Letter to the Editor

90d Exposure to Nonylphenol has Adverse Effects on the Spermatogenesis and Sperm Maturation of Adult Male Rats*

LU Wei Chen[1,2,&], WANG Ai Qing[3,&], CHEN Xiao Ling[1,4,&], YANG Guo[1], LIN Yao[1], CHEN Yi Ou[1], HONG Cheng Jiao[3], and TIAN Hai Lin[1,#]

This study was conducted to elucidate the reproductive effect of NP on testis, epididymis and epididymal sperm *in vivo*. Adult male *Sprague-Dawley* rats were gavaged with NP at 0, 40, 100, or 250 mg/kg body weight (bw) on alternate days for 90 d. The results showed that oral administration of NP may damage the structure and function of testis, induce apoptosis and oxidative stress in epididymis or even have cytotoxic effects on epididymal sperm.

Previous studies have collectively demonstrated that nonylphenol (NP) is associated with male reproductive damage in aquatic invertebrates and vertebrates. Han et al.[1] reported that NP at ≥125 mg/kg induced apoptosis in the seminiferous tubules of the testes. It has also been confirmed that NP exposure produces oxidative stress, enhancing reactive oxygen species (ROS) level and inhibiting the activity of antioxidant enzymes in testes, testicular cells and epididymal sperm[2-3]. Given that reproductive organs and sperm are the ideal action sites for antifertility agents, evidence is lacking whether NP administration could induce apoptosis, inactivate antioxidant enzymes of the epididymis or NP could affect the fertilization of epididymal sperm. The objective of the present study was to evaluate the adverse effects of NP *in vivo* on testis, epididymis and epididymal sperm function.

Adult male *Sprague-Dawley* (*SD*) rats (12 weeks old) were provided by the Experimental Animal Production Center of Soochow University (Suzhou, China). The animals were housed four/cage in the animal room under a light cycle of 10 h dark/14 h light with controlled ambient temperature of 22 °C -24 °C and a humidity of approximately 10%-50%. All rats were allowed access to standard chow and water ad libitum. Animals were randomly allocated into four groups (eight/group) and were allowed to acclimate to human contact for one week prior to the study. The four groups were given NP (4-nonylphenol; Shuangju, Changzhou Dyestuff Chemical Factory Co., China) dissolved in corn oil by gavage on alternate days at 0, 40, 100, or 250 mg(NP)/kg·bw starting from 12 weeks of age for 90 d. The control group was gavaged with corn oil (vehicle). The dosages used in this study were selected based on previous studies[1-2]. All animals were kept in accordance with the NIH Guide for Care and Use of the Laboratory Animals. The procedures were approved by the ethical committee of Soochow University. 24 h after the last gavage, all surgeries were performed under chloral hydrate anesthesia.

Blood samples were collected via the abdominal aorta. The testosterone concentration in the serum was estimated by ELISA according to the manufacturer's instructions (R&D, USA). Testes and epididymides were quickly excised and weighed. Histological changes of the testes were observed using HE stain and optical microscope (LEICA DFC 295). The cauda epididymides were separated, carefully cut and incubated for sperm suspension. The sperm concentration was determined by the Neubauer method. Sperm count was counted by two individuals and expressed as millions per milliliter. The live-cell system (LCS) was used to record sperm motility. Three different fields with two Z-layers per field were captured. Each Z-layer contained 6-8 layers. A total of 100 spermatozoa, including motile and non-motile cells, were evaluated to determine the percentage motility. Sperm smears were stained

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1. Department of Occupational and Environmental Health, School of Public Health, Soochow University, Suzhou 215123, Jiangsu, China; 2. KunShan Health Inspection Station, 458 Tongfeng Road, Kunshan 215301, Jiangsu, China; 3. Experimental Center of Medical College, Soochow University, Suzhou 215123, Jiangsu, China; 4. The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830000, Xinjiang, China

with eosin-Y. The number of morphologically abnormal sperm among 1000 spermatozoa was determined. The acrosomal status and sperm viability were assessed using a flow cytometer (Cytomics FC 500, Beckman Coulter, USA) by double-stain method that combined Alexa Fluor-488-PNA (Molecular Probes, Life Technologies, USA) (final concentration: 2 μg/mL) with propidium iodide (PI) (final concentration: 10 μg/mL). Each run on the flow cytometer counted 10,000 spermatozoa, and the data were analyzed with appropriate gating parameters by CXP Analysis software. Modified Kennedy method was used to measure the spermatozoa acrosin activity with the Human Spermatozoa Acrosin Activity Quantitative Assay Kit (Ruiaijin, Tianjin, China). The sperm intracellular reactive oxygen species (ROS) level was estimated by $DCFH_2DA$ (Sigma-Aldrich, Sigma, St. Louis, MO, USA) (final concentration: 10 μmol/mL) and flow cytometry. The left epididymides were homogenized and the activities of epididymal superoxide dismutase (SOD) and catalase (CAT) were determined by ELISA. The of apoptosis in epididymides was detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method and performed in accordance with the manufacturer's instructions (In situ Cell Death Detection kit, POD, Roche, USA; DAB, Roche, USA). The left caput epididymides were fixed in 4% formaldehyde and processed routinely. TUNEL-positive cells (brown) were counted in 5 randomly selected fields with the assistance of microscope (OLYMPUS, Japan) and Image-Pro Plus system (Media Cybernetics, USA). The incidence of apoptosis was expressed as the percentage of apoptotic cells. The statistical analysis was performed with SAS 9.0 using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test and statistical significance level was considered to be $P<0.05$.

In the present study, a reduction in the weight of the epididymides occurred at the 40 and 250 mg/kg dose, rather than in both of the reproductive organs (Table 1). Sperm count decreased ($P<0.05$) significantly in rats in the 100 and 250 mg/kg groups. The weights of the testis and epididymis largely depend on the mass of the spermatozoa and do not always change along with the body weight[4]. Here are three possible reasons for the outcomes of organ weights and sperm count. First, changes of the testis and epididymis weights may be attributable to NP-related suppression of spermatogenesis. NP has been confirmed to induce Sertoli cell dysfunction and increase the rate of apoptosis of testicular cells[1], which were followed by parallel decreases in sperm production and the mass of testicular cells. Second, a distinct increase in apoptosis of epididymal cells (40 and 250 mg/kg) in this study might explain the inconsistent alterations of epididymis weight and sperm count. Third, because *p*-nonylphenol was previously shown to be able to inhibit L-type Ca^{2+} channels in vascular smooth muscle cells and evoke an endothelium-independent relaxation of the coronary vasculature[5], we speculated that NP

Table 1. Effect of Nonylphenol on the Absolute Weights of the Testis and Epididymis and Sperm Parameters

Variabilities		**Treatment [nonylphenol (mg/kg·bw)]**			
		0	40	100	250
Absolute weight	Testes (g)	3.30±0.35	2.91±0.26	3.07±0.35	2.95±0.26
	Epididymides (g)	1.28±0.11	1.15±0.06**	1.23±0.13	1.10±0.08**
Cauda epididymis sperm	Sperm count (million/milliliter)	46.09±8.51	40.28±11.57	35.75±6.06*	31.81±4.94*
	Sperm motility (%)	36.90±3.69	32.52±5.13	22.49±7.06***	24.46±4.83***
	Sperm viability (%)	49.65±7.00	52.59±9.80	49.17±10.36	56.51±10.12
	Sperm abnormality (%)	3.62±1.46	4.40±1.88	5.60±1.01***	7.55±1.75***
Sperm acrosome integrity	Dead sperm with intact acrosome (PI+ PNA-)	43.82±6.80	39.70±9.28	41.82±9.87	34.95±12.13
	Dead sperm with ruptured acrosome (PI+ PNA+)	6.36±3.02	7.72±3.04	9.01±2.75	8.55±3.46
	Live sperm with intact acrosome (PI- PNA-)	47.66±7.35	50.11±10.15	45.66±10.78	52.90±10.26
	Live sperm with ruptured acrosome (PI- PNA+)	1.99±0.79	2.47±1.20	3.51±1.02*	3.61±1.30*

Note. Values are expressed as the mean±SD (n=8). Statistical significance is indicated as $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. PNA: Alexa Fluor-488-PNA, PI: propidium iodide.

could also inhibit the contraction of the smooth muscle of the seminiferous tubules, accounting for the decreased number of expelled spermatozoa.

Along with the NP dose, various degrees of damage in the testicular structure were observed (Figure S1), including distortion and atrophy of the seminiferous tubules, reduced thickness of the epithelium due to reductions in the number of germ cells and Sertoli cells, disordered arrangement of the spermatogenic cells, fewer spermatids and the presence of germinal cell debris in the lumina.

Testosterone has been found to influence the function of smooth muscle in the ductus deferens and seminal vesicles. Imbalance in circulating testosterone level would likely be associated with spermiation alteration and other male reproduction problems. This study showed that 250 mg/kg of NP decreased the serum testosterone level (*P*<0.05) (Figure 1). However, the precise effect of NP on testosterone level remains controversial and the way NP affects testosterone is still unclear. The decrease in serum testosterone herein may be due to negative feedback inhibition through the NP-disrupted hypothalamus-pituitary-gonad axis and/or NP-related detrimental effects on the Leydig cells. Moreover, NP-induced structural damage in testis in the present study could also influence its secretion function.

When rats were fed NP at 100 mg/kg or 250 mg/kg, the negative impact on sperm in the cauda epididymis was characterized by decrease in count (*P*<0.05) and motility (*P*<0.05) and increase in abnormality rate (*P*<0.05) (Table 1), indicating that

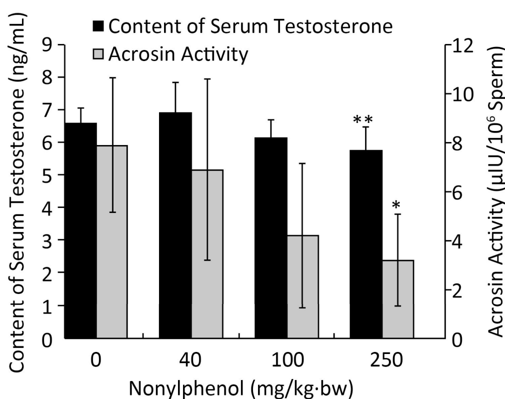

Figure 1. Effects of Nonlyphenol Exposure on Serum Testosterone and Acrosin Activity of Epididymal Sperm in rats. Statistical significance is indicated as $^{*}P$<0.05, $^{**}P$<0.01, and $^{***}P$ <0.001.

spermatogenesis and sperm maturation were disrupted by NP exposure. No significant response on sperm viability was noticed in all NP-treated groups. Consistent with our data, *in vivo* studies have demonstrated that NP has negative effects on basic sperm parameters, such as reductions in the cauda epididymal sperm concentration, decreases in sperm motility and accelerations in abnormal rate[2-3,6]. The reduction in motility could be associated with the fact that the transit time through the epididymis was accelerated, and the reduced transit time may be due to the decreased testosterone level. In males, spermatogenesis is primarily regulated by testosterone and sensitive to oxidative stress as germ cells contain abundant polyunsaturated fatty acids. Hence, the induction of abnormal spermatogenesis and sperm maturation by NP may be attributed to oxidative stress, lipid peroxidation and reduced testosterone levels[2,6], followed by consequent changes in Sertoli and germ cells. The premature release of germ cells due to NP-induced Sertoli cell dysfunction could influence spermatozoa morphogenesis. Besides, NP could directly impair sperm structure within 12 min[7].

During the process of sperm penetration into the zona pellucida and fusion with the oocyte, acrosome integrity and acrosin activity are of great importance. In the present study, the acrosomal statuses of PNA/PI double-stained spermatozoa were shown in dot plots divided into four populations of cells: live cells with intact acrosome (PI- PNA-), live cells with ruptured acrosome (PI- PNA+), dead cells with intact acrosome (PI+ PNA-), and dead cells with ruptured acrosome (PI+ PNA+) (Table 1). Treatment with 100 mg/kg or 250 mg/kg NP significantly increased the percentage of live spermatozoa with ruptured acrosome, which could impair male fertility (*P*<0.05). The reduction in spermatozoa acrosome integrity may be related to the membrane impairment caused by NP in combination with elevated ROS levels. ROS are now well established as regulators of normal sperm function; however, once overproduced, ROS will result in oxidative stress and attacking the plasma membrane and DNA. Significant increase of sperm ROS was obtained in the 250 mg/kg NP group (*P*<0.05) (Table 2). ROS are involved in sperm capacitation and acrosomal reaction. However, it is likely that the amount of ROS in sperm of 250 mg/kg group remarkably exceeded the appropriate level and damaged acrosome integrity. As for acrosin, our results are seemingly in conflict with those of previous

Table 2. Effect of Nonylphenol on Sperm ROS Level, Antioxidant Enzyme Activities of Epdidymis and the Percentage of Apoptotic Epididymal Cells

Variabilities	Treatment [nonylphenol (mg/kg·bw)]			
	0	40	100	250
Epididymal sperm ROS (*n*=8)	2.93±0.76	3.32±0.96	3.29±0.77	4.51±1.18*
Epididymal SOD acticity (nmol/min·mg) (*n*=8)	92.85±27.98	71.37±12.37	89.98±25.85	49.48±7.45***
Epididymal CAT activity (nmol/min·mg) (*n*=8)	49.03±9.32	36.58±7.10***	47.96±12.76	27.19±5.00***
Percentage of apoptotic epididymal cells (%) (*n*=3)	21.00±2.00	27.67±3.06**	25.67±2.52	35.00±3.00**

Note. Values are expressed as the mean±SD. Statistical significance is indicated as *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

reports[8-9] showing that E_2 and myricetin, a phytoestrogen, significantly increased the activity of acrosin, although NP has been confirmed to exert estrogenic activity. In the current study, acrosin activity was notably decreased in the 250 mg/kg group (Figure 1) and the inhibition of acrosin activity as well as the loss of acrosome integrity may be due to direct NP exposure and the overproduction of relevant ROS.

To eliminate the excess oxidation products and guarantee successful spermatogenesis and the sperm maturation process, there exists an antioxidant system composed of free radical scavengers and antioxidant enzymes. Table 2 indicated a notable decrease of epididymal SOD activity and an increase of sperm ROS in the 250 mg/kg group ($P<0.05$). The epididymal CAT activity of rats following treatment with 40 or 250 mg/kg NP decreased significantly ($P<0.05$). Hamdy et al.[2,10] reported similar findings that the H_2O_2 production and LPO significantly increased while the activities of SOD, CAT, and GSH-Px were inhibited in orally NP-treated adult rat's epididymal sperm at ≥100 mg/kg·d of 30 d. El-Dakdoky and Helal[3] also clarified that NP induced inactivation of SOD and GSH in the testes when the mice exposed to NP at ≥21.25 mg/kg·d for 35 d. These facts jointly reflect that NP could induce oxidative stress during spermatogenesis and sperm maturation, which may finally alter sperm fertility.

NP has been confirmed to cause apoptosis of testicular cells[1]. In mammals, spermatozoa get further maturation in epididymides. Results herein also revealed a distinct increase in the percentage of apoptotic epididymal cells (40 and 250 mg/kg, $P<0.05$, Table 2). The location of TUNEL-positive cells indicated that apoptosis occurred in the degenerating epithelium near the base of the epididymal duct (Figure S2). As is known, androgen is required to maintain the morphology and functions of the caput epididymis. Therefore, the observed epididymal apoptosis might be involved in the deterioration of the epididymis by NP-induced testosterone decrease. And apoptosis in epdidymides could accordingly affect sperm maturation and absolute weight of epdidymides.

SUPPLEMENTARY Details of Figure S1 and Figure S2 can be found in the websit of *www. besjournal.com*

#Correspondence should be addressed to: TIAN Hai Lin, associate professor, PhD, Tel: 86-512-65880070, E-mail: hltian@suda.edu.cn

&Authors with equal contribution.

Biographical notes of the first authors: LU Wei Chen, female, born in 1987, MM, majoring in environmental endocrine disruptors research; WANG Ai Qing, female, born in 1970, MM, majoring in health effects of environmental Radon exposure; CHEN Xiao Ling, female, born in 1987, MM, majoring in environmental endocrine disruptors research.

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SUPPLEMENTARY MATERIALS

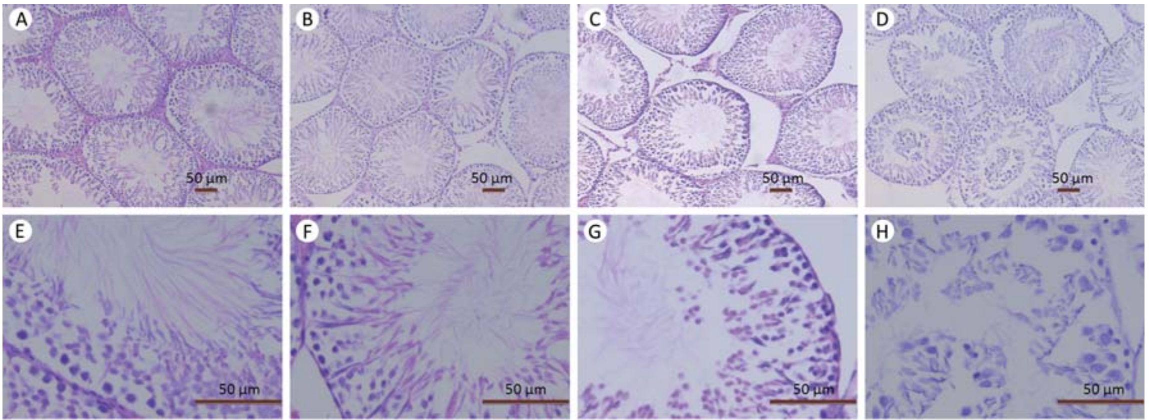

Figure S1. Histopathological changes in the testes in different groups. (A, B, C, and D: 100×; E, F, G, and H: 400×). A, B, C, and D represent for the control group, the 40 mg/kg group, the 100 mg/kg group, and the 250 mg/kg group, respectively. E, F, G, and H represent for the control group, the 40 mg/kg group, the 100 mg/kg group. and the 250 mg/kg group, respectively. Arrows in D and H show the germinal cell debris in the lumina of the rats in the 250 mg/kg group.

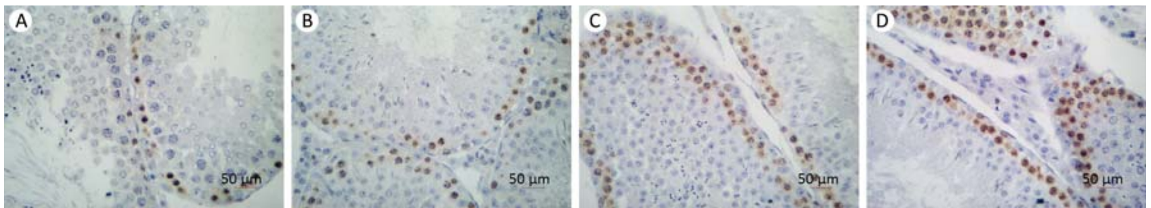

Figure S2. Effects of nonlyphenol exposure on apoptosis level of epididymides in rats. (A, B, C, and D: 400×). A, B, C, and D represent for the control group, the 40 mg/kg group, the 100 mg/kg group, and the 250 mg/kg group, respectively.