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Reproductive Toxicity of Male Mice after Exposure to Nonylphenol

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Alkylphenol ethoxylates, especially nonylphenol (NP) is widely used as lubricating oil additives, plasticizers and surface-active agents (Ahel et al. 1993). NPs have been documented to appear in the aquatic environment, particularly in sediment, and can reach up to 3,000 ppb in rivers and lakes (Ying et al. 2002). There are probably diverse routes of human exposure; not only via contaminated foods and drinking water, but also via dermal absorption or inhalation (Clark et al. 1992; Ahel et al. 1993).

Recent researchers have hypothesized that NP could induce reproductive abnormalities (Zha et al. 2007) and disrupt reproductive development in a number of animal studies; Kyselova et al. (2003) studied the multigenerational effects of NP on mice and found a negative effect on spermatogenesis and sperm quality. Male rats exposed to NP (200 mg/kg) during sexual maturation period suffered from damaged reproductive development (Qiu et al. 2005). The nature and the mechanism of action of NP on the male reproductive system remain unclear. Some researches have emphasized the estrogenic effect of NP might cause endocrine disruption (Kwack et al. 2002), others study focused on cytotoxicity (Han et al. 2004; Gong and Han 2006). In the present study we hypothesized that NP exposure in male mice would lead to oxidative stress by generating reactive oxygen species that damage cellular components. Accordingly, The purpose of this study was to investigate the effects of NP on sperm characteristics, fertility index, histopathological and biochemical changes related to oxidative stress in testes.

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Materials and Methods

p-Nonylphenol (4-Nonylphenol; C15H24O; mw220.36; with density 0.950 g/ml and purity 85%) was obtained from Sigma Chemicals Co., St. Louis, MO, USA.

Five-weeks-old male and female Swiss mice weighing 25–27 g were obtained from Central Animal House of the National Research Center, Egypt, kept under a 12:12 light cycle with a temperature of 23–25°C and relative humidity of 50–65%. Standard chow and water were available throughout the experiment.

In the present investigation, the acute intraperitoneal (i.p.) LD_{50} of nonylphenol (NP) was calculated for adult male mice as 170 mg/kg b.wt according to the simplified method of evaluating dose effect experiments (Litchfield and Wilcoxon 1949). In order to determine the minimal doses of NP capable of inducing any toxic effect on testis, daily doses of 5.31, 10.62, 21.25, and 42.50 mg/kg b.wt equivalent to 1/20, 1/10, 1/8 and 1/4 LD_{50} of NP were administered intraperitonealy for 2 weeks. The doses of 5.31 and 10.62 mg/kg were neglected as they did not exert any toxic effect on male reproductive organs weight and sperm characteristics.

In formal study, 40 adult males Swiss mice were divided into four groups of (n = 10): the oil, NP1, NP2 and control group, NP was dissolved in corn oil and administered i.p. to the oil, NP1 and NP2 groups at 0, 21.25 and 42.5 mg/kg b.wt./day, respectively. The control group did not receive any treatments. Animals treated for 35 consecutive days, this treatment time is selected to coincide with the time during which mice spermatogenesis occurs (Rees 1993). The route of administration (i.p.) was chosen so as to be sure that NP reached the reproductive system at high doses and to enhance the sensitivity of the assay. For the fertility test, at the end of

35 days each male was paired separately with normal untreated female for 7 days. Vaginal plugs and smears were examined daily. Females with semen plugs or sperm positive vaginal smears were considered pregnant, the day of detection was considered the first day of gestation. Then females were isolated and kept under observation till 18th day of gestation. The mating behavior and conception rate were determined by calculating the mating and fertility indices as follow:

Mating index = No. of confirmed mating/No. of mated × 100

Fertility index = No. of pregnant females/No. of confirmed mating × 100

To examine any adverse effects that may result on fetuses paternally treated with NP, pregnant mice were sacrificed on the day 19 of gestation. The uteri were dissected and the implantation sites, number of viable, resorbed and dead fetuses were recorded. The fetuses were examined morphologically to determine any external abnormalities.

After mating trials, male mice of all groups were anesthetized and sacrificed; the reproductive organs (testes, epididymis and seminal vesicles) were removed and weighed. One of the testes was homogenized, the homogenates were centrifuged and the supernatant was separated and used for oxidative stress analysis. Malondialdehyde (MDA) concentration, Glutathione reduced (GSH) level, and superoxide dismutase (SOD) activities were assayed according to the methods of Ohkawa et al. (1979), Beutler et al. (1963) and Minami and Yoshikawa (1979) respectively. For determination of sperm motility, concentration and sperm morphological abnormalities, the epididymal content of each mouse was obtained after cutting the tail of epididymis and squeezing it gently in sterile clean watch glass and examined according to the technique employed by Bearden and Fuquay (1980). For histological examinations, the right testes were fixed in 10% neutral formalin, dehydrated in graded series of alcohol, embedded in a paraffin wax, sectioned at 5–7 μm and stained with hematoxyline and eosin. The diameter and germinative cell layer thickness of the seminiferous tubule (ST) from ten different areas of testes were measured by the aid of "Leica Q500 MC" image analyzer computer system. The data obtained were subjected to statistical analysis.

All values were given as mean ± standard error of measurement (S.E.). To determine the differences among all groups in the whole parameters one-way analysis of variance (ANOVA) and post hoc LSD analysis were performed using the SPSS/PC computer program (version 10). Statistical significance was determined at the level of significance of $p < 0.05$.

Table 1 Testis, epididymis and seminal vesicle weights (mg)

Parameters	Right testis	Right epididymis	Seminal vesicle
Control	97.3 ± 4.6	48.4 ± 1.8	166.0 ± 6.9
Oil	96.6 ± 5.3	46.7 ± 2.8	165.4 ± 6.6
21.25 mg/kg NP	92.1 ± 3.6	43.0 ± 3.8	164.1 ± 6.1
42.50 mg/kg NP	81.7 ± 2.9*	39.7 ± 2.4*	161.8 ± 4.8

* Indicates significant compared to control group ($p < 0.05$)

Table 2 Epididymal sperm count, sperm motility and abnormal sperm rate

Parameters	Sperm count ($\times 10^6$) sperm/m^3	Sperm motility (%)	Sperm abnormal rate (%)
Control	27.6 ± 0.54	85.9 ± 1.6	4.23 ± 0.30
Oil	27.1 ± 1.02	85.7 ± 0.6	3.80 ± 0.12
21.25 mg/kg NP	26.3 ± 0.73	78.3 ± 2.8*	4.60 ± 0.37
42.50 mg/kg NP	25.6 ± 0.66*	72.7 ± 2.6**	4.90 ± 0.36

*, ** Indicates significant compared to control group at ($p < 0.05$), ($p < 0.01$), respectively.

Results and Discussion

The values of reproductive organs weight and sperm characteristics are shown in Tables 1 and 2, respectively. In the present study, the higher dose of NP significantly reduced testes and epididymis weight and sperm count ($p < 0.05$) and motility ($p < 0.01$), while the lower dose only decreased significantly sperm motility ($p < 0.05$) in comparing to controls. No changes in the seminal vesicles weight and in the sperm abnormal rate were noticed in all-experimental groups. These results are consistent with Qiu et al. (2005) and Han et al. (2004) who attributed the decrease in epididymal weight and sperm density to the dysfunction of Sertoli cells and the apoptosis of Sertoli and germ cells.

Male exposure to 1/8 and 1/4 LD_{50} NP for 35 days had no effect on mating behavior or pregnancy rate. Neither causes any significant changes in number of implantation per litter (No. impl./litter), live fetuses, fetal body weight or external visible abnormalities when compared to the control groups (Table 3). These may be attributed to NP had limited toxic effect on spermatogenesis which was not extended to the fertility.

Histological studies showed few necrotic and degenerative changes in the form of vacuolar degeneration in groups treated with NP. The diameter and thickness of the germinative cell layer of ST were significantly smaller in the NP exposed groups even at the low dose level (Table 4). These histological measurements further supported our finding of a low testicular mass. The current

Table 3 Mating index, fertility index and paternal effect on fetuses

Groups	Mating (%)	Fertility (%)	No. impl./litter	Live fetuses	Fetal body weight (g)
Control	100	100	8.3 ± 0.4	91.0 ± 5.71	1.35 ± 0.03
Oil	85.7	100	7.2 ± 0.9	89.2 ± 6.64	1.33 ± 0.07
21.25 mg	85.7	100	7.2 ± 1.1	89.0 ± 5.09	1.25 ± 0.06
42.50 mg	85.7	100	7.6 ± 0.8	86.1 ± 5.20	1.23 ± 0.07

Table 4 Mean values of diameter size and germinative cell layer thickness of seminiferous tubules in testes tissue (μm)

Parameters	Diameter size	Germinative cell layer thickness
Control	237.7 ± 2.81	58.2 ± 2.33
Oil	236.2 ± 5.02	57.5 ± 2.49
21.25 mg/kg NP	221.5 ± 2.20**	46.7 ± 3.52**
42.50 mg/kg NP	213.1 ± 4.27**	34.8 ± 2.22**

** Indicates statistical significant compared to control group at ($p < 0.01$)

Table 5 Mean values of MDA (η mol/g tissue), GSH (mmol/g tissue) and SOD (unit/g tissue)

Parameters	MDA	GSH	SOD
Control	76.0 ± 9.9	1.65 ± 0.07	357 ± 6.9
Oil	87.1 ± 9.4	1.64 ± 0.04	351 ± 8.1
21.25 mg/kg NP	108.2 ± 8.0*	1.11 ± 0.03***	224 ± 7.9***
42.50 mg/kg NP	117.3 ± 9.9*	1.16 ± 0.06***	220 ± 8.6***

*, *** Indicates statistical significant compared to control group at ($p < 0.05$), ($p < 0.001$), respectively

result is consistent with de Jager et al. (1999) and Han et al. (2004).

In our study, oxidative stress was found in testes tissue following NP exposure indicated by significant increase in testes MDA concentrations ($p < 0.05$) and decrease in GSH levels and SOD activities ($p < 0.001$) (Table 5). Chitra and Mathur (2004) reported similar findings, the activities of antioxidant enzymes SOD and glutathione reductase decreased significantly while the level of lipid peroxidation increased significantly in adult rat testis administered orally NP at 1, 10, and 100 μg/kg b.wt./day of 45 days. Also Gong and Han (2006) clarified that NP induces oxidative stress to rat sertoli cells when exposed in vitro to 10–40 μm NP for 24 h. In the present study, the observed deleterious effects on sperm characteristics and testicular tissue may be attributed to peroxidation of unsaturated fatty acids in the plasma membrane that may lead to alteration of membrane characteristics and function.

In conclusion, we suggested that adult male mice exposed to high dose of NP (1/4 LD_{50}) for 35 days had effects on some reproductive organs weight and sperm characteristics (count and motility), testicular MDA, GSH, and SOD but did not influence the mating behavior, male fertility and the developed fetuses. NP acts to increase the testicular oxidative stress, which may be responsible partially for such defects.

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