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Effects of nonylphenol administration on serum, liver and testis estrogen metabolism

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HIGHLIGHTS

- NP exposure exhibited general toxicity in rats. High-dose exposure induced the weigh, weigh gain, food unity and organ coefficient decreasing.
- On the way of estrogen resynthesis and decomposition, NP changes leads to the changes of hormones and enzymes both in the liver,serum and testis in rat
- In receptor binding pathway, NP can affect the transcription level of AR, ERα, ERβ genes in testis, then finally changing the free E2 and T levels.

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ABSTRACT

Purpose: Nonylphenol (NP) is one widely distributed representative of environmental estrogens that disturb reproductive activities, bone metabolism and brain function through interfering diverse signal pathways leading to hormone metabolic dysfunctions, immunologic derangement, and tumorigenesis. Few of previous studies have observed the subacute toxicity on rodents, and little has been focused on the mechanism underneath the toxicities observed.

Methods: The 32 male Sprague-Dawley (SD) rats were randomly divided into four groups, the negative control group (corn oil) NP low, medium and high dose groups [30, 90, 270 mg/(kg·d)]. SD rats administrated with different dosage of NP every other day for 28d. Elisa and RT-PCR was employed to observe estrogen metabolism markers or mRNA expressions.

Results: In serum, NP exposure caused testosterone (T) ($p < 0.001$), progesterone (PROG) ($p < 0.05$) and estrone (E1) ($p < 0.05$) increased. In testicle, NP exposure caused T ($p < 0.001$), PROG ($p < 0.05$), E1 ($p < 0.05$), 17β-estradiol (E2) ($p < 0.05$) and ERα mRNA ($p < 0.01$) increased, while P450 aromatizing enzyme ($p < 0.001$) decreased in NPL and ERβ mRNA ($p < 0.001$) decreased in NPM and NPH. In liver, NP exposure caused 17β-HSD2 mRNA ($p < 0.01$) increased, while P450 aromatizing enzyme decreased ($p < 0.05$).

Conclusion: NP exposure exhibited general and estrogenic toxicity in rats through disturbing estrogen secretion network and estrogen receptor expression network, inducing abnormal metabolism of estrogen, whether in serum, liver and testicle.

1. Introduction

Environmental estrogens (EEs) are compounds with estrogen-analogue characters that mimic the actions of female hormone, thus disturb regulating behaviors such as reproductive activities, bone metabolism and brain function through interfering diverse signal pathways, thus lead to hormone metabolic dysfunctions, immunologic derangement, and tumorigenesis in diverse species

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including human sapien (Lopez-Espinosa et al., 2009; Bhandari et al., 2015; Yu et al., 2017).

Alkylphenol compounds (APs), a diverse and abundant group of synthetic EEs compounds, are widely presented in global environment as results of permissive industrial discharge, typically as sewage sludge effluents from sewage treatment works, river water and sediments, soil and ground water (Soares et al., 2008). Among APs, NP was used extensively since first synthesized in 1940. Besides its wide use industrially, NP is also a toxic xenobiotic compound and typical representatives of APs, capable of interfering with the hormonal system in various organisms (Miles-Richardson et al., 1999; Puy-Azurmendi et al., 2014). According to Isobe et al., NP is widely distributed in the river sediments in rivers of Europe, America and Japan, profile of which can be traced back to the 1970s, indicating the refractory of degradation of NP (Isobe et al., 2001). Lopez-Espinosa and colleagues presented that NP residues were 100% detected in 20 adipose samples from individuals live in Southern Spain, reflecting the severity of NP's accumulative toxicity (Lopez-Espinosa et al., 2009).

Evidence both *in vitro* and *in vivo* has revealed the acute toxicity and genotoxicity of NP or its analogues. The initial controversial emerged in the 1980s, when Giger and colleagues found that NP presented even stronger toxicity to aquatic life forms than its precursors (Giger et al., 1984). Shortly after that in 1991, Soto and co-workers reported that NP could induce breast cancer cell proliferation. Lee and Lee concluded that NP exert its toxicity as a competitive inhibitor with E2, a natural hormone who functions by bind to receptor of natural estrogen, thus disrupt the inner homergy (Lee et al., 1996). Subsequently, more researches have been carried out to study further toxic effects of NP. In 2000, 2004, Laws et al. and Chitra et al. reported NP could damage sperm activity through reactive oxygen species (ROS) accumulation, or induce an uterotrophic response in prepubertal individuals as compared with ovariectomized adult individuals separately, both in rat models (Laws et al., 2000; Chitra et al., 2002). In 2006, Angelique and colleagues observed that NP altered hormone and estrogen receptor expression in Juvenile Rainbow trout model (Vetillard and Bailhache, 2006). In an investigation of several environment contaminants of human milk conducted in Italian women, calculated content of NP based on the sampled NP concentration is close to the tolerate daily intake, proposed by the Danish institute of safety and toxicology (Ademollo et al., 2008). It is well illustrated that NP pollution is in a desperate status and more moves await to be done.

Estrogen metabolism is a multi-path complex metabolic process mediated by a variety of key enzymes. Cholesterol is a direct ingredient in the synthesis of estrogen. PROG is a precursor of estrogen, which involves the synthesis and catabolic pathways shown in Fig. 1. The PROG of male animal testicular secretion, has a certain role in determination of the male reproductive function.

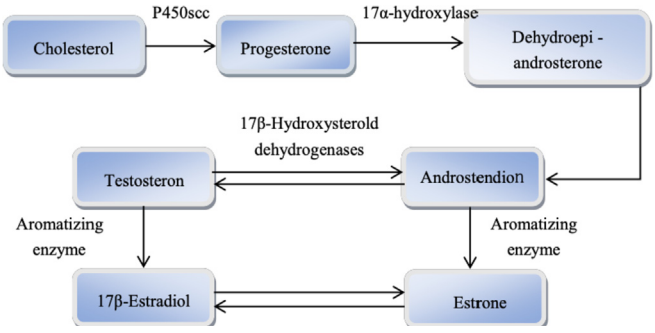

Fig. 1. The process of estrogen metabolism.

Androstenedione (ASD) is a direct precursor of T in the synthesis pathway of male hormones, and is also a precursor of E1 in the estrogen synthesis pathway. When T gets into the Sertoli cells, it converted to E2 under the action of aromatase (aromatizing enzyme, P450arom). The 17β-hydroxysteroid dehydrogenases (17β-HSD) is the last enzymes in the synthesis of sex hormones steps, and its role is to regulate the partial level of sex hormones. Like other steroid hormones, sex hormones must be mediated by the corresponding estrogen receptor (ER) and androgen receptor (AR).

However, to our knowledge, few of previous studies have observed the chronic toxicity on rodents, and little has been focused on the mechanism underneath the toxicities observed. According to Knaak et al. and Müller et al., estrogen analogues such as NP will be metabolized rapidly and thoroughly in organisms, in the meanwhile cause severe disturbance on estrogen-involved pathways elaborately (Knaak et al., 1966; Müller et al., 1998). How exactly did these metabolic dysfunctions progress remains unclear.

In our study, we hypothesized that the estrogen metabolism accompanied with violent biological disturbance might be attributed to the binding reaction between NP and various estrogen receptors (ERs). Our specific observational objectives are estrogen metabolite levels and estrogen metabolic mRNA expression levels.

2. Materials and methods

2.1. Animal grouping and treatment

All experiments were approved by the Animal Care Committee of the laboratory Animal Center of South China Agricultural University, Guangzhou. Thirty-two SPF male SD rats, weighing 170–210 g, were provided by the Guangdong Medical Laboratory Animal Center, and were housed under controlled conditions (12 h light/dark cycle, 22 ± 0.5 °C and 50–60% relative humidity). Rats were fed with a rodent chow and had tap water ad libitum. NP,4-*n*-nonylphenol, was purchased from Sigma. After 7d of adaptive feeding, the rats were randomly divided into four groups, each group consists of eight animals. The experiment was grouped as follows: repeated exposure to NP in low dosage [NP low-dose group, NPL, 30 mg/(kg·d)], middle dosage [NP medium-dose group, NPM, 90 mg/(kg·d)], and high dosage [NP high-dose group, NPH, 270 mg/(kg·d)], the solvent control group (control group, C). All animals were administered NP orally by gavage at 9:00 a.m. every other day. Rats in the control group were only given an equal volume of corn oil. The gavage volume was 1 mL. The utilization rate of food refers to the weigh grams gain that per 100 g feed consumption. Food consumption of animals was recorded every 4d during the experiment. On this basis, a formula of food utilization had been given. Food utilization rate = (weight gain)/[Food consumption during a stage (4d)] x 100.

Twelve hours after the last drug administration, the animals were weighed and blood was collected from the orbit, then sacrificed by cervical dislocation. Blood samples were collected and centrifuged at 3000 rpm at 4 °C for 15 min to obtain serum. Livers, testicles and serums were fully excised from the animal, all organs were washed in normal saline solution, sucked dry with normal filters, and then weighted. Organ coefficient is expressed as the ratio of organ weight (g) to body weight (g) (Huang et al., 2003).Precisely 1g liver or testicles was taken, rinsed with cold PBS, pH 7.4, to remove blood, dried with a paper wipe, placed in a 10-mL centrifuge tube, and then the tissue homogenate was centrifuged at 3000 rpm at 4 °C for 15 min. The supernatant was placed in a 10 mL centrifuge tube (namely 10% liver or testicles homogenate), immediately frozen in liquid nitrogen and stored

at −80 °C until use.

2.2. Determination of estrogen metabolites by enzyme linked immunosorbent assay (ELISA)

The levels of estrogen metabolites in the serum, liver and testicles were analyzed using relevant ELISA kits strictly according to packaging inserts. Commercial ELISA kits were provided by Jianglai biotechnology (Shanghai, China).

2.3. Analysis of estrogen metabolite gene expression by RT-PCR

The levels of estrogen metabolite mRNA were determined by real-time quantitative PCR. TRIzol reagent kit (Vazyme Biotech Co., Ltd, Nanjing, China) was used to extract total RNA. Total RNA purity was determined by the OD260/OD280 ratio that was measured with a k2800 trace spectrophotometer (Beijing koko, Beijing, China). The reaction mix (60 mL RNA, 20 mL DNase, 20 mL 10 × buffer) was digested for 30 min at 37 °C and inactivated for 10 min at 65 °C. An equal volume of phenol was added to extract the RNA, followed by centrifugation for 5 min at 9391 g, then the supernatant was taken. Next, an equal volume of chloroform was added (5 min at 9391 g), followed by isopropyl ketone (5 min at 12,000 g). The precipitated RNA was washed with 1 mL of 75% ethanol (12,000 g for 5 min), which was then discarded. The precipitate was dried and dissolved in 60 mL of DEPC water. The template RNA, primer mixture (12 mL), containing 1.5 mg of RNA, 0.5 mL of Olig (dT), 0.5 mL of AVP-RT primer and 0.5 mL of random primer, was incubated at 70 °C for 10 min, and then rapidly cooled in ice for 2 min. Next, the reverse reaction solution [20 mL containing the 12 mL Template RNA, primer mixture, 0.5 mL of 10 mM dNTP Mixture, 0.25 mL of RNase inhibitor (40 U), 4 mL of 5 × M-MLV buffer, and 0.5 mL of RTaseMMLV (RNase H-: 200 U/μl)] was placed in PCR tubes. The reaction solution was incubated at 42 °C for 60 min, followed by 72 °C for 15 min, and then stored at −80 °C until use. The PCR reaction volume was 20 mL, containing 2 mL of cDNA, 0.25 mL of upstream primer, 0.25 mL of downstream primer (Table 1), and 10 mL of SYBR ÒPremix Ex TaqTM (Tli RNaseH Plus). The 2 × PCR reaction procedure was: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Melting curve was the final step, which consisted of a temperature increase from 60 °C to 95 °C, rising by 0.5 °C increments in a span of 15 s. Relative expression levels were calculated using the 2 × DDCt method. In the present study, rRNA 18S was used as the housekeeping gene.

Primer sequence:

AR: TAAATTCATTGGGCTTGG/GAGCGAGCGGAAAGTTGTAG.

P450arom: CAAAGGGTTGGAAGGTGAGA/GGTTCAGCATCCAATAAGG.

ERα: GGTTCAGCATCCAATAAGG/GGCACTTCAGGAGACAT.

ERβ: ATCATCGCTCCTCTATGC/GCTTCCTCTTCAGTGTCT.

Table 1
Food utilization rate (%) (Mean ± S.D.) (n = 8).

Stage	Control	NPL	NPM	NPH
S1	33.84 ± 5.13	37.98 ± 3.70	36.26 ± 2.83	36.13 ± 6.95
S2	36.83 ± 7.94	36.49 ± 3.76	37.62 ± 4.63	36.53 ± 4.40
S3	35.64 ± 4.50	35.16 ± 4.36	36.81 ± 2.35	32.52 ± 4.03
S4	34.4107 ± 4.97	33.04 ± 2.93	32.19 ± 4.31	27.82 ± 9.99[a]
S5	25.18 ± 9.28	30.85 ± 4.30	29.77 ± 2.07	28.20 ± 5.00
S6	27.02 ± 10.95	23.93 ± 1.57	23.75 ± 4.71	22.90 ± 3.32
S7	24.17 ± 5.32	19.83 ± 2.26[a]	21.42 ± 3.62	18.99 ± 5.32[a]

NPL: NP low-dose group; NPM, NP middle-dose group; NPH, NP high-dose group.
S = Stage, every 4d is a stage, it includes 7 stages.
Food utilization rate = (weight gain)/[Food consumption during a stage (4d)] x 100.
[a] P < 0.05, compared with control group.

17β-HSD1: GGTTATGAGCAAGCCCTGAG/GGAAGCGGTTTGTGGAGAA.

17β-HSD2: CCTTTCGGTGCTCCAGAT/CCACAGCCCACAGTCCTT.

17β-HSD3: TCTTCTACAGCAGCACCA/CAACCCTAACTCTACCCAC.

β-actin: AACCCTAAGGCCAACCGTGAAAAG/CGACCAGAGGCATACAGGGACAAC (Forward/reverse).

3. Results

3.1. General toxicity of NP

No fatalities have occurred throughout experiment. Rats in NP administration group showed reduced quantity of activities and food intake, increased shedding, greater excitement and anxiety than rats of the control group.

According to our observation, though average weight increased consistently in each group, rats in NPH showed slowdown in weight gaining after the third weeks. As shown in Fig. 1, in the end of NP administration, average weight of NPH group was largely lower than that of control group ($P < 0.01$). No significant differences have been observed between C and NPL, NPM groups.

In accordance with comparison of weight gaining, reduced coefficient of food utilization has been observed in NPH since period 4 ($P < 0.05$). According to organ coefficient, it usually reflects the condition of animal organs affected for a long time. As shown in Fig. 2, there are no obvious changes in kidney and testicle coefficient. But in liver coefficient, NP administration led to large alteration on rat organ coefficients of liver, it has significant declined compared with controlled group.

As shown in Table 1, food utilization had a marked decline in NPL and NPH groups compared with controlled group in stage 7.

3.2. Toxic effects of NP on estrogen metabolites expression

ASD and T were then measured as part of estrogen metabolic network. As shown in Fig. 3A, ASD levels had no significant changes in serum group. Levels of testicle ASD in the exposure group had slight increases with the increase of NP dose. Fig. 3B indicated that T was dramatically increased in both serum and testicle in the NPM and NPL dose groups. But different from ASD, the peak production of T appeared in NPL group, both in serum and testicle.

As shown in Fig. 3C, E1 was increased in both serum and testicles dose-dependently. The serum E1 level in the NPH group and testicle E1 level in the NPM and NPH groups were significantly lower compared with control groups. Fig. 3D showed that first increase and then decrease in E2 production of serum and testicle, while the significant increase of E2 production was in NPM group of testicle.

As shown in Fig. 3E, the serum or testicle PROG concentration were both increased with the presence of various concentration of NP compared with control group. In serum, the peak PROG appeared in the high dose group. However, in testicles, the highest PROG concentration appeared in rats of low dose group.

3.3. Toxic effects of NP on mRNA expression

To make further observations, we performed tests on mRNA expression using RT-PCR. Since liver is one of the most critical organs where estrogen was decomposed, the homogenate of liver was used in the following tests. The level of critical enzymes throughout estrogen metabolism were measured. As shown in Fig. 4, the expression of P450arom mRNA were significantly decreased in NPL, NPM and NPH in a dose-dependent mode. 17β-HSD1 and 2 (Fig. 5) mRNA were measured but only 17-β-HSD2 was significantly increased by NP dose-dependently. Testicle is another

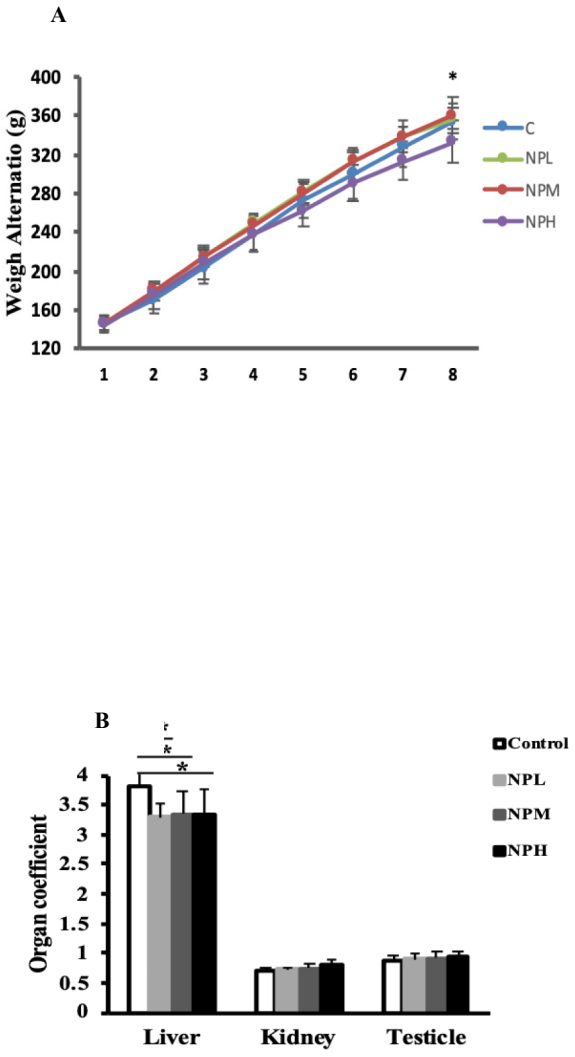

Fig. 2. Toxic effects of NP on weight alternation (A) and organ coefficient (B). NPL: NP low-dose group; NPM, NP middle-dose group; NPH, NP high-dose group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group. *: NPH group compared with control group); n = 8, every 4d is a stage, it includes 7 stages.

key organ where estrogen was metabolized. 17β-HSD3 (Fig.6) and P450arom mRNA were measured using testicle homogenate. As shown in Fig. 4, P450arom mRNA was significantly decreased in NPL group. ERα, ERβ and AR mRNA expression in testicle were detected as receptors. As shown in Fig. 6, ERα mRNA was increased in a dose-dependent behavior, while ERβ mRNA was decreased dose-dependently by NPM and NPH. In the meanwhile, we observed no alternation on the expression of AR mRNA in the presence of each concentration of NP.

4. Discussion

NP is a typical representative APs of industrial discharge and environment estrogens, the perniciousness of which have been stated elaborately. In this study, quaque die alterna (QD ALT) administration of NP in a total period of 28d caused severe toxic events in rats. A high dose of NP administration [270 mg/(kg·d)] induced irritability. Kimura et al. and Hao et al. have reported that NP induced the decrease of weight gaining in rodents from embryonic period to early adulthood and murine adipocyte differentiation (Kimura et al., 2006; Hao et al., 2012), however the toxic effects of NP on mature rodents eating habits and weight-gaining habits are not in agreement. As was reported by Aly et al. that 100–300 mg/(kg·d) in a total 30d administration led to no weight gain differences while administrated NP was lower than 300 mg on a model of Wistar rats (Aly et al., 2012), our results showed accordance with them. Our results showed that NP administration may cause reduction in coefficient of food utility in rodent model, the failure of food utilization may lead to reduced weight gaining.

Coefficient of organs alternation indicated that pathologic change happened in a histomorphologic level. In our study, coefficient of liver decreased in each NP groups, which may be attributed to atrophy or degenerative progress of liver. While kidney coefficient increased, which indicated that NP has chronic toxicity on kidney (Han et al., 2004). Coefficient of testicle have no significant changes compared with control group, this may be due to the low dosage, short feeding cycle and the fact that the effects on organs have not been reflected in their weight changes. In a study performed in Zoarces viviparus NP was proved to induce testicular abnormality through disturbance on E2 metabolism (Christiansen et al., 1998). As the vital organ where sex hormone metabolized in male objects, we assumed that the morphologic changes may trigger catastrophic alternation in estrogen metabolism.

Aside from general toxicity, we focused our observations on how NP displayed toxicity on estrogen metabolism on both expressional and metabolic level. To our knowledge, there's no comprehensive report on effect of dietary administration of NP on serums, hepatic or testicular estrogen metabolism in adult rat model.

PROG serves as a precursor of estrogen. Through interaction with dehydroepiandrosterone, 17β-HSD1/2 and P450arom, intermediate products such as ASD and T, or resultant such as E1 and E2 were catalyzed to presence. NP exposure induced increase of serum and testicular PROG/T concentration, serum and testicular E1 concentration, while the counterparts of E2 were decreased. Testicular P450arom mRNA expression was influenced in this study. The liver serves as another key organ where estrogen metabolism take place, and hepatic expression of 17β-HSD2 mRNA was dramatically enhanced, testicular expression of 17β-HSD3 mRNA was down-regulated, thus inhibiting E1 transforming to E2. We observed low-grade T secretion in various locations. According to Nagao et al., this may be attributed to mesenchymal cell lysis during NP exposure (Nagao et al., 2001). In their carp model, Lavado and colleagues observed a decrease in ovarian P450 activity (Lavado et al., 2004), which consistent with our result. This might suggest that NP may down-regulate E2 level by promoting P450 aromatase expression and ultimately achieve estrogenic effect.

On the other hand, receptor expression is another key step during estrogen metabolism. There are three subtypes of estrogen receptor, AR, ERα and ERβ, mainly. In our study, AR expression was non-affected, ERα mRNA expression was enhanced progressively in accompany with the increase of NPM and NPH concentration, while ERβ mRNA expression experience decrement. Free T in serum and testicle was both increased by NP, however this increasing trend encountered dilution with higher concentration of NP exposure. The reason for this paradox might be that low NP exposure induced T secreted excessively, while with concentration increasing, NP and over-produced T, which is the natural ligand of AR, competitively bound with its counterpart, up-regulated E2 production, thus lead to ER re-regulation, ERβ mRNA expression increased, while ERα mRNA expression decreased in low exposure environment. To the contrary, ERβ mRNA expression decreased, while ERα mRNA expression increased in high exposure environment.

Several studies reported NP induced ER mRNA up or down-regulation in liver and other organs (Yadetie et al., 1999; Sakazaki

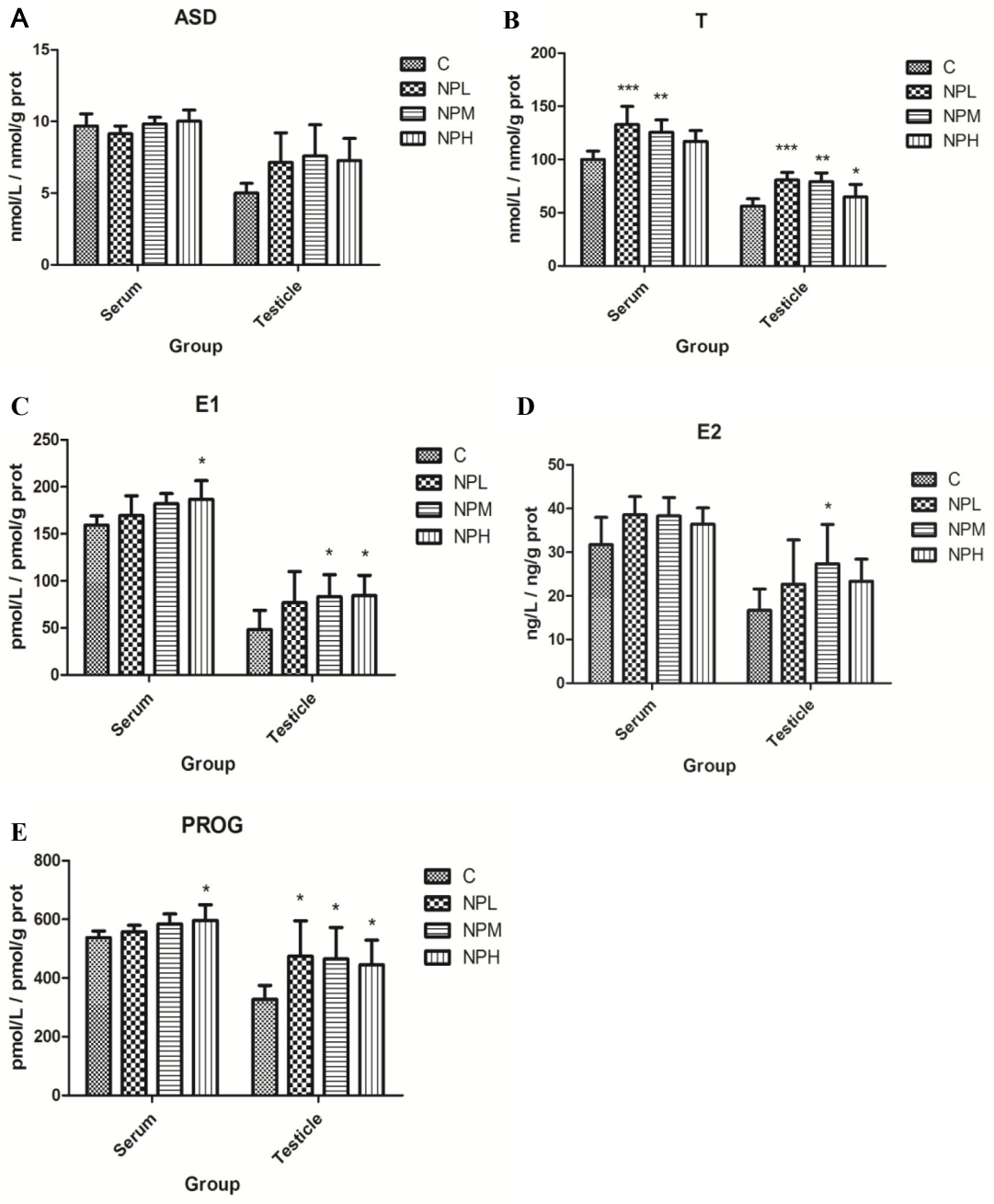

Fig. 3. Toxic effects of NP on ASD(A), T (B), E1 (C), E2(D), PROG (E) in serum and testicle were measured by ELISA. NPL: NP low-dose group; NPM, NP middle-dose group; NPH, NP high-dose group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different compared with control group.

et al., 2002). *In vitro* study also showed that NP could bind to ER, according to Scippo and co-workers (Scippo et al., 2004). As the most active estrogen, E2 was exhibit activity by binding to ER. NP could bind to ER competitively as well as bind to sex hormone-binding globulin (SHBG), substitute endogenous estrogen from SHBG, thus lead to increase of serum T and E2.

Endogenous steroid hormones were synthesized by lipase cytochrome P450 from cholesterol, specific isozymes catalyzed PROG to T, and finally E2 was generated from T by P450arom. The classical way of estrogen to produce effects can be concluded as such: estrogen form into ligand-receptor complex by combining with hormone-binding protein, while inducing dissociation between receptor and its chaperonin, dimerization between receptor and the dimer translocating into nucleus to bind with the corresponding responsive element, leading down-stream cascade reaction and ultimate physiological or pathological effects. At our observations, NP produced toxic effects through multilevel disturbance, including expressional level, levels of receptor and biochemical level.

In summary, various concentration of NP exposure exhibited

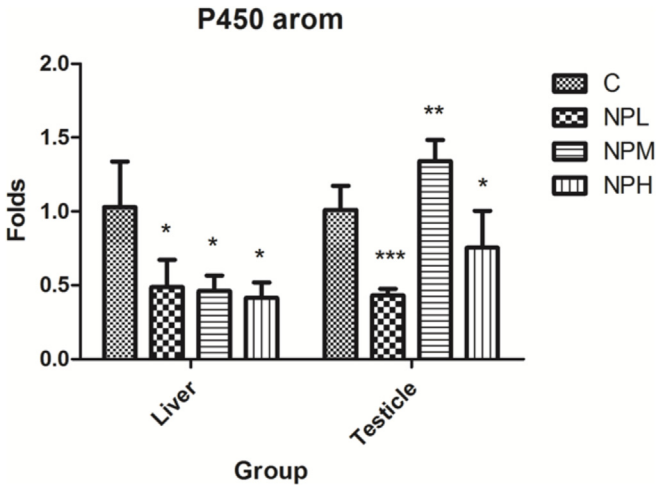

Fig. 4. Toxic effects of NP on testicular and liver estrogenic mRNA expression. Total RNA was prepared and levels of mRNA encoding P450arom. NPL: NP low-dose group; NPM, NP middle-dose group; NPH, NP high-dose group. *p < 0.05, **p < 0.01 and ***p < 0.001, significantly different compared with control group.

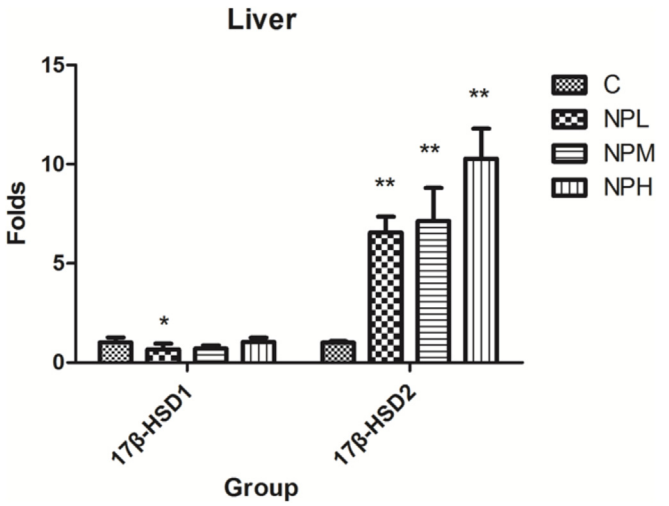

Fig. 5. Toxic effects of NP on liver estrogenic mRNA expression. Total RNA was prepared and levels of mRNA encoding 17β-HSD1 and 17β-HSD2 in liver were measured by RT-PCR. β-actin was used as an internal control. NPL: NP low-dose group; NPM, NP middle-dose group; NPH, NP high-dose group. *p < 0.05, **p < 0.01, significantly different compared with control group.

general and estrogenic toxicity in rats through disturbing estrogen secretion network and estrogen receptor expression network, inducing abnormal metabolism of estrogen, whether in serum, liver and testicle.

4.1. Statistical analysis

The SPSS (version 18.0) was employed for statistical analysis. One-way variance analysis was used for comparison of results among different groups. If the variances were homogeneous, the least significant difference (LSD) test was used. If not, the Games-Howell test was applied. Differences were considered to be statistically significant if $P < 0.05$.

Conflicts of interest

There are no conflicts to declare.

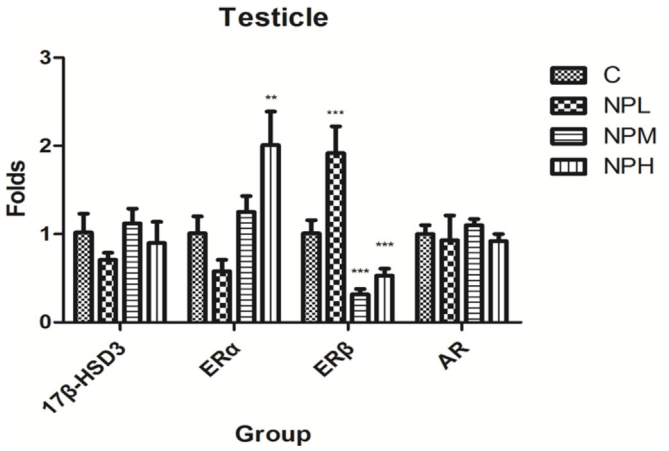

Fig. 6. Toxic effects of NP on expression of 17β-HSD3 and estrogenic receptor in testicle were measured by RT-PCR. β-actin was used as an internal control. Total RNA was prepared and levels of mRNA encoding AR, ERα and ERβ in testicle were measured by RT-PCR. β-actin was used as an internal control. NPL: NP low-dose group; NPM, NP middle-dose group; NPH, NP high-dose group. **p < 0.01, ***p < 0.001, significantly different compared with control group.

Author contributions

Conceptualization, Chunhong, Liu.; methodology, Tongwang, Yang.; software, Shaowen, Huang.; validation, Youting, Guo; formal analysis, Qingyi, Huang.; investigation, Lingling, Bu.; resources, Jie, Yang.; data curation, Tongwang, yang; writing—original draft preparation, Qingyi, Huang.; writing—review and editing, Lingling, Bu; visualization, Qingyi, Huang and Lingling, Bu.; supervision, Chunhong, Liu. and Yichao, Yang; project administration, Chunhong, Liu.; funding acquisition, Chunhong, Liu.

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