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# Combined effects of two environmental endocrine disruptors nonyl phenol and di-n-butyl phthalate on rat Sertoli cells in vitro

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ABSTRACT

In this study, our purpose is to analyze combined effects of nonyl phenol (NP) and di-n-butyl phthalate (DBP) for rat testicular Sertoli cell toxicity in vitro. Sertoli cells were isolated, purified, cultured, and identified with FSHR fluorescence staining. Then the purified Sertoli cells were treated with different doses of NP, DBP or NP + DBP. Although we did not find dramatic morphological changes, cell viability decreased significantly at high-dose NP and their mixture. The following Annexin V-PI staining demonstrated that DBP alone did not show apoptosis induction, the combination effect on apoptosis induction was due to NP, in addition, nucleus of Sertoli cell showed apoptosis morphological changes. In addition, increased LDH leakage was also observed in high-dose mixture. According to the above phenomena, we inferred that the combined effect of the two substances on Sertoli cell toxicity had an additive effect, and the induction of apoptosis may play an important role.



## 1. Introduction

Decline in human and wildlife reproductive health, which is mainly caused by environmental pollution, is receiving more and more attention from the public and the scientific community. So many chemicals as environmental endocrine disruptors have experimentally demonstrated to be able to affect endocrine processes to a great extent. Among them, nonyl phenol (NP) and di-n-butyl phthalate (DBP) are commonly found in water environment [1,2]. It has been observed that they are seriously threatening reproductive health of human and animals.

NP which has weak estrogenic activity is a primary degradation product of nonylphenol ethoxylate (NPEO), a major group of multipurpose nonionic surface active agents [3,4]. It has been found in preliminary studies that NP can interfere with reproduction in fish, reptiles, and mammals, induce the cell death in gonads, and change other reproductive parameters [3,5,6]. DBP belongs to phthalates which are man-made chemicals widely used in industry and commerce. It has been shown that DBP and its metabolite mono-n-butyl phthalate can cause such anti-androgenic effects as decreased anogenital distance (AGD), cryptorchidism, decreased testosterone levels, decreased sperm production, and infertility [7–9].

In general, level of human exposure to a single environmental endocrine disruptor is low, and it has much less potent than natural hormones. However, humans are often exposed to a mixture of these chemicals and combined effects should be considered to evaluate the human exposure risk. Therefore, Kortenkamp [10] claimed that epidemiology should focus on the mixed effects of multiple endocrine disrupters rather than a single endocrine disrupter. Since the above two compounds have effects on male reproductive system, it can be concluded that testis is one of the target organs of NP and DBP. They are widely and quickly consumed. As a result, they accumulate in the environment at an increasing level, and often appear together. Various channels are available for a mixture of NP and DBP to reach humans. Therefore, it is necessary to explore the combined effect of NP and DBP on male reproduction.

A series of studies on combined effects of mixture focused on the combinations of estrogenic, thyroid-disrupting, and anti-androgenic chemicals at low doses [11–16]. Existing data showed that if they were present synchronously, combined effects may result from the same endocrine disruption. However, there are few studies on the combined effects of a weak estrogenic chemical and an anti-androgenic chemical on reproductive toxicity in Sertoli cell in vitro. The two compounds are often present simultaneously and ranked the top two in the water environment. For example, in China, an analysis revealed that levels of NP and DBP in raw water from Yellow River were as high as 0.47 μmol/L and 0.021 μmol/L, respectively. After water treatment, water content of the residents was still 0.086 μmol/L and 0.008 μmol/L, respectively [2]. In Spain and Canada, it was also found that concentration of NP and DBP

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existence was of the order of magnitude μg/L in test samples from a number of water treatment plants [1,17]. In selected tissues from ewes and their lambs which were grazed on pastures fertilized with sewage sludge or with inorganic fertilizer, NP and phthalate also existed together in muscle and liver as well as fat tissue in treatment groups and there was also significant accumulation [18].

Sertoli cells exert important functions in supporting and nourishing germ cells as well as the constitution of blood–testicle barrier. Onset of spermatogenesis and the eventual production of a sufficient sperm number to insure fertility depend on Sertoli cells. Therefore, specific impairment of Sertoli cells will produce a parallel dysfunction in sperm production. In our laboratory, long-term research data on the Sertoli cell exposure to NP revealed that NP can induce oxidative stress and cytotoxicity in testicular Sertoli cell [19] and induce endoplasmic reticulum stress which may plays an important role in the induction of apoptosis [20]. Expression of 41 proteins among 63 protein spots identified by proteomic approach was altered in rat Sertoli cells after treated with low NP concentrations, similar to environmental conditions, for 24 h. Further analysis by Western blot found these proteins are mainly involved in the response of Sertoli cells to programmed cell death [21]. Additionally, study on the membrane dynamics of Sertoli cells indicated cellular membranes represented a plausible target for NP-induced cytotoxicity [22]. The study of Wang et al. [23] demonstrated that impairment of spermatogenesis caused by DBP should be partly due to the suppression of androgen binding protein (ABP) and inhibin (INH) alpha biosynthesis in Sertoli cells, and Sertoli cells should be one of the major toxic targets. All the above studies confirmed that Sertoli cells were the targets of the two chemicals [24]. Damage to Sertoli cells may lead to impairment of male reproduction.

Many related studies share an assumption that environmental endocrine disruptors disturb endocrine through hormone receptor, but some other studies have confirmed that this is not the case. Thus, it is particularly important to understand the mechanism of these compounds from a different perspective. The purpose of this study is to investigate combined effects in vitro of the aforementioned two environmental endocrine disruptors on primary rat Sertoli cells. It is elucidated whether a mixture of the two endocrine disrupters interacts in an additive, a synergistic, or an antagonistic way.

## 2. Materials and methods

### 2.1. Chemicals and reagents

NP (4-nonyl phenol, $C_{15}H_{24}O$, CAS: 25154-52-3) with 98% analytical standard was from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). DBP (di-n-butyl phthalate, $C_{16}H_{22}O_4$, CAS: 84-74-2) was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium-Ham's F-12 medium (DMEM-F12 medium), collagenase I, trypsin, C8H17N2O4Sna (HEPES sodium salt), penicillin, streptomycin sulfate, Hoechst 33342, fluorescein diacetate (FDA), propidium iodide (PI) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Anti-Follicle stimulating hormone receptor (FSHR) antibody and donkey anti-goat IgG-CY3 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Annexin V-PI apoptosis assay kit was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, Jiangsu, China). 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, Chain). CytoTox-ONE™ Homogeneous Membrane Integrity Assay was obtained from Promega Corporation (Madison, MI, USA).

NP and DBP were dissolved in 99% pure dimethylsulfoxid (DMSO, no. S 26740 916) from Schuchardt (Hohenbrunn, Germany) into stock solutions of 20 mM and 200 mM, respectively.

### 2.2. Primary culture of rat testicular Sertoli cells

Sprague–Dawley rats were purchased from Nanjing Medical University and kept in accordance with NIH Guide for the Care and Use of Laboratory Animals. The method in which primary Sertoli cell cultures were prepared from 3-week-old Sprague–Dawley rats by sequential enzymatic treatment have been used routinely in our laboratory as previously described [25]. Testes were aseptically removed, decapsulated, and washed twice, and tubules were carefully separated. The loosened tissues were transferred into 50 ml plastic tubes and sequentially digested in 0.25% trypsin in a rocking incubator (32 °C, 210 rpm, 30 min), followed by 0.1% collagenase I (34 °C, 150 rpm, 45 min). The homogenate was filtered through a 100-mesh stainless steel filter and the cell suspension was centrifuged at 200 × *g* for 5 min. Cells were washed twice in DMEM-F12 medium supplemented with 5% FBS. Isolated cells were plated on tissue culture dishes (10 mm in diameter, Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of $1.5 \times 10^6$ cells/ml in DMEM-F12 supplemented with 4 mM glutamine, 15 mM Hepes, 6 mM L-(1)-lactic hemicalcium salthydrate, 1 mM sodium pyruvate, antibiotics (final concentrations: penicillin, 100 IU/ml; streptomycin sulfate, 100 μg/ml) and 5% fetal bovine serum (FBS). Cells were maintained in a humidified atmosphere of 95% air/5% $CO_2$ (v/v) at 37 °C for 48 h. Sertoli cells attached to the bottom of dishes with only tiny dendrites protruding, but most of germ cells suspended in the medium and can be removed by changing the medium. Another 48 h later, the medium was changed again for second purification until Sertoli cells grow quickly to form a monolayer in new medium.

Cell purity was determined by immune fluorescence of Anti-FSHR antibody. After purification, cells were fixed with methanol for 5 min, washed three times in PBS. Then cells were incubated at 37 °C for 2 h with the primary anti-FSHR antibody. To block nonspecific binding, the primary antibody was diluted 1:100 with 1% BSA in PBS. Following incubation with the primary antibody, the cells were then incubated at 37 °C for 1 h with CY3-conjugated secondary antibody diluted in 1% BSA–PBS. Nuclear was stained with DAPI. The cells were washed and observed under the fluorescent microscopy (Nikon, Chiyoda-ku, Tokyo, Japan) with an appropriate barrier filter set.

### 2.3. Cell viability and toxicity assay

Cell viability assays were performed using CCK-8. The purified cells were digested with trypsin and plated in 96-well plates at $1 \times 10^4$ cells per well and cultured in the serum-free growth medium for 24 h. Then, Sertoli cells were exposed to vehicle (Control) or different concentrations of NP (N1 (0.1 μmol/L), N2 (1 μmol/L), N3 (10 μmol/L)), DBP (D1 (1 μmol/L), D2 (10 μmol/L), D3 (100 μmol/L)) or NP + DBP (N1 + D1, N2 + D2, N3 + D3). At the indicated time points, the cell numbers in sextuple wells were measured on an automated microplate reader (Bio-Rad, Japan) as the absorbance (*A*) (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Cell viability was calculated as follows:

$$\text{cell viability } (\%) = \frac{A(\text{treatment}) - A(\text{blank})}{A(\text{Control}) - A(\text{blank})} \times 100\%$$

### 2.4. Staining with FDA and PI for morphologic evaluation

A rapid, simultaneous double-staining procedure with fluorescein diacetate (FDA) and propidium iodide (PI) was used for morphologic evaluation [26]. This assay is based on the simultaneous determination of viable and dead cells with the detection of intracellular lipase activity by FDA and of plasma membrane integrity by PI, respectively. The purified Sertoli cells were transplanted into 96-well plates at $1 \times 10^4$ cells per well and exposed to vehicle (Control) or different concentrations of NP (N3 (10 μmol/L)), DBP (D3 (10 μmol/L)) or NP + DBP (N3 + D3) for 24 h, cells in tripartite wells were stained with 5 μg/ml PI and 4 μg/ml FDA for 5 min and observed under the fluorescent microscopy with an appropriate barrier filter set.

### 2.5. Flow cytometric assay

Annexin V-FITC/PI staining combined with flow cytometry was used to quantitatively determine the percentage of cells undergoing apoptosis and necrosis. The purified cells were digested with trypsin and plated in 6-well plates at $1 \times 10^6$ cells per well and treated with vehicle (Control) or NP (N3 (10 μmol/L)), DBP (D3 (10 μmol/L)) or NP + DBP (N3 + D3) for 48 h and then harvested. Then Sertoli cells in tripartite were stained with a saturating concentration of Annexin V-FITC and PI in Assay Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) at a concentration of $1 \times 10^6$ cells/ml in the dark for 20 min. Cells were pelleted and analyzed using a FACScan flow cytometer (Becton-Dickson, Immunocytometry System, San Jose, CA) immediately after staining.

### 2.6. In situ labeling of apoptotic cells

A DNA dye, Hoechst 33342 was used for examining nuclear morphology. The purified Sertoli cells were transplanted into 96-well plates at $1 \times 10^4$ cells per well and exposed to vehicle (Control) or NP (N3 (10 μmol/L)), DBP (D3 (10 μmol/L)) or NP + DBP (N3 + D3) for 48 h, cells in tripartite wells were stained with Hoechst 33342 according to the protocol of the kit. The result of staining was visualized under a fluorescent microscope that was excited at a wavelength of 350 nm and measured at 460 nm.