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# Very low concentration of cerium dioxide nanoparticles induce DNA damage, but no loss of vitality, in human spermatozoa

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## ABSTRACT

Cerium dioxide nanoparticles (CeO<sub>2</sub>NP) are widely used for industrial purposes, as in diesel, paint, wood stain and as potential therapeutic applications. The Organization for Economic Cooperation and Development included CeO<sub>2</sub>NP in the priority list of nanomaterials requiring urgent evaluation. As metal nanoparticles can cross the blood-testis barrier, CeO<sub>2</sub>NP could interact with spermatozoa. The genotoxicity of CeO<sub>2</sub>NP was demonstrated *in vitro* on human cell lines and mouse gametes. However, the effects of CeO<sub>2</sub>NP on human spermatozoa DNA remain unknown. We showed significant DNA damage induced *in vitro* by CeO<sub>2</sub>NP on human spermatozoa using Comet assay. The genotoxicity was inversely proportional to the concentration (0.01 to 10 mg·L<sup>-1</sup>). TEM showed no internalization of CeO<sub>2</sub>NP into the spermatozoa. This study shows for the first time that *in vitro* exposure to very low concentrations of cerium dioxide nanoparticles can induce significant DNA damage in human spermatozoa. These results add new and important insights regarding the reproductive toxicity of priority nanomaterials, which require urgent evaluation.

## 1. Introduction

Due to the extensive industrial production of nanoparticles, human exposure has been increasing exponentially. The reproductive toxicity of nanoparticles is a particularly important issue (Ema et al., 2010; Greco et al., 2015a), as toxic effects can be transgenerational. Indeed, Yoisingnern et al. showed that silver nanoparticles could be internalized into mouse sperm *in vitro* and subsequently alter fertilization and compromise embryo development (Yoisingnern et al., 2015). Moretti et al. incubated human sperm with high-concentration suspensions of silver and gold nanoparticles *in vitro*, observed a significant dose-dependent decrease of motility and viability, and described the internalization of Au-NP in sperm cells (Moretti et al., 2013). Taylor et al. recently emphasized the need for a better understanding of the reproductive toxicity of nanoparticles (Taylor et al., 2015).

Cerium dioxide nanoparticles (CeO<sub>2</sub>NP) are commonly used for industrial purposes, including as a diesel additive (Cassee et al., 2011; Park et al., 2008) or wood stain, and have potential medical applications such as protection against radiation-induced damage (Giri et al., 2013; Sack et al., 2014; Tarnuzzer et al., 2005). CeO<sub>2</sub>NP are on the priority list of nanomaterials requiring urgent evaluation as declared by the Organization for Economic Cooperation and Development (OECD's

guidelines, 2011). Indeed, the toxicity/safety assessment of CeO<sub>2</sub>NP is still incomplete, with only a few studies available. It has been shown in rats that CeO<sub>2</sub>NP can cross the blood-testis barrier and accumulate into the testis after *in vivo* exposure (Geraets et al., 2012); CeO<sub>2</sub>NP could then interact with spermatozoa. The impact of oral administration of citrate-coated 2–5-nm CeO<sub>2</sub>NP on semen parameters was recently investigated in aged rats *in vivo* (Kobyliak et al., 2015), and an improvement in sperm concentration, motility and morphology was observed in treated rats compared to control rats. Similarly, Falchi et al. reported no intracellular uptake and no impairment of the functional and morphological characteristics of ram sperm after *in vitro* exposure to high concentrations of CeO<sub>2</sub>NP (Falchi et al., 2016). Conversely, the genotoxicity of CeO<sub>2</sub>NP has been shown in human cell lines, mouse oocytes and spermatozoa (Benameur et al., 2015; Courbiere et al., 2013; Mittal and Pandey, 2014; Préaubert et al., 2015) after *in vitro* exposure. However, the effects of CeO<sub>2</sub>NP on human sperm DNA remain unknown. Our objective was to study the *in vitro* genotoxicity of well-characterized CeO<sub>2</sub>NP on human spermatozoa at low doses.

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## 2. Materials and methods

### 2.1. Physico-chemical characterization of CeO<sub>2</sub> nanoparticles

As the characterization of nanomaterial is of utmost importance for *in vitro* testing of toxicity (Love et al., 2012), we performed a thorough physico-chemical characterization of the cerium dioxide nanoparticles. CeO<sub>2</sub>NP (Rhodia chemicals) were synthesized by aqueous precipitation of Ce<sup>4+</sup>(NO<sub>3</sub><sup>-</sup>)<sub>4</sub> salt at an acidic pH (Spalla and Cabane, 1993). They are ellipsoidal crystallites with a mean diameter of ~7 nm and a specific surface area evaluated at 400 m<sup>2</sup>/g (Spalla and Cabane, 1993; Thill et al., 2006). Their hydrodynamic diameters were measured either in a stock suspension using dynamic light scattering (DLS) (NanoZS, Malvern Instruments® Inc., UK) with an optimal measurement range of 1 to 1000 nm or in FertiCult® medium using laser diffraction (Malvern3000, Malvern Instruments® Inc., UK).

The local atomic environment and oxidation states before and after incubation in FertiCult® were assessed by X-ray absorption spectroscopy at the Ce L<sub>3</sub>-edge (5723 eV). A concentration of 100 mg·L<sup>-1</sup> CeO<sub>2</sub>NP were incubated for 1 h in abiotic FertiCult® medium and then ultracentrifuged at 200,000g for 1 h. The solid phase was freeze-dried and analyzed by XAS (CRG-FAME beamline at the ESRF, France). Samples were diluted in BN, pressed into thin pellets, and analyzed at liquid helium temperatures in fluorescence mode with a 30-element solid-state Ge detector. The spectra were compiled from the merge of three scans. XANES (X-ray absorption near edge structure) data were obtained after performing standard procedures for pre-edge subtraction and normalization.

The dissolution of CeO<sub>2</sub>NP after a 1-h incubation in FertiCult® medium was assessed by inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer, Nexion 300 ×). The contaminated media (e.g., 0.01 mg·L<sup>-1</sup> to 10 mg·L<sup>-1</sup> of CeO<sub>2</sub>) were ultracentrifuged at 200,000g for 1 h and the cerium levels were detected in the supernatant.

### 2.2. Experimental design

According to the Organization for Economic Co-operation and Development guideline (OECD, 2014; Lovell and Omori, 2008; Wiklund and Agurell, 2003), we performed 3 independent *in vitro* experiments and analyzed 3 replicate slides from each experiment.

### 2.3. Culture media and reagents

We used FertiCult IVF® culture medium (FertiPro, Beernem, Belgium), which is specifically designed for *in vitro* human sperm and embryo culture. All other reagents were provided by Sigma-Aldrich® (St-Quentin-Fallavier, France) unless otherwise mentioned.

### 2.4. Collection and *in vitro* exposure of human spermatozoa

We used frozen human spermatozoa from 3 healthy fertile donors. The semen samples had been diluted in cryoprotectant medium according to the manufacturer's instructions (Spermfreeze; JCD, La Mulatiere, France), transferred to high security straws with a capacity of 300 µL (Cryo Bio System, L'Aigle, France), and then stored in liquid nitrogen until further use. These spermatozoa were purchased from GERMETHEQUE biobank, which obtained informed consent from each donor for inclusion of samples in the biobank and for their use in research experiments regarding human fertility in accordance with the 1975 Helsinki Declaration on human experimentation. The GERMETHEQUE biobank (BB-0033-00081 Marseille, France) Scientific Committee approved the present study design (number 20130102).

Straws were placed in a 37 °C water bath for 5 min; then, the cryoprotectant was removed by progressively diluting the thawed sample with 1 mL of FertiCult® culture medium at 37 °C. The preparation was aliquoted and centrifuged for 10 min at 1500 rpm (420 g), and

the supernatants were discarded. Then, 150 µL of the subsequent suspensions were carefully disposed on each pellet. As the genotoxicity of CeO<sub>2</sub>NP on human spermatozoa had not been studied before, we assessed a wide range of CeO<sub>2</sub>NP concentrations. The suspensions used for *in vitro* exposure of spermatozoa were a) FertiCult® culture medium (negative control); b) FertiCult® containing 0.01, 0.1, 1 or 10 mg·L<sup>-1</sup> CeO<sub>2</sub>NP; c) the supernatant obtained after ultracentrifugation (60,000 rpm (16,000g) for 1 h) of the suspensions from b); d) a 110 µM H<sub>2</sub>O<sub>2</sub> solution in FertiCult® (positive control); e) the same CeO<sub>2</sub>NP suspensions as described in b) with 5 mM L-ergothioneine. L-Ergothioneine (L-erg) is an anti-oxidant commonly used in toxicological studies as a powerful scavenger of free radicals to explore the involvement of oxidative stress in the induction of DNA damage (Franzoni et al., 2006). We incubated the preparations for 1 h at 37 °C in 5% CO<sub>2</sub>, after which we analyzed the supernatants containing motile-selected spermatozoa. We therefore studied spermatozoa selected by swim-up and included all motile sperm. The *in vitro* culture conditions we chose corresponded to the IVF-based culture conditions, which are close to those of the female genital tract environment.

For each CeO<sub>2</sub>NP concentration, 3 different conditions were studied: 1) CeO<sub>2</sub>NP suspensions in culture medium; 2) the supernatants of the same suspensions (containing dissolved Ce<sup>3+</sup>); and 3) CeO<sub>2</sub>NP suspensions with L-erg. Each condition of each concentration was repeated 3 times.

### 2.5. Sperm vitality

We assessed the viability of spermatozoa before performing each triplicate of the Comet assay to exclude sperm DNA damage associated with cytotoxicity. According to the World Health Organization manual, (World Health Organization, 2010), we combined 10 µL of a 0.5% eosin solution (Gilbert®, Hérouville-Saint-Clair, France) containing 0.9% NaCl with 10 µL of the exposed sperm sample and observed the preparation at ×400 under a contrast microscope. The percentage of live (unstained) and dead (red-stained) spermatozoa was assessed blindly from at least 100 evaluated cells in each condition.

### 2.6. Human sperm comet assay

The Comet assay is a common genotoxicity test that detects and quantifies DNA primary lesions of eukaryotic cells (Olive and Banáth, 2006). The Comet assay has been validated for the testing of chemicals to determine mutagenicity (Eastmond et al., 2009; Parry, 2000). It is highly sensitive, adaptable and does not require a large number of cells (Baumgartner et al., 2009). We performed a procedure adapted from Baumgartner et al. (2012), each condition and concentration in triplicates.

Each spermatozoa suspension obtained after *in vitro* exposure was mixed with an equal volume of 2% low melting point (LMP) agarose. The obtained suspension was spread on 3 glass slides that were pre-coated with 1% normal melting point (NMP) agarose. A third layer of 0.5% LMP agarose was added. Slides were immersed in a lysis buffer (100 mM EDTA, 10 mM Tris base, 2.5 M NaCl, 1% Triton X-100, 10% DMSO, 5 mM dithiothreitol, 0.01 mg·mL<sup>-1</sup> proteinase K; pH 10) for 1 h and then transferred to an electrophoresis tank. Slides were covered by freshly prepared electrophoresis buffer (1 mM EDTA, 300 mM NaOH; pH 13.5) at 4 °C, rested for 20 min for equilibration, and then electrophoresed at 25 V at 4 °C for 20 min in a 30 cm-long electrophoresis tank. Slides were rinsed with a Tris buffer (0.4 M Tris base; pH 7.4) and dehydrated with methanol.

#### 2.6.1. Scoring of comet slides

Slides were read blindly after staining with 0.1 mg·mL<sup>-1</sup> propidium iodide. For each condition, at least 50 spermatozoa were evaluated per slide (i.e., at least 150 spermatozoa over 3 slides per condition). All experiments were repeated three times (i.e., at least 450 spermatozoa in

3 independent experiments per condition). Quantitative image analysis was performed using a CCD camera (Andor Technology, Belfast, UK) attached to the microscope (Olympus, Rungis, France) and linked to the comet analysis software (version 6.0; Andor Bioimaging, Nottingham, UK). Sperm DNA damage was expressed as the percentage of tail DNA (% tail DNA), which is total DNA that migrates from the nucleus into the comet tail during electrophoresis (Baumgartner et al., 2012).

## 2.7. Transmission electron microscopy (TEM)

We performed TEM analysis on spermatozoa exposed to  $0.01 \text{ mg}\cdot\text{L}^{-1}$   $\text{CeO}_2\text{NP}$  for 1 h in order to explore the interaction between  $\text{CeO}_2\text{NP}$  and spermatozoa at the lowest studied concentration. Samples were washed two times in phosphate buffer, fixed with 2.5% glutaraldehyde for 30 min, dehydrated in a graded series of ethanol and finally embedded with an Embed-812 kit using a standard procedure. Ultrathin sections of 60 nm were examined with a JEOL/JEM 1400 apparatus, and images were obtained with a MegaView III CCD camera (SIS-Olympus, Munster, Germany).

## 2.8. Statistics

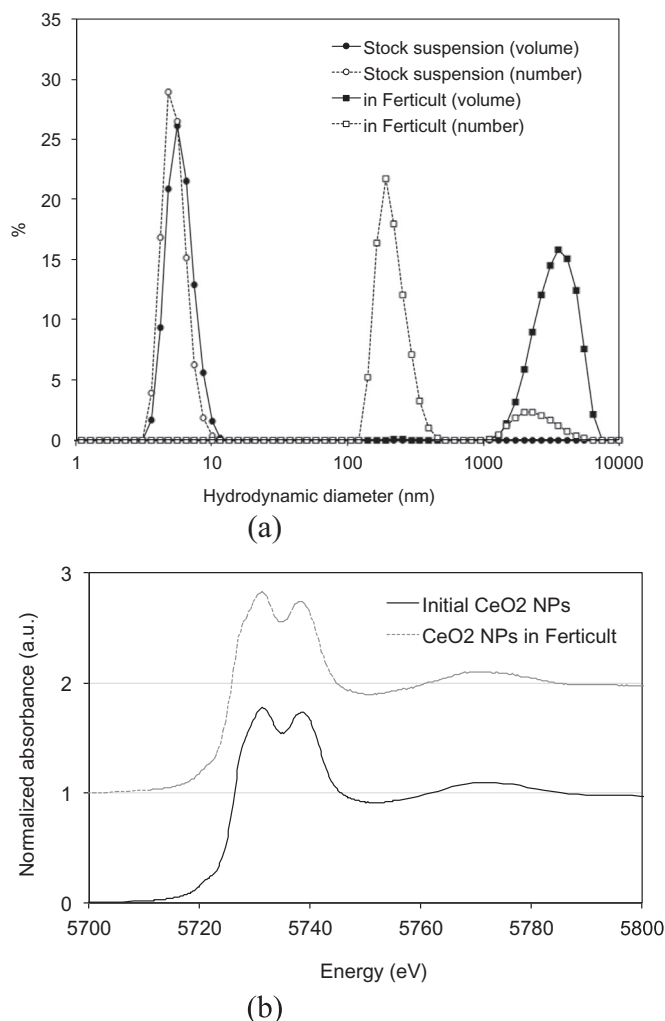
Each experiment contained at least 150 raw values of % tail DNA by condition and was replicated 3 times: the data for each condition are presented as the 3 means of % Tail DNA median values from the 3 independent experiments. For each condition, we summarized the values obtained for every experiment and performed a one-way ANOVA test using StatView 5.1 software (Abacus Concept, Berkeley, CA, USA), to compare DNA damage between the various concentration levels and control, between the various concentrations and, for each concentration, between the 3 conditions (Krzywinski et al., 2014). Differences were considered statistically significant at  $p < 0.05$ .

## 3. Results

### 3.1. Colloidal and chemical behavior of $\text{CeO}_2\text{NP}$ in FertiCult® medium

Fig. 1a shows the distribution of the hydrodynamic diameters ( $D_h$ ) of  $100 \text{ mg}\cdot\text{L}^{-1}$   $\text{CeO}_2\text{NP}$  after a 1-h incubation in FertiCult® medium. Although  $\text{CeO}_2\text{NP}$  are colloiddally stable in their stock suspension (with a  $D_h$  distribution centered at  $\sim 7 \text{ nm}$ ), significant aggregation occurred in the FertiCult® with a  $D_h$  centered at  $3.6 \mu\text{m}$  (volume distribution). Once expressed as a number, the distribution of most of these aggregates had a  $D_h$  centered at  $190 \text{ nm}$ . Even if this number distribution is based on assumptions regarding the shape, the density of the aggregates, etc., it highlights that most of the  $\text{CeO}_2\text{NP}$  interact with the spermatozoa as small aggregates.

The chemical stability of the nanoparticles was studied in FertiCult® medium in terms of dissolution and local-scale environment. The ICP-MS measurements show that  $< 2\%$  and  $0.02\%$  of the  $\text{CeO}_2\text{NP}$  were dissolved after 1 h from initial  $\text{CeO}_2$  concentrations of  $0.01 \text{ mg}\cdot\text{L}^{-1}$  and  $10 \text{ mg}\cdot\text{L}^{-1}$ , respectively (Fig. 3). This is in agreement with the low solubility expected for Ce oxy-hydroxide (as  $K_{sp}\text{Ce}(\text{OH})_3 = 6.3 \times 10^{-24}$  at  $25^\circ\text{C}$ ) (Söhnel and Garside, 1992). This chemical stability was also observed by XANES at the Ce  $L_3$ -edge (Fig. 1b). The experimental spectra of  $\text{CeO}_2\text{NP}$  before and after the 1-h incubation in FertiCult® are superimposed and indicate that the atomic structure of  $\text{CeO}_2\text{NP}$  is not affected. XANES is not sensitive to minor Ce species (i.e.,  $< 10\%$ ). Consequently, the detection of  $< 2\%$  Ce dissolution is not contradictory with the structural stability observed by XANES. Such local-scale stability and slow dissolution suggests that the  $\text{CeO}_2\text{NP}$  surface interaction with molecules in the FertiCult® medium is not associated with major surface complexation or reduction into Ce(III).



**Fig. 1.** Colloidal behavior of  $\text{CeO}_2\text{NP}$  in abiotic culture medium. (a) Distribution of the hydrodynamic diameters of  $\text{CeO}_2\text{NP}$  in their stock suspension and after 1 h in FertiCult® at  $100 \text{ mg}\cdot\text{L}^{-1}$  of  $\text{CeO}_2$ . (b) Structural stability of  $\text{CeO}_2\text{NP}$  after 1 h of incubation in FertiCult® medium as assessed by XANES at the Ce  $L_3$ -edge.

### 3.2. Human sperm comet assay

The spermatozoa viability rates were all  $> 58\%$ , which is the normality threshold for human spermatozoa as stated by the WHO criteria (World Health Organization, 2010).

DNA damage in the spermatozoa was quantified by % tail DNA. The variability of biological data in the 3 independent experiments and in each 3 replicate slide of each independent experiment is presented in Table 1.

The comet assay showed that compared to the negative control, there was a significant increase of DNA damage in human spermatozoa after *in vitro* exposure to  $\text{CeO}_2\text{NP}$  at all concentrations (Fig. 2), the supernatants of the  $\text{CeO}_2\text{NP}$  suspensions (Fig. 3) and the l-erg conditions (Fig. 2), ( $p < 0.0001$ ).

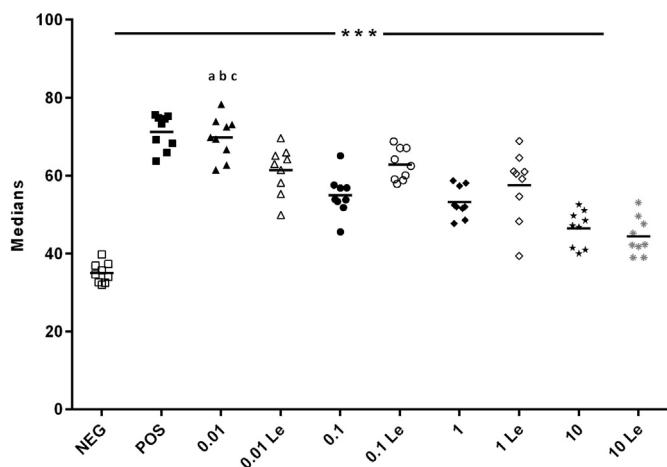
We detected a significant increase in DNA damage in spermatozoa exposed to the lowest nanoparticle concentrations, i.e.,  $0.01 \text{ mg}\cdot\text{L}^{-1}$   $\text{CeO}_2\text{NP}$  (mean median = 69.8) versus higher concentrations i.e.  $0.1 \text{ mg}\cdot\text{L}^{-1}$ ,  $1$  and  $10 \text{ mg}\cdot\text{L}^{-1}$   $\text{CeO}_2\text{NP}$  (55.0, 53.2 and 46.5, respectively) ( $p < 0.001$ ).

We observed no significant difference among the 3 conditions ( $\text{CeO}_2\text{NP}$ , supernatants and l-erg).

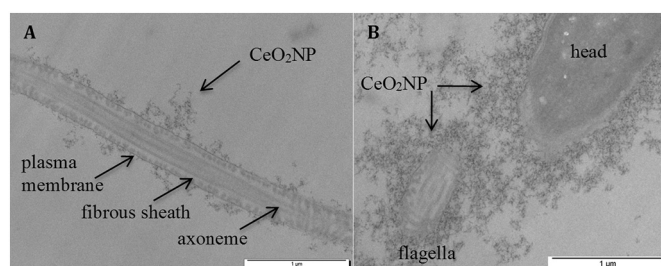
**Table 1**

Biological variability of the data. Median values of % Tail DNA of each slide (and means of median values of each independent experiment). Concentrations of CeO<sub>2</sub>NP suspensions (0.01, 0.1, 1, 10) are expressed in mg·L<sup>-1</sup>. S: exposure to supernatant (obtained after ultracentrifugation of the CeO<sub>2</sub>NP suspensions); L-erg: adjunction of L-ergothioneine (anti-oxidant) in the CeO<sub>2</sub>NP suspension.

		Negative control	Positive control	0.01	0.01 S	0.01 L-erg	0.1	0.1 S	0.1 L-erg	1	1 S	1 L-erg	10	10 S	10 L-erg
Experiment 1	Slide 1	36,9	74,5	73,9	62,3	49,9	65,1	62,4	62,5	52,0	55,0	54,7	40,0	48,6	45,2
	Slide 2	37,3	73,3	72,5	52,2	55,3	53,8	57,2	57,9	47,7	49,9	48,3	47,2	50,0	39,0
	Slide 3	32,5	75,6	69,4	65,7	64,2	53,4	59,7	58,8	51,6	59,2	61,0	52,6	41,4	42,2
	Mean of medians	35,6	74,5	71,9	60,0	56,4	57,4	59,8	59,7	50,4	54,7	54,7	46,6	46,6	42,1
Experiment 2	Slide 1	34,7	74,8	73,1	68,0	69,6	56,8	61,5	68,8	58,7	57,2	64,6	49,7	46,3	53,1
	Slide 2	35,7	75,3	78,3	55,5	65,9	56,9	57,8	59,1	52,5	64,7	59,2	51,1	34,1	41,8
	Slide 3	32,0	68,3	69,8	65,7	63,0	53,9	63,3	67,1	52,0	56,6	61,1	48,5	49,0	47,6
	Mean of medians	34,1	72,8	73,7	63,0	66,2	55,9	60,9	65,0	54,4	59,5	61,7	49,8	43,1	47,5
Experiment 3	Slide 1	32,6	69,2	62,7	52,7	58,2	57,6	62,5	64,2	48,6	53,9	39,4	41,0	44,6	39,0
	Slide 2	34,0	65,9	61,5	61,2	65,1	45,6	57,8	67,1	58,1	55,4	60,5	46,8	54,0	42,3
	Slide 3	39,8	63,7	66,7	60,9	61,5	51,8	50,9	60,1	57,4	56,1	68,9	41,5	36,6	49,6
	Mean of medians	35,4	66,3	63,6	58,2	61,6	51,7	57,1	63,8	54,7	55,1	56,3	43,1	45,1	43,6



**Fig. 2.** The comet assay shows, compared to the negative control, a significant increase of DNA damage in human sperm after a 1-hour *in vitro* exposure to CeO<sub>2</sub>NP at all concentrations as well as in the presence of an anti-oxidant, L-erg (\*) ( $p < 0.0001$ ). The results are presented as the 9 median values of % Tail DNA obtained from 9 different slides from 3 independent experiments for each condition. L-erg: L-ergothioneine (anti-oxidant). a: vs. 0.1 mg·L<sup>-1</sup> CeO<sub>2</sub>NP. b: vs. 1 mg·L<sup>-1</sup> CeO<sub>2</sub>NP. c: vs. 10 mg·L<sup>-1</sup> CeO<sub>2</sub>NP ( $p < 0.0001$ ). Positive control: 110  $\mu$ M H<sub>2</sub>O<sub>2</sub>.



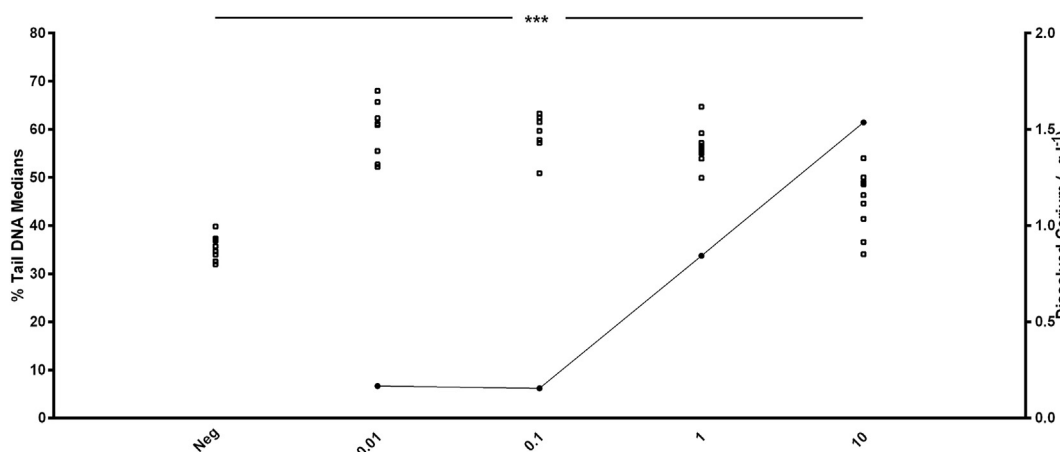
**Fig. 4.** The TEM aspect of human spermatozoa exposed to 0.01 mg·L<sup>-1</sup> CeO<sub>2</sub>NP *in vitro*. One-hour exposure at 0.01 mg·L<sup>-1</sup>: accumulation of nanoparticles (→) on the plasma membrane. A) Longitudinal section of the flagella. B) Transversal section of the flagella (on the left) and head.

### 3.3. TEM analysis of spermatozoa

After a 1 h incubation in 0.01 mg·L<sup>-1</sup> CeO<sub>2</sub>NP suspension in FertiCult® culture medium, TEM showed the accumulation of CeO<sub>2</sub>NP on the plasma membranes of exposed human spermatozoa (Fig. 4), particularly along the flagellum. We did not observe any internalization of CeO<sub>2</sub>NP in spermatozoa.

## 4. Discussion

We showed that *in vitro* exposure of human spermatozoa to CeO<sub>2</sub>NP significantly increased DNA damage at all concentrations as assessed by



**Fig. 3.** Chemical behavior of CeO<sub>2</sub>NP in the culture medium and genotoxicity of the corresponding supernatant. Release of Ce from the nanoparticles after a 1-h incubation in FertiCult® medium. [CeO<sub>2</sub>]<sub>initial</sub> = 0.01, 0.1, 1, or 10 mg·L<sup>-1</sup>; ambient temperature. The comet assay showed a significant increase in DNA damage in human sperm after a 1-h *in vitro* exposure to the supernatants (S, obtained after ultracentrifugation) of the CeO<sub>2</sub>NP suspensions compared to negative control (\*\*\*) ( $p < 0.0001$ ). The results are presented as the 9 median values of % Tail DNA obtained from 9 different slides from 3 independent experiments for each condition.



the comet assay. We did not observe any internalization of CeO<sub>2</sub>NP in spermatozoa. Interestingly, the lowest concentrations of nanoparticles were associated with the highest amount of DNA damage.

A strong point of our study design is the wide range of CeO<sub>2</sub>NP concentrations explored, including very low concentrations (0.01 to 10 mg·L<sup>-1</sup>). Indeed, the majority of recent studies about the genotoxicity of CeO<sub>2</sub>NP on human cells analyzed the impact of nanoparticle concentrations ranging from 0.1 to 100 mg·L<sup>-1</sup> (Franchi et al., 2015; Mittal and Pandey, 2014; Strobel et al., 2015; Verstraelen et al., 2014; Yoisungnern et al., 2015). We addressed lower exposure doses that are probably closer to the expected *in vivo* concentrations and remain poorly investigated. Nevertheless, to the best of our knowledge, no information is available about the expected human *in vivo* concentrations in the testes, seminal ducts, uterus or Fallopian tubes. Another strong point is that we performed an extensive chemical and physical characterization of the nanoparticles; this type of data are essential for toxicity studies *in vitro* and *in vivo* (Love et al., 2012).

The inverse dose-effect relationship observed has been described in previous nanotoxicity studies. Sergent et al. studied the toxicity of 100 nm SiO<sub>2</sub> nanoparticles (concentrations ranging from 10 to 150 mg·L<sup>-1</sup>) on the HT-29 human intestine cell line and showed inverse dose-dependent relationships between the nanoparticle concentration and cell viability and genotoxicity (Sergent et al., 2012). The authors hypothesized that the phenomenon of phagocytosis of damaged cells by undamaged cells could occur to maintain the integrity of the islet profile. Since sperm have no phagocytic abilities, it is unlikely that such a mechanism was involved in our study. Our results could be explained by dose-dependent changes in the physico-chemical behavior. At low doses, the probability of contact between two particles (hom aggregation) decreases, and it is likely that CeO<sub>2</sub>NP are more dispersed at lower concentrations. Indeed, agglomeration increases the contact surface between NP. Consequently, CeO<sub>2</sub>NP dispersion could lead to small aggregates with a large surface area available to interact with cells, which could enhance the biotransformation, biological and toxicological effects (Nel et al., 2009; Yoshida et al., 2012). Future work should include genotoxicity assessments at lower concentrations.

This inverse dose response could also be related to different genotoxicity mechanisms. At the lowest concentration (0.01 mg·L<sup>-1</sup>), the adjunction of an anti-oxidant (L-erg) in the exposure medium decreased DNA damage in the sperm. This indirectly highlights that oxidative stress is one mechanism involved in the observed DNA damage. With their high redox potential, CeO<sub>2</sub>NP can easily become reduced once in close contact with spermatozoa, thereby oxidizing the nearby organic molecules (Auffan et al., 2009a; Xu and Qu, 2014). Oxidative stress induced by *in vitro* exposure to CeO<sub>2</sub>NP has been described in human lung cells (leading to cytotoxicity (Mittal and Pandey, 2014)) and dermal fibroblasts (leading to DNA and chromosome damages (Auffan et al., 2009b)). Oxidative stress is known to induce DNA damage in sperm by oxidizing DNA bases (Aitken and Iuliiis, 2010). A higher oxidative stress at very low concentration could be related to a higher contact surface between sperm and CeO<sub>2</sub>NP, which could enhance the toxicological effect (Nel et al., 2009; Yoshida et al., 2012). These results are in agreement with previous nanotoxicity studies performed on mouse gametes (Greco et al., 2015b; Preaubert et al., 2015) and corroborate a mechanism of DNA damage occurring at low concentrations of nanoparticles (0.01 mg·L<sup>-1</sup>), thus requiring a close interaction between the cells and the CeO<sub>2</sub>NP.

At higher concentrations (0.1, 1 and 10 mg·L<sup>-1</sup>), no difference was observed between the applied nanoparticles and DNA damage induced among the 3 experimental conditions (exposure to CeO<sub>2</sub>NP suspension, supernatant or CeO<sub>2</sub>NP suspension + anti-oxidant), suggesting that the genotoxicity mechanisms are different and that oxidative stress is not involved. At all studied concentrations, the dissolved cerium concentration in the supernatant did not increase proportionally to the nanoparticles concentration: for example, whereas the concentration of nanoparticles increased 1000-fold, the dissolved cerium increased only

8-fold (Fig. 3). We assume that at all concentrations, dissolved cerium could be involved in the observed genotoxicity. Indeed, the Ce<sup>3+</sup> ions present in the nanoparticle stock suspension related to cerium dissolution in the culture medium could diffuse through the plasma membrane and indirectly stress the spermatozoa. Our results confirm the importance of a careful assessment of the physico-chemical behavior of nanoparticles to better understand the mechanisms of genotoxicity towards germ cells.

Our study is limited by its *in vitro* nature. However, these results of *in vitro* exposure are interesting, as spermatozoa can encounter nanoparticles in the female genital tract or in IVF plots. Indeed, metal nanoparticles can transfer to various organs in animals after *in vivo* exposure (Blum et al., 2012; Gao et al., 2012; Tassinari et al., 2014), and nanomaterials have been increasingly studied as future medical applications (Barkalina et al., 2014).

In mice, we previously found that *in vitro* exposure to CeO<sub>2</sub>NP was associated with decreased fertilization rates (Preaubert et al., 2015). DNA damage in spermatozoa could be partly responsible for the decreased fertilization rates. The present results show that very low concentrations of CeO<sub>2</sub>NP also induce DNA damage in human sperm. For obvious ethical reasons, the impact of CeO<sub>2</sub>NP on human *in vitro* fertilization could not be analyzed.

## 5. Conclusion

This study shows for the first time that *in vitro* exposure to very low concentrations of cerium dioxide nanoparticles induces significant DNA damage in human spermatozoa. These results add new and important insights regarding the reproductive toxicity of priority nanomaterials requiring urgent evaluation and warrant further *in vivo* animal studies examining exposure to low concentrations of CeO<sub>2</sub>NP.

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## Conflict of interest

The authors report no conflicts of interest.

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