4-[1-Ethyl-1-methylhexy]-phenol induces apoptosis and interrupts Ca²⁺ homeostasis via ROS pathway in Sertoli TM4 cells

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Abstract
Biological effect of an individual nonylphenol (NP) isomer extremely relies upon the side chain structure. This research was designed to evaluate the impact of NP isomer, 4-[1-ethyl-1-methylhexy]-phenol (NP₆₅), on Sertoli cells in vitro. Sertoli TM4 cells were exposed to various concentration (0, 0.1, 1, 10, or 20 μM) of NP₆₅ for 24 h, and the outcomes indicated that treatment of NP₆₅ induced reactive oxygen species (ROS) generation, oxidative stress, and apoptosis for Sertoli TM4 cells. In addition, it was found that NP₆₅ exposure affected homeostasis of Ca²⁺ in Sertoli TM4 cells by increasing cytoplasm [Ca²⁺]ᵢ, inhibiting Ca²⁺-ATPase activity and decreasing cyclic adenosine monophosphate (cAMP) concentration. Pretreatment with ROS scavenger, N-acetylcysteine (NAC), attenuated NP₆₅-induced oxidative stress as well as apoptosis for TM4 cells. Furthermore, NAC blocked NP₆₅-induced disorders of Ca²⁺ homeostasis by attenuating the growth of intracellular [Ca²⁺]ᵢ and the inhibition of Ca²⁺-ATPase and cAMP activities. Thus, we have demonstrated that NP₆₅ induced apoptosis as well as acted as a potent inhibitor of Ca²⁺-ATPase activity and resulted in disorder of Ca²⁺ homeostasis in Sertoli TM4 cells; ROS participated in the process. Our results supported the view that oxidative stress acted an essential role within the development of apoptosis and Ca²⁺ overload in TM4 cells as a consequence of NP₆₅ stimulation.

Keywords 4-[1, 2, 4-Trimethylhexyl]-phenol · Reactive oxygen species · Apoptosis · Ca²⁺ homeostasis · Sertoli TM4 cells

Abbreviations
cAMP Cyclic adenosine monophosphate
CGMP Cyclic guanosine monophosphate
DCF 2′,7′-Dichlorofluorescein
DCFH-DA 5(6)-Carboxy-2′-7′-dichlorofluorescein diacetate
DMEM/F-12 medium Dulbecco’s Modified Eagle’s Medium nutrient mixture F-12
HAM HAM

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Introduction
Nonylphenols (NPs), the major degraded products of nonylphenol polyethoxylate (NPEOs), were thought to be one of the endocrine disrupting chemicals (EDCs), which could interrupt the endocrine system function of human and animal (Lee et al. 2013; Merrill et al. 2020). Today, NPs and NPEOs have been employed widely in detergents, cosmetics, insecticides, surface-active reagents, food manufacturing, plastics, and polyvinyl chloride tubes, as
well as other industrial formula products as plasticizers or antioxidants (Bonefeldjørgensen et al. 2007; Cheng et al. 2017; Noorimotlagh et al. 2016). Studies have demonstrated that NPs were seriously threatening reproductive health (Malmir et al. 2020), immune function (Xia et al. 2013), and system of nerves (Li et al. 2019) in human and animals.

Disturbingly, NPs have been investigated in the majority of retailed food, and they might migrate from films for food packaging to simulants of food within the process of microwaving and cooking (Inoue et al. 2001; Kawamura et al. 2017). In addition, NPs could be uptaken by wildlife in aquatics, particularly by fish followed by accumulation into bodies of human via the food net (Coldham et al. 1998; Kookana 2002). The existence of NPs within the environment has become a growing worry due to their estrogenic effect in addition to the persistence and toxicity (Araujo et al. 2018; Malmir & Faraji 2020). At present, most of the studies were focused on industrial nonylphenol, which is a combination of para-substituted mono-alkylphenols containing numerous branched and isomeric nonyl groups. However, it was demonstrated that the biological effect of a distinctive nonylphenol isomer is greatly reliant on the side chain structure (Preuss et al. 2006; Kim et al. 2005). As far as we know, there have been few studies to observe the toxicity of NPs from the perspective of isomers specificity; the mechanism on toxicity of NP isomers has been barely studied.

Recently, some distinct NP isomers have been purified and used to analyze the toxicity as well as fate to environment of NPs. Estrogenic effects of some artificial isomers of NPs have been described (Boehme et al. 2010; Ying et al. 2012; Gabriel et al. 2008). To justify the relationship of distinct NP isomers in scientific research, Guenther et al. (2006) have built a system of numbering for entirely potential NP isomers, which respects the IUPAC rule for the description of alkylphenol substituents. For example, 4-[1-ethyl-1-methylhexyl]-phenol was abbreviated as NP65. Fortunately, Professor Guenther (Institute for Chemistry and Dynamics of the Geosphere, Research Centre Juelich, Germany) donated 12 NP isomers to our laboratory. This may lead us to investigate the environmental hazard of NPs from the perspective of isomer specificity. The effects of 4-[1, 2, 5-trimethylhexyl]-phenol (NP82) as well as 4-[1, 2, 4-trimethylhexyl]-phenol (NP41) on cell receptors and MAPK pathway in mouse Sertoli TM4 cells have been investigated in our laboratory (Liu et al. 2014).

Sertoli cells, the somatic cells of the testis, provide nutrition as well as morphogenetic support for germ cells during spermatogenesis. Thus, Sertoli cell is a potential target for environmental toxicant–induced reproductive dysfunction in male mice. Duan et al. found that 4-NP stimulates autophagy and inhibits mTOR-p70S6K/4EBP1 activity through AMPK activation in Sertoli cells (Duan et al. 2017), and 4-NP induces apoptosis, autophagy, and necrosis in Sertoli cells via ROS-mediated AMPK/AKT-mTOR and JNK pathways (Duan et al. 2016). At present, various Sertoli cell lines from mice and rats have been established, in order to study the regulation of Sertoli cell function and the influence of toxicants in vitro (Guttenbach et al. 2001). In current study, mouse Sertoli TM4 cells were used to determine the cytotoxicity induced by NP65.

Our previous results have indicated that NP65 suppressed the protein expression of estrogen receptor α, β, and activated Akt and JNK-MAPK pathways in mouse Sertoli TM4 cells (Liu et al. 2017). In recent years, it was testified that several EDCs play the role of chemical matter causing apoptosis of cells (Paulina et al. 2018; Su et al. 2018). In addition, studies have shown that apoptosis works as a mechanisms of reproductive damage (Wang et al. 2014; Liu et al. 2020). 4-n-NP applies a toxic effect on the development of reproductive system by inducing apoptosis of Sertoli TM4 cells, but whether other NP isomers cause the same problem is still unknown. In our research, we hypothesized that NP65 can damage the reproductive by inducing cell apoptosis and disturbing Ca2+ homeostasis in Sertoli cells. Therefore, the adverse effects of 0–20 μM NP65 on Sertoli TM4 cells and the underlying mechanism were evaluated in the existed research.

Materials and methods

Reagents

The 99.9% purity NP65 was from Prof. Guenther as a gift. The structure of NP65 is illustrated in Fig. 1 (Gabriel et al. 2008). NP65 was dissolved in ethanol as stock solution and diluted with medium, the final ethanol concentration in the medium was not more than 0.1% (v/v), and it showed no effect on the viability of Sertoli TM4 cells.

Phenol red-free Dulbecco’s Modified Eagle’s Medium nutrient mixture F-12 HAM (DMEM/F-12 medium, Cat. # SH30272), donor equine serum (Cat. # SH30074), and charcoal/dextran-treated fetal bovine serum (FBS, Cat. # SH30068) were purchased from Hyclone (Waltham, MA, USA). AnnexinV-FITC apoptosis kit was obtained from KeyGEN BioTECH (Jiangsu, China, Cat. # KGF001). ELISA kits were purchased from Westang (Shanghai, China). Streptomycin sulfate, 5(6)-carboxy-2’-7’-dichlorofluorescein diacetate (DCFH-DA), and penicillin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Ca2+-ATPase detection kit (Cat. # A070-4), malonic

Fig. 1 Structure of 4-[1-ethyl-1-methylhexyl]-phenol (NP65). * represents chiral carbon atom
dialdehyde (MDA) assay kit (Cat. # A003-1), and superoxide dismutase (SOD) assay kit (Cat. # A001-3) were obtained from Jiancheng (Nanjing, China). Analytical grade was applied to all other chemicals.

**Cell culture**

The mouse Sertoli TM4 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in F-12/DMEM medium free of phenol red, with 5% (v/v) donor equine charcoal stripped serum, 1% penicillin–streptomycin, and 2.5% (v/v) dextran-charcoal-stripped FBS in an atmosphere of 100% relative humidity as well as 5% CO2 at 37 °C. In the assay, experiment was set up into the following: (1) control group, (2) NP treatment group, and (3) NP + NAC treatment group. In the control group, cells were cultured normally. In the NP treatment group, cells were exposed to 0.1, 1, 10, or 20 μM of NP65 for 24 h. In NP + NAC treatment group, cells were pretreated with 5 mM N-acetylcysteine (NAC) for 30 min, followed by the exposure towards 20 μM of NP65 for 24 h. Treatment with 5 mM NAC for 30 min showed no effect to Sertoli TM4 cells, including cell viability, apoptosis, and ROS generation (Liu et al. 2019). Thus, NAC alone was not set up individually as a group in this study.

**Assay of reactive oxygen species production**

TM4 cells were seeded with a same number (2 × 10^4/ well in 200 μL of growth medium) for each group on a 96-well black microplate, and cultured for 6 h; NP65 was employed to the cells in 100 μL basic medium for 24 h, then the medium is aspirated and phosphatebuffered saline (PBS, NaCl 8.0 g, KCl 0.2012 g, Na2HPO4 1.38 g, KH2PO4 0.2722 g, and added water to 1.0 L, pH7.4) is used to flush the wells. The wells experienced incubation with 25 μM DCFH-DA dissolved in minimal medium for 1 h, then the relative fluorescent intensity was detected by Varioskan Flash (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 525 nm for emission and 485 nm for excitation.

**Determination of SOD activity and MDA content**

For detecting the activity of intracellular SOD as well as content of MDA, TM4 cells were seeded in a six-well plate with a number of 2 × 10^5/well. After 24-h culture, cells were treated with NP65 for 24 h, then cells were washed with ice cold PBS twice, followed by harvesting from the plates into PBS and homogenized. Whole-cell homogenate was harvested for 10 min by centrifugation at 4 °C, 12,000 g. The supernates were harvested and immediately assayed for SOD activity and MDA content with commercial kits with an automated microplate reader (Varioskan Flash, Thermo Scientific, USA), according to the instructions of manufacturer.

**Apoptosis analysis with Annexin V–FITC and PI staining**

Sertoli TM4 cells were seeded at a density of 2 × 10^5/well on 6-well plate and cultured for 24 h before NP65 exposure. Cells were harvested followed by washing with cold PBS thrice after exposed to NP65 for 24 h, and then the cells experienced staining with 5 μL Annexin V-FITC as well as 5 μL PI in 100 μL binding buffer based on the instructions of manufacturer. The flow cytometer was used for flow cytometry (FACS Calibur, Becton-Dickson, San Jose, CA).

**Determination for release of Ca^{2+} in TM4 cells**

The intra-cellular calcium ion ([Ca^{2+}]) was also determined by flow cytometry (Fengling et al. 2012). Sertoli TM4 cells were seeded at a density of 2 × 10^5/well on 6-well plate for 24 h following exposure to NP65 for 24 h. NP65-treated cells were harvested followed by washing thrice with cold PBS, then loading with 5 μM of Fluo-3/AM at 37 °C for 40 min at dark. Finally, the extracellular Fluo-3/AM dye was removed and the relative fluorescence intensity within the cells were detected by flow cytometry.

**Determination of Ca^{2+}-ATPase activity cAMP and cGMP concentration**

Sertoli TM4 cells were seeded at a density of 2 × 10^5/well on 6-well plate. The cells were cultured for 24 h and then were treated with NP65 for 24 h. NP65-challenged cells were collected and homogenized. Concentration of cAMP and cyclic guanosine monophosphate (cGMP in the supernatants were measured by ELISA kits (Westang, Shanghai, China). The Ca^{2+}-ATPase activity was also detected by a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China). The whole measurements were conducted according to the instructions of manufacturer, and the absorbance was determined via an automatic microplate reader.

**Reverse transcription polymerase chain reaction analysis**

Similarly, TM4 cells were seeded with a density of 2 × 10^5/well on 6-well plate and then treated with NP65
for 24 h. Trizol Reagent (Invitrogen, Carlsbad, CA, USA) was employed for extraction of RNA in total based on the protocol from manufacturer. After RNA extraction, the samples were treated with DNase I (Ambion, TX) at 37 °C for 30. Absorbance value of RNA was determined, the OD260/OD280 ratio between 1.8 and 2.1 was accepted. Two micrograms of the RNA sample was applied for synthesize cDNA by using ReverAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, MD, USA, Cat. # K1621). PCR amplification was carried out by employing approximately 1 μL cDNA as a template. The sequences for primer for PCR are shown within Table 1, and the primer pairs were designed and synthesized by professional biotechnology co., LTD. (Sangon Biotech, Shanghai, China). Polymerase chain reaction (PCR) program included 3 min at 94 °C, 30 s at 60 °C for annealing, 30 cycles of 30 s at 94 °C for denaturation, and 2 min at 72 °C for extension, plus 10 min at 72 °C as final extension. Products from PCR were examined on 2% (w/v) agarose gel by GoldView-agarose gel electrophoresis, followed by visualization with ChemDoc XRS + . The target genes’ signals were examined via scanning densitometry followed by normalization to β-actin with Quantity One software.

### Statistical analysis

Experiments were repeated at least three times for each test, and results from three independent experiments were combined. The statistical analysis was conducted on the software of SPSS with the version 19.0 for Windows. One-way analysis of variance with LSD test was employed for determination of the statistical differences among groups with different treatments after homogeneity of variance test. Non-parametric tests would be used if equal variances not assumed or data were not normally distributed. Values are presented as mean ± S.D.; the differences between two means were considered to be statistically significant if a value of P was less than or equal to 0.05.

### Results

#### Generation of ROS induced by NP65 in Sertoli TM4 cells

Pretreatment for cells was carried out with 5 mM NAC in 20 μM NP65 group for 30 min, followed by consecutive exposure to NP65, and later, the amount of ROS was analyzed via an automated microplate reader. Results indicated that comparing control group, NP65 increased generation of ROS in Sertoli TM4 cells were seeded in black 96-well plate and exposed to NP65 for 24 h. In NP+NAC treatment group, cells were pretreated with 5 mM NAC for 30 min, followed by the exposure towards 20 μM of NP65 for 24 h. ROS generation was determined by an automated microplate reader using DCFH-DA. Results with triplicate measurements are displayed as mean ± S.D.

#### Table 2 Effects of NP65 on the activity of SOD and MDA content

| NP65 concentration (μM) | NAC (μM) | SOD (U/mg protein) | MDA (μM/mg protein) |
|-------------------------|---------|--------------------|---------------------|
| —                       | —       | 15.86±2.48         | 19.31±2.92          |
| 0.1                     | —       | 15.58±0.20         | 19.22±3.34          |
| 1                       | —       | 10.61±1.76         | 19.61±1.03          |
| 10                      | —       | 8.66±3.92          | 20.14±2.41          |
| 20                      | —       | 8.44±3.48          | 21.84±3.07          |
| 20                      | 20      | 13.86±0.38         | 19.95±2.22          |

Sertoli TM4 cells were seeded with a density of 2×10^4/well on 6-well plate for 24 h and then treated with NP65 for 24 h. In NP+NAC treatment group, cells were pretreated with 5 mM NAC for 30 min, and then exposed to 20 μM of NP65 for 24 h.

Results are presented as mean ± S.D. with triplicate measurements

*P < 0.05 vs. control group

**P < 0.01 vs. control group

*P < 0.05 vs. 20 μM NP65 group
the cells in a manner of dose dependence, and achieved the maximum at 20 μM NP65. However, the increase was diminished by NAC pretreatment in 20 μM NP65 group (Fig. 2).

Effects of NP65 on SOD activity and MDA content

We thus further investigated the influences of NP65 upon the activity of SOD as well as MDA content in Sertoli TM4 cells. Compared with control group, 20 μM of NP65 treatment dramatically downregulated the level of SOD activity in cells ($P < 0.01$, Table 2). On the contrary, content of MDA was raised remarkably in 20 μM NP65 treatment groups comparing group of control (Table $P < 0.01$). Interestingly, pretreatment of NAC mitigated the decline of SOD activity and the growth of MDA content induced by NP65. Thus, we proposed that NP65 induced lipid peroxidation in TM4 cells, which finally resulted in oxidative stress in NP65 treated TM4 cells.

Induced apoptosis after NP65 exposure in TM4 cells

Apoptosis induced by NP65 was detected by flow cytometry in Sertoli TM4 cells (Fig. 3). Comparing control group, there was no sharp variation in the percentage of apoptotic cells in 0.1 as well as 1 μM of NP65 treatment groups. On contrary,
the portion of apoptotic cells was raised remarkably in 10 or 20 μM NP65 group (P < 0.05). In addition, NAC attenuated NP65-induced decrease of Bcl-2, which implied that ROS production or oxidative stress was related to the apoptosis process in TM4 cells, and that the process of apoptosis should be mediated via mitochondrial pathway.

**Impact of NP65 on intracellular Ca²⁺ in TM4 cells**

As illustrated in Fig. 5, comparing control group, fluorescence intensity of [Ca²⁺]i was raised in the groups treated with 1–20 μM NP65 (P < 0.05). Our data showed that NAC pretreatment attenuated NP65-enhanced [Ca²⁺]i increase in TM4 cells; although the attenuation was not significant, it still suggested that ROS is related to NP65-induced [Ca²⁺]i alteration in TM4 cells.

**NP65 inhibited the activity of Ca²⁺-ATPase and Serca expression in TM4 cells**

The influences of NP isomer upon Ca²⁺ homeostasis disorder were determined concerning Ca²⁺-ATPase activity and sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) function in TM4 cells. Ca²⁺-ATPase activity was downregulated by NP65 in a manner of dose dependence (P < 0.01, Fig. 6a). Furthermore, the data of this research indicated that the decrease of Ca²⁺-ATPase activity induced by NP65 was influenced by pretreatment of NAC; NAC significantly improved NP65-induced decrease of Ca²⁺-ATPase activity in the cells (P < 0.01). Consistent with the alteration of Ca²⁺-ATPase activity, the expression level of mRNA of Serca 2 in TM4 cells was significantly decreased by 20 μM of NP65, which was rescued by NAC (Fig. 6b and c).

**Concentration of cAMP and cGMP in Sertoli TM4 cells**

The results illustrated that the concentration of cAMP was significantly reduced by NP65 at 1, 10, and 20 μM (P < 0.05). However, the decline of cAMP content induced by NP65 at 20 μM was upregulated by NAC (Fig. 7a). On the other hand, it was found that NP65 showed no significant effects on cGMP secretion (Fig. 7b), indicating that the injury of NP65 to TM4 cells may be through inhibiting cAMP pathway but not cGMP pathway.

**Discussion**

NP was reported to cause intracellular accumulation of ROS in multiple cell types (Qi et al. 2013; Okai et al. 2004; Gong and Han 2006), and induced oxidative stress within testis of rats (Chitra and Mathur 2004). Our interesting findings...
implied that NP₆₅ treatment caused ROS over generation and oxidative stress in TM4 cells, as proven by rapid increase of MDA content and the marked decrease of SOD activity in NP₆₅-stimulated cells. Oxidative stress was thought to be the underlying mechanism for the death of apoptotic cells (Kitazawa et al. 2001). The current results demonstrated that NP₆₅ in TM4 cells could induce oxidative stress, which might lead to cellular apoptotic damages potentially. Apoptosis was accompanied by alteration in the mRNA expression of Bcl-2 family, suggesting that mitochondrial pathway should be contributed to NP₆₅-induced apoptosis in TM4 cells.

Ca²⁺, as the widely employed intracellular messenger, can encode a variety of cellular information based on the regulation of signals of Ca²⁺. Signals of Ca²⁺ have pivotal roles within various cellular behaviors including differentiation, proliferation, and transcription of genes (Bootman et al. 2012). In response to different stimuli, increase of the Ca²⁺ concentration within the cytosol induces plenty of types of events, which plays substantial role in cell apoptosis. Wang et al. (2005) have found that Ca²⁺ elevation and Ca²⁺-independent cell death could be induced by NP in MG63 human osteosarcoma cells. Studies have found that Ca²⁺ release and homeostasis disorder were involved in the apoptosis induced by alkylphenol in TM4 cells (Michel-angeli et al. 2008), and NP caused Ca²⁺-dependent apoptosis in SCM1 human gastric cancer cells (Kuo et al. 2010). Analysis of flow cytometry illustrated that NP₆₅ dramatically raised intracellular Ca²⁺ level in TM4 cells through a manner of dose dependence, and ROS scavenging contributed to weakening NP-induced Ca²⁺ overloading. The results suggested that ROS generation was involved in Ca²⁺ release in NP₆₅-challenged TM4 cells, and Ca²⁺ homeostasis disorder might act an important role in apoptosis of cells induced by NP₆₅.

SERCA has always been accepted as playing a central role in the mechanism of Ca²⁺ transport across the membrane, from the cell cytosol into the endoplasmic reticulum, and this is the reason why low levels of free cytosolic Ca²⁺ are maintained in the cells. Under stimulation, [Ca²⁺]ᵢ becomes higher and remains high if SERCA is blocked. Nonylphenol was reported to influence Ca²⁺ signaling mechanisms.
within cells by influencing transporters of Ca\(^{2+}\) such as Ca\(^{2+}\) pumps of SERCA (Hughes et al. 2000). Many estrogenic alkylphenols such as NP have been tested to inhibit the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase of skeletal muscle, which functions in a similar manner to the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Mason et al. 1993). Therefore, we hypothesized that the evaluation of Ca\(^{2+}\) induced by NP might be related to alteration of SERCA Ca\(^{2+}\) pumps function. The inhibition of Ca\(^{2+}\)-ATPase activity and SERCA-type Ca\(^{2+}\) pump expression in this study suggested that NP might act as an inhibitor of SERCA. We proposed that NP could release Ca\(^{2+}\) from intracellular stores by inhibiting the activity of SERCA Ca\(^{2+}\) pumps. Ca\(^{2+}\) channels were regulated by different kinases, calmodulin-dependent kinase, protein tyrosine kinase, and G protein subunits (Keef et al. 2001). Since cAMP and cGMP are important for regulation of protein kinase C, cGMP-dependent protein kinase, and cAMP-dependent protein kinase to exert vital functions of cell (e.g., differentiation, proliferation, transcription of genes, and cellular apoptosis) within all organisms, we concentrated our researches upon the effects of cAMP as well as cGMP in TM4 cells. The decrease of cAMP concentration could be one of the factors that were involved in the elevation of Ca\(^{2+}\)-ATPase (Mason et al. 1993). Therefore, we hypothesized that the evaluation of Ca\(^{2+}\) induced by NP might be related to alteration of SERCA Ca\(^{2+}\) pumps function. The inhibition of Ca\(^{2+}\)-ATPase activity and SERCA-type Ca\(^{2+}\) pump expression in this study suggested that NP might act as an inhibitor of SERCA. We proposed that NP could release Ca\(^{2+}\) from intracellular stores by inhibiting the activity of SERCA Ca\(^{2+}\) pumps. Ca\(^{2+}\) channels were regulated by different kinases, calmodulin-dependent kinase, protein tyrosine kinase, and G protein subunits (Keef et al. 2001). Since cAMP and cGMP are important for regulation of protein kinase C, cGMP-dependent protein kinase, and cAMP-dependent protein kinase to exert vital functions of cell (e.g., differentiation, proliferation, transcription of genes, and cellular apoptosis) within all organisms, we concentrated our researches upon the effects of cAMP as well as cGMP in TM4 cells. The decrease of cAMP concentration could be one of the factors that were involved in the elevation of...
Ca\(^{2+}\) level in TM4 cells, although the mechanism was still unclear. Interestingly, the Ca\(^{2+}\) disorder was moderately inhibited by NAC, no matter the inhibition of the activity of Ca\(^{2+}\)-ATPase or the decrease of cAMP content was weakened by NAC pretreatment. We concluded that ROS pathway was involved in NP\(_{65}\) disturbed Ca\(^{2+}\) signaling in TM4 cells.

Apoptosis and Ca\(^{2+}\) overload were related to the formation of ROS; several antioxidants can block cell apoptosis. NAC has been reported to alleviate damage of cells induced by toxic chemicals (Spagnuolo et al. 2006). In the present study, NAC was employed for investigation of the putative role of ROS production in apoptosis and loss of Ca\(^{2+}\) homeostasis induced by NP\(_{65}\). NP\(_{65}\)-induced apoptosis and loss of Ca\(^{2+}\) homeostasis were blocked by NAC, suggesting that ROS generation was involved in NP\(_{65}\)-induced apoptosis as well as loss of Ca\(^{2+}\) homeostasis. Thus, the current study built a direct linkage between NP\(_{65}\)-induced generation of ROS and apoptosis as well as Ca\(^{2+}\) disorder in TM4 cells.

It is known that the structure of the side chain may influence the estrogenic effect of a single NP isomer to a large extent. The estrogenic potency of NP\(_{65}\) was 6.1 \(\times\) 10\(^{-6}\), and 4-n-NP showed no estrogen effect (Preuss et al. 2006). Michelangeli et al. (2008) reported that the SERCA inhibition potency of alkylphenols was relevant to length of chain for linear chain alkylphenols, and branched chain alkylphenols in general had higher potencies compared with their counterparts of linear chain. There were different effects between NP\(_{65}\) and 4-n-NP on the apoptosis and Ca\(^{2+}\) disorder in TM4 cells, which further proved that the effects of NPs to organism were related to their structures or their potential xenoestrogenic activity.

In summary, this study has shown that the NP isomer, NP\(_{65}\), could trigger oxidative stress as well as mitochondrial pathway apoptosis in mouse Sertoli TM4 cells. In addition, NP\(_{65}\) was capable of elevating intracellular [Ca\(^{2+}\)] levels in Sertoli TM4 cells. Our study found that ROS was related to both the apoptosis as well as Ca\(^{2+}\) disorder induced by NP\(_{65}\) in TM4 cells. However, whether NP\(_{65}\) evoked Ca\(^{2+}\) disorder is related to NP\(_{65}\)-induced apoptosis is an important issue, which will be investigated in our further study. Nowadays, NPs have become an ever-present contaminant in the ecosystem where they negatively affect all forms of life. They mimic estrogen, thereby disrupting the normal functioning of the hormonal system in mammals, especially in males. Apoptosis reported as one of the main causes of male reproductive system injury (Wang et al. 2014; Liu et al. 2020). Ca\(^{2+}\) signaling is important in many cellular activities, such as cell division, cell proliferation, and differentiation (Bootman et al. 2012). NP\(_{65}\) induced apoptosis and interrupted Ca\(^{2+}\) homeostasis via ROS pathway in Sertoli TM4 cells. Taking all of these facts into consideration, it offered an alternative vision that NPs might disrupt the development of reproduction system by inducing apoptosis and disturbing Ca\(^{2+}\) relative signaling pathways in Sertoli cells. On the other hand, current study was proved that we could investigate the environmental hazard of NPs from the perspective of isomer specificity.

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Author contribution Xiaozhen Liu contributed to methodology and original draft. Fuxiang Li performed the research. Zhaoliang Zhu and Gaoyi Peng analyzed data. Danfei Huang contributed to discussion and resource provision. Mingyong Xie contributed to review, editing, and supervision. All authors approved the final manuscript.

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Data availability All data supporting the conclusions of this study will be made available by the authors, without undue reservation.

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Competing interests The authors declare no competing interests.

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