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Cytotoxicity and genotoxicity of CuO nanoparticles in sea urchin spermatozoa through oxidative stress

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ABSTRACT

Copper oxide nanoparticles (CuO NPs) are extensively used in various industrial and commercial applications. Despite their wide application may lead to the contamination of marine ecosystem, their potential environmental effects remain to be determined. Toxicity assessment studies have primarily focused on investigating the effects of CuO NPs on fertilization success and embryo development of different sea urchin species while the impact on sperm quality have never been assessed. In this line, this study aims to assess the effects of CuO NPs on the spermatozoa of the sea urchin *Paracentrotus lividus*.

After sperm exposure to CuO NPs, biomarkers of sperm viability, cytotoxicity, oxidative stress, and genotoxicity as well as morphology were evaluated. Results showed that CuO NPs exposure decreased sperm viability, impaired mitochondrial activity and increased the production of reactive oxygen species (ROS) and lipid peroxidation. Furthermore, CuO NPs exposure caused DNA damage and morphological alterations. Together with the antioxidant rescue experiments, these results suggest that oxidative stress is the main driver of CuO NP spermiotoxic effects. The mechanism of toxicity is here proposed: the spontaneous generation of ROS induced by CuO NPs and the disruption of the mitochondrial respiratory chain lead to production of ROS that, in turn, induce lipid peroxidation and DNA damage, and result in defective spermatozoa up to induce sperm cytotoxicity. Investigating the effects of CuO NPs on sea urchin spermatozoa, this study provides valuable insights into the mechanism of reproductive toxicity induced by CuO NPs.

1. Introduction

Nanotechnology allows the production of engineered nanoparticles (NPs) with peculiar characteristics which led to their wide employment in many industrial sectors as electronics, catalysis, cosmetics, medical diagnostics and pharmaceuticals. Although industrial nanoparticulate materials may exert some positive health effects (Buzea et al., 2007), health and environmental concerns on NPs have been highlighted in several reports (Commission, 2009; Pollution, 2008). Marine environments act as a sink for most contaminants and thus likely receive NPs from wastewater and trough the degradation of products containing NPs. Several studies highlighted the toxic effects of various NPs on the physiological processes of marine organisms (Baker et al., 2014; Baun et al., 2008; Canesi et al., 2012; Matranga and Corsi, 2012; Wong et al., 2010). In particular, the impact of NPs on fertilization success and embryo development has been widely investigated.

Copper oxide nanoparticles (CuO NPs) are widely used in several applications. They are employed as antifungal and antimicrobial additives in textiles, water treatment, and paints (Maisano et al., 2015). In particular, their use in anti-fouling paints is one of the most direct routes of entry into marine environment (Adeleye et al., 2016). It has been well documented that Cu ions are extremely toxic to marine animals (Tornero and Hanke, 2016); however, information on the impact of CuO NPs on marine organisms are still limited. Among them, it has been reported a reduction of bioluminescence or culturability on marine bacteria (Rossetto et al., 2014; Rotini et al., 2017), genotoxicity in bivalve molluscs (Gomes et al., 2013; Ruiz et al., 2015), body burden and behavioural disorders in marine worms (Buffet et al., 2013a; Dai et al., 2015), reproductive impairments in echinoderms (Minetto et al., 2016) and survival and moulting release in marine rotifers and crustaceans (Rotini et al., 2018).

Reproduction is the highly specialized process of cell interaction

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that marks the creation of a new and unique individual and is crucial for population maintenance and biodiversity conservation. Fertilization and specifically gamete interaction and activation are critical issues in reproduction, whose success strongly depends on gamete quality (Gallo, 2018; Tosti et al., 2011; Tosti and Ménézo, 2016).

Sperm quality is defined as the ability to successfully fertilize an oocyte and, subsequently, to allow the development of normal embryo. Assessment of sperm quality in marine species is now an important issue due to the increase of ecotoxicological studies looking at the impact of environmental pollutants on male reproductive health (Gallo and Tosti, 2015a; Tosti and Gallo, 2012). To date, sperm toxicity test is commonly performed to evaluate the adverse effects of NPs; however, in most cases spermiotoxicity is assessed by evaluating fertilization success and induction of transmissible damages to the offspring. Consequently, information on the underlying molecular mechanisms is still extremely scarce.

Most of the authors working on the biological effects of SiO_2, SnO_2, CeO_2, Fe_3O_4, Ag, TiO_2 and Co NPs found that the sea urchin sperm fertilization capability was not affected by exposure of these NPs while their effects were dramatically exerted on the offspring quality. In particular, developmental anomalies, as morphological alterations of the skeletal rods, were identified in embryos from the gastrula to pluteus stages after NP sperm exposure suggesting that NPs interfere with bio-mineralization process (Gambardella et al., 2013; Gambardella et al., 2016; Gambardella et al., 2015; Matranga and Corsi, 2012). Similarly, a decrease of fertilization success and a delay and disruption of the embryo development were reported in ascidian, sea urchin an mussel after sperm exposure to zero-valent iron NPs (Kadar et al., 2013). A slight spermiotoxic effect was exerted by ZnO NPs, followed by an early block of the regular larval development (Manzo et al., 2013), while nanoiron caused sperm DNA damage in *Mytilus galloprovincialis* (Kadar et al., 2011). However, these experimental protocols do not permit to clarify the mechanism of NP action (Gambardella et al., 2016).

Nowadays, ecotoxicological data on fertilization success and offspring quality under CuO NPs exposure are scarce and based mainly on sea urchin species (Cappello et al., 2017; Torres-Duarte et al., 2016; Torres-Duarte et al., 2017). Sea urchin is key-component of the marine ecosystems contributing to about 90% of the benthic biomass and is a model organism widely used in ecotoxicological assessment of marine pollution (Arslan and Karaaslan, 2016). Sea urchin has been so far the most studied group of marine invertebrates for assessing NP developmental toxicity. To date, two studies reported the sub-lethal toxic effects of CuO NPs on sea urchin development (Maisano et al., 2015; Torres-Duarte et al., 2016), while a first evaluation of CuO NP spermiotoxicity by assessing fertilization rate in sea urchin *Paracentrotus lividus* was reported in our previous study (Rotini et al., 2018). To our knowledge, no reports are available on the sensitivity of marine invertebrate spermatozoa towards CuO NP exposure. Therefore, the aim of the present study was to investigate the effects of CuO NPs on *P. lividus* spermatozoa by assessing biomarkers of cell viability, oxidative stress, and genotoxicity as well as sperm morphology, this can also provide insight on the possible mechanisms of CuO NP-induced developmental and reproductive toxicity.

2. Material and methods

2.1. CuO NPs and testing suspensions

The copper (II) oxide NPs were purchased from US Research Nanomaterials, Inc. as a water dispersion (20 wt%, purity of 99.95%) with a nominal particle size in the range of 25–55 nm. According to Rotini et al. (2018), CuO NP stock suspension (1 g/L) was prepared in 0.22 μm filtered milli-Q water from the 20% dispersion and sonicated for 15 min in a bath sonicator (60 watt, 47 kHz; Branson Ultrasonic Baths), then stored in the dark at 4 °C. CuO NP stock suspension was used, after 15 min sonication, to prepare the final testing suspensions in filtered (Millipore 0.22 μm; Milli Q, Medford, Ma) natural seawater (FNSW, pH 8.2 ± 0.1, Salinity 38 PSU). The final concentrations of CuO NPs used in this study are the following: 1, 2, 5, 10, 20 mg/L; they were chosen based on previous study (Rotini et al., 2018). Final testing suspensions prepared in seawater were vortexed prior to use, without sonication. CuO NPs characterization has been previously reported (Rotini et al., 2018) since the biomarker analyses were performed simultaneously to this previous study, by using the same CuO NPs and exposure medium used in the present study.

2.2. Sea urchin and spermatozoa collection

The *Paracentrotus lividus* (Lamarck) sea urchins were collected in the Gulf of Naples (Tyrrhenian Sea), from a location that is not privately-owned or protected in any way, according to Italian legislation of the Marina Mercantile (DPR 1639/68, 09/19/1980 confirmed by D. Lgs. 9/01/2012 n. 4). The field studies did not involve endangered or protected species. All animal procedures were in compliance with the guidelines of the European Union (Directive 609/86).

After collection, sea urchins were transported to Stazione Zoologica Unit of Marine Resources for Research laboratory within 1 h and maintained in tanks with circulating seawater at 18 °C. The animals were fed on *Ulva lactuca* and *Posidonia oceanica* and acclimated at least for 10 days until use. To induce gamete spawning, sea urchins were injected with 0.5 M KCl solution through the peristome membrane and then spermatozoa were collected dry and stored undiluted at 4 °C. Sperm concentration and motility were evaluated by a Makler counting chamber and, then, diluted to the desired concentration in FNSW.

2.3. Sperm viability

Sperm viability was evaluated using a double staining procedure including SYBR-14 and propidium iodide (PI) (LIVE/DEAD Sperm Viability Kit, Life technologies, Milan, Italy). This kit consists of SYBR 14 stock solution (1 mM in DMSO) and propidium iodide solution (PI; 2.4 mM solution in water). SYBR-14 permeates cells with preserved membrane integrity and emits green fluorescence. Contrastingly, PI permeates only spermatozoa that lost membrane integrity. Briefly, according to Gallo et al. (2018), aliquots of spermatozoa diluted at 5×10^6 cell/mL were added to final testing suspensions of CuO NPs and incubated for 1 h at 18 °C. After incubation, spermatozoa were stained by adding SYBR 14 (final concentration 100 nM) and incubated in the dark for 15 min. Following, PI (final concentration 12 μM) was added and incubated an additional 15 min. Samples were washed by adding FNSW, centrifuged at 2000 rpm for 10 min, resuspended in FNSW and then analyzed by spectrofluorimetry (Shimadzu RF-5301PC spectrofluorophotometer) using a quartz microtube (10 × 4 mm, high precision, Hellma Analytics, Mullheim, Germany). SYBR-14 and PI fluorescences were measured at 500–530 nm (live sperm) and at 570–650 nm (dead sperm), respectively. This experiment was performed ten times in duplicates.

2.4. Plasma membrane lipid peroxidation

Lipid peroxidation was evaluated using the fluorescent membrane probe C11-BODIPY$^{581/591}$. This probe is an oxidation-sensitive fluorescent fatty acid analog which is easily incorporated into membranes and sensitive to oxidation shifting from red (590 nm) to green (510 nm) fluorescence upon oxidation. Sperm suspensions were incubated for 30 min in the dark at 18 °C with 5 μM C11-BODIPY$^{581/591}$ in DMSO. After staining, spermatozoa were centrifuged at 2000 rpm for 15 min, the pellet was resuspended in FNSW and aliquots (2×10^7 spermatozoa/mL) were added to final testing suspensions of CuO NPs and incubated for 1 h at 18 °C. Later on, spermatozoa were centrifuged, the pellet resuspended in FNSW and transferred to quartz

cuvette for spectrofluorimetric analysis. Positive and negative controls were prepared by incubating the samples with two peroxidation promoters (150 mM ferrous sulfate and 750 mM vitamin C) and FNSW, respectively. The fluorescence intensity was measured at 488 nm excitation and 500–650 nm emission wavelengths. Then, a ratiometric analysis was performed by relating fluorescence emission peak value at ~520 nm to the sum of fluorescence emission peak values at ~520 and ~590 nm.

2.5. Intracellular reactive oxygen species (ROS) determination

ROS determination was performed using 2′,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA). This probe is freely permeable across cell membranes and is incorporated into hydrophobic regions of the cell where its acetate moiety is cleaved by esterase producing the impermeant non-fluorescent 2,7-dichlorodihydrofluorescein (H_2DCF). The H_2DCF is easily oxidized by H_2O_2 to the highly fluorescent 2,7-dichlorofluorescein (DCF) which emits fluorescence at 530 nm in response to 488 nm excitation wavelengths. Aliquots of spermatozoa diluted at 5×10^6 cell/mL were added to final testing suspensions of CuO NPs and incubated for 1 h at 18 °C. After incubation, spermatozoa were washed and then stained by adding H_2DCF-DA to final concentration of 10 μM. The mixture was incubated for 30 min in the dark at 18 °C, then samples were centrifuged for 15 min at 2000 rpm, the pellet was resuspended in FNSW and incubated for additional 30 min. Later on, the sperm suspensions were centrifuged for 15 min at 2000 rpm, the sperm pellet was resuspended in FNSW and transferred to the quartz cuvette for spectrofluorometric analysis. A positive and negative control was prepared by incubating the samples with hydrogen peroxide and FNSW, respectively. Fluorescence intensity was measured at an excitation wavelength of 488 nm and emission wavelengths of 500–560 nm.

2.6. Mitochondrial membrane potential (MMP)

To determine the MMP the vital mitochondrial dye JC-1 was used. This dye is freely permeable to cells and is a useful tool for investigating mitochondrial functional activity since it undergoes reversible transformation as MMP changes from an aggregate form to a monomer shifting the fluorescence emission from red (~595 nm) to green (~535 nm). Consequently, MMP value can be determined by red/green ratio. Aliquots of spermatozoa diluted at 1×10^6 cell/mL were added to final testing suspensions of CuO NPs and incubated for 1 h at 18 °C. Following exposure, spermatozoa were washed and then stained by adding 5 μM JC-1, diluted from a 7.7 mM stock solution in DMSO and incubating for 30 min at 18 °C. After staining, spermatozoa were centrifuged for 15 min at 2000 rpm, the pellet was resuspended in FNSW and transferred to quartz cuvette for spectrofluorometric analysis using 488 nm excitation and 500–650 nm emission wavelengths. The ratio between red and green fluorescence emission peak values was calculated. Controls were prepared by exposing JC-1 loaded spermatozoa to the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 5 μM) that disrupts MMP resulting in a shift from red to green fluorescence and FNSW.

2.7. Sperm DNA damage

Sperm DNA fragmentation was evaluated by using a commercially available kit (*In Situ* Cell Death Detection Kit, Fluorescein, Roche Diagnostics, Milan, Italy) following the manufacturer's instructions. Aliquots of spermatozoa diluted at 2×10^6 cell/mL were added to final testing suspensions of CuO NPs and incubated for 1 h at 18 °C. Following exposure, spermatozoa were washed, fixed in 2% paraformaldehyde for 1 h, and then permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. After centrifugation, the elongation reaction was performed by incubating sperm pellets in 50 μL of TUNEL reaction mixture (containing the TdT enzyme and the fluorescein-dUTP label solution) for 1 h at 37 °C in the dark. For a positive control, fixed and permeabilized spermatozoa were treated with DNase I (3 U/mL) for 10 min before incubation with the TUNEL reaction mixture. Negative controls were prepared by omitting the label solution from the TUNEL reaction mixture. Before spectrofluorometric analysis, spermatozoa were counterstained with 1 mg/mL DAPI. Fluorescein and DAPI fluorescence intensity peaks were measured at 488 excitation and 520–530 nm emission wavelengths and 358 nm excitation and 461 emission wavelengths, respectively. DNA fragmentation was calculated as a ratio between fluorescein and DAPI fluorescence emission peak values.

2.8. Intracellular pH (pH_i)

The pH sensitive fluorescent chromophore 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), a neutral lipophilic form of biscarboxyfluorescein freely diffusing through the plasma membrane, was used to evaluate pH_i. In the cell, this dye is hydrolyzed by esterases, releasing the intracellularly trapped indicator, BCECF, which is retained within the cytoplasm and its fluorescence intensity is dependent upon the pH. Aliquots of spermatozoa diluted at 1×10^6 cell/mL were added to final testing suspensions of CuO NPs and incubated for 1 h at 18 °C. Following exposure, the ester form of BCECF (5 mM BCECF-AM) was added to the sperm suspensions that were incubated in the dark at 18 °C for 30 min. Then, the suspensions were centrifuged for 15 min at 2000 rpm, the sperm pellet was resuspended in FNSW and transferred to the quartz cuvette for spectrofluorometric analysis. pH_i was measured by alternately exciting BCECF at 440 nm and 490 nm and recording the fluorescence emission peak value at 535 nm. Subsequently, the ratio between the two obtained emission peaks was converted to its respective pH_i by using a calibration curve. This was constructed on each experiment by incubating sperm suspensions in a calibration buffer solution (135 mM KCl, 5 mM HEPES, 290 mOsm) at pH 6.5, 7.0, 7.5 in presence of 5 μM nigericin, which acts as a potassium ionophore promoting K + /H + exchange.

2.9. Sperm morphology: Scanning electron microscopy (SEM)

Following exposure to CuO NPs, sperm were fixed for 1 h at RT in 2.5% glutaraldehyde solution in 0.2 M sodium cacodylate buffer and 20% FNSW. Samples were washed for 10 min once in sodium cacodylate buffer and twice in distilled water and then, post fixed for 1 h at RT in 1% osmium tetroxide in distilled water. After dehydration in an ascending ethanol series (30, 50, 70, 90, and 100%), samples were mounted on studs, then coated with palladium and examined under a scanning electron microcopy (JEOL JSM 6700F microscope).

2.10. Statistical analysis

Data were reported as the mean ± standard deviation (SD). Data were tested for normal distribution by performing the Shapiro-Wilk test and for variance homogeneity by using Leven's test. Since the two assumptions were accepted, the one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test was performed to test for significant differences between the control group and test concentrations and among the test concentration groups. In the case of values expressed as percentages, data were analyzed after arcsine transformation to achieve normality. For the pH, whose values are not characterized by a continuous distribution, a transformation in H^+ concentration was applied. The significance level was set at $p \leq 0.05$.

3. Results and discussion

Sperm quality is crucial to predict male reproductive success and depends on several abiotic and biotic factors; among these,

environmental conditions are considered key aspects (Gallo et al., 2011; Gallo and Tosti, 2013; Gallo and Tosti, 2015a; Gallo and Tosti, 2015b; Valdebenito et al., 2015). Many studies reported a negative effects of different pollutants on spermatozoa of marine organisms(Abascal et al., 2007; Au et al., 2000; Boni et al., 2016; Rurangwa et al., 2002); however, the effects of NPs have been poorly elucidated (Châtel et al., 2017; Gallo et al., 2016; Kadar et al., 2011). In ecotoxicological studies, fertilization success are commonly used as endpoint to evaluate the effects of sperm exposure to NPs(Arslan et al., 2007; Dinnel et al., 1989; Mwatibo and Green, 1997; Vaschenko et al., 1999; Volpi Ghirardini and Arizzi Novelli, 2001; Warnau et al., 1996). Even though NPs are known to induce various cellular defects in spermatozoa affecting fertilization success, ecotoxicological investigations rarely use cellular biomarkers to test spermiotoxicity of NPs (Kadar et al., 2013).

This study reports the first appraisal in marine animals of the impact of CuO NP *in vitro* exposure on diverse sperm quality parameters strictly related to fertilizing capability and provides significant insight into the possible mechanism for toxicity of these NPs in reproduction of marine organisms.

3.1. CuO NP characterization

The physico-chemical characterization of CuO NPs FNSW was previously provided (Rotini et al., 2018). Briefly, the instability of CuO NPs in seawater due to the high ionic strength, results in the formation of large, micrometric sized aggregates with a tendency to sediment. The Dynamic Light Scattering analysis detected a unimodal size distribution of the CuO NPs suspended in FNSW with a mean volume-weighted diameter of 488.6 ± 93.5 nm (polydispersity index = 0.04). The average sedimentation rate of CuO NPs and their agglomerates in FNSW was 1.02 ± 0.02 mm/h. The ionic copper released into seawater from CuO NPs used in this experiment was not negligible (0.15% of the initial copper concentration), meaning the observed toxic effects could be the result of free Cu ions. The released copper ions from CuO NPs has been suggested as a source of toxicity, even if the dissolution of copper ion may not be the major factor of toxicity from CuO NPs (Sun et al., 2017). We observed that exposure to copper nitrate pentahydrate (Cu $(NO_3)_2{\cdot}5\ H_2O$, Sigma Aldrich, ≥ 99%) did not significantly affect the sperm quality parameters analyzed in this study (data not shown) suggesting that the particles rather than the dissolved ions were the dominant source of CuO NP toxicity.

3.2. Sperm viability

Viability is a key determinant of sperm quality strongly influencing fertilization success. Sperm viability after NP exposure was investigated in mammals showing contrasting results. It was impaired in human but not in bovine and boar (Barkalina et al., 2014; Moretti et al., 2013; Taylor et al., 2014; Wang et al., 2017). In marine animals, sperm viability was assessed after exposure to different environmental cues (Akcha et al., 2012; Favret and Lynn, 2010; Le Goïc et al., 2013; Martínez-Páramo et al., 2013; Rolton et al., 2015; Suquet et al., 2016; Volety et al., 2016), but never after exposure to NPs. Our results showed that CuO NP exposure significantly affected sperm viability in sea urchin *P. lividus*. The percentage of live spermatozoa significantly decreased after exposure to 2 mg/L CuO NPs in comparison to control group (Fig. 1). Our previous study (Rotini et al., 2018) reported that the exposure of *P. lividus* spermatozoa to CuO NPs caused a decrease of fertilization rate. In the present study, we demonstrated that the fertilization failure is associated with decreased sperm viability.

3.3. Intracellular reactive oxygen species (ROS)

The production of ROS by spermatozoa is a normal physiological process. Low and controlled concentrations of ROS play a fundamental role in signal transduction mechanisms for sperm physiology, such as

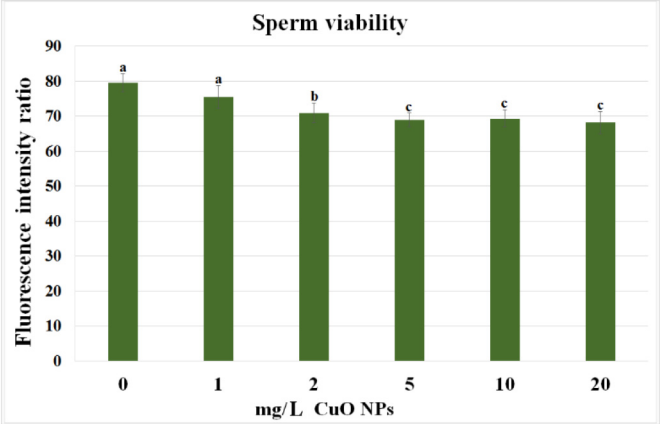

Fig. 1. Sperm viability after exposure to CuO NPs. The percentage of live spermatozoa significantly decreased after exposure to 2 mg/L CuO NPs. Data were reported as mean ± SD, $n = 10$. a *vs* b, a *vs* c, b *vs* c are significant differences, $p < 0.05$.

acrosome reaction, sperm motility and fertility (de Lamirande et al., 1997). However, when the intracellular ROS levels overcome the buffering capacity of the cell, this enters oxidative stress, potentially leading to damage of DNA, proteins and lipids (Guerriero et al., 2003). Oxidative stress has been so far identified as the main mechanism of action of different NPs (Canesi and Corsi, 2016; Gallo et al., 2016; Nel et al., 2006; Wang et al., 2017; Xia et al., 2006). NPs can lead to spontaneous ROS generation by mechanisms including prooxidant functional groups on the reactive surface of NP and active redox cycling on the surface of NP due to transition metal-based NP (Manke et al., 2013). Based on the current knowledge, CuO NPs are prone to exert oxidative damage. In the present study, we employed a fluorescent staining (DCF-DA) coupled with spectrofluorimetric analyses for detecting ROS production in the CuO NP treated spermatozoa demonstrating an increase of the intracellular level of ROS in spermatozoa of sea urchin *P. lividus* after exposure to 2 mg/L CuO NPs (Fig. 2). In the sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*, excessive production of ROS in spermatozoa was related to a reduction of fertilizing capability(Kazama and Hino, 2012) supporting that CuO NPs impair sperm fertilizing capability and offspring quality in *P. lividus* due to promoting intracellular ROS formation. This is the first experimental evidence of oxidative stress induced by CuO NPs in spermatozoa of marine animals.

3.4. Mitochondrial membrane potential (MMP)

Besides the spontaneous ROS generation, NPs can also promote ROS production due to their interaction with cellular components. A possible mechanism involves the disruption of the mitochondrial respiratory chain leading to ROS production and the interruption of ATP synthesis, which, in turn, causes apoptosis. Some studies suggested that the toxic effect of CuO NPs is due to the induction of oxidative stress (Buffet et al., 2013b; Gomes et al., 2013; Gomes et al., 2011; Kukla et al., 2017; Melegari et al., 2013; Moustakas et al., 2017; Sun et al., 2017) and/or to their ability to damage mitochondria, which can lead, in turn, to an increased ROS production and mitochondrial depolarization (Karlsson et al., 2009). Mitochondrial perturbation has been demonstrated in several cell lines following exposure to CuO NPs (Ahamed et al., 2015; Siddiqui et al., 2013) but there are no reports on marine animal spermatozoa. In the present study, the fluorescent dye JC-1 was used to estimate MMP in sea urchin spermatozoa showing that exposure to CuO NPs significantly affected mitochondrial activity. In fact, MMP was significantly reduced after incubation in 2 mg/L CuO NPs. By increasing CuO NP concentration, MMP underwent a further significant decline (Fig. 2).

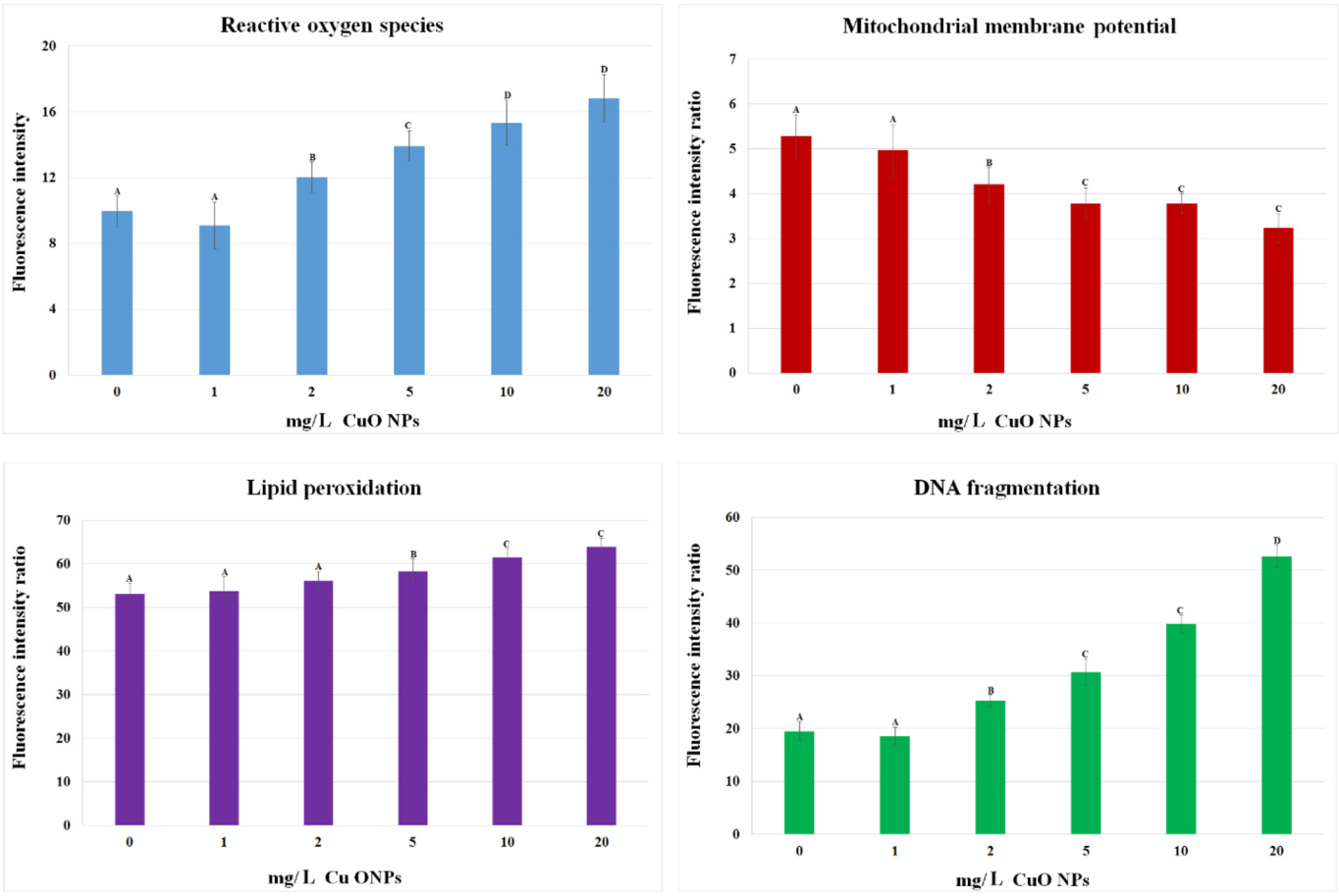

Fig. 2. CuO NP effects on different sperm quality parameters. Intracellular reactive oxygen species, mitochondrial membrane potential, plasma membrane lipid peroxidation and DNA fragmentation were evaluated by spectrofluorimetric analysis after 1 h sperm exposure to different CuO NP concentrations. Data were reported as mean ± SD, $n = 10$. A *vs* B, A *vs* C, A *vs* D, B *vs* C, B *vs* D, C *vs* D are highly significant differences, $p < 0.01$.

Mitochondrial status is an important trait of sperm quality related to motility, cell viability, and fertility (Cabrita et al., 2008; Garner et al., 1997; Kasai et al., 2002). The decrease in MMP induced by CuO NP sperm exposure could lead to a metabolic disorders in cells (AshaRani et al., 2009), which can determine reproductive impairment.

3.5. Plasma membrane lipid peroxidation

A consequence of ROS formation is lipid peroxidation, which often occurs in response to oxidative stress. Sperm plasma membrane contains high concentrations of polyunsaturated fatty acids, which confer to the plasma membrane the necessary fluidity for sperm motility and membrane fusion occurring at fertilization. However, this feature renders spermatozoa also highly susceptible to oxidative stress since polyunsaturated fatty acids could act as substrates for ROS (Aitken et al., 2004). Oxidant attack on plasma membranes results in lipid peroxidation, which affects sperm quality due to amplification of ROS production, alteration of integrity and fluidity of plasma membrane as well as impairment of ion exchanges and lipid-protein interactions (Tvrdá et al., 2011). CuO NP-induced lipid peroxidation was reported in different biological model(Ahamed et al., 2010; Akhtar et al., 2016; Fahmy and Cormier, 2009; Gomes et al., 2012; Siddiqui et al., 2013). In this study, we demonstrated that CuO NP exposure induces in spermatozoa a concentration-dependent increase of lipid peroxidation (Fig. 2).

3.6. Intracellular pH (pH_i)

The pH_i is of the utmost importance for sperm function. It is involved in sperm motility and acrosome reaction processes (Lee et al., 1983; Nishigaki et al., 2014; Tosti, 1994). A slight lowering of pH_i has been demonstrated in different cell lines and ascidian spermatozoa following exposure to CuO NPs, even if this variation seems to have no physiological relevance(Favret and Lynn, 2010; Moersdorf et al., 2010). In the present study, we demonstrated that CuO NPs did not significantly affect pH_i at any tested concentration (data not shown), probably due to the ability of spermatozoa to regulate their pH_i very efficiently and rapidly.

3.7. Sperm DNA damage

Sperm DNA integrity is necessary for the correct transmission of genetic material to the following generation. DNA damage, in fact, could result in an early arrest in embryo development or even prevent fertilization. Sperm DNA integrity assessment has gained special attention as an important sperm quality marker, linked with sperm fitness as well as with offspring quality.

The DNA integrity of spermatozoa in marine organisms is significantly affected owing to sperm exposure to genotoxic contaminants that are prevalent in the marine ecosystem. Sperm DNA damages were observed in marine animals after exposure to different xenobiotics and have been correlated with fertilizing capability reduction, embryo abnormalities and offspring damage (Lewis and Ford, 2012). NP-mediated genotoxicity has been previously observed for metal oxide NPs in

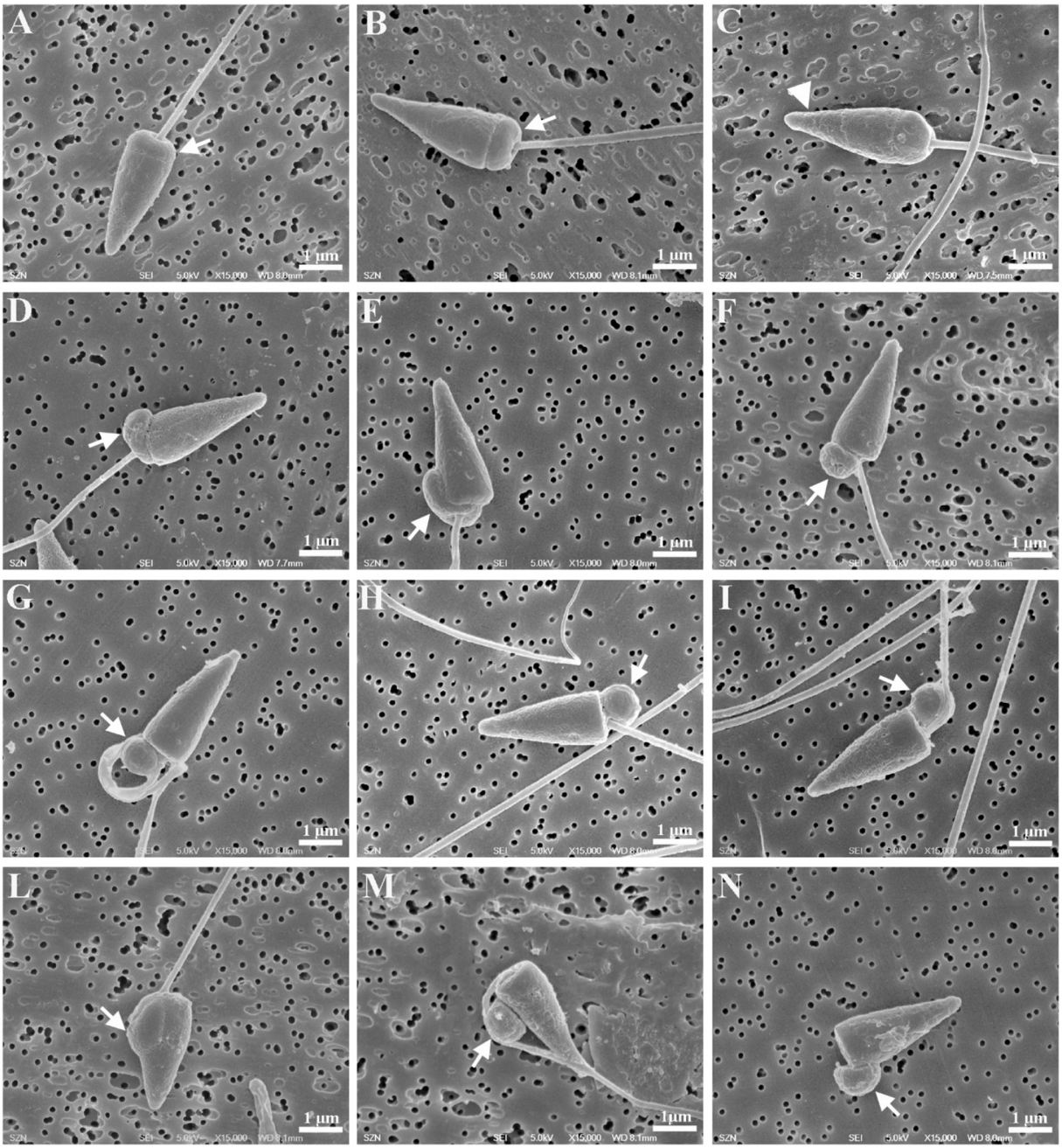

Fig. 3. SEM images of CuO NPs effects on sperm morphology. (A) control spermatozoon shows typical wrinkling of the head plasma membrane and thigh contact between head and mitochondrial ring indicated by arrow. Sperm exposed to 2 mg/L CuO NPs show the distortion of the mitochondrial ring indicated by arrow (B) and focal disintegration of the head plasma membrane indicated by arrow head (C); (D and E) sperm exposed to 5 mg/L CuO NPs show the initial detachment of the mitochondrial ring (arrow) causing the shift of the tail towards angled position; (F-I) sperm exposed to 10 mg/L CuO NPs show the detachment and agglomeration of mitochondrial ring indicated by arrow; (L-N) sperm exposed to 20 mg/L CuO NPs show total detachment of mitochondrial ring indicated by arrow, alteration of head morphology and loss of the tail.

spermatozoa of the mussel *Mytilus galloprovincialis* and the ascidian *Ciona intestinalis* (Gallo et al., 2016; Kadar et al., 2011). In this study, sea urchin sperm DNA damage was evaluated by using TUNEL assay. CuO NP-treated spermatozoa showed a concentration dependent increase in DNA fragmentation (Fig. 2). NPs can directly and/or indirectly promote DNA damage. Some NPs owing to their small size, are capable of reaching the nucleus and interact directly with DNA or they may also exhibit an indirect effect on DNA through their ability to generate ROS[78, 81]. In this study, ROS can be considered the major source of DNA damage. Here, we provide the first evidence for DNA damage assessed by TUNEL assay in sea urchin spermatozoa exposed to CuO NPs.

3.8. Sperm morphology

Normal morphology of a spermatozoon is considered an important factor in relation to the ability to fertilize an oocyte. The identification of morphological anomalies in mature spermatozoa is probably the oldest and the simplest way of assessing sperm quality and predicting sperm fertilization potential (Trivedi et al., 2010; Wyrobek et al., 1975). However, the evaluation of sperm morphology is scarcely used in toxicological and ecotoxicological studies for the screening of NP teratogenic effects. In particular, NP-induced abnormalities in spermatozoa have not yet been investigated in marine invertebrate species.

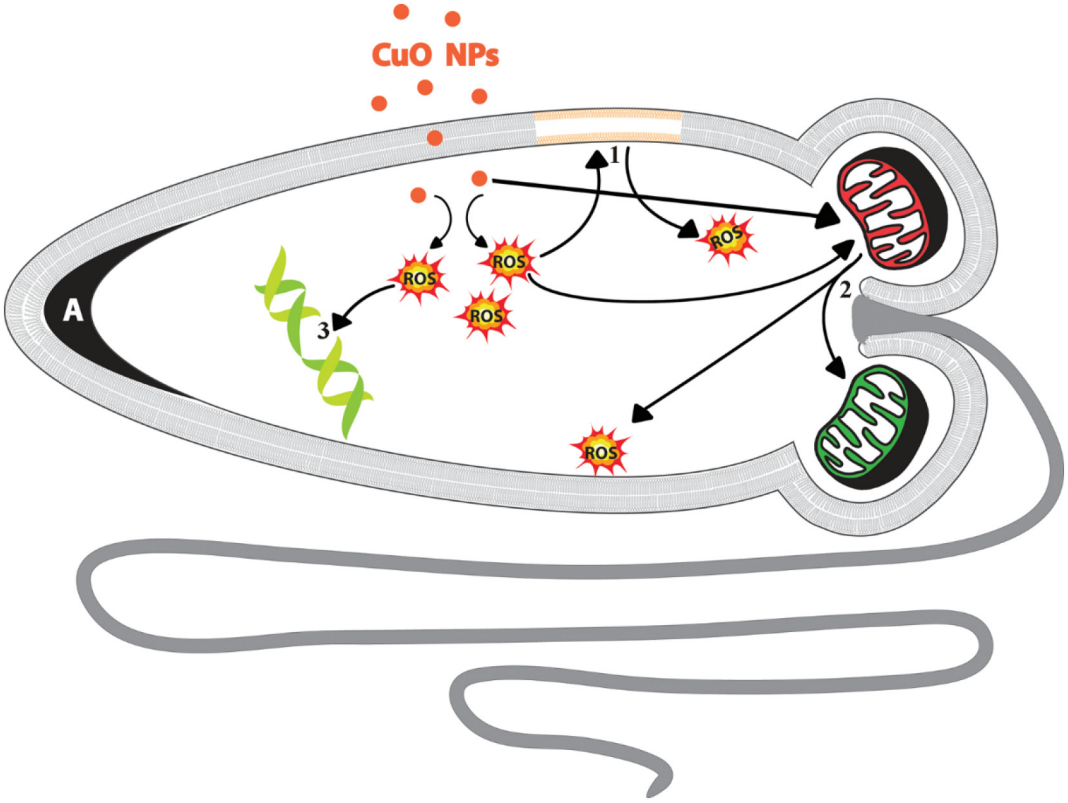

Fig. 4. CuO NPs spermiotoxicity mechanism. CuO NPs induce a spontaneous generation of ROS and mitochondrial damage, which in turn lead to increased ROS production. Intracellular ROS induce 1) plasma membrane lipid peroxidation, 2) mitochondrial dysfunction and 3) DNA fragmentation.

In sperm treated with CuO NPs, SEM analyses performed in this study revealed focal areas of head plasma membrane erosions, a fracture at the border between sperm head and mitochondrial ring. The extent and depth of these fractures increased at increasing NPs concentration. Furthermore, the detachment and/or distortion of the mitochondrial package caused the shift of the tail towards angled position causing a functional discontinuity between sperm head and the tail (Fig. 3). These results indicated that NPs induced dose-dependent alterations of sperm plasma membrane and morphology by deforming the normal arrangement of tail - mitochondria system that is necessary for sperm motility and fertilization capability. In the present study, we demonstrated that sea urchin sperm exposure to CuO NPs induces an increase in lipid peroxidation that, altering the organization of sperm membrane lipids, may cause defective spermatozoa identified.

4. Conclusion

Fertilization is a crucial process of sexual reproduction depending, among other factors, on sperm functionality that is related to various cellular parameters such as membrane and DNA integrity, mitochondrial activity and ROS production. Here, by performing an antioxidant rescue experiment (supplementary material), we demonstrated that ROS production is the upstream molecular event leading to the CuO NP induced damages. This elucidates the mechanism of CuO NP spermiotoxicity that involves the spontaneous generation of ROS and the disruption of the mitochondrial respiratory chain leading to production of ROS that, in turn, induce lipid peroxidation, DNA damage and result in defective spermatozoa (Fig. 4) leading ultimately to sperm mortality. Furthermore, CuO NPs impair sperm quality compromising fertilization success as reported in our previous study (Rotini et al., 2018). In sea urchin, CuO NP exposure is associated with a reduction in sperm quality and fertilization failure; such effects have potentially implications for specie fitness and survival. The effects of NPs on spermatozoa depend on NP nature and size and on the tested organisms; hence, further research on the impact of NPs on sperm quality of marine organisms will beneficial.

The present study highlights that spermatozoa are sensitive to NP exposure; consequently, spermiotoxicity assay need to be included when evaluating the overall toxicological impact of NPs in the marine environment. Furthermore, the integration of traditional spermiotoxicity tests with more specific assays, like those performed in the present study, is auspicable. Indeed, these assays represent useful tools to rapidly screen NPs for spermiotoxicity and may help understanding the major toxic mechanisms that could be relevant for different NP types in marine organisms.

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Authors' contributions

AG and RB designed and performed the experiments, and analyzed the data; LoM and AR contributed to the experiments; LuM provided nanoparticle solution; ET designed and supervised the project; AG and ET wrote the paper with input from all authors.

Conflict of interest

The authors declare no competing financial interest.

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