Bull. Environ. Contam. Toxicol. (2004) 73:620–627

DOI: 10.1007/s00128-004-0473-2

Bulletin of Environmental Contamination and Toxicology

Nonylphenol Induced Apoptosis in Rat Testis Through the Fas/FasL Pathway

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Received: 7 November 2003/Accepted: 15 July 2004

Nonylphenol polyethoxylates (NPEs) are non-ionic surfactants widely used as components of detergents, paints, herbicides, insecticides and many other synthetic products. A large number of NPEs are discharged into municipal and industrial water. NP is formed from NPEs by the action of bacteria as a final biodegradation product of NPEs (Kinnberg et al. 2000). NP has been shown to be estrogenic in both in vitro and in vivo screening assays (Kwack et al. 2002). It acted as a "xenoestrogen" to disrupt testicular development and male fertility (Hossaini et al. 2001). In the male, the testis is an organ vulnerable to xenobiotic toxicants (Pineau et al. 1999). And developmental exposure of male mammals to these toxicants may be responsible for reduction in sperm counts (Chapin et al. 1999). There are three possible routes by which xenobiotic toxicants can affecte spermatogenesis (Sharpe 1998): 1) an alteration of the hypothalamic-pituitary function; 2) the alteration of other functions which leads to adverse effects on the spermatogenetic process, and 3) the direct disruption of cells in testes, including the Germ cells, Sertoli cells, and Leydig cells. To a current study, in NP exposed male *medaka*, a six-fold greater extent of apoptosis in spermatocytes, Sertoli cells and Leydig-homologue cells was observed (Lynn et al. 2002). This result suggested that NP may cause testicular degeneration in fishes via increasing testicular cell apoptosis. However relevant research on male mammals is scarce now.

Our previous in vitro study revealed that NP increased Sertoli cell apoptosis dramatically (Wang et al. 2003). So we hypothesized that NP exposure in male rats would lead to direct injury of Sertoli cells. In our present study, male SD rats were exposed to NP for 60 days, and then TUNEL assay was used to quantitatively measure cellular apoptosis in seminiferous tubules. In testes, Sertoli cells express FasL, which can initiate killing Fas-expressing Germ cells (Lee et al. 1997). This mechanism is a key regulator of germ cell apoptosis in normal and injury-associated conditions. Therefore we used RT-PCR to investigate the expression of Fas and FasL messenger RNAs (mRNA) in testicular tissue, to investigate the possible mechanism of NP induced germ cell apoptosis.

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MATERIALS AND METHODS

Nonylphenol was obtained from Tokyo Kasei Kogyo CO., LTD (Tokyo, Japan), 17β-Estradiol (E_2) was purchased from Sigma Chemical Co. (St.Louis, MO.USA) Apoptosis Detection Kits was purchased from R&D systems (Minneapolis, MN, USA). Trizol regent was purchased from BioBasic Inc. (Scarborough Ontario, Canada). Taq polymerase enzyme, Moloney murine leukemia virus (M-MLV) Reverse Transcriptase and rRNasin (Ribonuclease Inhibitor) were from Promega (Madison, WI, USA). All other reagents were of the highest grade commercially available.

20-d-old male Sprague-Dawley (SD) rats were purchased from Nanjing Medical University and housed in groups at 24℃ in a humidity-controlled room with 12-h light/dark photoperiod. Standard rat chow and water were available throughout the experiment. Rats were allowed to adjust to the surroundings for 1 week prior to treatment.

For the dose-finding study, 32 SD rats were treated orally with NP at 0, 50, 200, 375 mg/kg/day for six weeks. Among the rats that were treated with NP at 375 mg/kg/day, four died, and a reduction in body weight gain and significant increases in liver and kidney weights showed a chronic and systemic toxic effect of NP, while no such effect was detected among the 50 and 200 mg/kg/day groups. So the doses for the formal study were set at 0, 125, 250, 300 mg/kg/day.

Because NP has been reported to demonstrate a weakly estrogenic potency, we choose 17β-estradiol as the positive control. For the dose-finding study of the positive controls, 48 SD male rats were allocated into 6 groups, administered 17β-Estradiol orally at concentrations of 0, 100, 10, 1, 0.1, 0.01 ng/kg/day. Six weeks later, significant changes on testosterone and E_2 levels were observed in the 1 ng/kg/day group compared to the negative control. What's more, no severe toxicity which was exerted at the concentrations of 10 and 100 ng/kg/day was exhibited. So we choose 1 ng/kg/day as the dose for the positive control.

In formal study, 40 SD rats were divided into 5 groups, i.e:, the control, NP1, NP2, NP3 and E2 group. NP was administered to the control, NP1, NP2 and NP3 group at 0, 125, 250 and 300 mg/kg/day, respectively. 17β-estradiol was administered to the rats at 1 ng/kg/day. Sixty days later, testes were gained after necropsy.

Total RNAs were isolated from testes by Trizol method. 2 μg total RNA was reverse-transcribed using reverse transcription-polymerase chain reaction (Perkin-Elmer Cetus DNA Thermal Cycler, Perkin-Elmer, CT, USA) in the presence of M-MLV reverse transcriptase and oligo-dT primers. After RT reaction, 2 μL of the incubation mixture was used as the template for the following

polymerase chain reaction. The following components were added to the mixture: 5 μL 10×PCR buffer, 1μL (10 pmol) of both sense and antisense primers(for Fas and β-actin, or for FasL and β-actin),1μL 10mM mixture of all four deoxynucleotide triphosphates, 1 μL Taq DNA polymerase, and 12 μL nuclease-free water to adjust the final volume to 25 μL. The primer sequences used for PCR are shown in the Table 1. After an initial incubation at 94 ℃ for 1 min, temperature cycling was started with each cycle as following: denaturation at 94 ℃ for 30 s, annealing at 65 ℃ for 1 min and elongation at 72 ℃ for 1 min. Then the reaction followed by a second round of 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 65 ℃ for 1 min and elongation at 72 ℃ for 1 min. And the reactions were terminated by incubation at 72 ℃ for 5 min. The PCR conditions and the number of cycles were carefully chosen and have been described in previous reports (Lee et al. 1997; Lee et al. 1999). The PCR products were then separated on 1.5% agarose gel containing 0.5μg/mL ethidium bromide. The gel was put on an UV-transilluminator and photographed. The Fas and FasL signal was measured by a densitometer and standardized against the β-actin signal using a digital imaging and analysis system.

Table 1. Primer sequences used for RT-PCR analysis.

Target gene	Primer sequence5'-3'	Excepted size (bp)
Fas forward primer	CTGTGGATCATGGCTGTCCTGCCT	969
Fas reverse primer	CTCCAGACTTTGTCCTTCATTTTC	
FasL forward primer	GGAATGGGAAGACACATATGGAACTG	238
FasL reverse primer	C	
	CATATCTGGCCAGTAGTGCAGTAATTC	
β-actin forward primer	AGGCATCCTGACCCTGAAGTAC	389
β-actin reverse primer	TCTTCATGAGGTAGTCTGTCAG	

Testicular tissues were fixed in 3.7% formaldehyde solution, embedded in paraffin and cut into 5 μm thin sections. Apoptotic cells in the testicular tissues were identified by TUNEL assay using the commercial Apoptosis Detection Kits according to the manufacture's instructions. TUNEL-positive cells in randomly picked 20 seminiferous tubules for each group were counted using an Olympus microscope with a SPOT digital camera (FR-988 Shanghai).

RESULTS AND DISCUSSION

In the E_2 group, the expression of Fas and FasL were significantly up-regulated

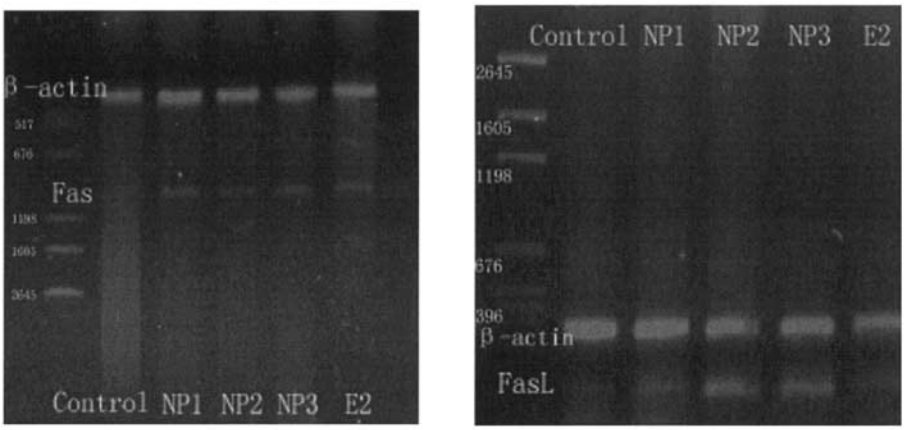

Figure 1. Analysis of polymerase chain reaction products in a 1.5% agarose gel stained with ethidium bromide.

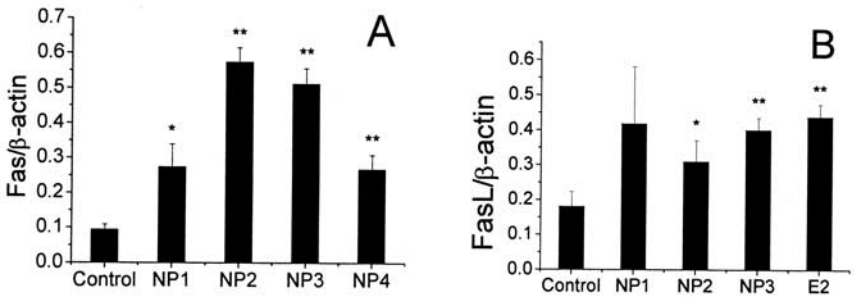

Figure 2. RT-PCR products of Fas and FasL mRNA were measured by a densitometer and standardized against the β-actin signal using a digital imaging and analysis system. Bars represents the mean±SEM. n=4, *: $p < 0.05$ **: $p < 0.01$

compared to the control ($p < 0.05$). And the same trend can be seen when rats were treated with NP at 125, 250, 300 mg/kg/d (Refer to Figure1 and Figure 2).

The number of TUNEL-positive cells in seminiferous tubules significantly increased compared to the control group when rats were treated with NP at 125 mg/kg/d. And the positive cells in tubules of NP2, NP3 and E2 groups increased dramatically (Refer to Figure3 and Figure4).

Previous studies on NP have mostly focused on aquatic wildlife. For example, exposure to nonylphenol caused changes in the number and size of Sertoli cells in fathead minnows, *Pimephales promelas* (Miles-Richardson et al. 1999), and produced marked effects on the cytology of Sertoli cells of eelpouts *Zoarces viviparous* (Christiansen et al. 1998). In conclusion, NP can alter testis structure and the conformation of Sertoli cells and germ cells in fishes. Until now more and

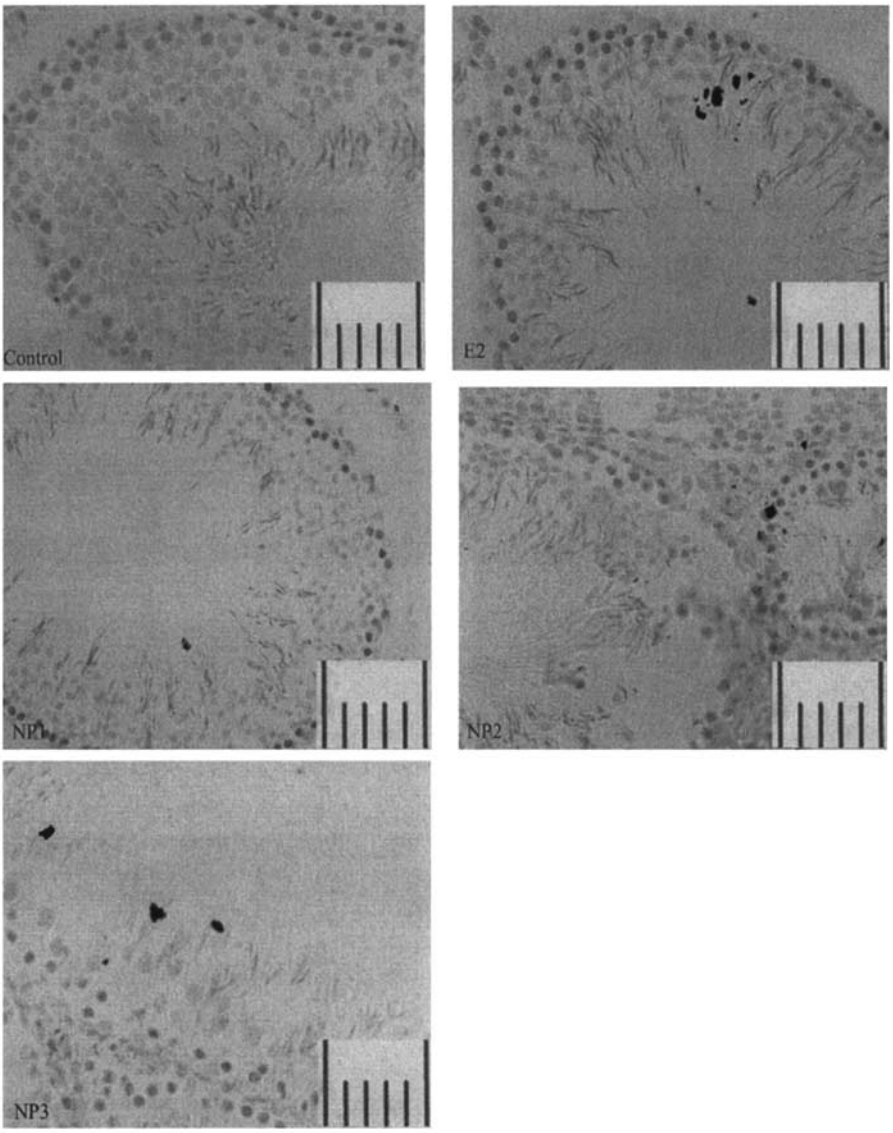

Figure 3. TUNEL staining of testes. Sections of treatments and controls testes from SD rats were probed with TUNEL assay. Positive cells are stained in brown, whereas negative cells are counterstained in green. All the sections are shown at 400×. The Scale Bar represents 50 μm.

more reports on the effect of NP in mammals are available. Nagao et al (2001) showed that early neonatal exposure to NP caused dysfunction of postpubertal reproductive function, as well as disrupted development of gonads in male and female rats. Chapin et al. (1999) revealed that epididymal weight and sperm density decreased in F2 male adults that were exposed to 650 and 2000 ppm NP.

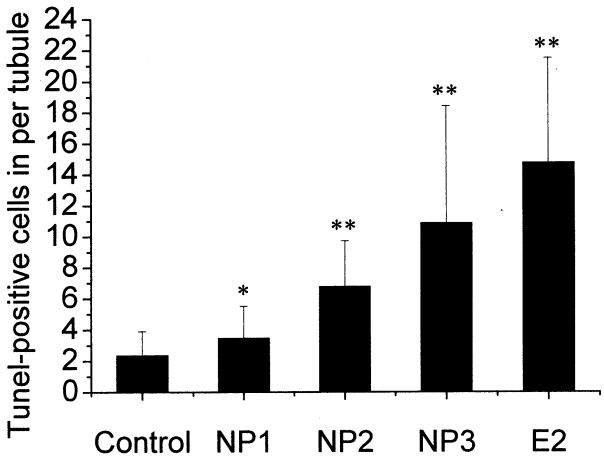

Figure 4. The number of TUNEL-positive cells per tubule. Bars represents the mean±SEM. n=20; *: $p < 0.05$; **: $p < 0.01$

Additionally, both spermatids per milligram testis and total spermatids per testis reduced at 2000 ppm NP exposure.

Though it is evident that NP decreases the quality and quantity of the spermatids, however, no relevant report has shown that by which pathway NP impacts the spermatogenesis. Our previous in vitro study has revealed that NP can significantly cause the apoptosis of Sertoli cells (Wang et al, 2003). In addition, NP has been shown to inhibit Ca^{2+}-ATPase of TM4 (a cell line of mammalian Sertoli cell) and cause physiological cell death (Hughes et al. 2000). So a possible mechanism has been suggested: NP maybe alters testis function via direct effects on Sertoli cells, including inducing the apoptosis of Sertoli cells. In our present study, it has been revealed that NP dramatically increased the number of TUNEL-positive cells in seminiferous tubules.

Additionally, increases of Fas and FasL mRNA were observed after NP exposure at all concentrations in our research. Recently, the Fas-signaling pathway between Sertoli cells and Germ cells has been highlighted as a crucial paracrine–signaling mechanism that responds to Germ cells apoptosis (Richburg 2000). In normal state, Sertoli cells maintain the homeostasis by providing positive support to most healthy Germ cells, and killing a few Fas-positive Germ cells with FasL. But after testicular injury, expression of Fas and FasL are significantly up-regulated. Sertoli cells increase FasL expression to eliminate inadequately supported Germ cells that express Fas. On the other hand, after Sertoli cell injury, the supporting capacity of

Sertoli cells is reduced. So it is obvious that the decadence of supporting capacity of Sertoli cells and the increases of FasL expression on Sertoli cells and Fas expression on germ cells result in apoptosis of Germ cells. And exposure to 17β-estradiol resulted in similar effects. However, further studies still have to be done concerning whether it is through the same pathway that NP and 17β-estradiol achieve the similar effects.

Acknowledgment. The authors are grateful to the foundation of the program: Project of Financing the Core Faculty in Higher Education Institutions.

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