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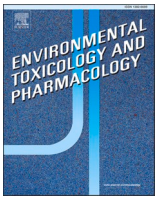

Silver nanoparticles and silver ions indistinguishably decrease sperm motility in Pacific oysters (*Magallana gigas*) after short-term direct exposure

Fátima Fernández-García [a], Ana Carvalhais [b], Ana Marques [b], Isabel B. Oliveira [c], Sofia Guilherme [b], Helena Oliveira [b], Catarina C.V. Oliveira [d], Elsa Cabrita [d], Juan F. Asturiano [a], Mário Pacheco [b], Cláudia Mieiro [b,*]

[a] Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Spain
[b] CESAM and Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
[c] CIIMAR, University of Porto, 4450-208 Matosinhos, Portugal
[d] CCMAR, University of Algarve, Campus Gambelas, 8005-139 Faro, Portugal

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ABSTRACT

The present study aimed to evaluate the reprotoxicity of environmental (0.25 μg.L^{-1}) and supra-environmental (25 μg.L^{-1} and 250 μg.L^{-1}) levels of silver nanoparticles (Ag NP) on the Pacific oyster (*Magallana gigas*), by determining sperm quality. For that, we evaluated sperm motility, mitochondrial function and oxidative stress. To determine whether the Ag toxicity was related to the NP or its dissociation into Ag ions (Ag^+), we tested the same concentrations of Ag^+. We observed no dose-dependent responses for Ag NP and Ag^+, and both impaired sperm motility indistinctly without affecting mitochondrial function or inducing membrane damage. We hypothesize that the toxicity of Ag NP is mainly due to adhesion to the sperm membrane. Blockade of membrane ion channels may also be a mechanism by which Ag NP and Ag^+ induce toxicity. The presence of Ag in the marine ecosystem is of environmental concern as it may affect reproduction in oysters.

1. Introduction

Reproduction is a key biological process that results in the generation of a new individual ensuring the continuum of the species. Reproductive features rely on several factors and lately there has been a growing awareness of this issue, particularly regarding factors affecting sperm competitiveness (Campbell et al., 2016; Kumar and Singh, 2022). Environmental factors (e.g. climate change and contamination) affect sperm performance and can shape sperm fitness (Marshall, 2006; Campbell et al., 2016; Kumar and Singh, 2022). This is particularly relevant for broadcast spawners since they release their gametes directly into the water where fertilization occurs (Lotterhos and Levitan, 2010). Most marine invertebrates are broadcast spawners and thus, recognized targets for environmental stressors with demonstrated harmful impacts on gamete quality and fertilization success (Au et al., 2001; Lewis and Ford, 2012; Esposito et al., 2020), even though without a wise understanding of the underlying toxic mechanisms.

Nanoparticles arise as emerging contaminants due to their wide distribution and release into the environment, with the marine environment constituting a sink. Silver nanoparticles (Ag NP) are widely used for their antimicrobial function in areas such as medicine (Islam et al., 2021), food, textile, cosmetics (Wei et al., 2023) and aquaculture (Abou-El-Sherbini et al., 2022). Their toxic effects are reported for marine bivalves (Ringwood et al., 2010; Buffet et al., 2013, 2014; Auguste et al., 2018; Efthimiou et al., 2021; Wang and Zhang, 2023), though, as far as our knowledge goes, scarce information is still available on spermiotoxicity. Nevertheless, Ag NP spermiotoxicity was already demonstrated in mice, humans (Brito et al., 2020; Wang et al., 2017) and echinoderms (Gambardella et al., 2015). This evident nano-spermiotoxicity demonstrates spermatozoa's vulnerability to these compounds, compromising the reproductive fitness and putting marine species at risk.

Sperm quality and quantity define fertilization ability (Kowalski and Cejko, 2019). Sperm quality comprises information about sperm motility (membrane integrity and mitochondria function), fertilization ability (membrane integrity), embryonic development and progeny fitness (DNA and RNA integrity) (Cabrita et al., 2014). Spermatozoa play a central role in broadcast spawners' reproductive process since intact

* Corresponding author.
E-mail address: cmieiro@ua.pt (C. Mieiro).

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male DNA must be delivered to the oocyte during fertilization, indicating that any impact on sperm is of utmost importance. Previous works highlighted sperm quality parameters as suitable biomarkers for the toxicological screening of emergent contaminants and for establishing their reprotoxicity (Gallo et al., 2020; Carvalhais et al., 2022; Oliveira et al., 2023).

The Pacific oyster is a broadcast spawner globally introduced for aquaculture purposes (Martínez-García et al., 2022), becoming one of the most produced bivalve species in Europe and worldwide (Hansen et al., 2023; Wu et al., 2023). It is also considered a keystone species due to its ecological role in improving water quality and reef formation and as a sustainable food, among others (Martínez-García et al., 2022). In addition, its biological features are highly studied, especially reproduction, setting the basis for using this species as a bioindicator of water contamination.

Accordingly, our goal was to disclose Ag NP and Ag ion contribution to spermiotoxicity in an ecologically and commercially relevant species, the Pacific oyster (*Magallana gigas,* formerly known *as Crassostrea gigas*), by evaluating sperm kinetics, membrane damage and mitochondrial function through an *ex vivo* exposure.

2. Materials and methods

2.1. Silver nanoparticles (Ag NP) suspensions and silver nitrate ($AgNO_3$) solutions

Silver NP dispersion (10 nm particle size, 0.02 mg.mL^{-1} in aqueous buffer, with sodium citrate as a stabilizer) was supplied by Sigma-Aldrich, and its structure was confirmed by Scanning Transmission Electron Microscopy (STEM).

Silver NP suspensions were prepared as described in Carvalhais et al. (2022). Briefly, a stock suspension (2.5 μg.mL^{-1}) in distilled water and Ag NP working suspensions (0.25, 25 and 250 μg.L^{-1}) were prepared in artificial seawater (ASW, 25 salinity). To understand the involvement of Ag ions (Ag^+) in the evaluated responses, $AgNO_3$ (purity ≥ 99%, ACS reagent, CAS# 7761–88–8, Sigma-Aldrich) solutions were also prepared. To obtain the same Ag^+ concentrations as for Ag NP, a stock solution of 4 μg.mL^{-1} Ag NO_3 ([Ag+] = 2500 μg.L^{-1}) was prepared in distilled water and working solutions (0.40, 40, 400 μg.L^{-1}, [Ag+] = 0.25, 25 and 250 μg.L^{-1}) in ASW (25 salinity). The lowest concentration (0.25 μg.L^{-1}) represents environmentally realistic concentrations (Deycard et al., 2017), while 25 and 250 μg.L^{-1} of Ag NP are considered supra-environmental levels. Evaluating Ag NP effects, ranging from realistic to supra-environmental levels, allows for establishing threshold levels for spermiotoxicity.

The mean dynamic particle size of Ag NP suspensions was determined by Dynamic Light Scattering (DLS) analysis.

2.2. Ex vivo experimental setup

Sexually mature males of Pacific oyster (*M. gigas*) were collected in June and July at Ria de Aveiro (North-West, Portugal). Sperm was directly retrieved from the gonad, diluted in artificial seawater (ASW, 25 salinity) and consecutively sieved by a 100 and 25 μM mesh to allow retain debris and impurities. Sperm (~2 ×10^{9}cells.mL^{-1}, n = 8) was directly exposed for 1 h, at room temperature, to Ag NP and Ag^+ as well as ASW 25 salinity (as control) with gentle agitation (each 15 min). After exposure, sperm was centrifuged in a refrigerated centrifuge (Eppendorf 5415 R) at 800 g for 15 min at 4 °C, and the supernatant was discarded to remove NP and seminal plasma (Carvalhais et al., 2022; Oliveira et al., 2023). Afterwards, the pellet was resuspended in ASW (25 salinity) and divided into aliquots for the evaluation of sperm quality (motility, metabolic function and oxidative stress).

2.3. Evaluation of sperm functionality: motility

Sperm motility was performed using a CASA system (ISAS - Integrated System for Sperm Analysis; Proiser, Valencia, Spain) as described by Oliveira et al. (2023). After sperm activation (5 μL of ASW [1100 mOsm·kg^{-1}]: 1 μL of the cell suspension), motility parameters (total motility - TM %, progressive motility – PM % and linearity – LIN %) and velocity (curvilinear velocity - VCL μm.s^{-1}) and straight-line velocity – VSL μm.s^{-1}) were assessed. Motility was considered when VCL was higher than 10 μm.s^{-1}.

2.4. Sperm energy metabolism: mitochondrial function

Mitochondrial function was evaluated according to Oliveira et al. (2009), as described in Carvalhais et al. (2022), using Rhodamine 123/propidium iodide (Rh123/PI) dual fluorescent staining. Ten μL of Rh123 (1 mg.mL^{-1} in methanol) were added to 400 μL of each cell suspension (1–2 ×10^7 cells.mL^{-1}) and incubated in the dark, at 4 °C for 30 min, to promote Rh123 accumulation in functional mitochondria. After incubation, samples were centrifuged for 10 min at 500 g, the supernatant was discarded, and the pellet was resuspended in 1 mL of ASW. Five minutes before analysing samples at the flow cytometer (Attune® Acoustic Focusing Cytometer, ThermoFisher Scientific), 10 μL of PI (0.1 mg.mL^{-1}) was added to label the unviable cells. For each sample, no less than 10,000 events were considered. The percentage of sperm with functional mitochondria was calculated as the ratio of cells positive for Rh123 (PI+Rh123 +/PI-Rh123 +) vs. total cell number.

2.5. Sperm oxidative stress: membrane damage

Samples were prepared for the determination of membrane damage by lipid peroxidation (LPO) assay as described by Martínez-Páramo et al. (2012), with some adaptations for oyster sperm. Briefly, 200 μL of the diluted sperm were incubated with 10 μL of sodium ascorbate solution (200 μM) and 10 μL of $FeSO_4$ (40 μM) for 30 min at 37 °C in the dark. Then, the LPO assay was based on Bird and Draper (1984) and adapted by Filho et al. (2001). The presence of thiobarbituric acid reactive substances (TBARs) was measured on a microplate at 535 nm, using a SpectraMax 190 microplate reader (Molecular Devices LLC, California, USA). LPO was expressed as nmol of TBARS formed/mg of protein (ε = 1.56 ×10^5 M^{-1} cm^{-1}).

The total protein content was determined in LPO aliquots according to Bradford (1976), using bovine serum albumin as a standard. Absorbance reading was done at 595 nm.

2.6. Statistical analysis

One-way ANOVA, followed by multiple comparison tests (Tukey test), was used to evaluate the effect of the treatments (independent variables) on the different dependent variables (motility, mitochondrial function, and membrane damage; N = 8 per treatment). Graphical validation tools were used to verify ANOVA assumptions. The results were expressed as mean ± standard deviation. The statistical analyses were performed using IBM.SPSS®, version 27.0.1, and all the tests were considered significant when $p < 0.05$.

3. Results

3.1. Silver nanoparticles (Ag NP) characterization

Ag NP were previously characterized by Carvalhais et al. (2022). STEM analysis confirmed the spheroid shape of silver dispersion (stock suspension) with a mean primary size particle diameter of 21.50 ± 8.27 nm and working suspension diameter ranging from 10 to 20 nm for the 25 μg.L^{-1} and 250 μg.L^{-1}, and 4–10 nm for the 0.25 μg.L^{-1}. The behavior of Ag NP in suspension in ASW was evaluated by DLS, showing

agglomerates with an average size of 142.73 ± 13.74 nm for the stock suspension (0.0025 mg.mL^{-1}) in distilled water, and 130.52 ± 7.89, 134.29 ± 42.28 and 230.04 ± 32.57 nm for 0.25, 25, and 250 μg L^{-1} working suspensions in ASW, respectively.

3.2. Sperm functionality: motility

Ex vivo direct exposure of sperm to Ag NP as well as to Ag^+ decreased their motility and velocity. The TM (Fig. 1) was more affected by Ag^+, decreasing in all the treatments ($F_{(3,28)} = 5.133$, $p = 0.006$), while for Ag NP just significantly decreased in the 25 μg.L^{-1} treatment ($F_{(3,28)} = 4.163$, $p = 0.015$). In contrast, the PM (Fig. 2) decreased in all Ag NP treatments ($F = 5.764$, $p = 0.003$) and just diminished after exposure to the highest Ag^+ concentration ($F_{(3,28)} = 4.174$, $p = 0.015$). The LIN (Fig. 3) was not affected neither by Ag NP nor Ag^+ ($F_{(3,28)} = 0.624$, $p = 0.606$).

The VCL decreased in all treatments for both Ag NP ($F_{(3,28)} = 6.973$, $p = 0.001$) and Ag^+ ($F_{(3,28)} = 5.598$, $p = 0.004$) (Fig. 4). The VSL was less affected (Fig. 5) as both environmentally realistic (0.25 μg.L^{-1}) and supra-environmental concentrations (25 μg.L^{-1}) of Ag NP decreased VSL ($F_{(3,28)} = 4.307$, $p = 0.013$), while Ag^+ just impaired the velocity at the highest supra-environmental concentration ($F_{(3,28)} = 4.433$, $p = 0.012$).

3.3. Sperm energy metabolism: mitochondrial function

No statistically significant alterations of the mitochondrial function occurred after exposure to both Ag forms (Fig. 6).

3.4. Sperm oxidative stress: membrane damage

Similarly to mitochondrial function, no oxidative stress occurred (Fig. 7), as no statistically significant membrane damage appeared.

4. Discussion

Silver NP can impair the health status of marine biota (Goncalves and Bebianno, 2021; Bai et al., 2023; Sıkdokur et al., 2020), even at environmentally realistic levels, suggesting that their presence in seawater may threaten marine ecosystems and that their release into this compartment must be monitored, allowing the establishment of adequate mitigation strategies. Our results showed that the presence of Ag NP or Ag^+ ion deteriorate the sperm qualitative parameters of TM, PM, VSL and VCL. As sperm motility is crucial for fertilization success,

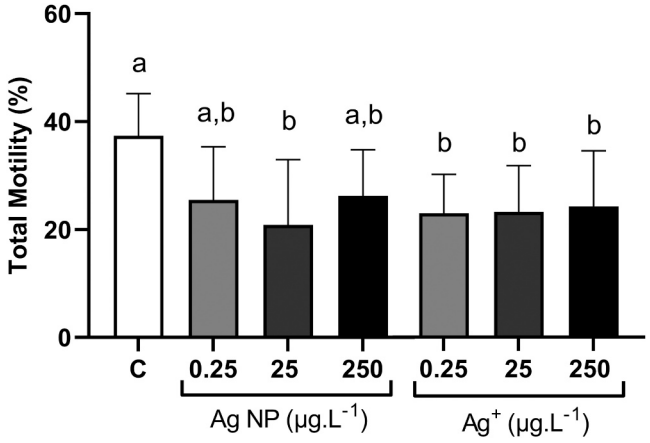

Fig. 1. Total motility (%) in sperm samples of *M. gigas* recorded 1 h after exposure to silver nanoparticles (Ag NP: 0.25, 25 and 250 μg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 μg.L^{-1}. Different lower-case letters denote significant differences ($p < 0.05$). Columns correspond to mean values and error bars represent the standard deviation. C - control.

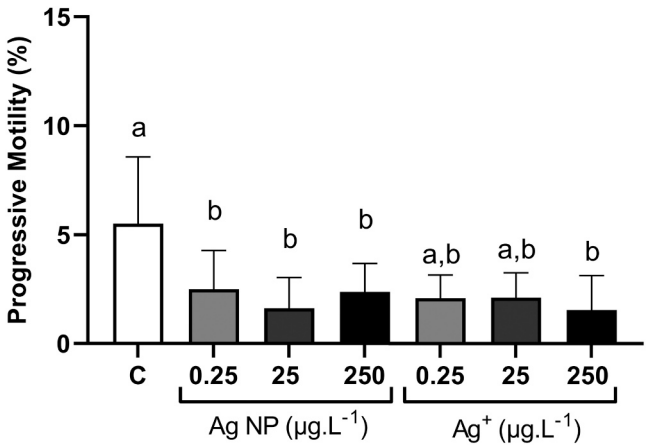

Fig. 2. Progressive motility (%) in sperm samples of *M. gigas* recorded 1 h after exposure to silver nanoparticles (Ag NP: 0.25, 25 and 250 μg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 μg.L^{-1}). Different lower-case letters denote significant differences ($p < 0.05$). Columns correspond to mean values and error bars represent the standard deviation. C - control.

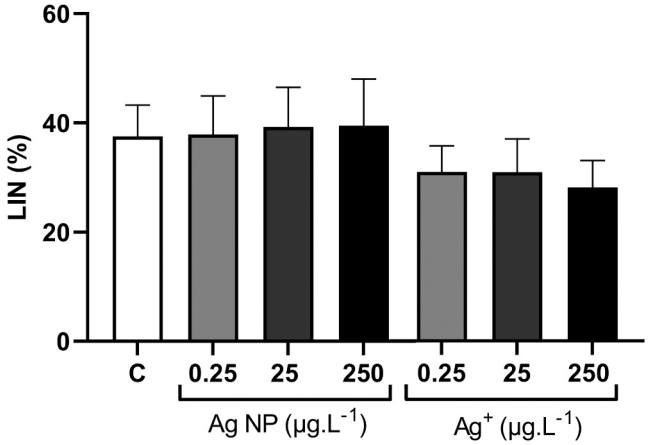

Fig. 3. Linearity (%) in sperm samples of *M. gigas* recorded 1 h after exposure to silver nanoparticles (Ag NP: 0.25, 25 and 250 μg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 μg.L^{-1}). Columns correspond to mean values and error bars represent the standard deviation. C – control.

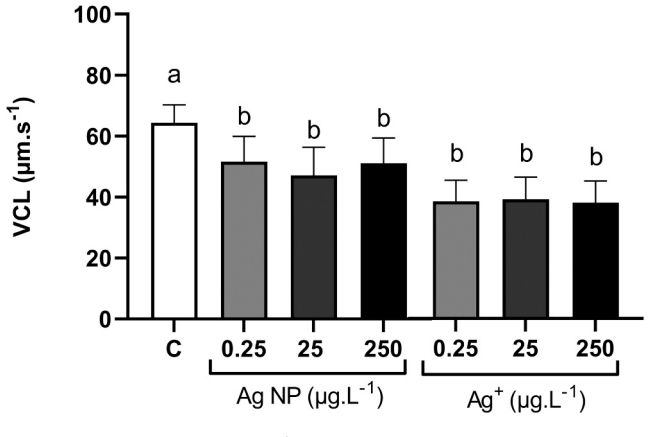

Fig. 4. Curvilinear velocity (μm.s^{-1}) in sperm samples of *M. gigas* recorded 1 h after exposure to silver nanoparticles (Ag NP: 0.25, 25 and 250 μg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 μg.L^{-1}). Different lower-case letters denote significant differences ($p < 0.05$). Columns correspond to mean values and error bars represent the standard deviation. C - control.

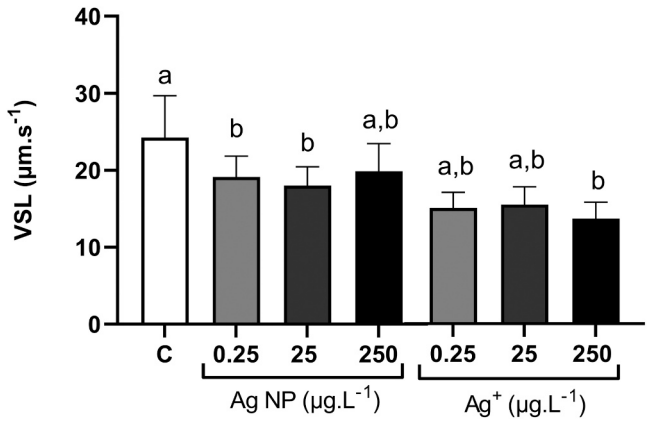

Fig. 5. Straight-line velocity (µm.s^{-1}) in sperm samples of *M. gigas* recorded 1 h after exposure to silver nanoparticles (Ag NP: 0.25, 25 and 250 µg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 µg.L^{-1}). Different lower-case letters denote significant differences ($p < 0.05$). Columns correspond to mean values and error bars represent the standard deviation. C - control.

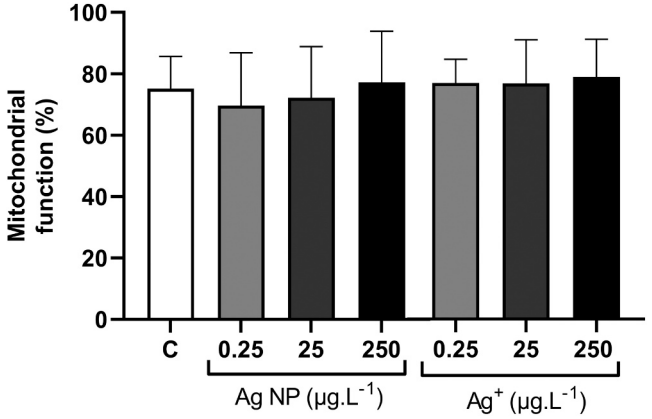

Fig. 6. Mitochondrial function (PI+Rh123 +/PI-Rh123 +) in sperm samples of *M. gigas* exposed for 1 h to silver nanoparticles (Ag NP: 0.25, 25 and 250 µg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 µg.L^{-1}). Columns correspond to mean values and error bars represent the standard deviation. C - control.

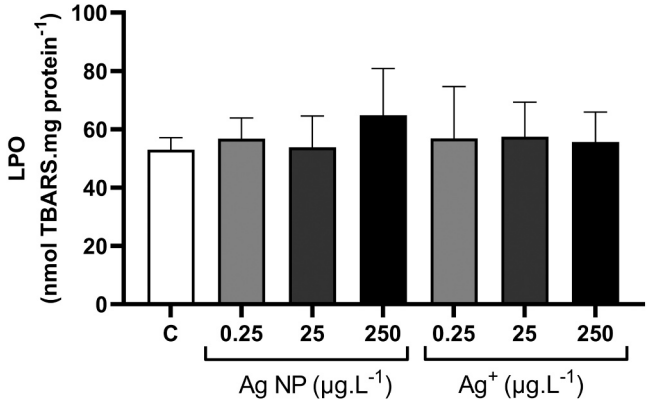

Fig. 7. Membrane damage measured as thiobarbituric acid reactive substances (TBARS) levels (nmolTBARS.mg $prot^{-1}$) in sperm samples of *M. gigas* exposed for 1 h to silver nanoparticles (Ag NP: 0.25, 25 and 250 µg.L^{-1}) or to silver as $AgNO_3$ ([Ag^+]: 0.25, 25 and 250 µg.L^{-1}). Columns correspond to mean values and error bars represent the standard deviation. C - control.

any alteration in these kinetic parameters may impair oysters' population growth and survival. Thus, the analysis of the sperm quality of the Pacific oyster may be decisive for predicting Ag NP spermiotoxicity in other broadcast spawners and contribute to establishing limits for their presence in the marine ecosystem.

4.1. Sperm kinetics

Sperm motility is considered one of the best-valued biomarkers of sperm quality known to date (Gallego and Asturiano, 2018). The evaluation of oyster sperm motility after exposure to environmental and supra-environmental levels of Ag NP and Ag^+ showed a loss of sperm quality translated into alterations in motility. The relatively low percentage of motile spermatozoa (TM) in the control group is typical of sessile species, which commonly have prolonged swimming periods but lower total motility (usually around 20%; Gallego et al., 2014). Specifically, the Pacific oyster spermatozoa are named "marathon racers" due to their low long-lasting motility and high distance traveled (Suquet et al., 2012). These sperm characteristics comply with the low motility observed in the control group, not the lack of sperm quality.

In general, the maximum sperm velocity for marine external fertilizers is reached at the moment of release into the environment (Browne et al., 2015), which according to our results can be affected by the lowest levels of Ag NP and Ag^+ (0.25 µg.L^{-1}) tested herein. Some studies with spermatozoa pointed to metal ions as more harmful than nanoparticles due to their ability to bioconcentrate and bind to ligands present in water that increase bioavailability (Kowalska-Góralska et al., 2019; Zhu et al., 2023). However, this was not observed in the present study as no evident differences were found between Ag NP and Ag^+. Yet, our results are in accordance with Gambardella et al. (2015), who demonstrated that Ag NP in similar and higher concentrations than those tested in this work, induced depletion of sea urchin sperm motility (percentage of rapid spermatozoa and VCL) after 1 h of direct exposure. The decrease of the Pacific oyster sperm motility (% of motile sperm and the velocity of the average path) was also reported after 1 h of exposure to nanopolystyrene beads (Tallec et al., 2020).

Contrarily to Ag NP, environmentally realistic levels of TiO_2 NP did not influence the Pacific oyster sperm motility (Oliveira et al., 2023). The different mechanisms of action between nanomaterials must be related to their specific mode of action and tested concentrations since the time of exposure was the same. Still, and despite the low number of studies about the effects of Ag NP in spermatozoa of marine invertebrates, the results suggest that Ag, both NP and ion form, are spermiotoxic for marine invertebrates at environmentally realistic and supra-environmental levels, reinforcing the need for further studying Ag releases into the environment and their effects on reproduction.

The uptake of NP in eukaryotic non-phagocytic cells occurs through endocytosis (clathrin-mediated for NP <200 nm and caveolae-mediated processes for NP around 500 nm). Therefore, the internalization of NP by spermatozoa is unlikely as spermatozoa lack the endocytosis machinery. Taylor et al. (2015) found that porcine spermatozoa are impermeable to NP, which is in line with the observed deposition of nanopolystyrene beads around the external membrane of the spermatozoa head, especially in the acrosomal area, without uptake into the cells of *M. gigas* (Tallec et al., 2020). The same authors found harmful effects of these nanomaterials without internalization, which complies with the hypothesis that NP can act as surface-acting agents inducing toxicity through adsorption (Handy et al., 2011).

In agreement we hypothesize that the reduction of sperm motility observed in our study may also be due to Ag NP deposition on the external membrane of the spermatozoa exerting mechanical interferences on sperm motility. Due to the lack of other effects on sperm (please see Sperm mitochondrial function and oxidative responses section), we also suggest that Ag NP might act by blocking calcium (Ca2 +) channels found in the cell, inhibiting the flow of ions across the membrane. Xu et al. (2018) had also reported that Ag NP affected Ca^{2+} signaling in zebrafish. A similar mechanism is anticipated for Ag^+, since

metals bind to the sulfhydryl groups of proteins and thus to cell membrane proteins responsible for modulating membrane permeability (Kone et al., 1990). Boulais et al. (2019) found that sodium and potassium are not required for triggering motility in *M. gigas*, though Ca^{2+} influx is critical for sperm motility activation. The increase in intracellular Ca^{2+} concentration initiates the signaling cascade for the onset of flagellar beating (Nichols et al., 2021). Any effect on Ca^{2+} channels, by physical blocking or altering membrane potential, severely compromises motility.

As previously mentioned, both environmental and supra-environmental levels of Ag (NP and ion) impair sperm motility, though no unequivocal dose-dependent impairment was found, parallel to the indistinct pattern for each parameter, which does not allow to disclose which silver form, nano or ion, are more toxic.

4.2. Sperm mitochondrial function and oxidative responses

Mitochondria play an important role in sperm motility. Flagellar beating is dependent of ATP consumption (Figueroa et al., 2019), so any impairment at this level would result in cellular dysfunction (Hatef et al., 2013; Rurangwa et al., 1998). In this study, mitochondrial function remained unaltered, despite the presence of Ag NP or Ag^{+}, even at supra-environmental levels. Carvalhais et al. (2022) also found no alteration of the mitochondria function in seabream (*Sparus aurata*) sperm exposed to Ag NP or Ag^{+}, with no alteration on motility parameters either. In contrast, in the present study, motility was affected without alteration in the mitochondrial function, which may be related to sperm species-specificities. Moreover, no oxidative stress occurred expressed as lipid peroxidation increased, despite Ag NP and Ag^{+} being recognized as inducing toxicity by oxidative stress mechanisms (Ramzan et al., 2022). Gallo et al. (2018) found that the spermatozoa of sea urchins exposed (1 h) to copper oxide NP showed impaired mitochondrial function along with an increase of ROS, LPO, DNA damage, and morphological alterations. They hypothesised that ROS disrupted sperm mitochondrial function and consequently increasing ROS production promoting membrane (LPO) and DNA damage and alteration of sperm morphology. These findings suggest that sea urchin vulnerability towards ROS production is higher than oysters, which may be related to the sperm motility duration since sea urchin sperm are motile for 20 min, while oysters are for up to 24 h (Gallego et al., 2014; Boulais et al., 2015). Moreover, *M. gigas* have a particular metabolic strategy in which the ATP necessary to keep the motility during the first 2 h is obtained by complementary metabolic processes to the oxidative phosphorylation, such as glycolysis, phosphagens and mobilization of stored ATP (Boulais et al., 2019). This suggests that in oysters, complementary metabolism should be evaluated to accurately assess the impacts of contaminants on the energy budget and mitochondrial function.

Plus, the fact that this species has additional strategies to obtain energy for sperm movement provides them adaptive advantages over other bivalves, which may anticipate that other bivalve species might be more susceptible to Ag cytotoxicity (Ag NP and Ag^{+}).

5. Conclusion

This study demonstrated that both environmental and supra-environmental levels of Ag NP and Ag^{+}, following *ex vivo* direct exposures, indistinctively induced spermiotoxicity by decreasing the kinetic parameters of *M. gigas* sperm cells. Though the impairment of sperm motility, which may compromise Pacific oysters' reproduction, no membrane damage or alteration of mitochondrial function occurred.

Our study highlights that the presence of Ag (NP and ion) in the marine ecosystem constitutes an environmental concern since environmental-realistic concentrations impair sperm motility.

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CRediT authorship contribution statement

Fátima Fernández-García: Investigation, Writing – original draft, Visualization. **Ana Carvalhais**: Formal analysis, Investigation, Data curation, Visualization. **Ana Marques**: Investigation. **Isabel B. Oliveira**: Investigation. **Sofia Guilherme**: Investigation. **Helena Oliveira**: Investigation, Resources. **Catarina C.V. Oliveira**: Investigation, Data curation. **Elsa Cabrita**: Conceptualization, Methodology, Resources, Writing – review & editing. **Juan F. Asturiano**: Conceptualization, Writing – review & editing. **Mário Pacheco**: Conceptualization, Resources, Writing – review & editing, Supervision. **Cláudia L. Mieiro**: Conceptualization, Methodology, Validation Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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