Research Article

Oxidative Stress and Apoptosis Contributed to Nonylphenol-Induced Cell Damage in Mouse NCTC Clone 1469 Cells

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Nonylphenol (NP) is considered an environmental toxicant and endocrine-disrupting compound. The present study aimed to investigate the effects of NP on NCTC Clone 1469, nonparenchymal hepatocytes, and to study the molecular basis of NP-induced liver injury. The results showed that NP decreased cell viability and induced nucleus crenulation and intracellular enzyme leakage in NCTC Clone 1469 cells. Additionally, NP-induced oxidative stress and apoptosis of NCTC Clone 1469 are accompanied by upregulating reactive oxygen species (ROS) production, increase of Bax, decrease of Bcl-2, activation of caspase-3 and caspase-12, and release of cytosolic free Ca^{2+} in the cells. ROS scavenger, N-acetyl-L-cysteine (NAC), prevented the intracellular enzyme leakage induced by NP. NP induced alteration of estrogen receptor- (ER-) α and ER- β expression, while ER antagonists, ICI 182,780, showed no effect on NP-induced intracellular enzyme leakage. We proposed that NP triggered cell damage via inducing oxidative stress and apoptosis in cells, but not estrogenic effect.

1. Introduction

Nonylphenol polyethoxylate (NPEO) is a kind of alkylphenol polyethoxylates, which are widely used as emulsifiers, plasticizers, and detergents [1]. Nonylphenol (NP), one of the degraded products of NPEOs, has also been used extensively in cleaners, cosmetics, pesticides, plastics, surface-active agents, polyvinyl chloride pipes, food processing and packaging industry, and other industrial and agriculturally formulated products [2–6]. NP has been detected in most of the retailed food, and it may be widespread in our daily food. Studies have shown that NP could be absorbed by aquatic wildlife species, especially by fish, and then accumulated in the human body through the food chain [7, 8]. In addition, NP can also migrate from food packaging films to food simulants during the process of cooking and microwaving

[9, 10]. Due to the bioaccumulative characteristic of NP, it can be harmful to the biological systems in a very low concentration.

Researches have indicated that the chemical structure of NP resembled the estrogenic hormone in animal and human and can cause the endocrine disorder [11]. Not only can NP disrupt endocrine but also it has toxic effects on systemic organs of the human body [12–14]. Several studies have shown that the gestational exposure of NP could damage the reproductive, neural, and digestive systems in rats [15]. The liver is an important immune organ and is the site of biological transformation and metabolism of exogenous chemicals. It is also the main target of chemicals for biological and chemical injury. NP administered by oral gavage was reported to be distributed to all tissues, and there was a higher distribution in the liver than other tissues [16].

However, until recently, most research has focused on the adverse effects of NP on the reproductive system, and little has been known regarding the effects of NP on liver injury. Consequently, this study was proposed to investigate the underlying mechanisms of NP toxicity on liver cells.

Apoptosis is a process of programmed cell death, which may happen in multicellular organisms [17]. In recent years, more and more researchers have been concerned about apoptosis induced by environmental pollutants. There have been a great number of reports suggesting that apoptosis is one of the most important reasons that cause the toxicity induced by environmental endocrine disruptors [18, 19]. It was reported that NP enhanced apoptosis in sperm cells, thymocyte cells, Sertoli cells, and human embryonic stem cells [20–22]. We previously showed that NP induced oxidative stress and apoptosis in TM4 cells, and the apoptosis may be mediated via MAPKs and Akt pathways [23]. Although many studies have provided evidence for NP-induced cell death, the detailed mechanisms or pathways of liver cell death remain unclear. Therefore, in this study, we assessed the toxicity of NP in a mice liver cell line, NCTC Clone 1469, with the specific aim of studying the role of oxidative damage and apoptosis in hepatotoxicity.

2. Materials and Methods

2.1. Reagents and Chemicals. NP (99.9% purity, CAS: 104-40-5), penicillin, streptomycin sulfate, and 5(6)-carboxy-2'-7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Inc., (St. Louis, MO, USA). Dulbecco's modified Eagle's medium-high glucose medium (DMEM-H medium) and fetal bovine serum (FBS) were obtained from Hyclone (Waltham, MA, USA). Fulo-3/AM was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Annexin V/PI apoptosis assay kit was purchased from Invitrogen Co. (Eugene, Oregon, USA). Antibodies against Bax, Bcl-2, ER- α , and ER- β were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Caspase-3 and caspase-12 activity assay kits were purchased from BioVision (Research, Mountain View, California). z-ATAD-fmk and z-DEVD-fmk were purchased from Becton Dickinson Company (Palo Alto, CA, USA). All other chemicals were analytical agents.

Nonparenchymal hepatocytes, NCTC Clone 1469 cells, were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in DMEM-H medium, supplemented with 1% penicillin-streptomycin, 10% (v/v) FBS in an atmosphere with 95% humidity and 5% CO₂ at 37°C.

2.2. Cell Cytotoxicity Assay. Cell viability was detected by 3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide (MTT) test. Briefly, cells were plated on a 96-well culture plate at 2×10^4 cells/well in 100 μ L culture medium. Cells were treated with NP, followed by the addition of 25 μ L MTT solution (5 mg/mL) to each well and further incubation for 4 h at 37°C. The supernatants were removed before adding 150 μ L DMSO to dissolve the formazan crystal. The absorbance was measured on an automated microplate

reader (Varioskan Flash, Thermo Scientific, USA) at 570 nm. Cell viability in each test group was expressed as a percentage of the control group. Cells in the control group were as 100% viable. In addition, after treatment with 60 μ M NP for 12 h, cells were observed by microscopy.

2.3. Leakage of LDH, ALT, and AST. Cells were challenged with NP at different concentrations (0, 1, 10, 20, 40, or 60 μ M) for 12 h and then the supernatant was collected for further analyses. LDH, ALT, and AST leakages were quantified according to the manufacturer's instructions of commercial enzymatic kits (Jiancheng Bioengineering Institute, Nanjing, China), respectively.

2.4. Determination of Intracellular ROS Production. NP-treated NCTC 1469 cells were collected and washed with cold phosphate-buffered saline (PBS). Washed cells were further incubated with 10 μ M DCFH-DA at 37°C for 20 min. Then, cells were washed with PBS and measured using a flow cytometer with excitation and emission settings of 488 and 530 nm, respectively.

2.5. Measurement of Malondialdehyde (MDA) Content and Enzyme Activities. To detect the intracellular MDA content and antioxidant enzyme activities, the NP-treated cells were washed twice with PBS, then harvested from the plates into ice-cold PBS (0.1 M, containing 0.05 mM EDTA), and homogenized. The homogenate was centrifuged at 4°C at 12,000g for 30 min. The resulting supernatant was used for the measurement of MDA content, superoxide dismutase (SOD) activity, and catalase (CAT) activity according to the instructions for the kits (Jiancheng Bioengineering Institute, Nanjing, China). Protein concentration was determined by the Bradford method, using bovine serum albumin as a reference standard.

2.6. Detection of Cytosolic-Free Calcium Levels. NP-treated cells were collected, loaded with 5 μ M of Fulo-3/AM for 30 min in the dark at 37°C, and then washed to remove extracellular Fluo-3/AM dye. Then, [Ca²⁺]_i in the cells were measured by using a flow cytometer.

2.7. Apoptosis Analysis with Annexin V-FITC and PI Staining. Cells at the density of 1×10^6 cells/well were incubated in the presence of NP for 12 h and then harvested. Specific binding of Annexin V-FITC was carried out by incubating the cells for 15 min at room temperature in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing a saturating concentration of Annexin V-FITC and PI, and then apoptosis was measured by a flow cytometer.

2.8. Expression Analysis of Bax and Bcl-2. The protein content was determined by using BCA protein assay kit (Beyotime, Jiangsu, China). Cell lysates were denatured by boiling in loading buffer (20 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 100 mM DTT, and 0.04% bromophenol

blue). Samples (approximately 40 μ g protein) were loaded onto 10% SDS polyacrylamide gel followed by electroblotting onto a nitrocellulose membrane. After blocking of nonspecific binding with 5% bovine serum albumin (prepared in TBS containing 0.1% Tween 20) for 2 h at room temperature, the membranes were then incubated with 1:1000 diluted antibodies against Bcl-2, Bax, and β -actin and washed 3 times in TBS-T. The membranes were incubated with appropriate HRP-labeled goat anti-mouse or goat anti-rabbit secondary antibody IgG for 2 h at room temperature and washed 3 times in TBS-T; then, the protein was detected using enhanced chemiluminescence (ECL). Densitometry was performed using the software Quantity One.

Bcl-2 and Bax mRNA expression levels were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was reverse transcribed from the RNA using First Strand cDNA Synthesis Kit (Thermo Scientific, Maryland, USA) and then was amplified based on the first cDNA template through polymerase chain reaction. The sequences of primers used for RT-PCR are shown in Table 1. PCR conditions were used as follows: 94°C for 3 min, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 60°C (β -actin), 54°C (Bcl-2), or 60°C (Bax) for 30 s, and extension at 72°C for 2 min, plus final extension at 72°C for 10 min. PCR products were analyzed by GoldView-agarose gel electrophoresis on 2% (w/v) agarose gel and visualised under UV light. The signals of Bcl-2 and Bax were measured by scanning densitometry using Quantity One software.

2.9. Caspase-3 and Caspase-12 Activities Assay. To investigate the mechanism of NP-induced apoptosis in NCTC 1469 cells, activities of caspase-3 and caspase-12 were detected in the present study. The assays were performed on 96-well microplates through incubating 10 μ L protein of cell lysate in 80 μ L reaction buffer (1% NP-40, 20 mM Tris-HCl, 137 mM NaCl, and 10% glycerol, pH 7.5) containing 10 μ L caspase-3 (Ac-DEVD-pNA) or caspase-12 (Ac-ATAD-pNA) substrate (2 mM). Lysates were incubated at 37°C for 4 h, and then samples were measured on an automated microplate reader with a 400 nm excitation and 505 nm emission. Caspase-3 inhibitor, z-DEVD-fmk, or caspase-12 inhibitor, ATAD-fmk, was also used to confirm whether NP-induced apoptosis was mediated by caspase activation. Inhibitors were added to the cell cultures half an hour before the NP treatment.

2.10. Analysis of Estrogen Receptors Expression. Protein expression of ER- α and ER- β was detected by western blot, mRNA expression levels of ER- α and ER- β were examined by RT-PCR, assays were performed as 2.9 described, and the annealing temperature for both ER- α and ER- β was 60°C.

ICI 182,780, a nonsteroidal selective estrogen receptor modulator, can compete with endogenous estrogens for receptor binding. In the current study, 100 μ M of ICI 182,780 (Fulvestrant, Sigma) was used to confirm whether the estrogenic effect was involved in NP-mediated liver cell injury. After the NCTC Clone 1469 were cultured for 24 h,

the reagents were added into the medium half an hour before the NP treatment. After 12 h of NP treatment, NCTC Clone 1469 were collected and used for assays.

2.11. Statistical Analysis. Results were expressed as mean $\pm$ S.D. One-way analysis of variance followed by the LSD test was used to determine the statistical significance between various groups (ANOVA) in SPSS (Windows version 11.9), and a value of $P < 0.05$ was accepted to be statistically significant.

3. Results

3.1. Cytotoxicity of NP. To evaluate the cytotoxicity of NP on NCTC Clone 1469, time course and dose response of cell viability were tested using the MTT method. As shown in Figure 1(a), no significant difference was observed in the 1–40 μ M NP groups, compared with the control group. However, cell viability significantly decreased in 60 μ M ($70.28 \pm 5.24\%$), 80 μ M ($63.84 \pm 4.09\%$), and 100 μ M ($50.46 \pm 6.18\%$) NP treatment groups. Additionally, the results showed that cell viability decreased in a time-dependent manner, and 0.1% DMSO showed no effects on cell viability. Besides, it was found that NCTC Clone 1469 presented nucleus crenulation after NP treatment, with being more and more serious over time (Figure 1(b)), suggesting that NP exerted damage effect to NCTC Clone 1469.

LDH, ALT, and AST are important intracellular enzymes, which are indicators of liver cell damage [24, 25]. After NCTC Clone 1469 was cultured with various concentrations of NP for 12 h, the activities of LDH, ALT, and AST in high NP treatment groups were increased compared with the control group. As shown in Figure 1(c), LDH activity significantly increased in high concentrations of 20 μ M ($P < 0.05$) and 40 and 60 μ M ($P < 0.01$) NP groups, ALT activity significantly increased in 40 and 60 μ M NP-treated groups ($P < 0.05$), and AST activity significantly increased in 60 μ M NP-treated group ($P < 0.05$).

3.2. NP-induced Oxidative Stress in NCTC Clone 1469. ROS generation and lipid peroxidation were indicators of oxidative stress. Recently, more and more studies have demonstrated that a balance between ROS production and antioxidants system was associated with cell death [26]. Thus, ROS generation, antioxidant enzyme activities, and MDA content were detected in the present study. Intracellular ROS was increased in a dose-dependent manner in NP challenged NCTC Clone 1469, which was statistically significant in 40–60 μ M NP groups with $P < 0.01$ (Figure 2(a)), and antioxidant N-acetyl-L-cysteine (NAC) attenuated NP-induced ROS production (Figures 2(a)–G). As shown in Figure 2(b), SOD activities in 40–60 μ M NP groups significantly decreased compared with the control group ($P < 0.01$), and CAT activities significantly decreased in 20 μ M ($P < 0.01$) and 40 μ M and 60 μ M ($P < 0.01$) NP groups. In contrast, MDA levels increased in 40 μ M or 60 μ M NP-treated NCTC Clone 1469 with $P < 0.01$. Moreover, NAC treatment prevented the decrease of SOD and CAT activities and the increase of MDA contents in 60 μ M NP treatment group

TABLE 1: Sequences of primers used for RT-PCR.

Gene name	Sequence (5'-3' direction)	Product size (bps)
ER- α	5'-GGCCTGACTCTGCAGCAGCAG-3'(F)	300
	5'-GTTGGGGAAGCCCTCTGCTTC-3'(R)	
ER- β	5'-GGCATTCTACAGTCCTGCTG-3'(F)	165
	5'-TCTGCATAGAGAAGCGATGA-3'(R)	
Bcl-2	5'-CGCCGGGCTGGGGATGACTTC-3'(F)	428
	5'-CACTTGTGGCCAGGTATGC-3'(R)	
Bax	5'-CTCCAACOAGGAAAATGATA-3'(F)	250
	5'-TCATGGAACGTATGATGAA-3'(R)	
β -Actin	5'-ATTGTAACCAACTGGGACG-3'(F)	533
	5'-TTGCCGATAGTGATGACCT-3'(R)	

(Figure 2(c)). These results implied that NP could significantly induce lipid peroxidation and oxidative stress in NCTC Clone 1469 cells, which may result in liver damage in final.

3.3. NP-Induced Apoptosis in NCTC Clone 1469. NP-induced apoptosis in NCTC Clone 1469 was analyzed by flow cytometry. After treatment with NP at various concentrations for 12 h, the proportion of apoptotic cells increased in a dose-dependent manner (from $3.21 \pm 1.66\%$ to $24.66 \pm 2.51\%$). The data showed that there was no significant difference in the percentage of apoptotic cells between the control group and the low concentration of NP treatment groups (1 and $10 \mu\text{M}$). However, the percentage of apoptotic cells increased dramatically by the treatment with NP at 20, 40, or $60 \mu\text{M}$ ($P < 0.01$). Caspases cascade played a central role in regulating various apoptotic responses [27]. DEVD-fmk or ATAD-fmk, caspase-3 or caspase-12 inhibitor, was used to substantiate whether caspases activation was involved in NP-induced apoptosis. The results showed that DEVD-fmk and ATAD-fmk significantly downregulated the proportion of apoptotic cells induced by $60 \mu\text{M}$ NP ($P < 0.01$, Figure 3).

The activities of caspase-3 and caspase-12 in NCTC Clone 1469 with or without NP treatment were examined. As shown in Figure 4, treatment of liver cells with NP caused remarkable activation of caspase-3 in 20– $60 \mu\text{M}$ NP groups, and activation of caspase-12 in 10– $60 \mu\text{M}$ NP groups.

Moreover, stress/toxicant-mediated apoptosis is also mediated by Bcl-2 family proteins [28, 29]. Thus, the mRNA and protein expression levels of Bcl-2 and Bax were determined in this study. NP significantly increased the mRNA level of Bax at 40– $60 \mu\text{M}$ ($P < 0.01$). In contrast, NP decreased the mRNA level of Bcl-2 in a dose-dependent manner, which was statistically significant in 20 ($P < 0.05$), 40, and $60 \mu\text{M}$ ($P < 0.01$) NP treatment groups (Figures 5(a) and 5(b)). Consistent with the mRNA expression, NP increased the protein expression of Bax and decreased that of Bcl-2 in 20– $60 \mu\text{M}$ NP groups (Figures 5(c) and 5(d)).

3.4. Intracellular Ca^{2+} Level. $[\text{Ca}^{2+}]_i$ change in NP treatment cells was analyzed by using flow cytometry with Fluo-3/AM. As shown in Figure 6, compared with the control group, $[\text{Ca}^{2+}]_i$ fluorescence intensity was increased in a dose-dependent manner in the groups treated with NP (from 4.35% to 36.41%) and showed a significant statistic difference in 40– $60 \mu\text{M}$ ($P < 0.01$). While pretreatment with caspase-3 or caspase-12

inhibitor, DEVD-fmk or ATAD-fmk, intracellular $[\text{Ca}^{2+}]_i$ overload reduced when compared with $60 \mu\text{M}$ NP group, which suggested that apoptosis resulted in $[\text{Ca}^{2+}]_i$ overload in the cells and the alteration of $[\text{Ca}^{2+}]_i$ may source from Ca^{2+} influx or endoplasmic reticulum Ca^{2+} release.

3.5. Protein and mRNA Expression of Estrogen Receptors. NP, considered as an environmental endocrine disruptor, acts by competing with hormone receptors [30]. In order to make it clear whether the estrogenic effect was involving NP-induced oxidative stress and apoptosis in nonparenchymal hepatocytes, RT-PCR and western blotting were performed to analyze the expression of ER- α and ER- β in NCTC Clone 1469. Compared with the control group, NP treatment significantly increased the mRNA expression of ER- α and ER- β , which were statistically significant at 10– $60 \mu\text{M}$ ($P < 0.01$) and 1– $40 \mu\text{M}$ ($P < 0.05$) NP groups, respectively. Interestingly, the mRNA expression of ER- β decreased at $60 \mu\text{M}$ NP treatment group (Figures 7(a) and 7(b)), which implied that NP may exert damage toxicity to NCTC Clone 1469 in high concentration. Meanwhile, the protein expression of ER- α increased remarkably in 40– $60 \mu\text{M}$ ($P < 0.01$), that of ER- β was distinctly increased in 10– $20 \mu\text{M}$ ($P < 0.01$) and $40 \mu\text{M}$ ($P < 0.05$) NP groups, and the protein expression of ER- β decreased in $60 \mu\text{M}$ NP group as well (Figures 7(c) and 7(d)). These interesting findings implied that NP may influence the expression ER- α and ER- β in NCTC Clone 1469.

3.6. Effects of NAC and ICI 182,780 on NP-Induced Cell Damage. To confirm whether NP exerted cytotoxicity by inducing oxidative stress and apoptosis in the cells, the ROS scavenger and NAC, were used to investigate the putative role of ROS in LDH, ALT, and AST upregulation in our experimental model. In our experimental model, the ROS scavenger, NAC, was used to investigate the putative role of NP stimulated ROS in LDH, ALT, and AST upregulation induced by NP. The release of LDH, ALT, or AST in NAC pretreatment group was remarkably inhibited when compared with $60 \mu\text{M}$ NP-treated group (Figure 8), suggesting that oxidative stress potentially contributed to NP-induced cell damage. However, there was no significant difference between ICI pretreated group and $60 \mu\text{M}$ NP exposure group in LDH, ALT, and AST activities (Figure 8), which indicated that toxic effects of NP on the liver cells may be not related to its estrogenic effect.

FIGURE 1: NP induced cytotoxicity to NCTC Clone 1469 cells. (a) Time- and dose-dependent cell viability. Cultured cells were exposed to different concentrations of NP (0, 1, 10, 20, 40, 60, and 100 μM) for 12 h or 60 μM NP for different time periods (2, 4, 6, 8, 10, 12, 24, 36, 48, 60, and 72 h). After the experiment, cell viability was examined by MTT assay. (b) Cell morphology was examined by microscopy with a photography system. (c) Effect of NP on LDH, ALT, and AST leakage in the supernatant. The activities of the LDH, AST, and ALT were expressed as % of control. Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus the control group.

4. Discussion

The potential hazard of NP to human health has attracted many researchers' attention. NP, a typical representative of endocrine disruptors, may cause damage to the body's endocrine system. However, when assessing the harm of NP to the body, we should consider its overall damage. Studies

have shown that maternal exposure to NP can cause liver tissue lesions in rats [31]. A study from the digestive tract of adult rats exposed to NP also found that the liver and kidney may be the primary target organs [32]. Hepatocyte culture *in vitro* was reported to be a simple and rapid way *in vitro* toxicology experimental system and a good way to study toxicology. NCTC Clone 1469 cell line provides an ideal

(a)

FIGURE 2: Continued.

FIGURE 2: NP induced oxidative stress in NCTC Clone 1469 cells. (a) Intracellular ROS levels detected in cells. After exposure to NP for 12 h, NCTC Clone 1469 were collected for DCFH-DA staining followed by flow cytometry analysis. (A–G) Flow cytometric plots. (H) Flow cytometric analysis result. (b) Effect of NP on intracellular SOD activity, CAT activity, and MDA content in NCTC Clone 1469. SOD activity, CAT activity, and MDA content are expressed as % of control. (c) NAC weakened NP-induced alteration of SOD activity, CAT activity, and MDA content. Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus the control group; # $P < 0.05$ versus 60 μ M NP group.

system for the in vitro analysis of function and responsiveness to biochemical/hormonal factors of this particular cell type. Estrogen receptors were expressed in NCTC Clone 1469, suggesting that this cell line could be useful for examining the relationship between liver injury and estrogenic actions. Now NCTC Clone 1469 cells were widely used in the research field about the effects of chemicals even alkylphenol on normal hepatocyte [33–35]. Thus, we cultured the NCTC Clone 1469 in vitro to investigate the underlying mechanisms of NP hepatotoxicity in the current study.

In the present study, we found that NP downregulated the viability of NCTC Clone 1469 in high concentration with 12 h incubation and shrunken cells revealed in microscopic observation, which indicated that NP showed stress or damage to NCTC Clone 1469. The amounts of LDH, ALT, and AST leakage in the supernatant were sensitive indicators to evaluate liver cell damage. LDH can catalyze the inter-conversion of lactic acid and pyruvic and regulate the body's normal physiological activity. LDH cannot get through the cell membrane under normal circumstances. However, when cells are damaged or poisoned, membrane

permeability increases and LDH is released into the medium [36]. ALT is mainly present in the cytoplasm of liver cells, and its activity could significantly elevate when the cell membrane permeability changes in cell injury. AST is mainly located in the mitochondria and is released to the medium when the mitochondria are seriously damaged. NP enhanced the leakage of LDH, ALT, and AST activities in NCTC Clone 1469, suggesting that NP at high concentrations strongly injured the cells, destructed the liver cell growth microenvironment, and caused an increase in membrane permeability.

Environmental contaminants can undergo redox cycling during the process of metabolism in organisms and produce large quantities of ROS. Oxidative stress induced by excess ROS can produce a series of toxic effects [37–39]. Previous studies have shown that NP increased intracellular ROS, regulated cell cycle, inhibited cell proliferation, and even led to cell death [40, 41]. Oxidative stress is caused not only by excessive intracellular ROS but also by the decrease of intracellular antioxidants. MDA is a product of lipid peroxidation reaction; its content often reflects the extent of lipid

FIGURE 3: NP induced apoptosis in NCTC Clone 1469 cells. After exposure to NP for 12 h, NCTC Clone 1469 were collected for Annexin V-FITC and PI staining followed by flow cytometry analysis. (a) Flow cytometric plots. (b) Flow cytometric analysis result. Results are presented as mean $\pm$ SD with triplicate measurement. ** $P < 0.01$ versus the control group; # $P < 0.05$ versus 60 μ M NP group.

peroxidation in organisms and the degree of cell damage indirectly [42, 43]. The level of intracellular SOD activity reflects the ability of scavenging free radicals in the body indirectly, and CAT is a receiver of H_2O_2 generated within cells, an important material for antioxidative defense in different regions inside cells [44]. In this study, we found that NP induced oxidative stress in NCTC Clone 1469, as evidenced by the increase of intracellular ROS level and MDA content and the decrease of intracellular SOD and CAT activities in NP challenged cells. NP mediated oxidative stress in NCTC Clone 1469 could potentially contribute to cellular damage induced by NP.

NAC is a typical antioxidant widely used for inhibiting oxidative stress [45]. In order to explore the relationship between oxidative stress and the toxic effects of NP on liver cells, NAC was used to investigate whether it could attenuate NP-induced damage in NCTC Clone 1469. NAC pretreatment significantly increased the activity of SOD or CAT and dramatically decreased MDA content, indicating that NAC as an antioxidant can prevent cells from damage through reducing the extent of intracellular oxidative stress caused by NP treatment. Additionally, NAC significantly blocked NP-induced LDH, ALT, and AST activities upregulation in cells, indicating that antioxidants may reduce the toxic effects of

FIGURE 4: Effect of NP on the activities of caspases-3 and caspase-12. Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus control group.

FIGURE 5: Protein and mRNA expression of Bax and Bal-2. (a) RT-PCR was performed for target genes and β -actin. (b) The histogram represents the quantification of Bax and Bcl-2 mRNA levels (levels of control cells/ β -actin defined as 1). (c) Western blot was performed for the target protein and β -actin. (d) The histogram represents the quantification of Bax and Bcl-2 protein levels (levels of control cells/ β -actin defined as 1). Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus the control group.

NP on liver cells, and oxidative stress may be one potential factor of cell toxicity caused by NP.

Apoptosis has a very important role in the maintenance of tissues, normal morphology, and function of the organ, and body homeostasis was maintained by cell proliferation and apoptosis mutually. Much research has indicated that

apoptosis is one of the reasons for toxic effects by environmental interference. It has been reported that the toxic effects of NP may be related to apoptosis in cells while there is still a lack of reports about apoptosis of mammalian liver caused by NP. Many studies have shown that excess of ROS can promote apoptosis [46]. Intracellular ROS generation

(a)
FIGURE 6: Continued.

FIGURE 6: NP-induced Ca^{2+} release in NCTC Clone 1469 cells. The appearance of Ca^{2+} release was detected by flow cytometry using Fluo-3/AM fluorescent probe. For each sample, 20,000 cells were analyzed. (a) Flow cytometric plots. (b) Flow cytometric analysis result. Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus the control group, # $P < 0.05$ versus 60 μM NP group.

FIGURE 7: Protein and mRNA expression of ER- α and ER- β . (a) RT-PCR was performed for target genes and β -actin. (b) The histogram represents the quantification of ER- α and ER- β mRNA levels (levels of control cells/ β -actin defined as 1). (c) Western blot was performed for the target protein and β -actin. (d) The histogram represents the quantification of ER- α and ER- β protein levels (levels of control cells/ β -actin defined as 1). Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus the control group.

FIGURE 8: Effects of NAC and ICI 182,780 on NP-induced alteration of LDH, ALT, and AST activities. Results are presented as mean $\pm$ SD with triplicate measurement. * $P < 0.05$, ** $P < 0.01$ versus the control group, # $P < 0.05$ versus 60 μ M NP group.

was observed in NP challenged NCTC Clone 1469; thus, apoptosis was detected to provide further insights in NP-induced hepatotoxicity. The process of apoptosis is regulated by a series of related genes, including Bcl-2 family. Bcl-2 is the first gene to be confirmed to inhibit apoptosis, mainly in the outer mitochondrial membrane and nuclear membrane, and is able to prevent the release of proapoptotic factors such as cytochrome c, maintain the stability of intracellular Ca^{2+} , decrease accumulation of free radicals, and finally inhibit apoptosis. Bax, a homologous protein of Bcl-2, can cause changes in mitochondrial permeability and release of cytochrome c, starting apoptosis reaction associated with Caspase. NP promoted the apoptosis of NCTC Clone 1469, accompanied by decrease of Bcl-2 and increase of Bax and intracellular free Ca^{2+} . Caspase is the core of apoptosis, caspase-3 called apoptosis executor is the final effect factor in apoptotic protein cascades reaction, and caspase-12 is a major regulator of apoptosis as well. In this study, NP at high concentrations significantly increased caspase-3 and caspase-12 activity. Meanwhile, the cell apoptosis level decreased remarkably because of the blocking of caspase-3 and caspase-12. These results revealed that NP could result in apoptosis through upregulating expression of Bax, down-regulating Bcl-2, and activation of caspase-12 and caspase-3. Therefore, our results suggested that NP could produce toxic effects on NCTC Clone 1469, but the exact intracellular transduction pathways were still undefined. Thus, additional studies are required to further elucidate on the detailed mechanisms by which NP causes toxic effects.

ICI 182,780 is a kind of ER antagonists that can compete with estrogens for receptor binding. NP has a similar chemical structure to 17 β -estradiol (E2), so it could inhibit competitively the binding of E2 and ER in the target organs. After NP and ER forming a ligand, they bind the response element in DNA binding domain and then induce or inhibit transcription of target genes, starting a series of estrogen-dependent physiological and biochemical processes [47]. ER has two subtypes: ER- α and ER- β ; both can be expressed at different levels in normal liver tissue [48]. NP altered the expression of ER- α

and ER- β in NCTC Clone 1469. However, we found that ICI 182,780 did not reduce LDH, ALT, and AST activities in the supernatant, suggesting that NP exerted potential estrogenic effect in liver cells, while the toxic effects of NP on the liver cells showed no direct relationship with the ER receptor.

In summary, the current study implied that NP showed cytotoxicity to NCTC Clone 1469 cells. Oxidative stress and apoptosis were involved in NP-induced cytotoxicity while this may not be mediated by estrogenic effect directly.

5. Conclusion

We found that NP exhibited toxic effects on NCTC Clone 1469 cells, including morphological change, the decrease of cell viability, and the leakage of intracellular enzymes. NAC weakened the effects of NP on NCTC Clone 1469 cells; we speculated that oxidative stress may be one of the mechanisms contributing to NP toxicity. Furthermore, NP induced apoptosis in NCTC Clone 1469, accompanied by decreasing expression of Bcl-2, increasing expression of Bax, upregulating intracellular Ca^{2+} level, and activating caspase-3/12 activities. Additionally, the results showed that NP affected the expression of ER- α and ER- β in NCTC Clone 1469. However, ICI 182,780 have no effects on NP-induced damage in NCTC Clone 1469. We concluded that NP induced damage to NCTC Clone 1469 cells via mediated oxidative stress and apoptosis in cells, which may not be related to the estrogenic effect of NP.

Abbreviations

ALT:	Alanine aminotransferase
AST:	Aspartate transaminase
CAT:	Catalase
DCF:	2'-7'-Dichlorofluorescein
DCFH-DA:	5(6)-Carboxy-2'-7'-dichlorofluorescein diacetate
DMEM-H:	Dulbecco's modified Eagle's medium-high glucose medium
EDCs:	Endocrine-disrupting compounds
ER- α :	Estrogen receptor α
ER- β :	Estrogen receptor β
HRP:	Horseradish peroxidase
LDH:	Lactate dehydrogenase
MDA:	Maleic dialdehyde
MTT:	3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide
NAC:	N-acetyl-L-cysteine
NP:	Nonylphenol
NPEOs:	Nonylphenol polyethoxylate
PBS:	Phosphate-buffered saline
ROS:	Reactive oxygen species
RT-PCR:	Reverse transcription-polymerase chain reaction
SOD:	Superoxide dismutase.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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