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Supplementation of cryodiluent medium with sulfated polysaccharides from green seaweeds in the freezing of *Colossoma macropomum* semen

Suplementação do meio criodiluidor com polissacarídeos sulfatados de macroalgas verdes na congelação do sêmen de *Colossoma macropomum*

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Highlights

Low concentrations of sulfated polysaccharides are recommended.
Sulfated polysaccharides maintained total motility of *C. macropomum* spermatozoa.
Sulfated polysaccharides maintain sperm membrane integrity of *C. macropomum.*

Abstract

This study proposes to investigate the addition of sulfated polysaccharides (SP) extracted from two species of green seaweeds, *Ulva lactuca* and *Caulerpa racemosa*, to *Colossoma macropomum* semen cryodiluent medium. Four concentrations of SP (1.0, 2.0, 3.0, or 4.0 mg mL^{-1}) of each seaweed were evaluated. Semen was collected during the month of September in Fortaleza - CE, Brazil. Fresh semen samples were analyzed for the parameters of total sperm motility, curvilinear velocity (VCL), straight line velocity (VSL), average

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path velocity (VAP), sperm morphology, membrane integrity, and DNA integrity. Then, the samples were cryopreserved in freezing medium containing 10% dimethyl sulfoxide (DMSO) + 5% glucose, which was supplemented with different concentrations of SP. An unsupplemented treatment was used as control. After 15 days, they were thawed in a water bath at 45 ºC for eight seconds and the same analyses of fresh semen were performed. Statistical analysis revealed that there were no significant differences ($p > 0.05$) between the different tested concentrations of SP for any of the evaluated parameters. Compared with the control, there was no difference in concentrations ($p > 0.05$) for total motility; however, for VCL, VSL, and VAP, the *U. lactuca* concentrations of 3.0 and 4.0 mg mL^{-1} were detrimental ($p < 0.05$). The same was observed with 4.0 mg mL^{-1} of *C. racemosa* for VSL and VAP. In terms of morphology, 1.0 and 4.0 mg mL^{-1} of *C. racemosa* reduced normal sperm ($p < 0.05$), whereas for the other concentrations there was no difference ($p > 0.05$). All concentrations of both seaweeds maintained plasma membrane integrity ($p > 0.05$). As for DNA integrity, only 4.0 mg mL^{-1} of *U. lactuca* produced lower results than the control ($p < 0.05$), whereas the other concentrations maintained the number of spermatozoa with intact DNA ($p > 0.05$). Based on the results, higher concentrations of SP are harmful to tambaqui sperm in the freezing medium, whereas lower concentrations maintain sperm parameters. Further research is warranted to better investigate the antioxidant potential of these polymers in cryodiluent medium for *C. macropomum* as well as other fish species.
Key words: Antioxidant. Cryopreservation. Glycans. Spermatozoa. Tambaqui.

Resumo

O estudo teve como objetivo avaliar a adição de polissacarídeos sulfatados (PS) extraídos de duas espécies de macroalgas verdes, *Ulva lactuca* e *Caulerpa racemosa*, no meio criodiluidor do sêmen de Colossoma macropomum. Para isso, foram avaliadas quatro concentrações de PS (1,0; 2,0; 3,0 ou 4,0 mg mL^{-1}), de cada macroalga. A coleta de sêmen foi realizada durante o mês de setembro, em Fortaleza, Ceará, Brasil. As amostras de sêmen fresco foram analisadas quanto aos parâmetros de motilidade total dos espermatozoides, velocidade curvilinear (VCL), velocidade em linha reta (VSL), velocidade média do trajeto (VAP), morfologia espermática, integridade de membrana e integridade de DNA. Em seguida, foram criopreservadas em meio de congelação contendo dimetilsulfóxido (DMSO) 10% + glicose 5%, e suplementadas com as diferentes concentrações de PS, tendo ainda um tratamento não suplementado como controle. Após 15 dias, foram descongeladas em banho-maria a 45 ºC por oito segundos, e as mesmas análises do sêmen fresco foram realizadas. Através da análise estatística, os resultados mostraram que não houveram diferenças significativas ($p > 0,05$) entre as diferentes concentrações de PS testadas para nenhum dos parâmetros avaliados. Já em relação ao controle, não houve diferença nas concentrações ($p > 0,05$) para a motilidade total, no entanto, para VCL, VSL e VAP, as concentrações de 3,0 e 4,0 mg mL^{-1} de *U. lactuca* foram prejudiciais ($p < 0,05$). O mesmo foi observado em 4,0 mg mL^{-1} de C. racemosa para VSL e VAP. Para a morfologia, 1,0 e 4,0 mg mL^{-1} de *C. racemosa* reduziram os espermatozoides normais ($p < 0,05$), enquanto para as demais concentrações não houve diferença ($p > 0,05$). Para todas as concentrações de ambas as macroalgas, a integridade de membrana plasmática foi mantida ($p > 0,05$). Quanto à integridade do DNA, apenas 4,0 mg mL^{-1} de *U. lactuca* foi inferior ao controle ($p < 0,05$), enquanto as demais concentrações mantiveram o número de espermatozoides com DNA íntegro ($p > 0,05$). De acordo com os resultados obtidos, concentrações mais elevadas de PS são

prejudiciais aos espermatozoides de tambaqui no meio de congelação, enquanto concentrações mais baixas mantiveram parâmetros espermáticos. Estudos posteriores são indicados para melhor avaliar o potencial antioxidante destes polímeros no meio criodiluidor do sêmen de *C. macropomum*, bem como de outras espécies de peixes.

Palavras-chave: Antioxidante. Criopreservação. Espermatozoides. Glicanos. Tambaqui.

Introduction

Colossoma macropomum (Curvier, 1816), popularly known as tambaqui, is a rheophilic fish of the order Characiformes that is native to the Amazon Basin. In addition to having meat that is appreciated in the market and possessing high commercial value, the species displays adaptability to the captive environment, good feed acceptance, and hardiness (Pereira et al., 2020; Garcez et al., 2021). In 2021, tambaqui led the production of native fish in Brazil, representing 31.2% of national production (Brazilian Association of Pisciculture [PeixeBr], 2022). The growing market demand has driven an increasing interest in research on this species (Sousa et al., 2016). One of the most important among such demands is the development of reproductive biotechnologies such as semen cryopreservation, which aims to preserve, protect, and maintain the genetic material of species with economic potential through the development of germplasm banks (Medina-Robles et al., 2019).

Cryopreservation of fish semen carries benefits such as the elimination of problems caused by asynchronous gonadal maturity between males and females; simplification of broodstock management at different times; ease in the transport of genetic material between fish farms; conservation of gametes from selected animals in breeding programs; and establishment of hybridization programs (Maria & Carneiro, 2012). However, despite the advantages shown by this biotechnique, sperm cells subjected to the freezing process suffer damage that significantly reduces their viability (Silva & Guerra, 2012). An example of this damage is the formation of intracellular ice crystals, which results in rupture of the plasma membrane due to thermal, mechanical, chemical, and osmotic stress exerted on the cell during freezing (Salmito-Vanderley et al., 2012). In addition, there may be toxicity of the cryoprotectant solution used or sperm sensitivity to reactive oxygen species (ROS) at high concentrations, generating oxidative stress (Palhares et al., 2021).

Oxidative stress configures an imbalance between oxidant and antioxidant substances in favor of oxidative agents, which causes disruption of redox signaling and molecular damage (Sies, 2015). Additionally, the presence of polyunsaturated fatty acids in large amounts in the plasma membranes of these cells makes them more susceptible to damage caused by ROS, especially lipid peroxidation (Martínez-Páramo et al., 2012). Thus, oxidative stress can lead to sperm dysfunctions such as reduced motility, DNA damage, as well as decreased cell viability and functionality (Félix et al., 2021).

Despite the various natural antioxidants present in seminal plasma, which are responsible for the protection of the spermatozoa, semen must be diluted in a cryoprotectant solution

during cryopreservation, which reduces the concentration of these antioxidants and favors imbalance, compromising the structural integrity and function of sperm (Silva & Guerra, 2012; Martínez-Páramo et al., 2012). Therefore, to mitigate the damage caused by ROS, substances with antioxidant potential have been added to the semen freezing media of several fish species, including *Rhamdia quelen* (Xavier et al., 2021), *Brycon orbignyanus* (Palhares et al., 2021), *Prochilodus brevis* (Almeida-Monteiro et al., 2017), *Cyprinus carpio* (Linhares et al., 2017), and *C. macropomum* (Lopes et al., 2018).

Among the additives with antioxidant potential for semen are sulfated polysaccharides (SP), polymers formed by repetitive units of sugars that are negatively charged due to the presence of sulfate radicals (Anand et al., 2016). Sulfated polysaccharides are found in several species of animals, plants, algae, bacteria, fungi, and other microorganisms (Negreiros et al., 2015). Because the chemical structure of these polymers varies according to the specific source in which they are found, they can exist in different forms, with changes in the nature of their components (Vasconcelos et al., 2015). Nonetheless, only marine seaweeds can synthesize SP in large amounts and with high iron chelating activity, which makes them potential sources of SP chelants and consequently, potential antioxidants (Presa et al., 2018).

Sulfated polysaccharides can appear in very heterogeneous ways in different seaweed species depending on the arrangement of their monomeric chains, which suggest particular interactions in the different biotechnological processes to which they can be applied (Rodrigues et al., 2012). In green seaweeds (Chlorophyta), these polymers may be constituted by different monosaccharides, and for the genus *Caulerpa,* these SP are generally sulfated galactans whose main sugar is galactose, whereas xylose, glucose, and mannose are other common components (Wang et al., 2014). In Ulvales species, the observed SP are composed of rhamnose, xylose, glucose, uronic acid, and sulfates (Yaich et al., 2015).

Previous studies examined different applications of these biomolecules, e.g. the antitumor, anticoagulant, antithrombotic, antinociceptive, anti-inflammatory, antiparasitic, and antioxidant potential of seaweeds of the genera *Caulerpa* and *Ulva* (Guidara et al., 2021; Barcellos, et al., 2018; Rodrigues et al., 2012; Lehnhardt-Pires et al., 2013; Tian et al., 2019). Studies on their application in semen freezing medium are still scarce (Pereira et al., 2020); however, SP are suggested to have a strong ability to eliminate ROS, possibly holding the potential to improve the quality of thawed semen (Pereira et al., 2020). In this scenario, the present study was undertaken to evaluate the use of different concentrations of sulfated polysaccharides extracted from *Caulerpa racemosa* and *Ulva lactuca* in the supplementation of tambaqui semen cryodiluent medium.

Material and Methods

The experiment was conducted in the Marine Biochemistry Laboratory at the Pici *campus* of the Federal University of Ceará (UFC); and in the Fish Reproduction Biotechnology Laboratory at the Itaperi *campus* of the State University of Ceará (UECE).

Algae collection and extraction of sulfated polysaccharides

The green seaweeds *C. racemosa* and *U. lactuca* were collected at Flecheiras beach, in Trairí - CE, located in the Brazilian northeast. A sample of each alga was deposited in the Prisco Bezerra Herbarium at the Department of Biological Sciences at UFC, Brazil. The seaweeds were taken to the Marine Biochemistry Laboratory at the Center for Biotechnology Applied to Aquaculture of the Department of Fishery Engineering at UFC for the extraction of sulfated polysaccharides.

The seaweeds were separated from the epiphytes and then dehydrated at room temperature. The sulfated polysaccharides were obtained following Farias et al. (2000). Briefly, a 5-g sample of each seaweed tissue was cut into small pieces, suspended in 100 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 510 mg of papain, 5 mM EDTA, and 5 mM, and incubated at 60 °C for 24 h. The incubation mixture was then filtered and the supernatant saved.

The SP present in the solution were precipitated with 16 mL of a 10% cetylpyridinium chloride solution at room temperature for 24 h. The mixture was then centrifuged at 9,560 × g for 20 min. Next, the sediment containing the SP was washed with 100 mL of a 0.05% cetylpyridinium chloride solution, dissolved in 100 mL of a 2M NaCl:ethanol (100:15) solution, and precipitated for 24 h at 4 °C with 100 mL of commercial ethanol. The obtained precipitate was centrifuged (9,560 × g for 20 min) and washed twice with 100 mL of 80% ethanol and once with the same volume of commercial ethanol. Finally, the SP were dried in a forced-air oven (60 °C, 6 h) and yield was expressed as a percentage (%) of dry matter.

All extracted SP were freeze-dried. After extraction, the SP were diluted in distilled water and subsequently aliquoted into the cryopreservation medium in a representative amount of each concentration used in this experiment, namely, 1.0, 2.0, 3.0, or 4.0 mg mL^{-1}.

Experimental animals and semen collection

The study was submitted to and approved by the Animal Use Ethics Committee at UECE (approval no. 09279405/2021). The experiment was carried out in Fortaleza - CE, Brazil.

The experimental animals were acquired from the LBRP-UECE herd, kept at the Center for Biotechnology Applied to Aquaculture at UFC. They consisted of 30 *C. macropomum* males with an average weight of 5 kg that exhibited traits indicative of reproductive maturity, such as a hyperemic urogenital papilla and the release of a detectable amount of semen under mild abdominal pressure (Oliveira et al., 2016).

Initially, the animals were hormonally induced to spermiation by applying carp pituitary extract (CPE), at a rate of 2.5 mg kg^{-1} of live weight, via the intracoelomic route. Semen was collected after 14 h of application. For this, the animals were sedated in a Eugenol solution (Sigma-Aldrich®), at a ratio of 1:10:10000 (eugenol:alcohol:water), until they showed loss of equilibrium. Twenty-five animals showed release of semen, which was thus collected in graduated polyethylene tubes through abdominal

massage in the craniocaudal direction. The material was then transported in thermal boxes to the Laboratory of Fish Reproduction Biotechnology at UECE, where the samples were analyzed for the formation of semen pools and, later, cryopreserved. To this end, the fresh semen of each animal was analyzed individually for sperm motility, and samples that showed motility below 30% were discarded. Subsequently, five pools were formed (n=3); afterwards, the fresh semen was further analyzed for sperm kinetics, morphology, membrane integrity, and DNA integrity.

Semen cryopreservation and thawing

The *pools* (n = 5) were diluted (1:9, sperm:diluent) and frozen in a solution of 5% glucose (diluent) and DMSO (10% dimethyl sulfoxide (cryoprotectant) that was supplemented with different concentrations (1.0, 2.0, 3.0, or 4.0 mg mL^{-1}) of SP from the green seaweeds *C. racemosa* or *U. lactuca.* An unsupplemented solution was used as a control.

Subsequently, the samples were filled into 0.25-mL French straws, which were sealed with polyvinyl alcohol and left to equilibrate for 10 min at approximately 10 ºC. Afterwards, the straws were transferred to the *dry shipper,* where they remained for 15 min for freezing in nitrogen vapor (-170 ºC), and then stored in liquid nitrogen cylinders (-196 ºC). After 15 days, the samples were thawed by immersion in a water bath at 45 ºC for eight seconds. Thawed semen was evaluated for sperm kinetics, morphology, membrane integrity, and DNA integrity.

Fresh and thawed sperm analysis

Sperm kinetics was evaluated based on the analysis of the parameters of total motility (%), curvilinear velocity (VCL, μm s^{-1}), straight line velocity (VSL, μm s^{-1}), and average path velocity (VAP, μm s^{-1}). The analyses were carried out using the Computer-Assisted Sperm Analysis System (CASA) in Sperm Class Analyzer software (SCA, Microptics®), adopting the settings indicated for fish. For this, 5 μL of semen were pipetted over a Makler chamber, activated with 100 μL of NaCl (125 mM), and immediately subjected to analysis.

For the analysis of sperm morphology, the semen was fixed in a formalinized 4% citrate solution (10:100; semen:fixative) and then stained with Rose Bengal at a ratio of 1:10 (dye:fixed semen). Two slides were prepared per pool using smears, and 100 spermatozoa were evaluated per slide with an optical microscope (400x). Sperm cells were classified according to Miliorini et al. (2011) with adaptations.

The plasma membrane integrity of the spermatozoa was evaluated by the eosin-nigrosin staining method. One slide was prepared per pool at a 1:2:2 ratio (semen:eosin:nigrosin), and 10 μL of this mixture were used for the preparation slides, by the smear method. Using a light microscope (400x), 200 spermatozoa were analyzed per slide and considered to have an intact membrane when they were colorless, or with a ruptured membrane when stained pink or red, according to the method by Nunes et al. (2016).

DNA integrity analysis was performed using the SCD (Sperm Chromatin Dispersion) test, based on the sperm chromatin

fragmentation rate, according to Fernandez et al. (2005) and following adaptations by Almeida-Monteiro et al. (2020). Two and five microliters of fresh and thawed semen, respectively, were diluted in 1.5 mL of phosphate-saline buffer (PBS) and kept in a water bath at 37 °C until later use. Then, 25 μL of semen-PBS solution were mixed with 50 μL of low-molecular-weight agarose (Sigma-Aldrich, St Louis, MO, USA) and 2-μL aliquots of this mixture were deposited in each of 10 spots on a slide previously prepared with ultrapure agarose (NA Agarose; Sigma-Aldrich, St Louis, MO, USA). Then, the slides were placed on a metallic surface cooled at 4 °C for 5 min. Subsequently, they were subjected to baths in different solutions: acid solution (hydrochloric acid (HCl) and Milli-Q water, for 7 min); lysis solution (sodium chloride (NaCl), sodium dodecyl sulfate (SDS), triton X, ethylene diamine tetraacetic acid (EDTA), β-mercaptoethanol, Tris-HCL solution, and distilled water, for 25 min); distilled water (for 5 min); and 70% alcohol, 90% alcohol, and absolute alcohol, respectively (for 2 min each). After the bathing procedure, the samples were stained with a panoptic kit (RenyLab Chemical and Pharmaceutical Ltd., Barbacena County, MG, Brazil); each slide was immersed in each dye for 10, 20, and 20 s, respectively. Finally, the slides were washed in distilled water and dried at room temperature. Then, 200 spermatozoa were evaluated per pool using a camera-coupled phase-contrast microscope (200; Nikon Eclipse 50i, Tokyo, Japan) to investigate the incidence of halo around the sperm head. In this analysis, cells with an external halo indicated sperm chromatin dispersion (intact DNA), whereas cells without a halo indicated fragmentation of sperm DNA.

Statistical analysis

All data are expressed as mean ± standard error of the mean, and the significance level adopted was $P < 0.05$. For statistical analyses, R statistical language environment software (R Core Team [R], 2021) was used. Because they are proportion measures, the variables of total motility (%), membrane integrity (%), normal morphology (%), and DNA integrity (%) were converted by an arcsine square root function, according to the equation $y = \sin^{-1}\sqrt{x} \times 180/\pi$. The other variables, namely, VAP (μm s^{-1}), VCL (μm s^{-1}), and VSL (μm s^{-1}), were not transformed. Analysis of variance (ANOVA) was performed using the MASS package (Venables & Ripley, 2002) followed by a post-hoc test to evaluate the significant mean difference by Tukey's test, using the *agricolae* package (Mendiburu & Yaseen, 2020) and Dunnett's test to compare the treatments with the control using the *DescTools* package.

Results and Discussion

Fresh semen

The total sperm motility rate of fresh semen was 24.4 ± 3.4%. VCL, VSL, and VAP were 29.4 ± 5.3, 14.1 ± 4.8, and 20.6 ± 5.8, respectively. In morphology, the normal spermatozoa averaged 67.4 ± 3.3%, while membrane integrity averaged 66.9 ± 3.0% and DNA integrity 82.2 ± 2.7%. In the present study, semen collection took place during the month of September, a period outside the reproductive season for this species, which generally occurs between the months of November and April. For this reason, the

results of the parameters evaluated in the still-fresh semen are presented with a percentage relatively below the average, when compared with those described by other authors (Oliveira et al., 2016; Pereira et al., 2021). However, a previous study carried out in the same region on the seasonality of sperm characteristics of tambaqui semen found that despite a variation between the months of the year, this fact does not preclude the use of semen from this species throughout the year, especially under hormonal induction (Vieira et al., 2011).

Thawed semen

In the present study, the different tested concentrations of SP from the green seaweeds U. lactuca and C. racemosa did not differ significantly ($p > 0.05$) for any of the evaluated parameters. When we compare the different SP concentrations of the two seaweeds with the control treatment, composed only of 10% DMSO and 5% glucose, no significant differences were detected ($p > 0.05$) for total sperm motility (Table 1).

Table 1
Kinetic parameters of *C. macropomum* sperm cryopreserved in freezing medium supplemented with SP from green seaweeds at different concentrations (n = *5 pools*)

Sulfated polysaccharides (mg mL^{-1})	Motility (%)	VCL (µm s^{-1})	VSL (µm s^{-1})	VAP (µm s^{-1})
Control	14.2 ± 1.1	20.1 ± 1.3	5.6 ± 1.2	9.7 ± 1.5
U. lactuca 1.0	13.2 ± 1.1	17.5 ± 0.8	3.2 ± 1.0	7.0 ± 0.9
U. lactuca 2.0	14.0 ± 0.4	17.2 ± 1.1	3.0 ± 1.0	6.6 ± 1.1
U. lactuca 3.0	13.8 ± 0.9	**15.8 ± 0.3**	**1.8 ± 0.2**	**5.1 ± 0.4**
U. lactuca 4.0	12.6 ± 1.4	**16.0 ±0.5**	**2.0 ± 0.4**	**5.1 ± 0.5**
C. racemosa 1.0	15.2 ± 0.9	17.2 ± 0.5	2.9 ± 0.8	6.3 ± 0.8
C. racemosa 2.0	14.8 ± 0.9	16.8 ± 0.4	2.5 ± 0.4	**5.8 ± 0.5**
C. racemosa 3.0	13.4 ± 1.0	18.2 ± 0.9	3.4 ± 1.0	6.8 ± 1.1
C. racemosa 4.0	16.2 ± 1.2	17.9 ± 1.4	**2.2 ± 0.3**	**5.8 ± 0.7**

Data expressed as mean ± standard error. Statistical significance level of P > 0.05.
Values in bold represent a significant difference compared with the control (P > 0.05).

However, for the other kinetic parameters (VCL, VSL, and VAP), the *U. lactuca* SP concentrations of 3.0 and 4.0 mg mL^{-1} showed inferior results to the control treatment ($p < 0.05$), whereas at the lower concentrations (1.0 and 2.0 mg mL^{-1}) it exhibited no differences ($p > 0.05$). For SP extracted from *C. racemosa,* the concentrations of 2.0 and 4.0 mg mL^{-1} showed a lower VAP than the control treatment ($p < 0.05$), whereas the other concentrations did not differ ($p > 0.05$). At the concentration of 4.0 mg mL^{-1}, VSL was lower than in the control treatment ($p < 0.05$) (Table 1).

In terms of sperm kinetics, therefore, higher concentrations (3.0 and 4.0 mg mL^{-1}) of *U. lactuca* SP considerably reduced the post-thawing velocities of *C. macropomum* sperm. This finding may indicate a low affinity of tambaqui spermatozoa with the SP of this seaweed species, or even a negative effect of SP on sperm cells. According to Lopes et al. (2018), an excess of substances with antioxidant action can lead to increased oxidative stress and inhibit important physiological functions of ROS. This corroborates the assertions of Félix et al. (2021) and Salas-Huetos et al. (2019), who described the occurrence of oxidative stress caused by the exacerbated administration of antioxidants as an "antioxidant paradox".

Considering the SP from *C. racemosa,* changes in VSL occurred at the highest concentration (4.0 mg mL^{-1}), similar to what was found in *U. lactuca*, which possibly indicates the action of the antioxidant paradox. As for VAP, the variation found at the lower concentrations (2.0 and 4.0 mg mL^{-1}) may be because, among the CASA parameters, this velocity is the one that most varies between biological samples, which is possibly due to characteristics inherent to each animal (Sanches et al., 2013).

Despite the large number of studies that evaluated the different applications of SP, its use in the composition of cryodiluent medium for the sperm of Characiformes is still recent. In a study with *P. brevis* using different concentrations of SP from green and red seaweeds (*Ulva fasciata* and *Gracilaria domingensis*, respectively), the concentration of 1.0 mg mL^{-1} was superior, regardless of the seaweeds, for all sperm velocities (Nascimento, 2021). In *C. macropomum,* SP from green and red seaweeds (*Caulerpa cupressoides, Solieria filiformes,* and *Acantophora muscoides*) were added at concentrations lower than those in the present study and no differences were observed for motility, VCL, VSL, or VAP (Pereira et al., 2021).

Among the structural integrity parameters of spermatozoa, sperm morphology is directly related to the fertilizing capacity of these gametes and, as such, it is fundamental for the assessment of semen quality (Leite et al., 2018). In the present study, when the *C. racemosa* concentrations of 1.0 and 4.0 mg mL^{-1} were used, sperm morphology revealed a lower percentage of normal spermatozoa than the control treatment ($p < 0.05$) whereas for the other treatments there were no significant differences ($p > 0.05$) compared with the control (Table 2). The fact that 1.0 mg mL^{-1} also reduced the number of normal spermatozoa may indicate that the lowest concentration of this seaweed was insufficient to preserve normal sperm morphology; the highest concentration, on the other hand, was possibly toxic. Additionally, this may be related to the high variability of the biological samples used, which can interfere even after the semen is distributed in pools (Lopes et al., 2018).

Table 2

Structural integrity parameters of *C. macropomum* sperm cryopreserved in freezing medium supplemented with SP from green seaweeds at different concentrations (n = 5 *pools*)

Sulfated polysaccharides (mg mL^{-1})	Normal morphology (%)	Membrane integrity (%)	DNA integrity (%)
Control	61.4 ± 2.7	64.9 ± 1.7	87.6 ± 3.2
U. lactuca 1.0	54.1 ± 3.7	59.5 ± 2.2	80.2 ± 6.6
U. lactuca 2.0	59.3 ± 5.5	61.8 ± 2.1	79.0 ± 5.8
U. lactuca 3.0	50.4 ± 5.1	61.4 ± 4.5	82.4 ± 4.2
U. lactuca 4.0	43.6 ± 4.0	62.9 ± 2.1	**59.2 ± 10.3**
C. racemosa 1.0	**42.7 ± 7.8**	65.0 ± 2.1	76.8 ± 12.7
C. racemosa 2.0	50.5 ± 3.7	60.9 ± 1.7	78.4 ± 4.9
C. racemosa 3.0	48.1 ± 4.0	64.3 ± 2.4	83.2 ± 4.7
C. racemosa 4.0	**41.0 ± 3.6**	62.3 ± 2.2	90.4 ± 1.0

Data expressed as mean ± standard error. Statistical significance level of P > 0.05.
Values in bold represent a significant difference compared with the control (P > 0.05).

Regarding sperm plasma membrane integrity, there were no significant differences ($p > 0.05$) for any of the seaweed SP concentrations used between treatments or in comparison with the control (Table 2). The results obtained for this parameter were satisfactory, which indicates the absence of detrimental effects of SP on sperm membranes. The plasma membrane can be largely affected by the attack of ROS, which lead to a reduction in its fluidity and make the sperm susceptible to damage such as phospholipid peroxidation, protein oxidation, damage to the midpiece, in addition to mitochondrial deficiencies and DNA fragmentation (Costa & Streit Jr., 2019; Félix et al., 2021).

With regard to sperm DNA integrity, the *U. lactuca* SP concentration of 4.0 mg mL^{-1} showed a lower percentage of spermatozoa with intact DNA than control treatment ($p < 0.05$). There were no significant differences for the other concentrations (Table 2), reinforcing the results discussed for sperm kinetics in the present study. DNA integrity is a crucial factor for germ cells, especially spermatozoa, since their DNA does not have the same repair mechanisms found in oogenesis, which makes them more likely to suffer injuries. Moreover, sperm chromatin is very sensitive to the stress generated by several factors that can increase oxidative stress (Fernández-Díez et al., 2016).

The deleterious effects observed with the addition of higher concentrations of SP on some sperm parameters of *C. macropomum* in the present study indicate a possible toxicity of these molecules in excess. Besides the concentration, a good interaction must be achieved between the semen and the type of SP, since the characteristics of these compounds change depending on the degree of sulfation, position of the sulfate, and composition of its sugars (Jiménez-Escrig et

al., 2012). Another aspect to be considered is the presence of sulfur in the composition of these polysaccharides, since this element can decrease the pH of the freezing medium (Muthmainnah et al., 2018). In addition to the plurality in the composition and action of these molecules, fish semen is assumed to respond in a species-specific manner to the composition of the freezing media. Therefore, different diluents and antioxidant substances act better in certain species than in others, thus warranting studies on the seminal characteristics of each species (Salmito-Vanderley et al., 2012, 2016).

Nevertheless, the maintenance of some sperm parameters at lower concentrations indicates a potential of SP to be exploited, since polymers of biological origin are easily found in nature, in addition to being biocompatible, non-toxic, and having an affordable cost, as mentioned by Pires et al. (2015). Moreover, environmental changes are believed to limit the reproductive success of most fish species in Brazil, which follow an annual cyclical reproductive behavior (Leite et al., 2018), suggesting a need for further studies on *C. macropomum* semen using SP in the cryodiluent medium with collection performed during the breeding season.

Conclusions

The results regarding supplementation of *C. macropomum* semen cryodiluent medium with sulfated polysaccharides extracted from *C. racemosa* and *U. lactuca* showed variations in the concentrations used in comparison with the control. Higher concentrations, especially 4.0 mg mL^{-1}, are not indicated due to the probable toxicity caused in sperm cells. As observed for the parameters of kinetics, membrane integrity, and DNA integrity, the concentration of 1.0 mg mL^{-1} of both seaweeds is viable for tambaqui semen, which suggests the need for further research during the breeding season, using other lower concentrations as well as SP from other seaweed species. Furthermore, the use of these SP in the present study opens up possibilities for the application of these polymers in cryodiluent media for semen of other fish species, given the current scarcity of studies with this purpose.

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