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# Cerium dioxide nanoparticles did not alter the functional and morphologic characteristics of ram sperm during short-term exposure

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ABSTRACT

The aim of the study was to investigate the interaction and the short-term effects of increasing doses of cerium dioxide nanoparticles ($CeO_2$ NPs) on ram spermatozoa, stored at 4 °C for up to 24 hours, on the main functional and kinematic parameters. Spermatozoa were incubated with 0, 22, 44, and 220 μg/mL of $CeO_2$ NPs at 4 °C and submitted at 0, 2, and 24 hours to the following analyses: (1) intracellular uptake of $CeO_2$ NPs by the spermatozoa; (2) kinematic parameters; (3) acrosome and membrane integrity; (4) integrity of DNA; (5) mitochondrial activity; (6) ROS production. The results indicated that the exposure of spermatozoa to increasing doses of nanoceria was well tolerated. No intracellular uptake of NPs by the cells was observed and both kinematic parameters and status of the membranes were not affected by the incubation with NPs ($P > 0.05$). Moreover, no influence on the redox status of spermatozoa and on the levels of fragmentation of DNA was reported among groups at any time ($P > 0.05$). The data collected provide new information about the impact of $CeO_2$ NPs on the male gamete in large animal model and could open future perspectives about their biomedical use in the assisted reproductive techniques.



## 1. Introduction

There is an increasing interest toward nanoparticles and their potential applications in everyday life. Engineered NPs are defined by the SCENIHR (Scientific Committee of Emerging and Newly Identified Health Risk) as particles which size ranges from 1 to 100 nm in diameter, conferring them novel chemical, physical, and biological characteristics. Because of their peculiar physicochemical properties, NPs are also nowadays widely studied for their use in biomedicine, e.g., as drug delivery systems, drug targeting, diagnostic imaging, and for treatments of a wide range of pathologies [1].

However, a better knowledge of their effects at cellular and tissue levels is necessary, and little information is available about their activity in the reproductive system. It has been demonstrated that NPs can accumulate in the ovaries [2] and can cross the hemato-testicular barrier [3–5]. However, their potential effects have not been clearly demonstrated, and they may depend to the chemical composition, cell type, and species sensitivity. Recently, the effects of gold and silver NPs on porcine oocytes and spermatozoa have been evaluated. Gold NPs did not have any effect on oocytes-cumulus complex and spermatozoa,

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whereas silver NPs were detrimental for oocytes-cumulus complex but not for spermatozoa [6]. Similarly, gold and silver NPs did not affect vitality and motility parameters of human spermatozoa [7]. On the contrary, bovine spermatozoa incubated with gold NPs displayed alterations in the chromatin decondensation ability [8], and exposure to silver led to cytotoxicity and genotoxicity of testicular cells in the mouse [9].

Among others, cerium dioxide, an inorganic compound of cerium, a rare earth element of lanthanide series, and its NPs (cerium dioxide nanoparticles [$CeO_2$ NPs]) are recently exerting an interest for their industrial applications such as catalysis, solid oxide fuel additives, sensor technology, and UV filters. This great interest is also related to the high surface area, which confers to $CeO_2$ NPs the ability to change the oxidation state [10]. This property is comparable to that of biological antioxidants such as superoxide dismutase [11,12] and catalase [13]. This unique scavenger property of $CeO_2$ NPs [10] has been recently investigated in several biological systems and cell types, representing promising applications in biomedicine [14–17]. On the other side, negative effects of $CeO_2$ NPs in several cell types of have been reported [18–21] indicating that this compound, as many other free radical scavenging substances, can have a paradox activity.

Little is known about the potential effects of $CeO_2$ NPs in the reproductive system and gametes. In female germ cells of mice, it has been reported that $CeO_2$ NPs are able to penetrate into follicular cells and to be trapped in zona pellucida inducing dose-dependent oxidative stress and consequent DNA damage [22]. Moreover, a recent study that investigated the effects of $CeO_2$ NPs on IVF in mice, reported genotoxicity in female and male gametes and a deleterious effect on fertilization rates [23]. Conversely, in rats, it has been shown that the supplementation of $CeO_2$ NPs in the diet leads to an improvement in progressive motility (PM) and viability of spermatozoa through an antioxidant activity [24].

To our knowledge, no reports are available nowadays on the interactions and biocompatibility of $CeO_2$ on spermatozoa in large animal model. Therefore, the aim of the present study was to investigate the interaction and the short-term effects of increasing doses of $CeO_2$ NPs on ram spermatozoa, stored at 4 °C for up to 24hour, on the kinematic parameters, membrane status, DNA fragmentation, mitochondrial activity, and reactive oxygen species (ROS) production.

## 2. Methods

### 2.1. Synthesis and characterization of cerium dioxide nanoparticles ($CeO_2$ NPs)

Cerium (III) nitrate hexahydrate ($Ce[NO_3]_3\ 6H_2O$, ABCR 99.9%), urea ($CH_4N_2O$, Aldrich 99%), 2-propanol (99.7%, Carlo Erba), 1-M hydrochloric acid (HCl, Aldrich), 5-M aqueous ammonia ($NH_4OH$, Aldrich) were used as received without further purification. Urea was used as coordinating agent, $NH_4OH$ and $Ce(NO_3)_3$ as inorganic precursors. $7\times10^3$ mg of $Ce(NO_3)_3\ 6H_2O$ were dissolved in 20 mL of 2-propanol, then 0.5 mL of hydrochloric acid were added to it and left under stirring until a homogeneous solution was obtained. In a separate vial, $2\times10^3$ mg of urea and 0.5 mL of HCl were dissolved in 20 mL of 2-propanol and left under stirring for 5 minutes. The urea solution was then added, dropwise, to the $Ce(NO_3)_3$ solution under stirring, and soon after, 14 mL of $NH_4OH$(aq) were added to the mixture to form a precipitate that was exposed to microwaves (4 times at 600 W for 10 seconds), washed with water and centrifuged at 10,000 × *g*. A light yellow milky dispersion was obtained and then diluted with water to reach a concentration of 56 mg $CeO_2$ per mL (nanoceria stock suspension). X-ray diffraction (XRD) pattern of $CeO_2$ NPs was collected by a Bruker D8 "Discover" in grazing incidence geometry with a Cu Kα line ($\lambda = 1.54056$ Å); the X-ray generator worked at a power of 40 kV and 40 mA. The patterns were recorded in 2θ mode ranging from 20° to 80° with a step size of 0.02° and a scan speed of 0.5 seconds until to achieve an optimal signal-to-noise ratio. MAUD software was used to analyze the XRD data according to the Rietveld method [25]; following isotropic model, average crystallite size and lattice strain were separated from the total broadening assuming a dependence of microstrain from the reflection order following an isotropic model. Dynamic light scattering (Malvern Instruments Zetasizer Nano S90 with a He-Ne laser 633 nm) was used to evaluate the NPs aggregation states after 0, 2, and 24 hours of incubation in the OVIX cell medium (IMV Technologies) at different concentrations (22, 44, and 220 μg/mL).

### 2.2. Experimental design

Ram semen was collected and submitted to an initial assessment: only those ejaculates that scored at least three for mass motility and had a concentration of $3 \times 10^9$ cells/mL were chosen for the experiment. After collection, each ejaculate was temporarily stored in a water bath at 30 °C and processed by computer assisted sperm analysis (CASA; Ivos, Hamilton Thorne, Biosciences). The ejaculates were pooled, diluted in OVIX cell to reach a final concentration of $10^8$/mL, and analyzed for the integrity of acrosomal and cytoplasm membranes (Propidium iodide/*Pisum sativum* agglutinin [PI/PSA]), kinematic parameters (CASA), oxidative stress ($H_2$DCFDA and Mitotracker), and DNA integrity (SCSA) to assess the initial conditions of the semen before the exposure to $CeO_2$ NPs (fresh control, 0 hours). The diluted samples were divided into four aliquots supplemented with increasing doses of $CeO_2$ NPs (0, 22, 44, and 220 μg/mL), allowed to gradually cool to 4 °C for 2 hours and stored at 4 °C for up to 24 hours. Analysis on the interaction among sperm cells and nanoparticles, integrity of acrosome and cytoplasm membranes, kinematic parameters, oxidative stress, and DNA integrity were carried out on the four treatment groups at 2 and 24 hours of exposure. The experiment was carried out in six replicates.

### 2.3. Animal management and semen collection

For the present experiment, ejaculates were collected from five healthy adults (2–6 years old) Sarda rams (Genetic Center of AGRIS, Agenzia Regionale per la Ricerca in Agricoltura, Bonassai, Italy). The animals were of proven