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Mutual promotion of apoptosis and autophagy in prepubertal rat testes induced by joint exposure of bisphenol A and nonylphenol☆

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ABSTRACT

BPA and NP are both typical endocrine disruptors, the exposed populations are widespread, and the health risks mustn't be ignored. However, the interactions between them on spermatogenesis are rarely mentioned. And the underlying mechanism is unclear yet. In the present study, prepubertal SD rats were exposed to different low doses of BPA and NP separately or jointly for 4 weeks. The results indicate that the joint exposure induced excessive apoptosis and autophagy in the testes, as proved by a series of characteristics such as chromatin condensation and autophagosomes formation. Besides, endocrine disorders and oxidative stress were also caused by the exposure. Apoptosis was mediated by the mitochondrial apoptosis pathway, since the Bax and Caspase-3 gene expressions significantly increased with a prominent decrease of Bcl-2. While autophagy was caused by the inhibition of the Akt/mTOR pathway, as the expressions of the downstream genes Beclin-1, Atg5, Atg12 and the split of LC3 protein increased altogether. Worse yet, autophagy and apoptosis might reinforce each other and make the situation more severe in the joint group. What's more, remarkable histopathological changes such as spermatogenic epithelium atrophy, germ cell loss, and various ultrastructural modifications were strongly related to the apoptosis and autophagy. In aggregate, this study shows the enormous risk on male reproductive system brought by the interactions between BPA and NP. The findings provide a broader vision to understand the roles of apoptosis and autophagy induced by the joint exposure in the aggravation of spermatogenesis impairment, which could be a reference for the situation of complex EDCs exposure-induced male reproductive toxicity, and possibly inspire us to find new ideas for preventive and therapeutic treatments.

1. Introduction

Bisphenol A (BPA) and nonylphenol (NP) are phenolic substances used in high volumes worldwide. BPA has primarily been employed in manufacturing polycarbonates and epoxy resins, due to the extensive uses of these two kinds of plastics, BPA lurks in numerous products (Ehrlich et al., 2014; FitzGerald and Wilks, 2014; Mendonca et al., 2014). It is estimated that a considerable portion of these products stand a chance of contacting our food directly (Liao and Kannan, 2014). Regarding NP, it's an intermediate product from the process of nonylphenol ethoxylate (NPEO) synthesis. In industry, NPEO is widely used to produce plastics, textile, and agricultural chemicals. In our house, it's commonly involved in various daily chemicals, such as detergents, paints, insecticides, and cosmetics. During the courses of production and usage, NPEO will finally migrate into water body and release NP (Guenther et al., 2002).

Because of the high production and widespread usage, BPA and NP are almost ubiquitous and coexist frequently in various environmental medium including our food and beverage

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(Gyllenhammar et al., 2012; Lee et al., 2015; Lu et al., 2015). Li et al. (2010, 2013) once detected the levels of BPA and NP in bottled water and tap water in Guangzhou, and the results showed that BPA and NP existed in 80.95% and 100% bottled water samples, with concentrations ranging from 17.6 to 324 ng/L and 108–298 ng/L, respectively. And the highest concentrations of BPA and NP were up to 317 and 1987 ng/L in tap water, respectively. It's estimated that if an adult drinks 2 L tap water every day, 148 ng BPA and 1410 ng NP would be ingested (Li et al., 2010). By the 2007 total diet study (TDS), Niu et al. (2015) reported that BPA and NP were detected in 50% and 99.31% of the 144 TDS samples, respectively. The highest concentrations of them reached up to 267 μg/kg and 1268 μg/kg, respectively. For children aged 0–6 years who take formula as staple food, the exposure dose of NP can be as high as 17 μg/kg bw/day.

On account of the general exposures, even if BPA and NP exist in different sources singly, substantial evidence has indicated that large crowds are co-contaminated. For example, Calafat et al. (2005) measured the urinary BPA and NP levels of 394 adults in the USA, BPA and NP were detected in 95% and 51% of the samples, respectively. After detecting the levels of BPA and NP in 287 urine samples from Guangzhou, Li et al. (2013) reported that without exception, the respondents of 3–24 years old were all contaminated by BPA and NP simultaneously.

Because of their high bioavailability, diverse endocrine-disrupting effects, and potential bioconcentration, BPA and NP have long since been defined as endocrine disrupting chemicals (EDCs) and of special focus over the years. Remarkably, both epidemiological data and laboratory studies have revealed their negative effects on male reproduction (Jin et al., 2014; Peretz et al., 2014). Our previous researches have also come to the same conclusion. Furthermore, we have demonstrated that either BPA or NP can exert male reproductive toxicity *in vivo* and *in vitro* by disturbing the normal regulations of the Akt/mTOR signaling pathway and inducing autophagy/apoptosis in rat testicular cells (Huang et al., 2016; Duan et al., 2017; Quan et al., 2017). However, although we explored the possible toxicology mechanisms, there are two main drawbacks in our previous studies. First, the dosages used in the studies were comparatively high (mg/kg bw/day), but a new risk assessment for the low dose effects of EDCs exposure is very pressing (Vom and Hughes, 2005). Second, considering the actual exposure state of the population, the interactions of BPA and NP on spermatogenesis mustn't be ignored. So we designed this study to explore the joint effects of BPA and NP on prepubertal rat testes, and pay focused attention on the changes of the Akt/mTOR signaling pathway after the exposure. We hope this study could help to uncover the mechanisms of the interactions between BPA and NP on spermatogenesis, and provide new insights into the prevention of the male reproductive hazards induced by BPA and NP.

2. Materials and methods

2.1. Chemicals

Bisphenol A (CAS No. 80-05-7, 99.5% purity) was acquired from Dr. Ehrenstorfer GmbH (Augsburg, Germany). NP (CAS No. 84852-15-3; mixture of isomers, 99% purity) was from ACROS Organics Co. (Geel, Belgium). All other chemical reagents were of analytical grade from formal manufacturers.

2.2. Animals and treatments

Seventy prepubertal male Sprague-Dawley rats aged 4 weeks (68–103 g) and standard rodent chow were purchased from the Experimental Animal Center of Huazhong University of Science and Technology (HUST, Wuhan, China). Animals were fed *ad libitum* and maintained in a holding facility with constant temperature (24 ± 2 °C) and humidity (55–60%), as well as a regular 12 h light/dark circle. The entire laboratory workflow was approved by the Ethics Committee of HUST, and all animal experiments were conformed to the guidelines of the National Health Commission of the People's Republic of China.

After a week of adjustment, animals were weighed and randomly divided into seven groups as follows: solvent control group (tocopherol-stripped corn oil, Sigma-Aldrich, St. Louis, MO), two BPA-treated groups (5 and 50 μg/kg bw/day), two NP-treated groups (15 and 150 μg/kg bw/day), and two joint exposure groups (Low dose mix (M1 group): 5 μg/kg BPA + 15 μg/kg NP, high dose mix (M2 group): 50 μg/kg BPA + 150 μg/kg NP). Namely, there were ten 5-week-old animals in each group at the start of the chemical exposure. All the doses were given by oral gavage, and the injection volume was 5 mL/kg body weight.

The World Health Organization (WHO) has estimated the mean dietary daily intake of BPA for adults to be 0.4–1.4 μg/kg bw, with a worst case scenario of 4.2 μg/kg bw (FAO/WHO, 2011). Nielsen et al. (1999) have proposed a tolerable daily intake (TDI) of 5 μg/kg bw for NP, but for children aged 0–6 years, the actual intake could be much higher (17 μg/kg bw/day) (Niu et al., 2015). Considering the no observable adverse effect level (NOAEL, both 50 mg/kg bw/day) (Tan et al., 2003), the dosages used in the previous studies (Jubendradass et al., 2012), and possible exposures from other sources together, the dosages in this study were determined.

Rats were euthanized after the 4 weeks treatment, their serums were extracted to detect the levels of sex hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T); the epididymides were weighed and collected for hematoxylin-eosin (HE) staining; the right testes were collected for oxidative stress indexes detection, histopathological examination, and transmission electron microscopy (TEM) examination. The left testes were frozen in liquid nitrogen instantaneously, and then stored in an ultra-low temperature freezer for Western blotting and RT-PCR analysis.

2.3. HE staining

Paraformaldehyde fixed testes and epididymides were embedded in paraffin blocks and cut into slices of 4 μm thickness. The sections were stained with hematoxylin and eosin after deparaffinization and rehydration, then mounted with neutral balsam and examined under a light microscopy. Three rats were randomly selected from each group for the examination.

2.4. Immunohistochemical (IHC) and immunofluorescent (IF) staining

After deparaffinization and rehydration, testicular sections were incubated in 3% H_2O_2 for 10 min to block the endogenous peroxidase activity, then applied with p-AMPKα rabbit mAb (Thr172) (CST, Beverly, MA) at 4 °C for 12 h. The negative control was incubated with PBS instead of p-AMPKα antibody. After an incubation of peroxidase-conjugated secondary antibody (Santa Cruz, CA) for 60 min, the sections were washed with PBS, and then stained with diaminobenzidine (DAB) and hematoxylin. P-Akt rabbit mAb (Ser473), p-mTOR rabbit mAb (Ser2448), and LC3A/B rabbit mAb (CST) were employed as primary antibodies for IF assay, and the secondary antibody was Cy3-conjugated (KPL, Gaithersburg, MD). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Nonspecific IgG antibody was used as first antibody in negative control. Three sections were analyzed for each