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Melatonin production improves Senegalese sole sperm motility at night, but fails as a supplement during cryopreservation

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ABSTRACT

Melatonin is a powerful antioxidant present in fish seminal plasma. This study aimed to understand melatonin's endogenous and exogenous effects on first-generation Senegalese sole sperm quality for sperm management applications. In the first experiment, samples were collected at mid-light (ML) and mid-dark (MD) daytimes, to evaluate the effects on sperm motility. In a second experiment, using confocal microscopy and melatonin-FITC, spermatozoa permeability to melatonin was evaluated and, after showing that it enters the nucleus and mitochondria by passive diffusion, exogenous melatonin toxicity and antioxidant potential during a cryopreservation assay were performed. The toxicity assay tested different melatonin concentrations (0.01, 0.1, 1, and 10 mM) and exposure times (3, 5, 15 and 30 min), and sperm motility parameters were measured (TM, PM, VCL, VSL, LIN) using CASA system. The best conditions (0.1 and 10 mM) were selected for the cryopreservation assay, and a set of post-thaw sperm quality analyses were performed (motility, viability, reactive oxygen species, lipid peroxidation, and DNA fragmentation). The motility analyzed at ML and MD showed significant differences in all parameters, mainly on velocities (VCL, VSL, VAP), that were significantly higher at MD. Supplemented melatonin did not influence spermatozoa motility, MDA content or DNA fragmentation, although a lower percentage of viable cells was obtained on the 10 mM treatment. Altogether, Senegalese sole spermatozoa motility was enhanced at night, putatively by endogenous melatonin through direct or indirect mechanisms, whereas supplemented melatonin did not confer extra protection during cryopreservation.

1. Introduction

Senegalese sole (*Solea senegalensis*) is a species produced in aquaculture farms in the Iberian Peninsula [17], but has reproductive constraints when males are born and raised in captivity (G1, first-generation animals) [13]. In this species, gonadal maturation and spermatogenesis occur through a semi-cystic and asynchronous process [14], but spermiation in G1 males is compromised having this males low sperm density, volume and quality [32], which ultimately affect fertilization success [12]. Moreover, typical courtship behaviour is also affected [18]. Despite decades of research, the causes of such reproductive dysfunction are still under study. To overcome this obstacle, artificial fertilization and sperm cryopreservation are being used by the industry because they 1) solve the female and male gametes release asynchrony, and 2) allow the usage of quality selected G1 sperm material. However, the cryopreservation protocol to store G1 Senegalese sole sperm needs to be optimized, since the only published protocol was performed with sperm from wild males [40], which has higher sperm volume, quality and, consequently, higher cryo-resistance.

Melatonin is a multifunctional molecule believed to be present in all living organisms [38]. It is known to be produced at night by the pineal organ [1], but other extra-pineal locations have been discovered to also produce melatonin, contributing to its increasing circulating levels at night [16]. In mammals, melatonin exhibits hormonal, immunological, anti-inflammatory and antioxidant properties [27,44,48]. In fish, melatonin is involved in the regulation of important physiological processes [15,35] and, in seasonal breeding species, it triggers reproduction and maturation in both female and male gonads [26,43]. However, high variability in melatonin response can be found in the literature [3].

Moreover, as a natural multipotent antioxidant compound [39], melatonin has been used in a different range of applications, such as nutrition, reproduction, immunology, and toxicology [1,36,49]. In *in vivo* experiments with fish, the animals are normally exposed to melatonin via oral administration [6], injections [9,31], implants [3]

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or diluted in water [25]. Recently, melatonin has been tested as an antioxidant supplement also in *in vitro* studies, like cryopreservation [21]. Its ability to scavenge free radicals, modulate antioxidant enzymatic activity and detoxify cells at the mitochondrial level is well documented in the literature, including in fish studies [2,26]. As far as we know, in fish, the only published study evaluating the impacts of endogenously produced melatonin on fish sperm quality is from our group [19]. According to their results, endogenously produced melatonin at night could be a natural way of improving G1 Senegalese sole sperm resistance to the damage provoked by the cryopreservation procedure. Besides, cryopreservation is a technique that, despite its practical advantages, exposes the cells to oxidative stress through the freezing and thawing processes [28], reason why some antioxidant additives are normally employed in the protocol. Apart from that, melatonin proved to be a suitable additive for sperm cryopreservation of several species, such as bull [4,7], ram [42], rooster [34], chicken [5,47] and rabbit [50]. More recently, melatonin also started to be tested as a supplement during sperm cryopreservation in fish species, like in seabream [19] and the freshwater species *Prochilodus lineatus* [33], *Brycon orbignyanus* [37] and *Prochilodus brevis* [45]. The results of melatonin action in protecting and increasing spermatozoa quality have been very divergent, not only because it depends on the administration method, melatonin concentration and time of exposure, but also because it acts in a species-specific manner [21].

In this study, we aimed to understand both the *in vivo* effects of day/night changes in G1 Senegalese sole sperm quality, and the *in vitro* effects of the exogenous melatonin supplemented during sperm cryopreservation. Based on the natural melatonin production by the fish at night, the known presence of melatonin in Senegalese sole seminal plasma and its ability to scavenge free radicals [20], we hypothesize that melatonin can act as an antioxidant during cryopreservation and improve the viability and quality of G1 Senegalese sole spermatozoa after thawing.

2. Materials and methods

2.1. Ethic statement

All the experimental procedures followed the ARRIVE guidelines, directives 86/609/EU and 2010/63/EU of the European Parliament and Council, and the Portuguese legislation for the use of laboratory animals (PORT 1005/92) from the *Direção Geral de Alimentação e Veterinária* (DGAV) attested with a specific approvement reference (003289) for experiments with germ cells. Additionally, all technicians and researchers participating in animal handling and sampling hold a FELASA B or C category certification, recognized by DGAV. This declaration testifies that all animals used in the current investigation were maintained in accordance with the best practices.

2.2. Broodstock management and samplings

Fish were acquired from IFAPA *El Toruño* (Cádiz, Spain) and properly transported to the University of Algarve research facilities at *Ramalhete* research station (Faro, Portugal). After quarantine, the fish with approximately 900 ± 216 g of body weight were distributed into 6 fibber-glass tanks of 5.89 m^3 and kept in a semi-open water system, six months before the onset of the experiments. Each tank contained 16–18 animals established at a density lower than 5 kg/m^2. The broodstock was kept under natural light: dark (LD) photoperiod oscillations of 12L:12D (March) to 15L:9D (July), with a 2:1 ratio of male to female, necessary for the reproductive experiments with this species. Fish were sampled for sperm collection from March to July, during two consecutive breeding seasons, coinciding with temperatures ranging from 16.7 to 22.3 °C in the first year, and from 16.4 to 20.4 °C in the second one. In each breeding season, fish were sampled every 15 days, so they could recover from stress caused by handling. For sperm collection, males were anesthetized with 300 ppm phenoxyethanol, and genital pore was cleaned with PBS (a neutral saline solution) and dried-out to ensure that all anesthesia was removed, avoiding sperm contamination. The collected samples were kept at 10 °C and immediately taken to the laboratory for sperm quality analysis.

2.3. In vivo experiment: effects of endogenous melatonin on sperm motility

In the first breeding season, from April to July, samples were collected at two different moments of the day, mid-light (ML, n = 42) and mid-dark (MD, n = 36), to assess the impact of the endogenously produced melatonin on sperm motility. Fish were randomly selected in both moments of the day.

2.4. In vitro experiment: melatonin supplementation

In the second year, the permeability of the spermatozoa membrane to melatonin was evaluated. Afterwards, from March to June, sole samplings were performed during the day, always at mid-light, so that endogenous melatonin production at night would not interfere with the results from the *in vitro* assays. Samples were collected for the melatonin toxicity test, using different exposure times and concentrations, so we could predict possible toxicity and/or beneficial effects on reproductive cells, and for the cryopreservation assays.

2.4.1. Confocal microscopy

To see if melatonin was able to move across the spermatozoa membrane, melatonin FITC conjugated was used in confocal microscopy. The final molecule had a molecular weight of 548.6 g/mol and a purity degree of 94.1 %. A sperm sample was centrifuged (1200 g, 1 min, 4 °C) to remove any interfering substances from seminal plasma, and cells were resuspended in 270 mOsm/Kg Ringer solution (40.23 mM KCl, 111.22 mM NaCl, 2.7 mM CaCl2, 2.38 mM NaHCO3) to get a final concentration of 1-2 x 106 cells/mL. After, the sperm sample was incubated at 4 °C with 2 μM melatonin-FITC, 1 μM MitoTracker Deep Red (Invitrogen), and 2 μM 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, 1:1000). After incubation (10 min), the sperm sample was washed (1200 g, 4 °C, 1 min) and resuspended in Ringer solution. Finally, 30 μL of the resuspended cells were placed in a glass slide with a cover slide and sealed with transparent polish. Once the preparation was dried, the stained sample was observed on the confocal microscope (LSM 800, Zeiss). Different images were recorded with Z-stack and taken with 100x objective under previously optimized settings (pinhole of 1, gain voltage of 750 V, scan speed 6).

2.4.2. Melatonin toxicity test

For the toxicity test, individual sperm samples (n = 11) were diluted according to the dilution rate 1:2 (v/v) in an extender solution containing 10 % dimethyl sulfoxide (DMSO) in 1 % NaCl medium (control), and in extender solutions with different concentrations of melatonin (0.01, 0.1, 1, and 10 mM, from Sigma-Aldrich). All the solutions were prepared using the same control solution on the day of analysis and covered with aluminum foil, due to rapid melatonin degradation and sensitivity to light. Using the CASA software (see 2.5.1 section), sperm motility parameters [total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight-line velocity (VSL), and linearity (LIN)] were evaluated at 15 s (s) post-activation, after 3, 5, 15 and 30 min of equilibration time, for each melatonin concentration tested. The best conditions were chosen for the cryopreservation assays.

2.4.3. Sperm cryopreservation

The cryopreservation assays were performed according to the protocol established by Riesco et al. [40]. The best melatonin concentrations from the first trial (0.1 and 10 mM) were used as a supplement to the cryoprotectant media (10 % DMSO + 10 % Egg Yolk). Sperm samples were collected as previously described and pooled using samples

from 10 to 14 males for each pool (n = 11). After, sperm samples were diluted in the respective solutions (control, 0.1 mM, and 10 mM melatonin) and loaded into 250 μL French straws along the 2 min of equilibration time. The straws were immediately placed on a horizontal rack 2 cm above liquid nitrogen, for 10 min, and then submerged in liquid nitrogen. After cryopreservation, samples were kept in a nitrogen container until thawing. Before sperm analysis, the thawing process was performed in a water bath at 25 °C for 15 s.

2.5. Sperm quality analysis

2.5.1. Spermatozoa motility analysis

For motility analysis, sperm samples were activated by mixing 1 μL of sperm in 10 μL of artificial seawater (pH 8.2). The activation was performed in a Makler chamber using a phase contrast microscope (Nikon 200) with a 10x negative contrast objective connected to a digital camera (ISAS 782M, Proiser R + D, S.L.) set for 25 frames per second (fps). Sperm motility was analyzed after activation using a computer assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser R + D, S.L.). For experiment 1, sperm motility was recorded at 15, 30, 45, and 60 s post-activation; and for experiment 2, sperm motility was characterized at 15 s post-activation. In all experiments, the parameters monitored were spermatozoa total motility (TM, %), spermatozoa moving in a progressive manner - progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s) and linearity (LIN, %). Only samples or pools with total motility above 40 % were selected to be used in the experiments.

2.5.2. Cell viability and reactive oxygen species (ROS)

For the analysis performed using flow cytometry, the Senegalese sole spermatozoa detection settings were previously adjusted considering cell size (forward scatter – FSC) and granularity (side scatter – SSC), defining the cell gate in the cytometer. Cell viability analysis was performed by staining post-thaw sperm samples with propidium iodide (PI) (Sigma-Aldrich) in the following proportions: 500 μL of 1 % NaCl solution, 3 μL of thawed sperm, and 1 μL of PI (1 mg/mL). After the incubation for 5 min in the dark, the fluorescence emitted by the samples was measured in a flow cytometer (BD FACSCalibur™, BD Biosciences, CA) equipped with a 488 nm laser for PI detection with a 585/42 nm filter (FL2). The ROS detection was performed by combining post-thaw sperm with dihydroethidium (DHE) and SYTOX® green (Invitrogen™, ThermoFisher) in the following proportions: 500 μL of 1 % NaCl solution, 3 μL of thawed sperm, and 0.5 μL of DHE (0.5 mM). After 5 min of DHE incubation, 0.5 μL of SYTOX® green (1 μM) was added. The samples were analyzed after 10 min of total incubation time in the dark in a flow cytometer equipped with a 488 nm laser for SYTOX® green detection with a 530/30 nm filter (FL1) and for DHE detection with a 585/40 nm filter (FL2). For both techniques, the collection of data was performed using the BD CellQuest Pro software (version 8.7, BD Biosciences, CA), and a total of 30.000 events were collected from each sample (n = 11 pools). For cell viability, the CellQuest software determined the percentage of non-viable cells, and thus viable cell determination was calculated by subtracting this value from the sperm population. For ROS determination, the software determined the percentage of non-viable cells, non-viable cells producing ROS, live cells producing ROS, and live cells. Only the subpopulation of live cells producing ROS was considered to infer about ROS production.

2.5.3. Lipid peroxidation

Lipid peroxidation was assessed by quantifying the concentration of malondialdehyde (MDA) through a colorimetric assay (kit BIOXYTECH LPO-586™, OxisResearch) and following the manufacturer protocol with small adjustments in terms of volumes, according to the protocol described by Riesco et al. [40]. The absorbance was read in a microplate reader (Synergy 4, Biotek Instruments. Inc.) at 586 nm, and MDA concentration was determined from a standard curve calculation. The results were presented in nanomoles of MDA per million spermatozoa (n = 11 pools).

2.5.4. DNA fragmentation

Comet assay was performed to detect DNA fragmentation in spermatozoa, following the protocol described by Cabrita et al. [10] and adapted by Riesco et al. [40] for Senegalese sole. For comets observation, 10 μL of diluted PI (20 μM) was placed in each sample and covered with a coverslip. A total of 11 pools for each treatment were analyzed by observing the slides in a fluorescence microscope (Nikon Eclipse E200). Images were captured and recorded with a digital camera (VisiCam 5 Plus, VWR), and at least 100 cells per slide were analyzed using imaging Komet v6.0 software (Andor Technology, Ltd.). The percentage of tail DNA (tDNA) was the parameter used to determine the amount of DNA fragmentation since it is related to both the amount and size of the DNA fragments.

2.6. Statistical analysis

After being logarithmic or arcsine square-root transformed, data were statistically analyzed using IBM SPSS statistics v26 software. To the motility data from the endogenous melatonin assay, comparing ML and MD moments, a *t*-Student test was applied. Data from the toxicity test was analyzed using a two-way ANOVA, followed by the *post hoc* test Student-Newman-Keuls (SNK) to evaluate exposure time, melatonin concentration, and interaction between effects on sperm motility. Data from the cryopreservation assay was analyzed by a Two-way ANOVA followed by SNK, for the motility data, and for the other quality assays a one-way ANOVA, followed by SNK, was applied to verify statistical differences within treatments. Differences were considered significant when $p < 0.05$ and significant outliers were removed (extreme outlier was considered when a sample was on the 1st or 3rd quartile ±3 x interquartile range).

3. Results

3.1. In vivo experiment: night effect on sperm motility

The results from motility analysis between samples collected at both moments of the day revealed that the moment of the day for collection of samples and the time post-activation of sperm had a significant effect, without interaction, in all motility parameters, with exception for TM (two-way ANOVA, $p < 0.05$) (Supplementary Table S1). Regarding the influence of the moment of the day, at each post-activation point, significant differences (*t*-Student, $p < 0.05$) were found in all the motility parameters analyzed (Fig. 1). The TM and LIN were significantly higher at MD (16 % and 60 %, respectively) than at ML (9 % and 53 %, respectively) only at 60 s post-activation (Fig. 1A and C). After sperm activation, PM was also generally higher at MD, but only significant at 45 s (MD: 8 %, ML: 4 %) and 60 s (MD: 4 %, ML: 1 %) post-activation (Fig. 1B). Moreover, all the spermatozoa velocities (VCL, VSL and VAP) were strongly influenced by the moment of the day, showing to be significantly higher at MD than ML from the beginning of activation until the 60 s post-activation (Fig. 1D, E and 1F).

3.2. In vitro experiment: melatonin permeability and toxicity test

The exposure of spermatozoa to melatonin-FITC revealed that melatonin enters the spermatozoa, since it crosses the plasma membrane, being homogeneously spread inside the nucleus and in the mitochondria area (Fig. 2). The original figure, edited on ZEN 3.4 software, is available in the supplementary material (Fig. S1).

Motility results from fresh sperm exposed to different melatonin concentrations and exposure times revealed that only the concentration factor had a significant effect (two-way ANOVA, SNK, $p < 0.05$) on

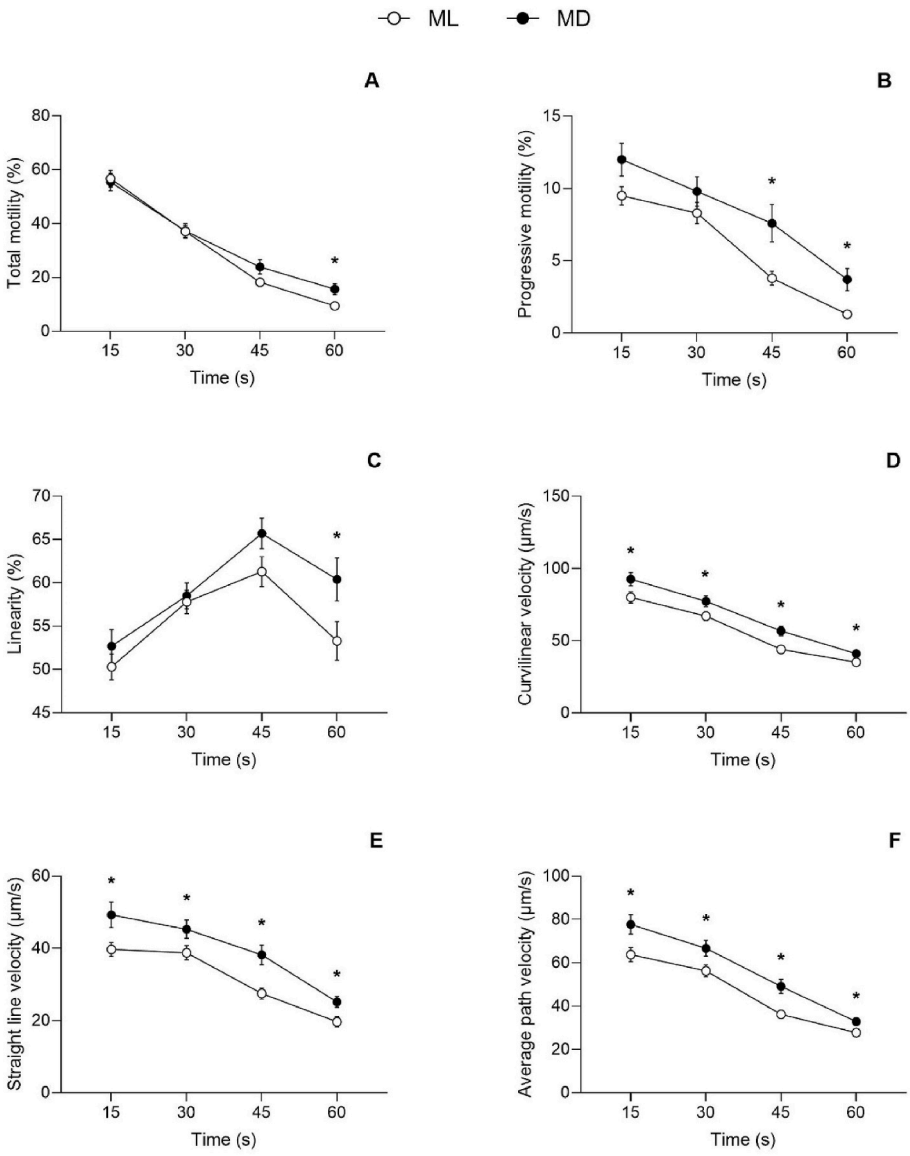

Fig. 1. Spermatozoa total motility (A), progressive motility (B), linearity (C), curvilinear velocity (D), straight-line velocity (E) and average path velocity (F) of G1 *S. senegalensis* samples collected at mid-light (white dots) and mid-dark (black dots), registered at 15, 30, 45 and 60 s post-activation. Results are expressed as mean ± std. error. Significant differences between ML (n = 42) and MD (n = 36) are identified with an asterisk (*) (Students' *t*-test, $p < 0.05$).

sperm motility parameters, specifically, on TM ($p = 0.005$) and PM ($p = 0.022$) (Supplementary Table S2). Regarding TM, the melatonin concentrations of 0.1 and 10 mM (both 34 %) did not differ from the control (43 %) in the percentage of motile cells (Fig. 3A). Similarly, PM did not differ from the control (7 %) in spermatozoa exposed to 0.1, 1 and 10 mM melatonin (6 %, 6 % and 8 %, respectively) (Fig. 3B).

Considering these results, both concentrations of 0.1 and 10 mM were chosen for cryopreservation experiments, since they did not differ from the control on TM and PM analysis. In addition, the fact that time of exposure to melatonin concentrations did not reveal any influence on the analyzed sperm, cryopreservation protocol was performed according to the protocol described previously by Riesco et al. [40].

3.3. *In vitro experiment: sperm cryopreservation with supplemented melatonin*

3.3.1. *Spermatozoa motility*

The motility registered in cryopreserved samples after thawing did not show significant differences (One-way ANOVA, $p < 0.05$) between treatments in any of the analyzed parameters (Fig. 4). The obtained TM was low (Ctr: 16 %, 0.1 mM: 11 %, 10 mM: 16 %), as PM and LIN (Fig. 4A). The same was observed on all the spermatozoa velocity parameters, showing no differences between treatments (VAP – Ctr: 43 µm/s, 0.1 mM: 49 µm/s and 10 mM: 54 µm/s) (Fig. 4B).

3.3.2. *Cellular and biochemical analysis*

The results from cellular and biochemical analysis demonstrated significant differences between melatonin concentrations (One-way ANOVA, SNK, $p < 0.05$) only on sperm viability, with the control treatment showing the highest percentage of viable cells (42 %), followed by the treatments 0.1 mM (38 %) and 10 mM (33 %) (Fig. 5A). On the reactive oxygen species assay, the percentages of live cells with ROS were very similar between all treatments: Ctr (37 %), 0.1 mM (38 %) and 10 mM (41 %) (Fig. 5B), as it happened on the measurement of MDA content, in which the control and 0.1 mM melatonin had the same concentration of MDA in their samples (136.8 and 136.9 nM/million spz) and 10 mM melatonin showed fewer MDA content (115.7 nM/million spz), even though without significant differences (Fig. 5C).

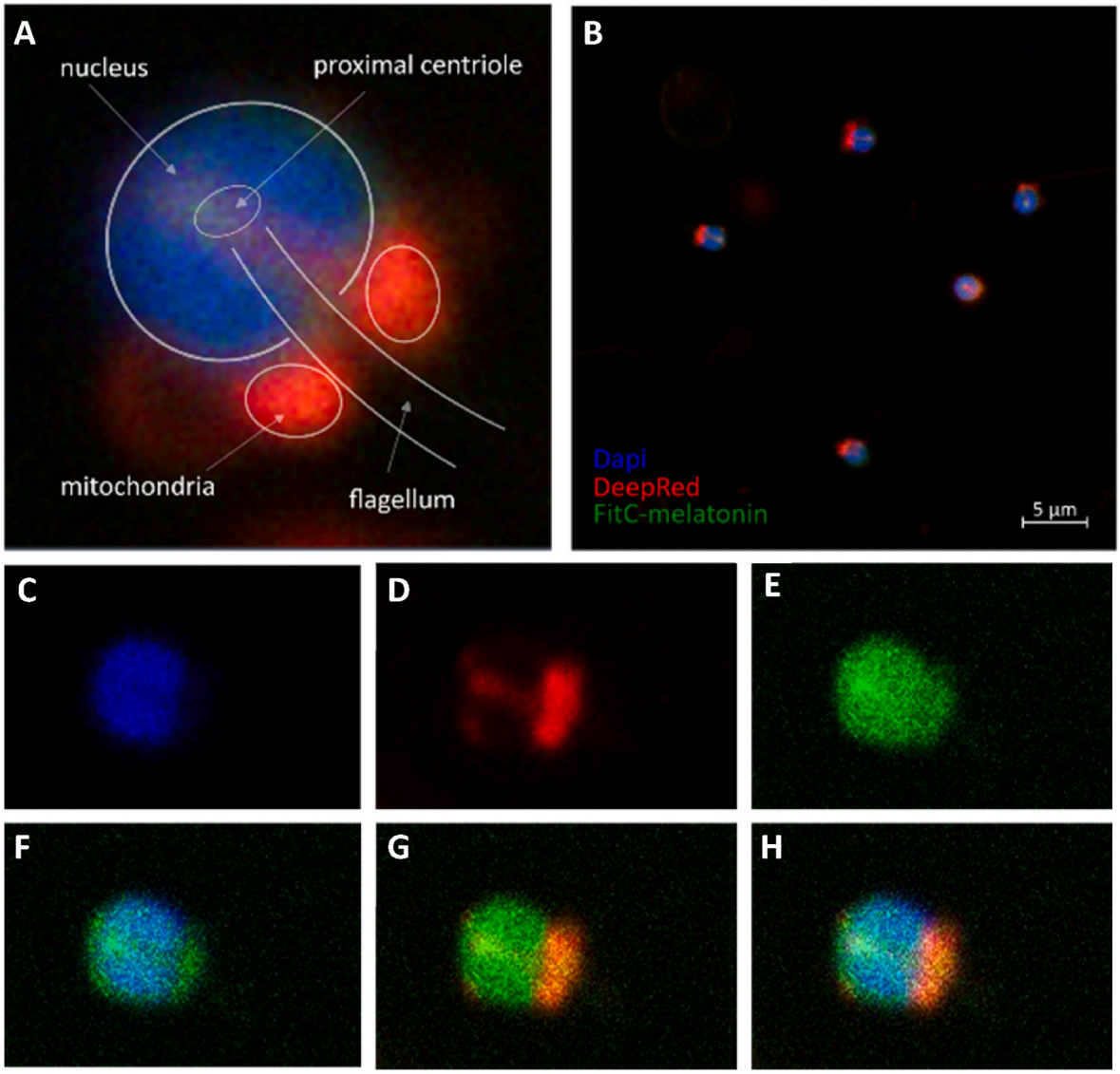

Fig. 2. Localization of intracellular melatonin in *S. senegalensis* spermatozoa using confocal microscopy. Diagram of stained Senegalese sole spermatozoa (A), and spermatozoa stained with DeepRed (red) for mitochondria identification, Dapi (blue) for nucleus and melatonin-FITC (green) for melatonin localization (B and H). For better visualization, zoomed images of spermatozoa stained with Dapi (C), DeepRed (D), melatonin-FITC (E), and combining Dapi and melatonin-FITC (F), DeepRed and melatonin-FITC (G), and all the three fluorophores (H) are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Regarding the Comet assay, tail DNA percentages obtained were similar between all treatments and the control (11.9 % on control, 10.9 % on 0.1 mM and 11.8 % on 10 mM) (Fig. 5D).

4. Discussion

Given that melatonin is a powerful antioxidant involved in many fish physiological processes, the present study aimed to comprehend the effects of day/night changes (led by endogenous melatonin production at night) and exogenous melatonin in G1 Senegalese sole reproduction, especially in terms of sperm quality and applicability as a supplement in cryopreservation. The objective of the first experiment was to understand if melatonin produced at night could directly or indirectly improve sperm quality, as suggested for other species such as gilthead seabream [19]. An improvement in some sperm quality parameters was observed in sperm motility assessed in samples collected at mid-dark (MD). Overall, the night effect had more impact on spermatozoa velocity parameters (VCL, VSL, VAP), once all parameters were significantly higher at MD throughout sperm movement (until 60 s). In a study performed on killifish (*Fundulus heteroclitus*) exposed via water to 1 μM melatonin for 8 days, it was found that sperm VCL, VSL and VAP were enhanced in comparison to the control group [25], which were the same velocity parameters enhanced in our experiment. Also, melatonin added to the cryoprotectant medium enhanced the same sperm velocity parameters of *Prochilodus brevis* fish after thaw [45]. Since melatonin is produced during the night and its blood concentration peaks at MD in this species [20], this indolamine may modulate spermatozoa motility parameters not only by exerting its antioxidant properties, as pointed out previously by our group, but also through the activation of 5' AMP-activated protein kinase (AMPK) phosphorylation, as described in the literature [41, 50], explaining the increased sperm kinetic parameters. This phenomenon was also described in gilthead seabream (*Sparus aurata*) sperm, in which endogenous melatonin is suggested to be responsible for the improvement of post-thaw TM, velocity parameters and increased cell concentration [19]. The moment of highest sperm motility for Senegalese sole is about 15 s post spermatozoa activation and, normally, it

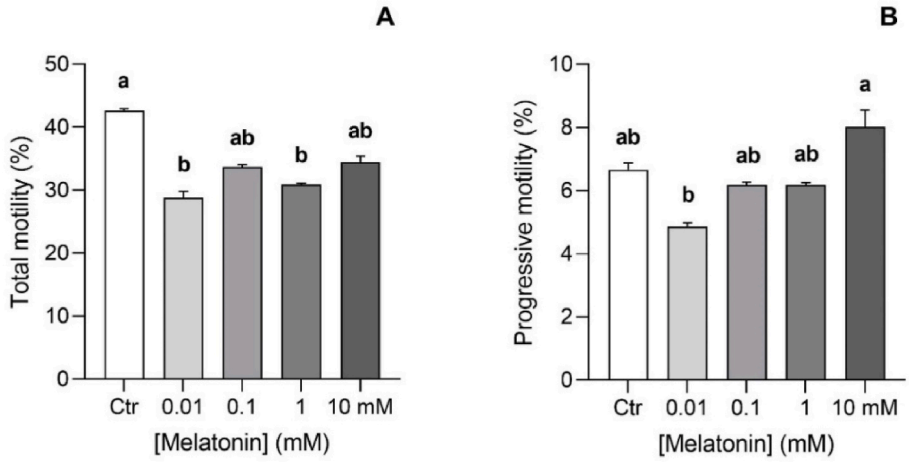

Fig. 3. Total motility (A) and Progressive motility (B) of *S. senegalensis* sperm (n = 11) exposed to different melatonin concentrations [control (Ctr), 0.01, 0.1, 1, and 10 mM melatonin], at 15 s post-activation. Results are expressed as mean ± std. error. Significant differences between treatments are identified with different letters (ANOVA, SNK, $p < 0.05$).

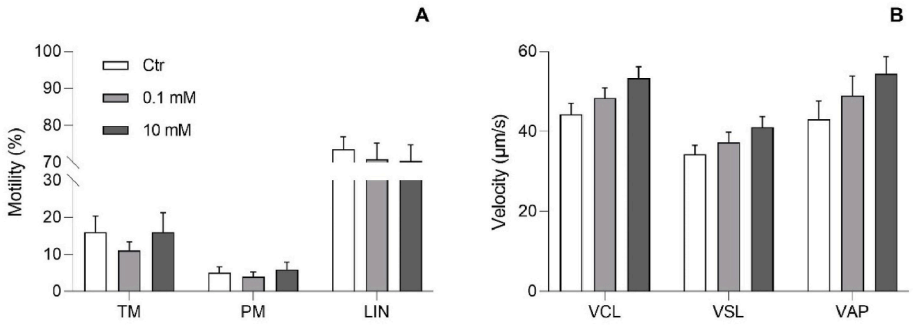

Fig. 4. Spermatozoa motility (A) and velocity (B) parameters of *S. senegalensis* G1 spermatozoa after thawing, at 15 s post-activation. Results from total motility (TM), progressive motility (PM), linearity (LIN), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) are expressed as mean ± std. error. No significant differences found (one-way ANOVA, SNK, $p < 0.05$).

lasts approximately 60 s with slow or vibrating movement, although in wild males it is described that it can reach 1.30 min [12]. Therefore, any improvement of sperm kinetic parameters can be crucial to attain fertilization. Our study also suggests that at night, when endogenous melatonin concentration is higher [20], spermatozoa had extra resistance, since not only kinetic parameters were improved but the percentage of total motile cells and progressive cells were enhanced at MD on the last recorded time-points (45 and 60 s), compared to the ML sampling. Similar effects were previously described in paddlefish (*Polyodon spathula*) sperm, in which 0.5 μM melatonin was enough to prolong spermatozoa motility after 24 h of storage at 4 °C [23]. Spermatozoa motility and velocity are important sperm quality biomarkers due to their direct correlation with gamete viability and consequent fertilization success [22]. In fact, in the above-mentioned study with killifish, spermatozoa from the fish group exposed to melatonin had not only the motility parameters enhanced, but also a higher fecundity ability [25]. Particularly in G1 Senegalese sole, it would be interesting to further explore the reported impact of melatonin in motility duration and if it could be transduced into higher fertilization success. Apart from that, it may exert an important effect on cryo-resistance since freezing higher-quality samples has been shown, specifically in fish, to promote higher cryopreservation success [11].

To understand the mechanism through which melatonin interacts with the spermatozoa, we further explored if melatonin could act inside the cell as a cryoprotectant, or if it would rather exert some external protection by linking with putative membrane receptors [30]. Confocal microscopy analysis revealed that melatonin can easily cross the spermatozoa plasma membrane, once it was possible to observe melatonin-FITC widely spread in the spermatozoa head and mitochondria area. In the present study, this imaging approach contributed to new knowledge about melatonin mechanisms of action in fish reproduction, proving that melatonin acts inside the cell, reaching its target through passive diffusion due to its lipophilic properties and low molecular weight [15]. Also, the fact that it strongly links to mitochondria, can help to explain the improvement in the fresh sperm velocity parameters. According to the literature, melatonin can protect mitochondria from ROS and reactive nitrogen species (RNS), improving electron transport chain activity (ETC) and reducing the damage in mitochondrial DNA (mtDNA) [39]. Through this process, melatonin boosts mitochondrial respiration and adenosine triphosphate (ATP) synthesis [24], essential for sperm movement. Further analysis of mitochondrial activity would be of interest to prove this mechanism of action in Senegalese sole spermatozoa supplemented with melatonin, either in short or long-term storage.

In production, it is difficult to coincide the daily practices, like gametes' collection and fertilization, with the biological rhythms of the animals without alterations in the daily photoperiod. For that reason, exogenous melatonin supplementation to sperm samples collected during the day may allow to mimic the natural increase of melatonin in the animal body at night. The sperm toxicity test revealed that only melatonin concentration had a significant effect on motility parameters, namely in TM and PM, and no effect of the exposure time was observed. From all concentrations tested, the lowest concentration (0.001 mM) showed the worst results compared to the control group. In addition, 0.1

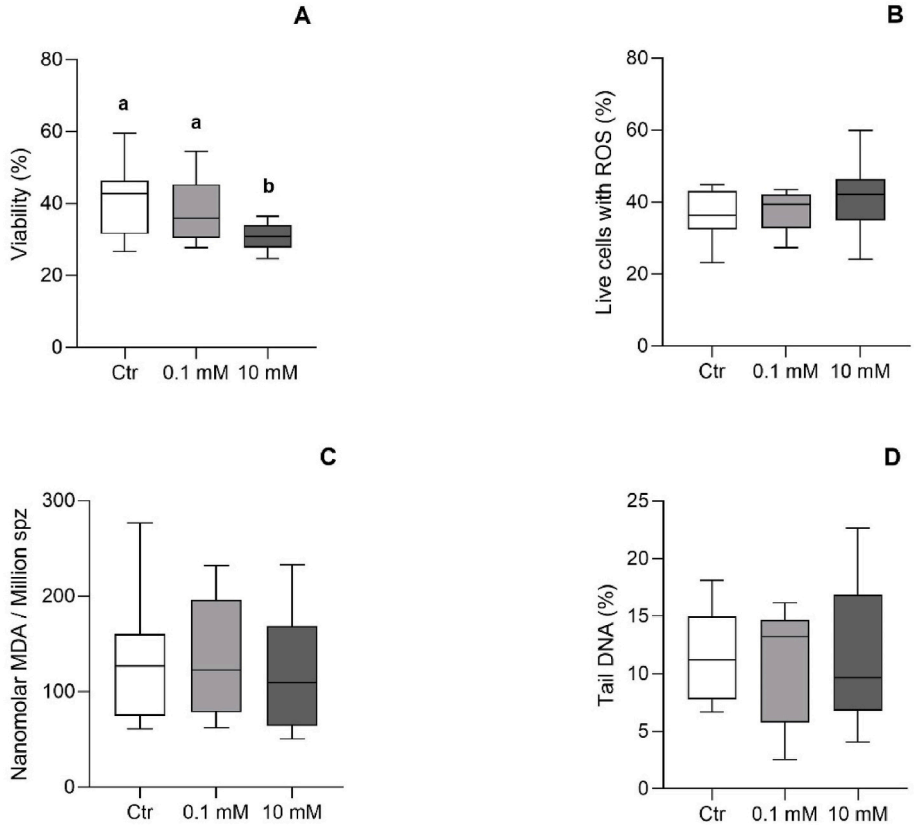

Fig. 5. Viability (A), ROS (B), lipid peroxidation (C) and DNA fragmentation (D) of *S. senegalensis* sperm cryopreserved with control (Ctr), 0.1 and 10 mM melatonin (n = 11). Results are expressed in box plots with max and min values. Statistical differences are identified with different letters (One-way ANOVA, SNK, $p < 0.05$).

and 10 mM melatonin did not differ from the control or showed even better results in progressive motility. Based on that, it was presumed that for Senegalese sole sperm, melatonin toxicity depends on the concentration used and, in an adequate proportion, it can have positive impacts on some sperm motility parameters. It is worth mentioning that melatonin effects are species-specific, as generally reported in the literature [1,16,20], thus the concentrations used need to be tested and adapted, via toxicity tests, considering the species under study. In fish, there are few studies about melatonin putative effects in fresh sperm. Besides the previously mentioned experiment in paddlefish sperm [23], in a study with *Capoeta trutta* sperm exposed to titanium dioxide nanoparticles (TiO2-NPs), the co-exposure of spermatozoa to 0.1 mM melatonin decreased the toxic effects of TiO2-NPs by improving spermatozoa velocity parameters [36].

Sperm cryopreservation is a tool for reproductive management in some species and would be of great importance to have an optimized protocol for G1 Senegalese sole. In this second experiment, several quality analyses were performed after sperm thawing to assess the effects of melatonin supplementation. The overall motility parameters displayed lower values in the cryopreservation assay, compared to fresh sperm. The percentage of TM obtained after thaw was considerably lower (approximately 18 %) than the TM of fresh samples (approximately 60 %). This can be explained by 1) the cryopreservation process itself, which diminishes sample quality due to water crystals formation and rearrangement during freezing and thawing processes, compromising cell structures, such as membranes and organelles [46]; or 2) the usage of a Senegalese sole G1 male broodstock, known for presenting lower sperm quality and poor resistance to artificial procedures when comparing with the wild breeders [12]. In fact, to the best of our knowledge, there is no other published cryopreservation assay with G1 Senegalese sole sperm quality analyses performed to compare our results. Still, in a previous study from our group with wild Senegalese sole, testing different cryoprotectants, the post-thaw total motility ranged from 30 to 60 % according to the cryoprotectant tested [40], being generally much higher than the percentage obtained in the present experiment with G1 males, which reinforces the need for optimizing sperm cryopreservation protocols for these animals. Regarding melatonin supplementation, it did not provide extra protection to the cryopreserved cells, neither in terms of motility or velocity, nor in terms of lipid peroxidation, ROS determination or DNA integrity. From all gamete quality analyses performed only cell viability showed significant differences, being lower in samples cryopreserved with 10 mM melatonin. Although in toxicity tests this concentration did not have negative impacts on the motility parameters, after cryopreservation it showed to negatively affect spermatozoa viability, reinforcing the importance of doing as much quality analysis as possible in fresh sperm. In other cryopreservation studies with supplemented melatonin, it also did not improve cell quality after sperm thawing. In a study with curimba (*Prochilodus lineatus*), a freshwater species, all the melatonin concentrations tested (1, 2 and 3 mM) showed to be inefficacious in cell protection against cryodamage [8], as it also happened with gilthead seabream sperm (0.001, 0.01 and 0.1 mM melatonin) [19]. In mammals, there are also examples of melatonin species-specificity and inefficacious during cryopreservation, as such for boar spermatozoa [29]. Although supplemented melatonin did not work as a cryoprotective agent, the fact that better sperm quality was observed in fresh sperm samples collected at MD leaves space for the industry to shift the animals' photoperiod to coincide the MD with their working hours, so sperm management procedures, such as cryopreservation, could benefit from this natural increase of melatonin and sperm quality. Moreover, we intend to encourage further research on cryopreservation at MD, because those samples collected at "shifted MD" could be more resistant to the cryopreservation process, and this would have even more economic impact on the industry, once it allows better broodstock management.

5. Conclusions

In this study, night-time (characterized by literature as the melatonin production time) and exposure to exogenous melatonin seemed to modulate spermatozoa motility of G1 Senegalese sole. The overall velocity motility parameters were enhanced at MD, coinciding with the endogenous melatonin peak for this species, which may act directly (through antioxidant properties) or indirectly (through activation of AMPK phosphorylation and mitochondrial activity) on fish sperm quality. On the other hand, in *in vitro* experiments, it was possible to observe on confocal microscopy that melatonin can easily cross the plasmatic membrane of spermatozoa by passive diffusion and spread through the cytoplasmatic and mitochondrial areas, wherein it may exert its antioxidant properties. Nonetheless, the exogenous melatonin influenced motility according to the concentration used, but melatonin supplemented in a cryoprotectant medium did not grant additional sperm protection against cryodamage.

CRediT authorship contribution statement

F. Félix: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **L. Ferrão:** Investigation, Formal analysis, Data curation. **V. Gallego:** Investigation, Formal analysis, Data curation. **C.C.V. Oliveira:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Conceptualization. **E. Cabrita:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have nothing to declare.

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Appendix A. Supplementary data

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