Toxicology in Vitro 26 (2012) 1114–1121

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Comparative evaluation of nonylphenol isomers on steroidogenesis of rat Leydig Cells

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ARTICLE INFO

Article history:
Received 6 March 2012
Accepted 28 June 2012
Available online 5 July 2012

Keywords:
Nonylphenol isomers
Leydig cell
Estrogenic potency
Steroidogenesis

ABSTRACT

Nonylphenol (NP) has been proven to be one of the most investigated xenohormones interacting with the estrogen receptor. Technical nonylphenol (t-NP) contains at least 20 para-substituted isomers. It has been shown that NP isomers vary in their estrogenic potency. So the use of mixtures or impure substances can lead to misinterpretations and unsatisfying conclusions. In the present study, experiments were performed to examine effects of NP isomers on steroidogenesis of rat Leydig cells. Primary cultured Leydig cells were exposed to NP isomers (*p*33-NP, *p*262-NP, *p*353-NP, *p*363-NP) at the optimized inhibitory concentration 5 μmol/L for 6 h. NP isomers showed various degrees of inhibition of testosterone biosynthesis, with *p*363-NP leading to the most significant decrease and others sharing the similar efficacy. The expression of *3b-HSD*, *Cyp11a1*, *Star* and the apoptosis of Leydig cells were further measured to investigate the underlying mechanisms. We demonstrated that NP isomers can affect the steroidogenesis of rat Leydig cells, at least in part, through their influence on gene expression and cell apoptosis, but varied in their individual degree. However, the final results were not completely coincident with their estrogenic potency tested *in vitro*, which implies that effects of NP isomers on steroidogenesis appear to be mediated through some other underlying mechanisms besides their various estrogenic potency.

1. Introduction

Nonylphenol (NP) is the biodegradation product of nonylphenol polyethoxylate (NPE), which has been proven to be one of the environmental endocrine disrupting chemicals(EDCs), binding to the estrogen receptor (ER) and mimicking estrogenic action *in vivo* and *in vitro* (Han et al., 2004; Hossaini et al., 2001; Kwack et al., 2002; Nimrod and Benson, 1996). Due to its wide usage, a large amount of nonylphenol was discharged into ecosystem, especially into drinking water, in which the concentration of NP was measured up to 55.3 μg/L (Berryman et al., 2004). Moreover, Vazquez-Duhalt et al. (2005) has summarized that several endocrine alterations can be induced after exposure to NP at concentration as low as 0.1 μg/L, which evokes a public health risk. Sequential studies have reported adverse effects of NP exposed perinatally or directly to adults on the male reproductive system, including reduced reproductive organ weights, decreased sperm production and testicular abnormalities (Chapin et al., 1999; Hossaini et al., 2001; Lee et al., 1999).

Since these endpoints are sensitive to hormone levels, particularly the level of testosterone, which is responsible for the maintenance of spermatogenesis and secondary sexual characteristics in the male, several studies (Gong and Xiaodong, 2006; Murono et al., 1999; Wu et al., 2010) have expounded biphasic effects of nonylphenol and octylphenol (OP) on steroidogenesis of rat Leydig cells with an increase at low concentrations and a decrease at high concentrations. The mechanism by which NP modulates steroidogenesis has not been well-defined, but it can be partially explained by the activation or inhibition of the enzymes required for the biosynthesis of testosterone in Leydig cells, including CYP11A1, 3β-HSD, and 17β-HSD (Laurenzana et al., 2002; Payne and Sha,

Abbreviations: NP, Nonylphenol; t-NP, Technical nonylphenol; *p*33-NP, 4-(3′-methyl-3′-octyl)phenol; *p*262-NP, 4-(2′,6′-dimethyl-2′-heptyl)phenol; *p*353-NP, 4-(3′,5′-dimethyl-3′-heptyl)phenol; *p*363-NP, 4-(3′,6′-dimethyl-3′-heptyl)phenol; OP, Octylphenol; hCG, LH/human chorionic gonadotrophin; 3β-HSD, 3β-hydroxysteroid-dehydrogenase.

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http://dx.doi.org/10.1016/j.tiv.2012.06.016

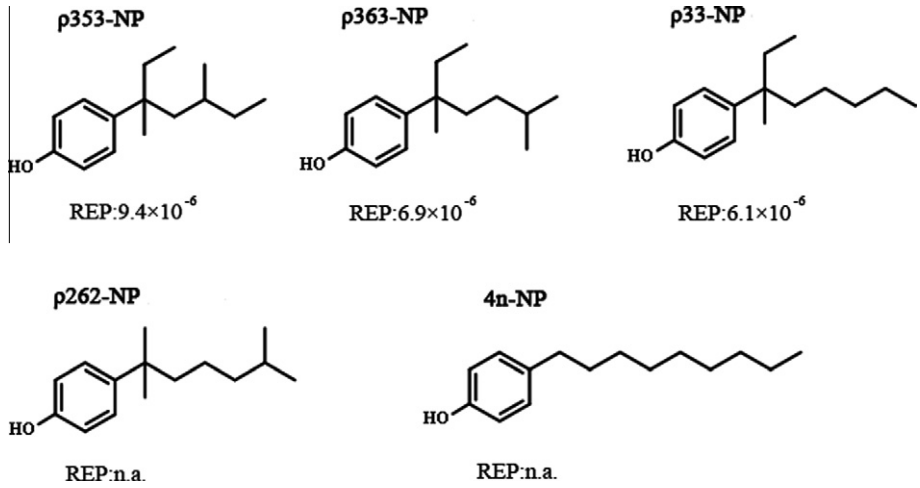

Fig. 1. The structures of NP isomers and their relative estrogenic potency (REP). NP, nonylphenol; REP, the relative estrogenic potency of NP isomers calculated by the EC20 for the MVLN assay (transformed MCF-7 human breast cancer cell line) and the EC50 for the E-screen (proliferation test using MCF-7 cells); n.a, no estrogenic potency (Preuss et al., 2006).

1991). Majdic et al. (1996)) have shown that mRNA levels, protein, and activity of cytochrome P450 17alpha-hydroxylase/C17-20-lyase (P450c17) were reduced in testes from neonatal rats treated with OP during gestation. Meanwhile, Han et al. (2004) also illustrated that nonylphenol exposure resulted in testicular degeneration and increased cell apoptosis in a dose-dependent manner.

Actually, the main source of nonylphenol in the natural environment is technical nonylphenol (t-NP)that contains at least 20 para-substituted isomers (Kim et al., 2004; Thiele et al., 2004; Vinken et al., 2005). Considering the limitation in separating and quantifying all isomers, previous studies are usually performed using the t-NP mixture or the linear form 4n-NP, which is not present in the technical mixture. The structures of NP isomers (Fig. 1) resemble the structure of natural hormone 17β-estradiol (E2), resulting in mimicking or blocking endogenous hormones with agonistic or antagonistic effects at estrogen receptors (Preuss et al., 2006, 2010).

Nowadays, NP isomers have been shown to vary in their estrogenic potency *in vitro* (Gabriel et al., 2008; Preuss et al., 2006; Shioji et al., 2006). Previous studies suggested that estrogenic potency of alkylphenols is linked to a tertiary branched α-carbon and the length of the side chain at that position. In the MVLN assay, in which expression of the exogenous reporter gene luciferase under control of the estrogen response element (ERE) is used as a sensitive measure of potency, it was reported that p353-NP exhibited the same relative potency as the t-NP mixture, whereas p262-NP, p22-NP and 4n-NP caused no measurable luciferase activity (Preuss et al., 2006). The same potency pattern was shown by the E-screen assay (Preuss et al., 2006) and the YES-assay (Gabriel et al., 2008), but surprisingly, in these two assays, p262-NP, p22-NP and 4n-NP also exhibited a clear estrogenic response. Although no unified conclusion has been reached, we can state that different NP isomers vary in their estrogenic potency.

The differences in the estrogenic potency of the isomers may be due to differences in receptor affinity, receptor activation/deactivation or some non-receptor mediated pathways. It is worth noting that the use of mixtures or impure substances can also lead to misinterpretations and unsatisfying conclusions, which strongly favors that synthetic isomers are preferred to be chosen. In 2007, Lalah et al. first used a synthetic NP isomer, a branched *p*-nonylphenol isomer p363-NP, on *Lymnae stagnalis* to study its effects on embryogenesis (Lalah et al., 2007). However, presently there seem to be few reports using synthetic isomers in a mammalian model.

Since NP isomers vary in their estrogenic potency, we hypothesized that the responses to administration of different NP isomers in a mammalian model would be diverse. In the present study, we investigated effects of NP isomers on steroidogenesis of rat Leydig cells to evaluate their environment risk and discussed possible mechanisms underlying the differences in their reproductive toxic effects.

2. Materials and methods

2.1. Chemicals and reagents

The technical nonylphenol (t-NP) was purchased from Sigma (Shanghai, China). The linear isomer (4n-NP) was purchased from Alfa Aesar (Shanghai, China) with >98% purity. The NP isomers: 4-(3-methyl-3-octyl) phenol (p33-NP), 4-(2,6-dimethyl-2-heptyl)phenol (p262-NP), 4-(3,5-dimethyl-3-heptyl) phenol (p353-NP) and 4-(3,6-dimethyl-3-heptyl) phenol (p363-NP) were chemically synthesized as described previously (Shan et al., 2011), of which the purity (>99%) was examined via gas chromatography-electron impact mass spectrometry (GC-EIMS).

Testosterone Radioimmunoassay (RIA) kits were purchased from Beijing North Institute of Biological technology. (Beijing, China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Japan). Column Animal RNAout for total RNA extraction was purchased from TianDZ, Inc (Beijing, China). EasyScript First-Strand cDNA Synthesis SuperMix was obtained from Beijing TransGen Biotech Co. Ltd. (Beijing, China). 2×Taq PCR Master Mix was purchased from Nanjing Bioedify Biotech Co. Ltd. (Nanjing, China). All the reagents were of analytical grade.

2.2. Animals and housing

Adult male Sprague-Dawley rats (approximately 250 g) were purchased from Experimental Animal Center of the Academy of Military Medical Science Institute, China. The rats were housed in the animal facility of School of Medicine, Nanjing University with a 12 h: 12 h light–dark cycle and allowed ad libitum access to feed and filtered water for 7 days prior to experiments. Animals were handled in accordance with the directions in the ethics approval obtained from the Experimentation Ethics Review Committee of Nanjing University (ethics approval No. A9089).