A linear increase was observed from 250ppm, peaked at 1000ppm, and then declined at 2000ppm for both sperm viability and membrane integrity, although these values remained higher than those of the control group (Table 1).

Sperm DNA fragmentation and ROS levels: Sperm DNA fragmentation was evaluated using the TUNEL assay (Table 2), revealing significant differences (P<0.05) between the control and all treatment groups (250, 500, 1000, and 2000 ppm). Non-significant differences in sperm DNA fragmentation were found among the 250, 500 and 2000ppm extract groups in post-hoc test, while the 1000 ppm group exhibited significantly lower DNA fragmentation than the control and other treatment groups (P<0.05). The lowest sperm DNA fragmentation was recorded in the 1000ppm group, with the control group showing the highest DNA fragmentation. A significant negative correlation (r=-0.871; P<0.01) was observed between sperm DNA fragmentation and PRM-1 protein levels, indicating that increased PRM-1 expression was associated with reduced DNA damage in the sperm.

Reactive oxygen species (ROS) levels (Table 2) were significantly reduced in the 500, 1000, and 2000ppm extract treatment groups compared to the control group and 250ppm extract group (P<0.05). The difference in ROS levels between the control and 250ppm extract group was not significant. Similarly, the differences in ROS levels among the 500, 1000, and 2000ppm extract groups were not significant. A significant positive correlation (r=0.596; P<0.05) was observed between ROS levels and malondialdehyde (MDA) concentrations, suggesting that elevated oxidative stress was associated with increased lipid peroxidation.

Table 1: Effects of different concentrations of extract on sperm motility, viability and membrane integrity post-thawing

Brown Macroalgae extract concentration (ppm)	Frozen semen (Post-thawing)		
	Motility (%)	Viability (%)	Membrane integrity (%)
0	36.25±4.94^{a}	65.03±3.33^{a}	68.17±5.30^{a}
250	42.96±4.90^{b}	71.03±2.55^{b}	71.57±6.67^{ab}
500	43.96±5.00^{b}	72.70±3.48^{b}	74.66±5.81^{b}
1000	49.13±6.66^{b}	76.56±3.06^{c}	82.31±2.20^{c}
2000	47.00±3.95^{b}	72.61±2.57^{b}	75.78±3.76^{b}

Values with different superscript letters within the same column indicate significant differences among the groups (P<0.05).

Table 2: Effects of different concentrations of extract on sperm DNA fragmentation and ROS levels in frozen semen (post-thawing)

Brown Macroalgae extract concentration (ppm)	Frozen semen (Post-thawing)	
	DNA fragmentation (%)	ROS (%)
0	6.60±1.04^{a}	52.91±6.00^{a}
250	5.04±0.71^{b}	48.79±4.44^{a}
500	4.75±0.53^{b}	39.29±6.02^{b}
1000	3.35±0.16^{c}	40.72±5.57^{b}
2000	4.28±1.23^{b}	42.34±4.42^{b}

Values with different superscript letters within the same column indicate significant differences among the groups (P<0.05).

MDA and PRM-1 concentrations: As shown in Table 3, significant differences (P<0.05) in MDA levels were observed between the control and all extract treatment groups (250, 500, 1000, and 2000ppm). The MDA level in the 250ppm group was higher than in the 1000ppm group, and lower than that in the 2000ppm group (P<0.05), but did not differ significantly from in the 500ppm group. The 500ppm group showed significantly lower MDA levels than the 2000ppm group (P<0.05) but did not differ significantly from the 1000ppm group. Significant differences were also found between the 1000 and 2000ppm groups (P<0.05), with levels being higher in the 2000ppm than in the 1000ppm group. The lowest MDA levels were found in the 1000ppm group, while the highest levels were recorded in the control group, indicating

extensive lipid peroxidation in sperm cells in the control group.

Table 3: Effects of different concentrations of extract on sperm MDA and PRM-1 protein levels in frozen semen (post-thawing)

Brown Macroalgae extract concentration (ppm)	Frozen semen (Post-thawing)	
	MDA (ng/mL)	PRM-1 protein (ng/mL)
0	175.44±17.47[a]	0.50±0.01[a]
250	139.03±14.11[c]	0.71±0.27[b]
500	131.60±9.91[cd]	0.76±0.02[c]
1000	120.16±13.22[d]	0.90±0.02[d]
2000	160.37±21.11[b]	0.74±0.03[c]

Values with different superscript letters within the same column indicate significant differences among the groups (P<0.05).

PRM-1 protein levels were significantly (P<0.05) lower in the control group than in all treatment groups (Table 3). The mean PRM-1 protein levels increased significantly as the concentration of the extract increased from 250ppm to 1000ppm, followed by a significant decrease at 2000ppm (P<0.05). The highest PRM1 protein levels were observed in the 1000ppm group, with the lowest levels observed in the control group (P<0.05).

DISCUSSION

In the present study, post-thaw examination of semen frozen in extenders supplemented with different concentrations of Brown Macroalgae extract was conducted to assess the improvement in the quality of frozen semen after thawing. According to the standards used in Indonesia, the minimum progressive motility of post-thaw semen should be 40% (Indonesian National Standard (SNI) No. 4869-1-2017). In this study, semen diluted in extender supplemented with four concentrations of the Brown Macroalgae extract also exhibited sperm motility above 40%, whereas the control group frozen in extender without Brown Macroalgae extract showed sperm motility below 40%. The highest sperm motility (49.13±6.66%) was observed in the 1000ppm extract treatment group. According to previous studies, a sperm motility of 68.17% was recorded in Simmental bull semen when green tea extract at 0.1 mg/100 mL of extender was used (Susilowati *et al.*, 2020), 43.33% when bull semen extender was supplemented with 5% strawberry extract (El-Sheshtawy and El-Nattat, 2018), and 51.67% after supplementing buffalo semen extender with 10µg/mL spirulina extract (Badr *et al.*, 2021). However, Saberivand *et al.* (2022) recorded 89.66% sperm motility by adding 1000ppm of an alcoholic extract of *Caulerpa sertolarioides* alga into the tris-egg yolk-based Simmental bull sperm freezing media. These studies consistently showed increased sperm motility after adding antioxidants to semen extenders.

The post-thaw sperm motility recorded in this study was lower than that reported in earlier investigations. This might be due to variations in the natural materials used, which affect the amount of antioxidant compounds present in the natural materials. Furthermore, the technique used for estimating sperm motility (CASA or traditional method) can also affect the results.

As an extra antioxidant source, *Sargassum ilicifolium* extract supplemented semen in this study demonstrated higher motility than that in the control group. According to Tuncer *et al.* (2021), semen extenders containing antioxidant additives improved sperm motility compared to groups without supplements. Similar effects have been observed in other species, such as cattle (Tuncer *et al.*, 2021), sheep (Hameed *et al.*, 2024), and goats (Batool *et al.*, 2024). Because of their interactions with Ca^{2+}-ATPase, an essential enzyme that controls sperm motility, polyphenols present in antioxidants such as *S. ilicifolium* extract may have a protective effect on sperm. However, high concentrations of the polyphenol quercetin can inhibit Ca^{2+}-ATPase activity, thereby increasing intracellular Ca^{2+} levels and decreasing sperm motility (El-Khawagah *et al.*, 2020). This may explain the decreased sperm motility observed in the 2000ppm treatment group.

Previous studies have shown 70.67% sperm viability in Simmental bull semen when green tea extract at 0.1 mg/100 mL extender was used (Susilowati *et al.*, 2020), 85.33% when bull semen extender was added with 1% strawberry extract (El-Sheshtawy and El-Nattat, 2018), and 70.30% after supplementing bull semen extender with 1000ppm green macroalgae extract (Saberivand *et al.*, 2022). In the current study, extender supplementation with brown macroalgae yielded the highest sperm viability of 76.56±3.06% at 1000ppm, which corresponds with sperm membrane integrity results showing the highest value of 82.31±2.20% for the same concentration, followed by a decline at a higher extract concentration of 2000 ppm. El-Khawagah *et al.* (2020) noted that high doses of quercetin in semen extenders had adverse effects on sperm viability and membrane integrity, potentially due to pro-oxidant activity at higher concentrations of quercetin. A similar effect may explain the decreased sperm viability and plasma membrane integrity in the 2000ppm treatment group recorded in this study.

Freezing and thawing induce alterations in the ratio of polyunsaturated fatty acids (PUFA) to cholesterol, which destabilizes the sperm membrane and affects sperm viability and motility. Damage to the sperm plasma membrane is associated with increased ROS production, reduced acrosome integrity, elevated DNA fragmentation, and protamine deficiency (Handarini *et al.*, 2024). Therefore, maintaining membrane integrity is crucial for preserving sperm function after cryopreservation. Consistent with our findings, Saberivand *et al.* (2022) highlighted that plant-derived antioxidants rich in flavonoids and phenolics help protect sperm during freezing by scavenging free radicals. In particular, Sargassum extracts significantly inhibited sperm lipid peroxidation and effectively scavenged hydroxyl radicals at high concentrations, which likely contributed to the improved sperm quality observed in this study. Our results align with those of previous studies, suggesting that the antioxidant properties of Sargassum extracts play a beneficial role in minimizing oxidative damage during cryopreservation.

The lowest DNA fragmentation in this study was 3.35% in the 1000ppm group. Dogan *et al.* (2015) established a DNA fragmentation threshold of 7%–10% as critical for successful artificial insemination. All treatment groups in this study showed DNA fragmentation of < 7%. Begum *et al.* (2021) demonstrated that fucoxanthin from brown macroalgae protects cells from DNA damage following oxidative stress. ROS-induced damage to sperm viability and motility precedes DNA fragmentation, likely

because ROS induce DNA base modifications rather than direct DNA strand breaks. Hence, DNA fragmentation was lower than motility and viability decline during freezing. Excessive ROS production also leads to DNA base modifications, the most common DNA base addition which is 8-hydroxy-2'-deoxyguanosine (8-OHdG). As spermatozoa possess only the initial enzyme of base excision repair, 8-oxoguanine DNA glycosylase (OGG1), complete repair is not possible, leaving single-strand DNA damage (Ribas-Maynou *et al.*, 2022). According to Fatima *et al.* (2020), excessive ROS production and limited antioxidant defense induce oxidative stress in spermatozoa. Elevated ROS levels affect proteins, DNA, and PUFAs, causing DNA damage and death of sperm cells (Imani *et al.*, 2021). Sperm DNA is damaged when the plasma membrane is also damaged. The quality of chromatin in the nucleus also determines the status of protamines bound to DNA, which protects DNA (Prihantoko *et al.*, 2020).

The lowest ROS level of 39.29±6.02% in this study was observed in the 500ppm extract treatment group, which is lower than 59.75%reported by Kumaresan *et al.* (2017) in bulls with average fertility. This reduction may result from the antioxidant effect of *S. ilicifolium* extract, which supports endogenous antioxidant enzymes in controlling ROS during freezing-thawing (Susilowati *et al.*, 2020). According to Wang *et al.* (2020), oral administration of fucoxanthin extract from brown macroalgae at 100, 200, and 500 mg/kg protected sperm mitochondrial membranes, thereby decreasing ROS levels in hamsters. Furthermore, fucoxanthin significantly reduced H_2O_2 and O_2^- levels in sperm by inhibiting NADPH oxidase-4 (NOX-4), an enzyme responsible for ROS generation (Kong *et al.*, 2019).

The lowest MDA level of 120.16±13.22ng/mL in this study was recorded in the 1000ppm extract treatment group compared to the control and treatment groups ($P<0.05$). All groups supplemented with *S. ilicifolium* extract had lower MDA levels than those in the control group. El-Khawagah *et al.* (2020) also showed that quercetin supplementation reduced MDA levels compared to the control in human, goat, rabbit, and pig sperm. Oral fucoxanthin supplementation in hamsters also lowered sperm, plasma, and testicular MDA levels (Kong *et al.*, 2019; Wang *et al.*, 2020). According to Batubara *et al.* (2016), the antioxidant activity in brown macroalgae depends on the harvest season, with summer-harvested *Sargassum* sp. (April) contained higher antioxidant levels than those of rainy season (October) harvests. Other bioactive antioxidant compounds in brown macroalgae include flavonoids and phenolic compounds. Flavonoids and phlorotannins possess multiple phenolic rings, each with hydroxyl (OH) groups and conjugated double bonds that are critical for scavenging free radicals, particularly hydroxyl and peroxyl (ROO) radicals. These compounds donate hydrogen atoms from phenolic OH groups to free radicals, forming less reactive flavonoid phenoxyl radicals (FIO). The conjugated double bonds and resonance-stabilized structures of flavonoid and phlorotannin phenoxyl radicals allow electron delocalization and free radical neutralization (Batubara *et al.*, 2016).

The highest mean PRM-1 protein level of 0.90±0.02ng/mL in this study was recorded in the 1000ppm treatment group, with significant differences ($P<0.05$) between the control and treatment groups. A significant negative correlation ($r=-0.871$; $P<0.01$) was recorded between DNA fragmentation and PRM-1 levels, which is consistent with the findings of Dogan *et al.* (2015) and Handarini *et al.* (2024), who also reported negative correlation between DNA fragmentation and protamine-deficient sperm populations in bovines. The freeze–thaw process can disrupt the disulfide bridge bonds in protamines, leading to increased DNA damage (Hitit *et al.*, 2020). PRM-1 plays a key role in chromatin condensation, which is essential for normal sperm function. Abnormal PRM expression has been associated with reduced sperm count, motility, and morphology, as well as increased chromatin damage (Pardede *et al.*, 2020). Protamine binds to and compacts sperm DNA, protecting it from oxidative stress. However, the disruption of disulfide bonds during the freeze–thaw process compromises the protective function of protamine (Susilowati *et al.*, 2022). In this study, supplementation with the brown macroalgae (*Sargassum ilicifolium*) extract improved PRM-1 protein levels. According to Riwanti *et al.* (2024), the phenolic compounds present in *Sargassum ilicifolium* neutralize or inhibit free radicals by transferring hydrogen atoms from hydroxyl groups through a mechanism involving hydrogen atom donation from phenol to free radicals. In addition, Gazali *et al.* (2019) stated that certain phenolic compounds can also act as electron donors. Flavonoids are another group of bioactive antioxidant compounds present in brown macroalgae. These flavonoids are polyphenolic compounds composed of 15 carbon atoms arranged in a C6–C3–C6 configuration, consisting of two C6 units (benzene rings) connected by a three-carbon aliphatic chain (C3). The antioxidant activity of flavonoids is associated with their distinct chemical structures, including hydroxyl groups, ortho-dihydroxy substitution on the B-ring, a double bond between C2=C3 in conjugation with a carbonyl group at position 4 on the C-ring, and O-methylation of the C-ring. Structural features associated with antioxidant activity include catechol groups, O-methylation, C2=C3 double bonds, 3-hydroxyl groups, and 4-carbonyl groups (Platzer *et al.*, 2022). Macroalgae contain bioactive components with potentially high antioxidant properties owing to the presence of up to eight interconnected polyphenol rings (Debnath *et al.*, 2020).

Conclusions: This study demonstrated that semen samples frozen in extenders supplemented with different concentrations of *Sargassum ilicifolium* extract exhibited decreased spermatozoa damage, as indicated by better sperm motility, viability, membrane integrity parameters, and reduced DNA fragmentation due to lower ROS production compared to the control group. The best results in terms of sperm motility, viability, membrane integrity, and lower DNA fragmentation were observed in the 1000ppm treatment group. ROS levels in the treatment groups were associated with lower MDA and increased PRM-1 levels. Moreover, DNA fragmentation was inversely related to PRM-1 levels. Overall, the best results across multiple parameters were observed in the 500–1000ppm extract treatment range. Further studies are needed to fully explore the potential of *S. ilicifolium* extract in maintaining semen quality during the freeze–thaw process.

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REFERENCES

Arguelles EDLR, 2021. Evaluation of antioxidant capacity, tyrosinase inhibition and antibacterial activities of brown seaweed, *Sargassum ilicifolium (Turner) c. Agardh* 1820 for cosmeceutical application. J Fish Environ 45(1): 64-77.

Arguelles EDLR and Sapin AB, 2022. Bioactive properties and therapeutic potential of *Padina australis Hauck (Dictyotaceae, Ochrophyta)*. Int J Agri Technol 18(1): 13-34.

Badr M, Rawash Z, Azab A, *et al*., 2021. *Spirulina platensis* extract addition to semen extender enhances cryotolerance and fertilizing potentials of buffalo bull spermatozoa. Anim Reprod 18(2): e20200520.

Batool I, Fayyaz MH, Hameed, *et al*., 2024. Quercetin in semen extender improves frozen-thawed spermatozoa quality and *in-vivo* fertility in crossbred Kamori goats. Front Vet Sci 11: 1385642.

Batubara L, Kristina TN and Rachmawati B, 2016. Effectiveness of brown algae extract to reduce serum malondialdehyde and protein carbonyl levels in *Streptozotocin*-induced Sprague Dawley rats. Sains Med J Kedokt dan Kesehat 7(2): 43-48.

Bilodeau JF, Chatterjee S, Sirard MA, *et al*., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev 55(3): 282-88.

Begum R, Howlader S, Mamun-Or-Rashid ANM, *et al*., 2021. Antioxidant and signal-modulating effects of brown seaweed-derived compounds against oxidative stress-associated pathology. Oxid Med Cell Longev 2021(1): 9974890.

Debnath T, Kim EK, Lee KG, *et al*., 2020. Antioxidant compounds from marine seaweeds and their mechanism of action. J Marine Res 78(2): 131-48.

Dogan S, Vargovic P, Oliveira R, *et al*., 2015. Sperm Protamine-status correlates to the fertility of breeding bulls. Biol Reprod 92(4): 92. doi: 10.1095/biolreprod.114.124255.

El-Khawagah AR, Kandiel MM and Samir H, 2020. Effect of quercetin supplementation in extender on sperm kinematics, extracellular enzymes release, and oxidative stress of egyptian buffalo bulls frozen–thawed semen. Front Vet Sci 7: 604460.

El-Sheshtawy RI and El-Nattat WS, 2018. Effect of tris-extender supplemented with various concentrations of strawberry (*Fragaria spp*.) on bull semen preservability. Asian Pac J Reprod 7(2): 93-96.

Fatima S, Alwaznah R, Aljuraiban GS, *et al*, 2020. Effect of seminal redox status on lipid peroxidation, apoptosis and DNA fragmentation in spermatozoa of infertile Saudi males. Saudi Med J 41(3): 238.

Gangwar C, Saxena A, Patel A, *et al*., 2018. Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. Anim Reprod Sci 192: 171-78.

Gazali M, Zamani NP and Nurjanah N, 2019. The potency of green algae *Chaetomorpha crassa Agardh* as antioxidant agent from the coastal of Lhok Bubon, West Aceh. IOP Conf Ser Earth Environ Sci 278(1): 012029; doi: 10.1088/1755-1315/278/1/012029.

Goshme S, Asfaw T, Demiss C, *et al*., 2021. Evaluation of motility and morphology of frozen bull semen under different thawing methods used for artificial insemination in North Shewa zone, Ethiopia. Heliyon 7(10): e08183; doi: 10.1016/j.heliyon.2021.e08183.

Hameed N, Akhter S, Souza-Fabjan JMG, *et al*., 2024. Effects of different extenders, storage temperatures, and antioxidant supplementation on chilled semen quality: A review. Trop Anim Health Prod 56(2): 85: doi: 10.1007/s11250-024-03930-2.

Handarini R, Baharun A, Rahmi A, *et al*., 2024. Correlation of sperm motility, acrosome integrity, protamine deficiency, and DNA fragmentation in proven and unproven Friesian Holstein bulls. J Adv Vet Anim Res 11(3): 796.

Handayani E, Supriatna I, Tumbelaka LI, *et al.,* 2021. Analisis komparatif kualitas semen beku yang telah dan belum bersertifikasi standar nasional Indonesia (comparative analysis of quality SNI certified and non SNI certified frozen semen). J Veteriner 22(2): 207-15.

Hezavehei M, Sharafi M, Kouchesfahani HM, *et al*., 2018. Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. Reprod Biomed Online 37(3): 327-39.

Hitit M, Ugur MR, Dinh TTN, *et al*., 2020. Cellular and functional physiopathology of bull sperm with altered sperm freezability. Front Vet Sci 7: 581137.

Imani M, Talebi AR, Fesahat F, *et al*., 2021. Sperm parameters, DNA integrity, and protamine expression in patients with type II diabetes mellitus. J Obstet Gynaecol 41(3): 439-46.

Ismaya I, 2017. Bioteknologi Inseminasi Buatan Pada Sapi dan Kerbau (Biotechnology of Artificial Insemination on Cattle and Buffaloes). Gadjah Mada University Press, Yogyakarta, Indonesia; pp:53-60.

Kong ZL, Sudirman S, Hsu YC, *et al*., 2019. Fucoxanthin-rich brown algae extract improves male reproductive function on streptozotocin-nicotinamide-induced diabetic rat model. Int J Mol Sci 20(18): 4485.

Kritaniya D, Yadav S, Swain DK, *et al*., 2020. Freezing-thawing induces deprotamination, cryocapacitation-associated changes, DNA fragmentation, and reduced progesterone sensitivity in buck spermatozoa. Anim Reprod Sci 223:106628.

Kumaresan A, Johannisson A, Al-Essawe EM, *et al*., 2017. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above-and below-average fertility bulls. J Dairy Sci 100(7):5824-5836.

Lutfia FNL, Isnansetyo A, Susidarti R, *et al*., 2020. Chemical composition diversity of fucoidans isolated from three tropical brown seaweeds (*Phaeophyceae*) species. Biodiversitas 21(7): 3170-77.

Pardede BP, Agil M and Supriatna I, 2020. Protamine and other proteins in sperm and seminal plasma as molecular markers of bull fertility. Vet World 13(3): 556.

Peris-Frau P, Soler AJ, Iniesta-Cuerda M, *et al*., 2020. Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. Int J Mol Sci 21(8): 2781.

Platzer M, Kiese S, Tybussek T, *et al*., 2022. Radical scavenging mechanisms of phenolic compounds: A quantitative structure-property relationship (QSPR) study. Front Nutr 9: 882458.

Prihantoko KD, Kusumawati A, Widayati DT, *et al*., 2020. Effects of storage duration on mitochondrial activity and DNA fragmentation of post-thawed spermatozoa from several Ongole grade bulls in Indonesia. Vet Pract 21(2): 264-268.

Prihantoko KD, Arif M, Kusumawati A, *et al*., 2022. Evaluation of sperm DNA fragmentation using TUNEL assay in different animal species. Adv. Anim. Vet. Sci 10(1): 14-19.

Ribas-Maynou J, Delgado-Bermúdez A, Mateo-Otero, *et al*., 2022. Determination of double-and single-stranded DNA breaks in bovine sperm is predictive of their fertilizing capacity. J Anim Sci Biotechnol 13(1): 105.

Riwanti P, Mahmiah M and Juniar K, 2024. Antioxidant activity of brown algae extract (*Sargassum sp*): a review. Sci Midwifery 11(6): 962-970.

Saberivand A, Sarvarzadeh F, Peighambarzadeh SZ, *et al,* 2022. The effect of *Caulerpa Sertularioides* extract on bull sperm freezability and subsequent embryo development. Theriogenology 189: 167-176.

Susanto E, Fahmi AS, Agustini TW, *et al*., 2017. Effects of different heat processing on fucoxanthin, antioxidant activity and colour of Indonesian brown seaweeds. IOP Conf Ser Earth Environ Sci 55(1): 012063.

Susilowati S, Sardjito T, Mustofa I, *et al*., 2020. Effect of green tea extract in extender of Simmental bull semen on pregnancy rate of recipients. Anim Biosci 34(2): 198.

Susilowati S, Mustofa I, Wurlina W, *et al*., 2022. Green tea extract in the extender improved the post-thawed semen quality and decreased amino acid mutation of Kacang buck sperm. Vet Sci 9(8): 403.

Tuncer PB, Sariözkan S, Bucak MN, *et al*., 2021. Antioxidant supplementation ameliorates bull sperm parameters and fertilizing ability following the freeze-thaw process. Turk J Vet Anim Sci 45(3): 457-462.

Upadhyay VR, Ramesh V, Dewry RK, *et al*., 2021. Implications of cryopreservation on structural and functional attributes of bovine spermatozoa: an overview. Andrologia 53(8): e14154.

Wang PT, Sudirman S, Hsieh MC, *et al*., 2020. Oral supplementation of fucoxanthin-rich brown algae extract ameliorates cisplatin-induced testicular damage in hamsters. Biomed Pharmacother 125: 109992.

Yánez-Ortiz I, Catalán J, Rodríguez-Gil JE, *et al*., 2022. Advances in sperm cryopreservation in farm animals: cattle, horse, pig and sheep. Anim Reprod Sci 246: 106904.

Yoon SJ, Kwon WS, Rahman MS, *et al*., 2015. A novel approach to identifying physical markers of cryo-damage in bull spermatozoa. PLoS One 10(5): e0126232.