

# CeO<sub>2</sub>NPs relieve radiofrequency radiation, improve testosterone synthesis, and clock gene expression in Leydig cells by enhancing antioxidation

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**Introduction:** The ratio of Ce<sup>3+</sup>/Ce<sup>4+</sup> in their structure confers unique functions on cerium oxide nanoparticles (CeO<sub>2</sub>NPs) containing rare earth elements in scavenging free radicals and protecting against oxidative damage. The potential of CeO<sub>2</sub>NPs to protect testosterone synthesis in primary mouse Leydig cells during exposure to 1,800 MHz radiofrequency (RF) radiation was examined in vitro.

**Methods:** Leydig cells were treated with different concentrations of CeO<sub>2</sub>NPs to identify the optimum concentration for cell proliferation. The cells were pretreated with the optimum dose of CeO<sub>2</sub>NPs for 24 hrs and then exposed to 1,800 MHz RF at a power density of 200.27  $\mu$ W/cm<sup>2</sup> (specific absorption rate (SAR), 0.116 W/kg) for 1 hr, 2 hrs, or 4 hrs. The medium was used to measure the testosterone concentration. The cells were collected to determine the antioxidant indices (catalase [CAT], malondialdehyde [MDA], and total antioxidant capacity [T-AOC]), and the mRNA expression of the testosterone synthase genes (*Star*, *Cyp11a1*, and *Hsd-3 $\beta$* ) and clock genes (*Clock*, *Bmal1*, and *Rora*).

**Results:** Our preliminary result showed that 128  $\mu$ g/mL CeO<sub>2</sub>NPs was the optimum dose for cell proliferation. Cells exposed to RF alone showed reduced levels of testosterone, T-AOC, and CAT activities, increased MDA content, and the downregulated genes expression of *Star*, *Cyp11a1*, *Hsd-3 $\beta$* , *Clock*, *Bmal1*, and *Rora*. Pretreatment of the cells with 128  $\mu$ g/mL CeO<sub>2</sub>NPs for 24 hrs followed by RF exposure significantly increased testosterone synthesis, upregulated the expression of the testosterone synthase and clock genes, and increased the resistance to oxidative damage in Leydig cells compared with those in cells exposed to RF alone.

**Conclusion:** Exposure to 1,800 MHz RF had adverse effects on testosterone synthesis, antioxidant levels, and clock gene expression in primary Leydig cells. Pretreatment with CeO<sub>2</sub>NPs prevented the adverse effects on testosterone synthesis induced by RF exposure by regulating their antioxidant capacity and clock gene expression in vitro. Further studies of the mechanism underlying the protective function of CeO<sub>2</sub>NPs against RF in the male reproductive system are required.

**Keywords:** CeO<sub>2</sub>NPs radiofrequency radiation, Leydig cell, testosterone synthesis, clock genes, antioxidant

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## Introduction

Exposure to radiofrequency (RF) radiation at frequencies of 850–2,100 MHz, which are used for cellular phones, is increasing rapidly in modern life.<sup>1</sup> Consequently, public concerns about the effects of RF radiation on health are

also increasing rapidly throughout the world.<sup>2</sup> Infertility is common in approximately 15% of the couples globally, and this is attributed to environmental factors, including exposure to RF radiation.<sup>3–5</sup> In humans, the testis is located in the area of RF exposure because men usually carry their mobile phones in their trouser pockets or waist-bands or keep computers in their laps. Reports have suggested that RF exposure affects testicular development, damages the seminiferous tubules, and reduces the number of Leydig cells in rats.<sup>6</sup> Testosterone, the hormone secreted by Leydig cells in testes, plays a pivotal role in sustaining structural and functional integrity of male reproductive organs.<sup>7</sup> The effects of RF exposure on testosterone, male sex hormone, in animals were inconsistent: some studies showed decreased levels in rabbits and rats while others reported increased levels in mice and humans.<sup>8–11</sup> The observation in our previous studies indicated that RF exposure inhibited the testosterone synthesis and that clock gene *Rora* was involved in its regulatory mechanism.<sup>12</sup> There were suggestions that free radical-mediated pathway might be involved in the adverse effects of RF exposure on testosterone secretion.<sup>13</sup>

Cerium oxide nanoparticles (CeO<sub>2</sub>NPs) are oxides of rare earth elements and attracted much attention in their biological applications due to the switch between Ce<sup>3+</sup> and Ce<sup>4+</sup> on the surface of CeO<sub>2</sub>NPs structure.<sup>14</sup> The preparation method, particle size, exposure route, and cell type studied have been found to influence the biological effects of CeO<sub>2</sub> NPs.<sup>15</sup> Particles with smaller size have a larger surface area per mass unit and hence, they were found to be potentially more active as antioxidant and UV shield.<sup>16,17</sup> In our previous studies, CeO<sub>2</sub>NPs was found to improve the antioxidant capacity of tissue in mice.<sup>18</sup> There were reports suggesting that some strong antioxidant may act as a protective agent and keep the male reproductive system from adverse effects of the RF exposure through the elimination of the highly reactive free radicals.<sup>19,20</sup> Hence, in this study, the antioxidant capacity of CeO<sub>2</sub>NPs was investigated on testosterone synthesis and adjusting the clock genes expression in primary Leydig cells under RF exposure.

## Materials and methods

### CeO<sub>2</sub>NPs

The CeO<sub>2</sub>NPs were obtained from Sigma-Aldrich (Shanghai, China) (Product Number: 544,841, APS: <25

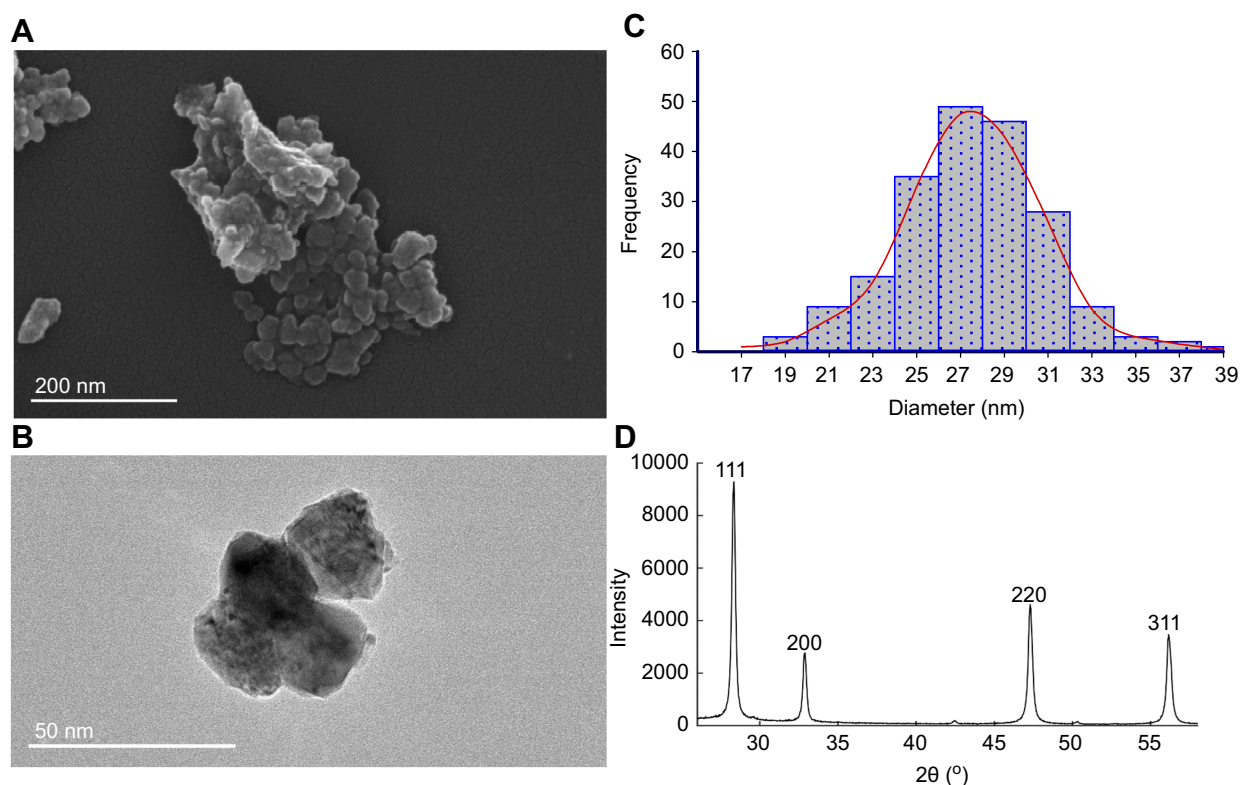
nm and purity >99% trace metal basis). Their characteristics were detected by scanning electron microscopy (SEM, Quanta FEG 250, Hillsboro, OR, USA), transmission electron microscope (TEM, JEOL 2100, Tokyo, Japan), and X-ray powder diffractometry (XRD8 Advance X-ray diffractometer; Bruker AXS Endeavor, Billerica, USA) and presented in Figure 1A, B and D. The X-ray diffraction of the precipitated material showed cubic crystals. The intense peaks from the XRD test corresponded to the diffraction peak of CeO<sub>2</sub>. The size distribution of CeO<sub>2</sub>NPs with a 18–40 nm size range was analyzed from the transmission electron microscopy images as shown in Figure 1C, and the average size of CeO<sub>2</sub>NP was 27.62 ± 3.01 nm.

### Primary Leydig cells separation and identification

The experiments were approved (number, 201701A323) by IACUC (Institutional Animal Care and Use Committee) of Soochow University, China. All procedure for the animal experiment was conducted according to the guidelines of IACUC of Soochow University. Primary Leydig cells used in the experiments were isolated from the testis of 9-week-old C57 male mice who exhibited circadian rhythmicity by 0.05% Collagenase I. Approximately 5.0 × 10<sup>6</sup> cells were mixed with 3 mL complete medium consisting of 1:1 DMEM/F-12 (Invitrogen, Thermo Fisher, Shanghai, China), 2.5% fetal bovine serum (FBS) and 5% horse serum (Gibco, Grand Island, NY, USA) and cultured in an incubator maintaining 37°C, 5% CO<sub>2</sub> atmosphere, and 95% air. The cells were cultured in vitro for 24 hrs at which time they were processed for the cell purity identification using HSD-3β-specific histochemical staining method.<sup>21</sup> Those with dark blue particles were identified as viable in Leydig cells.

### Experimental design

Firstly, cell proliferation assay was used to evaluate the most suitable addition dose of CeO<sub>2</sub>NPs according to cell proliferation rate. Secondly, cells which were pretreated with/without CeO<sub>2</sub>NPs (the optimal addition level obtained from the first step) for 24 hrs were then exposed to 1,800 MHz RF for 1 hr, 2 hrs, or 4 hrs. Immediately after RF exposure, the culture medium was collected to measure testosterone concentration. The cells were kept to determine the antioxidant levels (MDA, CAT, and T-AOC) and to extract the total RNA for real-time PCR to examine mRNA expression of testosterone synthesis



**Figure 1** Characterization of test material CeO<sub>2</sub>NPs. **(A)** Scanning electron micrographs of CeO<sub>2</sub>; **(B)** transmission electron micrographs of CeO<sub>2</sub>; **(C)** size distribution histogram of CeO<sub>2</sub>NPs; **(D)** the results of XRD test.

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; XRD, X-ray powder diffractometry.

and clock genes. The samples were investigated from these groups of Leydig cells as Table 1.

## Cell proliferation assay

Approximately  $5.0 \times 10^6$  Leydig cells were seeded in a separate sterile 35-mm petri dish in quadruplicate, and semi-confluent cells were starved in DMEM/F12 medium (without serum for 16 hrs) to synchronize at G0/G1 phase. Then, the cells were cultured in complete medium with 2.5% FBS and 5% horse

serum for 8 hrs and then distributed into separate 96-well plates, 10  $\mu$ L CeO<sub>2</sub>NPs solution diluted by dispersant (PBS with 0.5% DMSO) was added to the medium of cells by 10 addition gradients as following: 0 (Blank), 0 (Solvent), 4, 8, 16, 32, 64, 128, 256, 512, and 1,024  $\mu$ g/mL in quadruplicate. MTT assay [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-based colorimetric method] was performed to find the optimal concentration of CeO<sub>2</sub>NPs for the proliferation of Leydig cells. Twenty microliters MTT (Sigma-Aldrich, China) solution (5 mg/mL in PBS) was added, and the cells were further incubated for 4 hrs at 37°C. Then, 150  $\mu$ L DMSO was added to dissolve of formazan crystals. Finally, the absorbance in each well was measured at 490 nm using a microplate reader (Beckman, San Jose, CA, USA). Cell proliferation rate was used as the evaluation index of the most suitable addition dose of CeO<sub>2</sub>NPs: Cell proliferation rate (%) = (OD value in the experimental group/OD value in the solvent group)  $\times$  100.

## RF exposure system and dosimetry

In this experiment, the RF exposure system consists of RF generator (E4438C ESG; Agilent Technologies Inc., Palo Alto, CA, USA) and narrow band amplifier (SN1012; HD

**Table 1** Experimental design groups

Groups	Solvent	CeO <sub>2</sub> NPs	RF exposure
Blank	–	–	–
Solvent Control	+	–	–
CeO <sub>2</sub> NPs	+	+	–
RF 1 hr	+	–	+
RF 2 hrs	+	–	+
RF 4 hrs	+	–	+
CeO <sub>2</sub> NPs + RF 1 hr	+	+	+
CeO <sub>2</sub> NPs + RF 2 hrs	+	+	+
CeO <sub>2</sub> NPs + RF 4 hrs	+	+	+

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation.

Communications Corp, New York, NY, USA). The sensors and fans of the exposure system were connected to a PC that monitors the power densities ( $200.27 \mu\text{W}/\text{cm}^2$ ) during RF exposure and maintain a constant temperature and environment ( $37^\circ\text{C}$ ,  $5\% \text{CO}_2$ ). The RF exposure system was set to produce an electromotive force similar to that emitted by the global system for mobile communications mobile phones with a frequency of 1,800 MHz. As shown in Table 1, the cells were pretreated with/without  $\text{CeO}_2$  NPs for 24 hrs, and then exposed to 1,800 MHz RF radiation at  $200.27 \mu\text{W}/\text{cm}^2$  power densities for 1 hr, 2 hrs, or 4 hrs, respectively. The special absorption rate (SAR) was evaluated and computed using the 3D-FDTD full-wave electromagnetic simulation software, Sim4life 4.0 (ZMT Zurich MedTech, Zürich, Switzerland), and the specific absorption rate distribution inside the culture medium is shown in Figure 2. As shown in Figure 2, the maximum SAR was  $0.519 \text{ W/kg}$ , and the mass-average SAR was  $0.116 \text{ W/kg}$  simulated in Sim4life 4.0.

### Testosterone assay

Cell culture medium was collected after RF exposure for 1, 2, and 4 hrs. The medium was centrifuged at 3,000 rpm for 10 mins, and the supernatants were kept to evaluate for testosterone concentration using the Beckman DTX-880 Multimode Plate Reader (Beckman Coulter, San Jose, CA, USA). The testosterone levels were obtained using enzyme-immunoassay ELISA kit (Elabscience, Wuhan, China), and the absorbance was read at 450 nm.<sup>22</sup> All samples were read in duplicate, and the coefficient of

variation was less than 12% within and between measurements. Duplicate samples were used for each assay and repeated three times.

### Antioxidant levels

Cells were collected after RF exposure for 1, 2, or 4 hrs with trypsinization, washed and centrifuged at  $1,000\times g$  at  $4^\circ\text{C}$  for 20 mins. Then, the cells were sonicated to disrupt the cell wall, centrifuged at  $8,000\times g$  for 10 mins at  $4^\circ\text{C}$ , the supernatant was kept on ice to determine the CAT activities, MDA content, and T-AOC levels using kits according to manufacturers' instructions (Suzhou Comin, Suzhou, China), respectively.

### Real-time PCR

The Leydig cells were collected after RF exposure for 1, 2, or 4 hrs for real-time PCR. Total RNA was isolated using the Mini Kit (Qiagen, Duesseldorf, Germany) and, the quality and quantity were determined using BioPhotometer (Analytik Jena AG, Jena, Germany). Quantitative real-time PCR was conducted on the QuantStudio™ 7 Flex Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) using 2.5X SYBR Green Abstart One Step RT-PCR Mix (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. Primers were designed and synthesized by Invitrogen (Life Technologies, Shanghai, China) and the forward and reverse sequences are presented in Table 2. All values were normalized to a mice housekeeping gene  $\beta$ -actin. The fold change in mRNA was calculated by the  $\Delta\Delta\text{Ct}$  method ( $\text{fold} = 2^{\Delta\Delta\text{Ct}}$ ). All samples were tested in duplicates.

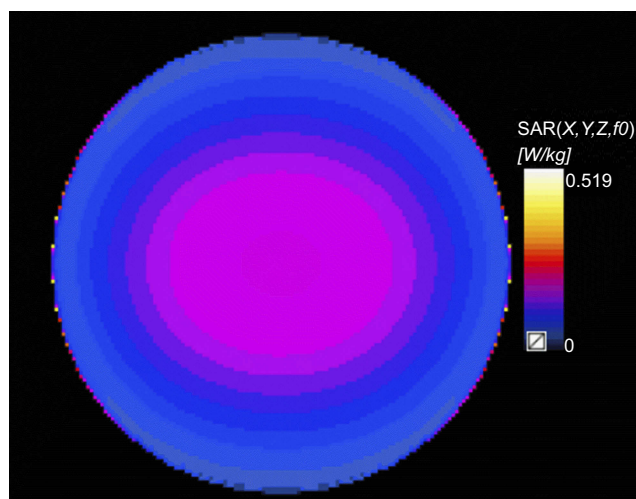
### Statistical analysis

All data were presented as mean  $\pm$  SD (standard deviation). Statistical analyses were performed by one-way analysis of variance between the Blank/Solvent/ $\text{CeO}_2$  NPs/RF/ $\text{CeO}_2$  NPs+ RF groups with SPSS 22 (SPSS Inc, Chicago, IL, USA). When appropriate, LSD test was carried out. A statistical  $P$ -value of  $<0.05$  between two groups was indicative of significant difference.

## Results

### Identification of primary Leydig cell

Figure 3A and B shows the Leydig cells stained with HSD-3 $\beta$ -specific dye after culturing in vitro for 24 hrs. The cytoplasm of most cells was blue-black with dark blue particles, and more than 95% of the cells are stained. Thus, the purity of primary Leydig cells was above 95%.



**Figure 2** Distribution of SAR of the culture medium inside a standard petri dish (35-mm) exposed to 1,800 MHz radiofrequency fields (power densities,  $200.27 \mu\text{W}/\text{cm}^2$ ).  
**Abbreviation:** SAR, specific absorption rate.



**Table 2** Primer sequence for real-time PCR

Genes	Primer sequence
<i>Star</i>	Sense: 5'-GGCATACTCAACAACCAGAAGGC -3' Antisense: 5'-CTCCATGCGGTCCACAAGTTCTTC -3'
<i>Cyp11a1</i>	Sense: 5'-CCGTGGATAACAGCAGCAGGAAC -3' Antisense: 5'-CCAGCACAGATGGTCGCAGATAC -3'
<i>Hsd-3β</i>	Sense: 5'-TCCACACTGCTGCTGTCATTGATG -3' Antisense: 5'-AGAAGATGAAGGCTGGCACACTTG -3'
<i>Bmal 1</i>	Sense: 5'-CTCGGTACATCCTACGACAAAC -3' Antisense: 5'-CACAGAAGCAAATACAAGCCAAC -3'
<i>Clock</i>	Sense: 5'-TGGTGACTGCCTATCCTACCTTCG -3' Antisense: 5'-TGCTGCTGCTGCTGCTGTTG -3'
<i>Rora</i>	Sense: 5'-CCACCTACTCCTGTCTCGTCAG -3' Antisense: 5'-CTTCTGCACCTCGGCGTACAAG -3'
<i>β-actin</i>	Sense: 5'-TGGAATCCTGTGGCATCCATGAAAC-3' Antisense: 5'-TAAACGCAGCTCAGTAACAGTCCG-3'

### Proliferation of Leydig cells affected by different doses CeO<sub>2</sub>NPs

The proliferation rate of Leydig cells which were treated with different addition doses CeO<sub>2</sub>NPs [0 (Blank), 0 (Solvent), 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 µg/mL] is shown in Figure 4. In terms of the cell proliferation rate, there is no statistical difference between Blank and Solvent groups. The proliferation rate was significantly increased to 127.72%, 181.22%, and 148.53% in cells treated with 64, 128, and 256 µg/mL CeO<sub>2</sub>NPs, respectively, compared with the solvent (0 µg/mL) group (100%). There was no statistical difference in other CeO<sub>2</sub> NPs-treated groups. Therefore, the addition concentration of 128 µg/mL CeO<sub>2</sub>NPs was considered optimum for the

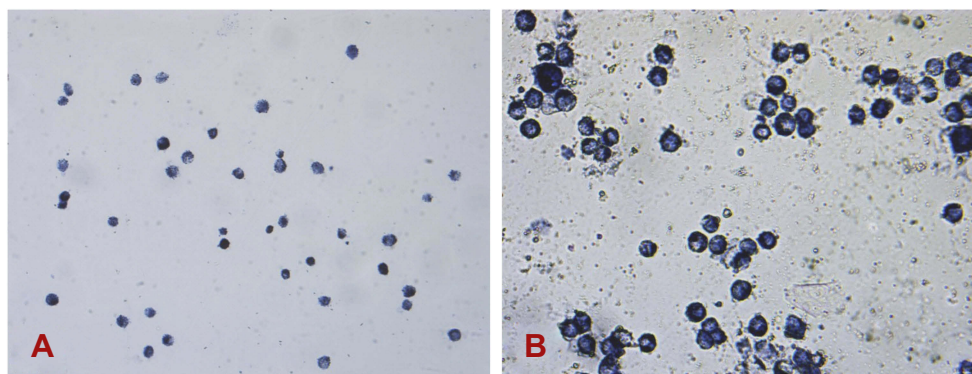
proliferation of Leydig cells and also as the protective dose for RF exposure in the next experiments.

### Testosterone

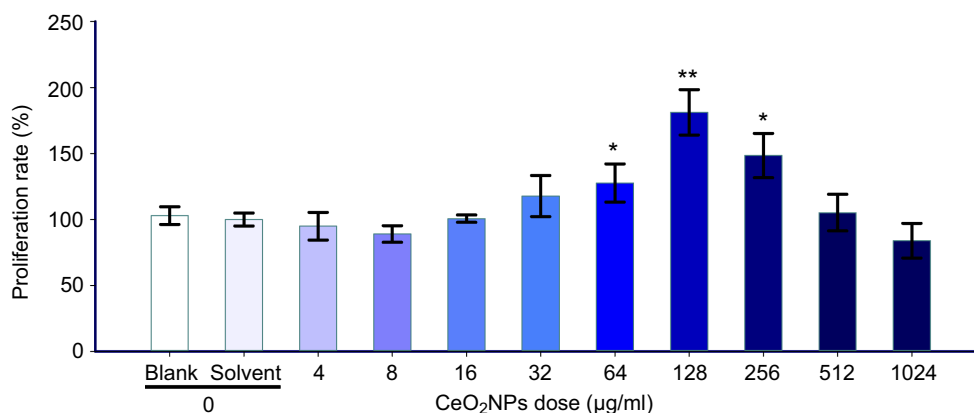
Testosterone synthesis reflects the function of primary Leydig cells, which is easily affected by environmental factors. In this study, for testosterone concentrations in cells medium, solvent control group is no statistical difference from the blank group. Compared with the solvent control group, CeO<sub>2</sub>NPs (128 µg/mL) addition increased the testosterone concentrations, while RF exposure for 1, 2, and 4 hrs significantly diminished testosterone levels. Cells pretreated with 128 µg/mL CeO<sub>2</sub>NPs for 24 hrs and then exposed to RF for 1, 2, and 4 hrs, showed increased testosterone concentration compared to that of RF exposure alone groups, respectively (Figure 5). The results showed 128 µg/mL CeO<sub>2</sub>NPs pretreatment could protect RF exposure-induced toxicity on testosterone synthesis.

### Antioxidant levels (CAT, MDA, and T-AOC)

The data in Figures 6–8 show the results of CAT activities, MDA content, and T-AOC levels. For three antioxidant parameters, there is no difference between the blank group and the solvent control group. There were significant differences between solvent control and CeO<sub>2</sub> NP/RF-exposed cells. Cells treated with 128 µg/mL CeO<sub>2</sub>NPs showed increased the CAT activities and T-AOC levels, reduced MDA content in primary Leydig cells, while RF exposure induced the opposite change on antioxidant levels. Compared to cells exposed to RF for 1, 2, and 4 hrs groups, CeO<sub>2</sub>NPs + RF each group reduced the MDA content, increased CAT activities and T-AOC levels. The changes in CAT activities, MDA content, and T-AOC levels observed

**Figure 3** Stain of HSD-3β on primary leydig cells (A, 20×; B, 40×).

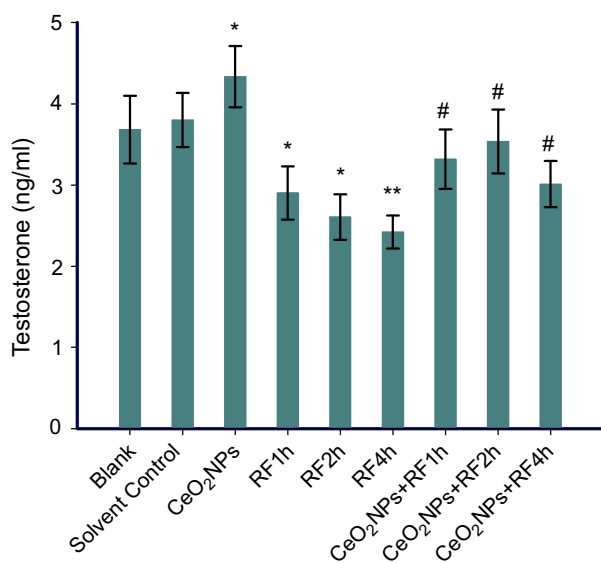
**Abbreviation:** HSD-3β, 3β-Hydroxysteroid dehydrogenase.



**Figure 4** Effects of different dose CeO<sub>2</sub>NPs on the proliferation rate of primary Leydig cells.

**Notes:** Compared with the 0 dose level (Solvent), the difference was significant as, \* $P < 0.05$ , \*\* $P < 0.01$ .

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation.



**Figure 5** Testosterone concentration in Leydig cells medium treated by CeO<sub>2</sub>NPs, RF and CeO<sub>2</sub>NPs + RF.

**Notes:** Compared with the Solvent Control group, the difference was significant as, \* $P < 0.05$ , \*\* $P < 0.01$ ; Compared with the RF group at same exposure time, the difference was significant as, #  $P < 0.05$ .

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation.

after RF exposure 1, 2, and 4 hrs were reversed by pretreatment with 128 µg/mL CeO<sub>2</sub> NPs for 24 hrs.

## Testosterone synthase genes expression

The data in Figure 9 show the results of the real-time PCR test for *Star*, *Cyp11a1*, and *Hsd-3β*. From the results, there is no difference between the blank group and the solvent control group in the three gene expression. Compared to the solvent control group, exposure of the cells to 128 µg/mL CeO<sub>2</sub> NPs quite significantly increased the mRNA expression of testosterone synthesis genes *Star*, *Cyp11a1*, and *3β-Hsd* in primary

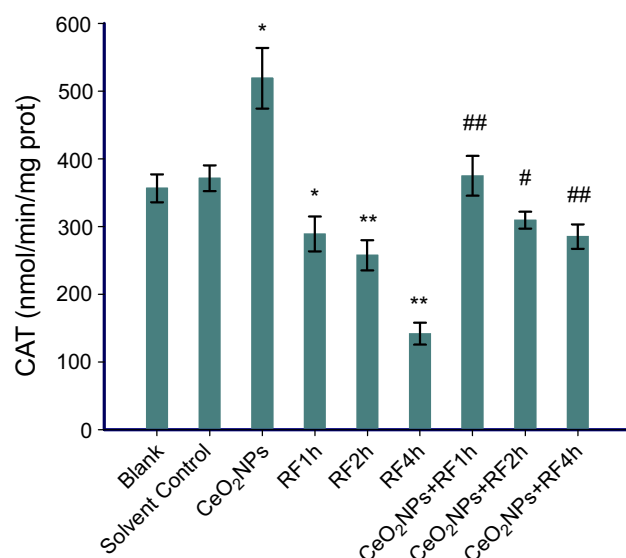
Leydig cells, while RF exposure for 2 or 4 hrs induced the distinct downregulation of the three testosterone synthesis genes expression. CeO<sub>2</sub>NPs + RF groups at all RF exposure times distinctly enhanced the genes expression of testosterone synthesis *Star*, *Cyp11a1*, and *Hsd-3β* in primary Leydig cells compared to RF exposure alone for 1, 2, and 4 hrs, respectively. The downregulation effects of RF exposure effect on the mRNA expression of testosterone synthesis genes, *Star*, *Cyp11a1*, and *Hsd-3β* could be effectively reversed by 128 µg/mL CeO<sub>2</sub>NPs pretreatment for 24 hrs.

## Clock genes expression

The results of real-time PCR for clock genes *Baml1*, *Clock*, and *Rora* mRNA expression are shown in Figure 10. As reported above indicators, no difference appeared between the blank group and solvent control group in the expression of clock genes. As compared to the solvent control group, *Rora*, *Clock*, and *Baml1* mRNA expression remarkably increased in CeO<sub>2</sub> NPs treatment group. There are significant decreases on the mRNA expressions of *Baml1*, *Clock*, and *Rora* in the RF exposure 2 or 4 hrs group compared to the solvent control cells, and RF exposure 1 hr also reduced the gene expressions of two clock genes *Clock* and *Rora* except for *Baml1* expression. As for the genes expression of *Rora*, *Clock*, and *Baml1*, CeO<sub>2</sub>NPs + RF each group is higher than that of RF exposure alone for 1, 2, and 4 hrs, respectively. The downregulation toxicity of RF exposure on the mRNA expression of clock genes *Rora*, *Clock*, and *Baml1* could be effectively reversed by 128 µg/mL CeO<sub>2</sub>NPs pretreatment 24 hrs.

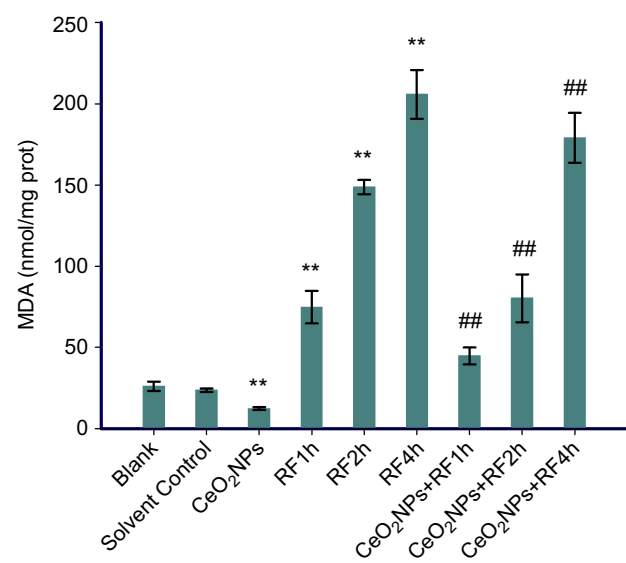
## Discussion

There were several reports suggesting RF exposure effects on various cellular systems, and highly reactive free



**Figure 6** CAT activity in Leydig cells treated by CeO<sub>2</sub>NPs, RF, and CeO<sub>2</sub>NPs + RF. **Notes:** Compared with the Solvent Control group, the difference was significant as, \**P*<0.05, \*\**P*<0.01; Compared with the RF group at same exposure time, the difference was significant as, # *P*<0.05, ## *P*<0.01.

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation; CAT, catalase.



**Figure 7** MDA content in Leydig cells treated by CeO<sub>2</sub>NPs, RF, and CeO<sub>2</sub>NPs + RF. **Notes:** Compared with the Solvent Control group, the difference was significant as, \*\**P*<0.01; compared with the RF group at same exposure time, the difference was significant as, ## *P*<0.01.

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation; MDA, malondialdehyde.

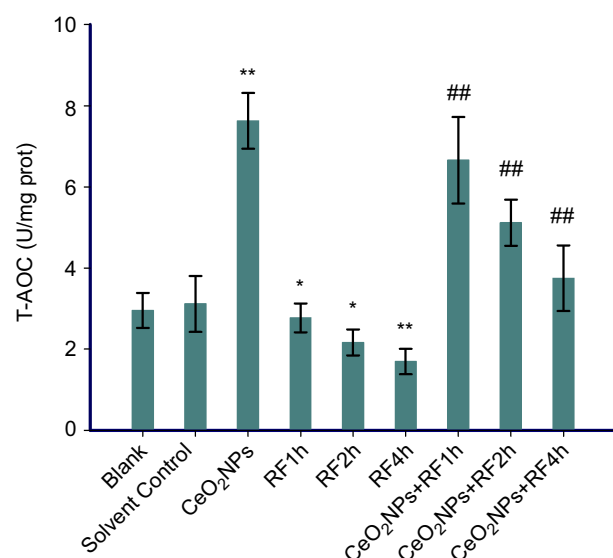
radicals were implicated in adverse biological effects (oxidative damage) induced by RF.<sup>23–26</sup> The aims of this study were to examine whether 1,800 MHz RF radiation induces oxidative stress and promote lipid peroxidation and also to investigate the role of CeO<sub>2</sub>NPs, which have antioxidant

properties, against possible testosterone synthesis toxicity in primary Leydig cells induced by 1,800 MHz RF radiation in vitro.

Leydig cells are located in interstitial of seminiferous tubules in testis and play an important role in the paracrine regulation of spermatogenesis and, are responsible for the synthesis of testosterone.<sup>27</sup> Testosterone has significant effects during the growth and development of the male reproductive organs.<sup>28</sup> Long-term exposure to environmental toxins was suggested to be responsible for the decline of testosterone that further induced the defective sperm.<sup>29</sup> It is generally considered that Leydig cells are sensitive to the environmental stress, such as heat, radiation, and exposure to heavy metals.<sup>30,31</sup> There were reports indicating RF radiation from cell phone, and other electronic equipment may have a negative influence on Leydig cells.<sup>25,32</sup> In our previous studies, we found 1,800 MHz RF exposure reduced the level of testosterone synthesis in mice and rats.<sup>12,33</sup> The results in the present study showed that 1,800 MHz RF exposure for 1, 2, and 4 hrs negatively affected testosterone synthesis in primary Leydig cells.

In order to investigate the potential mechanisms of RF exposure effect on primary Leydig cells, the expression of genes involved in testosterone synthesis (*Star*, *Cyp11a1*, *Hsd-3β*) was examined, and all were found to be altered. Steroidogenic acute regulatory protein (StAR) is able to mediate the production of steroid hormones which is initiated by translocation of cholesterol through the mitochondrial membrane from the outer to the inner.<sup>34</sup> Cholesterol was metabolized into pregnenolone by cytochrome P450 Family 11 subfamily A member 1 (CYP11A1), and the hormonally and developmentally regulated expression of CYP11A1 is mainly driven by a variety of trans-acting factors.<sup>35</sup> Due to the close correlation with HSD-3β, pregnenolone transfers from the mitochondria into the smooth endoplasmic reticulum.<sup>36</sup> In this study, the mRNA expression levels of *Star*, *Cyp11a1*, and *Hsd-3β* in Leydig cells exposed to RF radiation for 2 and 4 hrs decreased significantly, which could be responsible for the inhibition of testosterone synthesis.

In our previous study, we found that RF exposure inhibited testosterone synthesis through CaMKI/RORα signaling pathway. RORα (the retinoid-related receptor alpha) is a critical member of the core clock gene machinery, its ROR response elements (ROREs) have been identified in several clock genes including *Bmal1* and *Clock*, indicating that ROREs are essential for rhythmic transcriptional regulation of *Bmal1* and *Clock*.<sup>37</sup> BMAL1 as an essential



**Figure 8** T-AOC levels in Leydig cells treated by CeO<sub>2</sub>NPs, RF, and CeO<sub>2</sub>NPs + RF. **Notes:** Compared with the Solvent Control group, the difference was significant as, \* $P<0.05$ , \*\* $P<0.01$ ; Compared with the RF group at same exposure time, the difference was significant as, ##  $P<0.01$ .

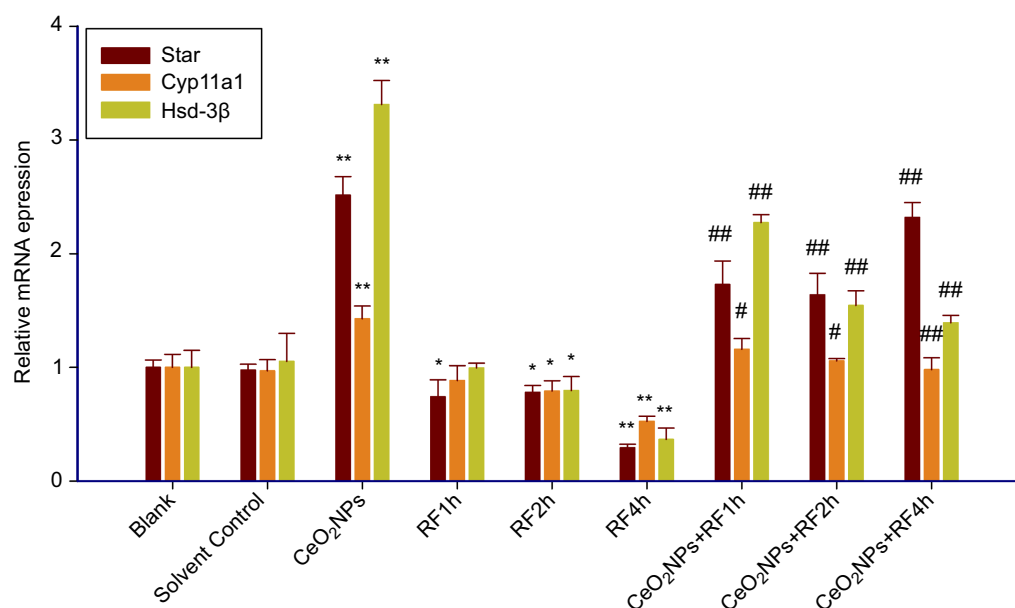
**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation; T-AOC, total antioxidant capacity.

component of the circadian pacemaker in mammals can regulate the rate-limiting step of steroidogenesis by enhancing the transcription of *Star* gene.<sup>38</sup> Biological clock genes also regulate the expression of cytochrome P450 (CYP) gene family members in which Cyp11 family including

CYP11A1.<sup>39</sup> The gene expression of *Hsd-3 $\beta$*  is also under transcriptional control of the circadian clock.<sup>40</sup> Thus, testosterone synthesis can be changed by clock genes expression. In this present study, the clock genes *Rora*, *Clock*, and *Baml1* as well as the three steroidogenic genes, viz., *Star*, *Cyp11a1*, *Hsd-3 $\beta$* , were downregulated in mouse primary Leydig cells exposed to 1,800 MHz RF radiation for 1, 2, and 4 hrs.

Damage in biological systems induced RF emitted from cellular mobile phones may be due to the accumulation of free radicals which enhance oxidative stress, and change the antioxidant defense systems of tissues.<sup>41</sup> Under oxidative stress, circadian rhythmicity is usually affected and the expressions of the key circadian gene are directly inhibited.<sup>42</sup> In the present study, 1,800 MHz RF exposure negatively affected testosterone synthesis and clock genes expression, decreased the CAT and T-AOC levels, increased the MDA content, changed the antioxidant capacity of primary Leydig cells, and induced the oxidative stress. Thus, the results demonstrated that the inhibition on testosterone synthesis induced by RF exposure may be due to oxidative stress and downregulation of clock genes.

CeO<sub>2</sub>NPs have antioxidant properties due to their variable particle sizes, crystal structures, and surface chemistries, and have also been demonstrated that are able to quench ROS produced and protect cells against oxidative damage due to its free radical-scavenging properties.<sup>43,44</sup>

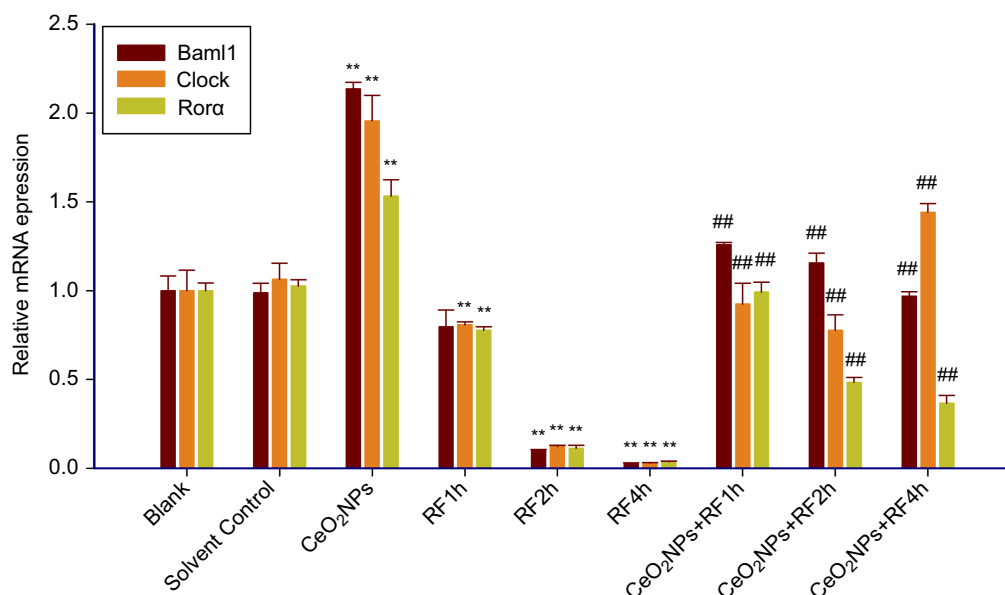


**Figure 9** The mRNA expression of testosterone synthase genes (*Star*, *Cyp11a1*, *Hsd-3 $\beta$* ) in Leydig cells treated by CeO<sub>2</sub>NPs, RF, and CeO<sub>2</sub>NPs + RF.

**Notes:** Compared with the Solvent Control group, the difference was significant as, \* $P<0.05$ , \*\* $P<0.01$ ; Compared with the RF group at same exposure time, the difference was significant as, #  $P<0.05$ , ##  $P<0.01$ .

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation.





**Figure 10** The clock genes (*Clock*, *Bmal1*, *Rora*) mRNA expression from Real-time PCR in Leydig cells treated by CeO<sub>2</sub>NPs, RF, and CeO<sub>2</sub>NPs + RF.

**Notes:** Compared with the Solvent control group, the difference was significant as, \*\**P*<0.01; Compared with the RF group at same exposure time, the difference was significant as, ## *P*<0.01.

**Abbreviations:** CeO<sub>2</sub>NPs, cerium oxide nanoparticles; RF, radiofrequency radiation.

In the present study, application alone of CeO<sub>2</sub> NPs increased the CAT activities and T-AOC levels, decreased the MDA content, which agreed with the literature that reported CeO<sub>2</sub>NPs reduced the oxidative stress.<sup>45</sup> Meanwhile, these nanoparticles further relieved the oxidative stress induced by RF, accompanied by the upregulation of clock genes *Rora*, *Clock*, and *Bmal1* in this present study. These results were consistent with the report which indicated circadian clock is involved in regulating the response of a cell to oxidative stress.<sup>46</sup>

Previous studies reported that CeO<sub>2</sub>NPs materials have positive effects on reproductive cells in sheep and are capable of promoting reproductive performance in rabbits.<sup>47,48</sup> It is also reported citrate-stabilized CeO<sub>2</sub>NPs help to accelerate the proliferation of primary mouse embryonic fibroblasts in vitro.<sup>49</sup> In the present study, CeO<sub>2</sub>NPs treatment alone promoted the proliferation of mouse primary Leydig cells, enhanced the testosterone synthesis and clock genes expression, and increased the antioxidant capacity. Further, we found that pretreatment of the cells with CeO<sub>2</sub>NPs for 24 hrs reduced the oxidative damage and the clock genes downregulation caused by RF exposure and thus, improved the testosterone synthetase genes expression and testosterone secretion.

As there are always two sides to everything, CeO<sub>2</sub>NPs also showed some toxicities.<sup>50,51</sup> According to our previous

studies in animals, it must be noted that doses play a crucial role in the benefits or toxicity of CeO<sub>2</sub>NPs on the reproductive system.<sup>18,52</sup> In the present study, we set ten dose gradients to screen the optimum addition dosage of CeO<sub>2</sub> NPs by proliferation rate assay of Leydig cells. The results found that the cell proliferation rate was increased most significantly at the dose 128 µg/mL but then gradually decreased above the 128 µg/mL. It suggested there is an optimum addition for the proliferation of mouse primary Leydig cells, and CeO<sub>2</sub>NPs above the optimum addition level may induce a declining cell proliferation rate, even inhibit the cells proliferation as the dose constantly increases.

## Conclusion

In summary, our study provided some evidence that 1,800 MHz RF exposure may first induce the oxidative damage in primary Leydig cells and then, weaken the expression of clock genes *Clock*, *Bmal1*, and *Rora* which in turn, downregulates its target genes involved in testosterone synthesis, including *Star*, *Cyp11a1*, and *Hsd-3β*, resulted in reduced testosterone production. Preexposure of the cells to CeO<sub>2</sub>NPs also reduced RF radiation effects, improves testosterone synthesis and clock genes expression in Leydig cells via enhancing antioxidant levels.

## Disclosure

The authors report no conflicts of interest in this work.

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