Bull. Environ. Contam. Toxicol. (2003) 70:898–904

DOI: 10.1007/s00128-003-0067-4

Bulletin of Environmental Contamination and Toxicology

Effect of Nonylphenol on Apoptosis of Sertoli Cells in Vitro

X. Wang, X. Han, Y. Hou, G. Yao, Y. Wang

Reproductive Immunology Laboratory, Nanjing University Medical School, Nanjing, Jiangsu 210093, People's Republic of China

Received: 15 July 2002/Accepted: 30 January 2003

Chemical contaminants influence human reproductive health. Nonylphenol (NP) is the final biodegradation product of nonylphenol polyethoxylates which are non-ionic surfactants widely used in detergents, paints, and pesticides (Burkhardt-Holm et al. 2000). It presents as a pollutant in oceans, rivers and lakes (Naylor et al. 1992) and has proven to be an environmental estrogen (Johling and Sumpter 1993). In addition, it has been proposed that developmental exposure to estrogenic chemicals in the environment may be responsible for the proposed reduction in sperm counts (Carlsen et al. 1992). Studies with NP have mostly focused on aquatic wildlife, altering testis structure and the conformation of Sertoli cells and germ cells in fishes (Christiansen et al. 1998). Few reports on the effect of NP in mammals are available. Nagao et al. (2000) 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 at 650 and 2000 ppm NP in F2 (second developmentally exposed generation) male adults. Additionally, both spermatids per milligram testis and total spermatids per testis were reduced at 2000 ppm NP In addition, testosterone levels dramatically decreased when rats were exposed to NP at 250 mg/kg/day (Nagao et al. 2001). The purpose of our in vitro study was to find out how NP impacts spermatogenesis, by examining the growth, development, and maturation of Sertoli cells in mammals. We chose Sertoli cells as our objective for their crucial function in spermatogenesis, including fostering the development and maintaining the viability of germ cells by secreting hormonal and nutritive factors. The present study, used Sertoli cell cultures prepared from 20 days rats, which are just beginning puberty, to examine Sertoli cell proliferation and apoptosis after exposure to various NP concentrations.

MATERIALS AND METHODS

Primary cultures of Sertoli cells were prepared from testes of 20-d-old

Correspondence to: X. Han

Sprague-Dawley (SD) rats. The following materials, with suppliers, were obtained for this research: 20-d-old Sprague-Dawley (SD) rats (Nanjing Medical University), Ham's F-12 nutrient mixture (F-12), Dulbecco modified Eagle medium (DMEM), fetal calf serum (FCS) (GibcoBRL), insulin, Trypsin, collagenase I, transferrin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraolium bromide (MTT), HEPES ($C_8H_{17}N_2O_4SNa$), epidermal growth factor (EGF) (Sigma), and NP ($C_{15}H_{24}O$) (Sigma-Aldrich).

Testes were cleared from their capsule, washed twice and finely chopped in Hanks Balanced Salt Solution (HBSS). The chopped tissues were suspended in HBSS and centrifuged at 900 rpm for 4 min. After the supernatant was discarded, the pellets were sequentially digested in 0.25% Trpsin (32 ℃, 210 r/min, 30 min), followed by 0.1% collagenase I (34 ℃, 150 r/min, 30 min). The homogenate was filtered through a 100-mesh stainless steel filter and the cell suspension was centrifuged at 800 rpm for 10 min. Cells were washed twice in Ham's F12 nutrient mixture (F12) and Dulbecco modified Eagle medium (DMEM) (1:1, v/v) supplemented with 10% FCS. Sertoli cells were cultured at 1.5×10^6 cells/ml in tissue culture dishes (60 mm in diameter, Miniplasi) in F12 and DMEM (1:1, v/v) with 10% FCS, containing sodium bicarbonate (1.2 mg/L), HEPES (15 mmol/L), insulin (10 μg/ml), transferrin (5 μg/ml), EPO (2.5 ng/ml), penicillin (100 IU/ml), and streptomycin (100 IU/ml). Cells were maintained in a humidified atmosphere of 95% air, 5% CO_2 at 34 ℃ for 36 hr. Thereafter, cells were hypotonically treated with 20 mM Tris, pH 7.4, for 1.5 min to lyse the unwanted germ cells. Sertoli cell cultures, with more than 90% purity, were then washed twice, and suspended in serum-free medium, cultured in 34 ℃ incubator Viability of the cells was judged using the trypan blue exclusion assay, and more than 90% of cells were viable. Cells were allowed to recover for 24 hr at 34 ℃.

After the cells reached attachment, two experiments were conducted to evaluate the effect of NP on Sertoli cells. In the initial experiment, the cells were collected and transferred to 96-well culture plates at a density of 5.0×10^4 cells/90 μL in every well. To each well, 10 μL medium containing NP was added. The final NP concentrations in 100 μL medium were 0, 200, 1000, 3000, or 5000 ppb respectively. Cultures were performed in six wells per concentration and incubated at 34 ℃ in a humidified atmosphere of 95% air, 5% CO_2 for 24, 48, 72 hr. In the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraolium bromide] assay, solution was added to each well and plates were incubated at 34℃ for 4 hr The assay was stopped using 100 μL 0.01 M HCl containing 20% Sodium dodecyl sulfate (SDS). An automated microplate reader (Bio-Rad Model 550) was used to measure the light absorbance at 570 nm.

A second experiment was conducted to examine apoptosis in Sertoli cells by flow cytometry. After attachment, the medium was discarded and replaced with fresh medium containing final concentrations of 0, 3000, 4000, or 5000 ppb NP. All the experiments were conducted in triplicate. Cells were cultured at 34 ℃ in a humidified atmosphere of 95% air, 5% CO_2 for 72 hr. Then cells were harvested and a single cell suspension in Phosphate Balanced Solution (PBS) was prepared. Cells were washed twice and resuspended at 1×10^6 cells/ml, and fixed in 70% cold ethanol for at least 4 hr and centrifuged at 1000 rpm for 5 min. Then cells were washed twice and suspended in 1ml PBS containing ribonuclease (RNase) (50 μg/ml) and propidium iodide (PI) (50 μg/ml) , incubated at 4℃ for 30 min. Samples were stored at 4 ℃ until analyzed by flow cytometry.

Two-way analysis of variance was used to compare the mean values between treatments and the control after ascertaining the homogeneity of variance between the groups. Mean values between two groups were compared by calculating least significant differences using t-values.

RESULTS AND DISCUSSION

Nonylphenol exposure induced a concentration- and time-dependent decrease in Sertoli cell proliferation (Table 1). The 200 and 1000 ppb concentrations did not show significant changes in Sertoli cell proliferation, but at 3000 ppb, the largest reported concentration in the environment (Nagao et al. 2001), such proliferation was significantly ($p < 0.05$) decreased after 72 hr of exposure. Moreover, at 5000 ppb, proliferation of Sertoli cells was significantly ($p < 0.05$) decreased as early as 24 hr and was further inhibited at the 48 and 72 hr timepoints ($p < 0.01$).

Apoptosis of Sertoli cells, as detected by flow cytometry, increased with increasing NP concentration (Table 2, Figures 1 – 4). After 72 hr treatment, apoptosis of Sertoli cells was significantly increased ($p < 0.05$) at all concentrations when compared with the control.

Nonylphenol is an environmental estrogen as it may disturb normal endocrine and reproductive functions in aquatic wildlife. A number of in vivo studies have demonstrated that NP is harmful to the male reproductive system in fishes. The quantity and quality of spermatids was reported to have been affected (Johling and Sumpter 1993; Weber et al. 2002). Kinnberg et al. (2000a) noted the severe effect of NP on testis structure of male platyfish, with treated fish having degenerated seminiferous lobules and squamous Sertoli cells. Electron microscopy revealed greater numbers of phagocytized spermatozoa in these Sertoli cells. Christiansen et al. (1998) reported similar findings.

Table 1. Effect of NP on Sertoli cells proliferation

NP (ppb)	Optical Density (570nm)		
	24h	48h	72h
Control	0.13±0.03	0.14±0.04	0.14±0.02
200ppb	0.13±0.03	0.13±0.04	0.12±0.04
1000ppb	0.13±0.04	0.15±0.05	0.12±0.04
3000ppb	0.13±0.04	0.15±0.04	0.10±0.03*
5000ppb	0.10±0.02*	$0.05\pm6.4\times10^{-4}$**	$0.05\pm6.8\times10^{-4}$**

Values are means±S.D., n=6 * P<0.05, ** P<0.01 vs Control

Table 2. Effect of NP on apoptosis of Sertoli cells after 72 hr treatment

	NP (ppb)			
	Control	3000	4000	5000
Apoptosis (%)	31.6±2.2	39.0±4.2*	56.3±5.8**	66.2±4.4**

Values are means±S.D., n=3 * P<0.05, ** P<0.01 vs Control

Of relevance to our findings in mammals, Weber et al. (2002), using the TUNEL assay, reported apoptosis in spermatocytes, Sertoli cells and Leydig-homologue cells in the testis of NP-exposed male *medaka* versus the controls. Since no significant differences were found in apoptosis of intestine, liver or kidney cells between the controls of these same fish and those of treated fish, it was suggested that NP is a reproductive toxicant, perhaps through an unknown estrogenic mechanism. Kinnberg et al. (2000b) assumed that NP altered testis structure via direct or indirect effects on Sertoli cells. Furthermore, multigeneration studies in rats showed adverse effects of nonylphenol on spermatogenesis (Chapin et al. 1999; Nagao et al. 2001).

Sertoli cells play an important role in spermatogenesis since they foster the development of germ cells and maintain viability of those cells by secreting hormonal and nutritive factors into a specialized compartment. Significant shedding of germ cells from Sertoli cells occurred when mixed cultures of Sertoli cells and germ cells were treated with Pb (Neeta et al. 2000). In addition, the viability of detached germ cells decreased compared to the control. In contrast though, viability of detached germ cells was not decreased by mono-(2-ethylhexyl) phthalate (MEPH) (Gray and Beamand 1984), so this agent may damage Sertoli cells directly, thereby decreasing their supporting role and leading to germ cell detachment (Boekelheide and Lee 1988).

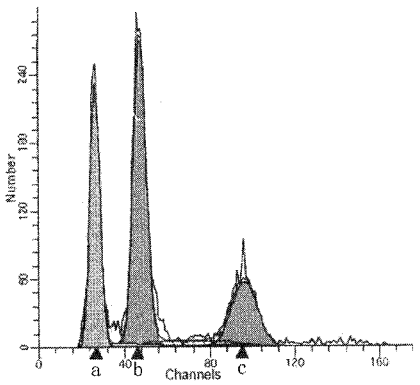

Figure 1

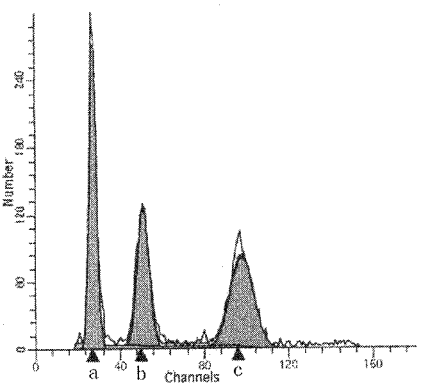

Figure 2

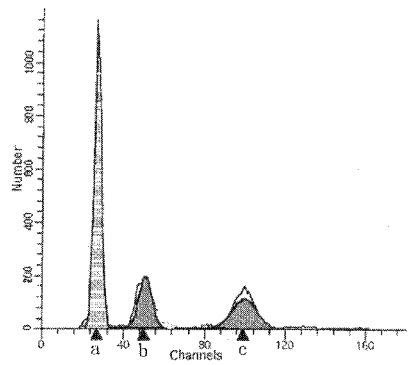

Figure 3

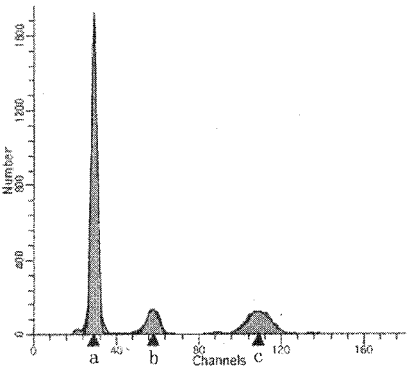

Figure 4

Figure 1-4: Analysis curve of cell cycles (The analysis of the cell cycle in each stage is number of cells.) a: Apoptosis; b: G_0+G_1; c: G_2+M. Figures 1-4 are the control, 3000 ppb, 4000 ppb, and 5000 ppb treatments, respectively.

Our study revealed that NP caused significant apoptosis in Sertoli cells of rats. The MTT results confirmed that after 72 hr treatment at 3000 ppb, the reported largest concentration detected in environment (Nagao et al. 2001), proliferation of Sertoli cells was significantly decreased compared to the control. Proliferation of Sertoli cells in 5000 ppb treatment decreased significantly by 24hr. Results from flow cytometry revealed significant increases in apoptosis of Sertoli cells exposed for 72 hr to 3000, 4000, and 5000 ppb NP. This result is consistent with the MTT results, since reduced proliferation and increased apoptosis of Sertoli

cells directly affect the quality and quantity of germ cells. Developmental exposure to an estrogenic chemical in the environment may be responsible for reduction in sperm counts, as proposed previously (Carlsen et al. 1992). Therefore, we conclude that apoptosis of cultured Sertoli cells may be the major reason for the adverse effects of NP on spermatogenesis in mammals, so NP should be used with care in modern life. Alsoof note, cultures of Sertoli cells and mixed Sertoli and germ cell cultures can be useful in assessing male reproductive system toxicants.

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

REFERENCES

Boekelheide K, Lee J (1998) Expression of Fas system-related genes in the testes of the golden hamster. Toxicol Lett 102-103: 503-508

Burkhardt-Holm P, Wahli T, Meier W (2000) Nonylphenol affects the granulation pattern of epidermal mucous cells in rainbow trout *Oncorhynchus mykiss*. Ecotoxicol Environ Saf 46(1): 34-40

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE (1992) Evidence for decreasing quality of semen during the past 50 years. Br Med J 305: 609-613

Chapin RE, Delaney J, Wang YF, Lanning L, Davis B, Collins B, Mintz N, Wolfe G (1999) The effects of 4-nonylphenol in rats: A multigeneration reproduction study. Toxicol Sci 52: 80-91

Christiansen T, Korsgaard B, Jespersen A (1998) Effects of nonylphenol and 17-beta-oestradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout *Zoarces viviparous*. J Exp Biol 201: 179-192

Gray TJB, Beamand JA (1984) Effect of some phthalate esters and other testicular toxins on primary cultures of testicular cells. Food Chem Toxicol 22(2): 123

Johling S, Sumpter J P (1993) Detergent components in sewage effluents are weakly oestrogenic to fish: an in vitro study using rainbow trout hepatocytes. J Aquat Toxicol 27:361-372

Kinnberg K, Korsgaard B, Bjeeregaard P, Jespersen A (2000a) Effects of nonylphenol and 17-beta-estradiol on vitellogenin synthesis and testes morphology in male platyfish *Xiphophorus maculatus*. J Exp Biol 203:171-181

Kinnberg K, Korsgaard B, Bjeeregaard P (2000b) Concentration-dependent effects of nonylphenol on testes structure in adult playfish *Xiphophorus maculatus*. Mar Environ Cell Tissue Res 267: 583-590

Nagao T, Saito Y, Usumi K, Nakagomi M, Yoshimura S, Ono H (2000) Disruption of the reproduction system and reproductive performance by administration of nonylphenol to newborn rats. Hum Exp Toxicol 19(5): 284-296

Nagao T, Wada K, Marumo H, Yoshimura H, Ono H (2001) Reproductive effects of nonylphenol in rats after gavage administration: a two-generation study. Reprod Toxicol 15: 293-315

Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR (1992) Alkylphenol ethoxylates in the environment. J Am Oil Chem Soc 69: 695-703

Neeta A, Neelima S, Saxena DK (2000) Effect of lead on Sertoli-germ coculture of rat. Toxicol Lett 116(1): 45

Weber LP, Kiparissis Y, Hwang GS, Niimi AJ, Janz DM, Metcalfe CD (2002) Increased cellular apoptosis after chronic aqueous exposure to nonylphenol and quercetin in adult medaka (*Oryzias latipes*) Comp Biochem Physiol 131: 51-59