Original

Effects of Maternal Exposure to Nonylphenol on Growth and Development of the Female Reproductive System and Uterine Carcinogenesis in Rats

Midori Yoshida¹, Takasumi Shimomoto¹, Sayumi Katashima¹, Tomoyuki Shirai², Dai Nakae¹, Gen Watanabe³, Kazuyoshi Taya³, and Akihiko Maekawa¹

¹Department of Pathology, Sasaki Institute, 2–2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–0062, Japan

²Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya, Japan

³Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu, Japan

Abstract: Effects of maternal exposure to nonylphenol (NP) on growth and development of the female reproductive system and uterine carcinogenesis in Donryu rats were investigated. Dams were administered 0, 0.1, 10 or 100 mg/kg NP daily by gavage from gestation day 2 up to the day before weaning. The treatment with NP did not influence the reproductive ability of the dams. In their female offspring, there were no significant effects on the reproductive system such as uterine growth and development, vaginal opening, and hormonal secretion until puberty. Moreover, NP had no apparent influence on estrous cyclicity after maturation, morphology of the reproductive organs, and uterine carcinogenesis initiated by N-ethyl-N'-nitro-N-nitrosoguanidine. Regarding biotransfer of NP, the chemical was detected at low levels in the milk of dams given NP at 10 and 100 mg/kg/day in a dose-dependent manner, but not in the serum. In the offspring also, NP was not detected in the liver in any of the treated groups. Taken together, maternal exposure of rats to 0.1 – 100 mg/kg NP did not have any effects on the female reproductive system of offspring from puberty up to 15 months of age. NP at 10 mg/kg and 100 mg/kg doses was transferred from dams to their offspring via the milk, but with these doses no accumulate in the liver of offspring was evident.

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Key words: nonylphenol, endocrine disrupting chemicals, female reproductive system, maternal exposure

Introduction

Alkylphenolic compounds are derived from biodegradation of nonionic surfactants, alkylphenol ethoxylates, which are widely used as lubricating oil additives, plasticizers, resins, detergents, and surface-active agents. The nonylphenol group of ethoxylates is broken down into nonylphenol (NP), mainly found in rivers¹. NP exerts weak estrogenic activity *in vivo* and *in vitro*^{2,3}, binding to both estrogen receptors (ER) α and ER β with low affinity⁴. Uterotropic effects of NP at high doses have been reported in immature or ovariectomized female rats^{2,5,6}, as well as with octylphenol^{7–9}.

The most serious issue with endocrine disrupting chemicals (EDCs) is potential effects of prenatal and/or neonatal exposure on offspring. Inappropriate exposure to

endogenous and/or exogenous estrogens is known to induce irreversible change in the reproductive system, with an influence on uterine carcinogenesis^{10–15}. However, the perinatal effects of EDCs on the reproductive organs in rodents are very complex and the underlying mechanisms remain to be determined in detail. In reproductive toxicity studies, high-dose NP exposure resulted in estrogenic-effects on pubertal development in male and female rats^{16–18}. However, relatively low dose maternal or perinatal exposure to NP demonstrated no adverse effects on the reproductive tract in rodents¹⁷, although perinatal treatment with estrogenic EDCs at doses comparable to human exposure levels has been reported to exert an influence^{19–22}. For human risk assessment, it is very important to determine the effects of actual exposure to EDCs on reproductive organs, but the studies so far conducted in accordance to the test guidelines for safety evaluation did not demonstrate adverse effects or the results were controversial. One reason for the latter is the lack of established endpoint markers to detect maternal or perinatal effects of EDCs in rodents, especially females. It has been reported that a 'delayed' influence of EDCs on the reproductive system of rodents exposed

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Mailing address: Midori Yoshida, Department of Pathology, Sasaki Institute, 2–2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–0062, Japan
TEL: 81-3-3294-3286 FAX: 81-3-3294-3290
E-mail: midoriy@sasaki.or.jp

perinatally may be manifested after puberty or sexual maturation^{15,23,24}. Thus, relatively long-term comprehensive studies of endocrinological and morphological aspects might be necessary for determination of the impact of perinatal treatment with EDCs. At the same time, with maternal exposure it is crucial to examine biotransfer of chemicals from dams to offspring, because the effects of EDCs on the target organs are fundamentally related to serum levels⁸. Little information is available regarding test compound transfer from dams to offspring via the placenta or milk.

The purpose of the present study was to investigate the effects of maternal exposure to NP at low to high exposure-doses, with reference to growth and development of the female reproductive tract and also uterine carcinogenesis in rats. For these purposes a relatively long-term period from prepuberty up to 15 months of age was employed with many parameters to detect effects of EDCs reported previously. In addition, we assessed transfer of NP to offspring via the placenta and milk.

Materials and Methods

Animals, NP treatment, and housing conditions

Thirty seven pregnant female Crj:Donryu rats at gestation day (GD) 2, checked by plugs and sperm in the vagina and judged to be pregnant by the breeder, were purchased from Charles River Japan (Kanagawa, Japan) and allocated into four groups: 0 mg/kg/day (vehicle controls, 10 dams), 0.1 mg/kg/day NP (Tokyo Kasei Kagaku, Tokyo, Japan, 10 dams), 10 mg/kg/day NP (10 dams), and 100 mg/kg/day NP (7 dams). The highest dose is known to have adverse effects, with uterotrophic- and cell-proliferative activity observed in the uterus and mammary glands in rats when given by gavage²⁵. The middle-dose was selected as near the no observed effect level in multiple generation reproductive study using rats¹⁶ and the lowest dose as relevant to human daily intake of isoflavones in Germany (1 mg/kg), because uterotrophic activity of NP was reported to be 10 times stronger than that of daidzein, a major isoflavone²⁶. NP was suspended in 0.05% carboxymethylcellulose (CMS) solution (Wako Pure Chemicals, Osaka, Japan) for this purpose. The females were orally administered NP or vehicle solution (0.05% CMC), every morning from GD 2 to the day before weaning (21 days after delivery) by gavage. The treatment period was selected to observe effects of maternal exposure to NP as long as possible. Commercial pellet diet (CRF-1, Oriental Yeast, Co., Japan) and drinking water stored in plastic containers were available *ad libitum* throughout the study. The day of birth was designated postnatal day (PND) 0. After delivery, dams with offspring were housed in plastic cages containing wooden chips, and litter sizes were adjusted to 8–10 pups/dam at PNDs 4 or 6. All pups were weaned at PND 21 and female pups in the same treatment group were housed together in cages (3 or 4 pups per cage). Animals were maintained in air-conditioned animal rooms under constant conditions of $24 \pm 2^\circ\text{C}$ and $55 \pm 10\%$

humidity with a 12-h light/dark cycle. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Examination of dams

Body weights of dams were checked once a week during the pregnancy and lactating periods. All dams were observed at least twice a day for morbidity, mortality and treatment-related clinical signs. Dams were euthanized at weaning (PND21 of their offspring) and the numbers of implantation sites in the uterus were recorded after complete necropsy. The uterus, vagina, ovaries, pituitary, adrenals, liver, and kidneys were fixed in 10% neutral buffered formaldehyde solution and examined histopathologically.

Examination of offspring

Body weights, sex, the number of offspring and external abnormalities were checked at PNDs 1, 7, 14 and 21.

Uterine growth and development at prepuberty and ovulation: To investigate uterine growth and development of female offspring up to puberty, 3 or 4 animals per group being different littermate were euthanized by decapitation at PNDs 10, 14, 21, and 28, the individual animals at each time-point being derived from different dams. After the uteri were weighed, the numbers of uterine glands were histopathologically quantified. Briefly, the uteri were fixed in 10% neutral buffered formalin solution, 21 cross sections per uterine horn were taken from upper, middle and lower parts of the bilateral uterine horn, and examined histopathologically. To assess ER α expression and cell proliferative activity in the developing uteri, serial uterine sections from slices used for measurement of uterine gland-genesis were incubated with anti-ER α and anti-proliferating cell nuclear antigen antibodies (Dako, Kyoto, Japan), for immunohistochemical comparison with control animals. In the morning of the estrus stage at 8 weeks of age, 4 animals per each group were euthanized and the numbers of ova in the oviduct were counted. The ovaries, vagina and other representative organs were fixed in 10% neutral buffered formaldehyde solution at PNDs 10, 14, 21, and 28 and 8 weeks of age and processed routinely for histopathological examination.

Hormonal profiles at prepuberty: Blood from the same animals used for histopathological examination was collected after decapitation and serum was stored at -80°C until assayed. Up to PND 14, pooled serum samples were used, since volume of serum per single animal was too small to allow analysis. Serum follicle stimulating hormone (FSH) and inhibin (INH) levels at PNDs 10, 14, 21, or 28 were measured using NIDDK radioimmunoassay kits for rat FSH^{27–29}.

Vaginal opening and estrous cyclicity: After weaning, female pups were checked daily for vaginal opening. After this was confirmed, estrous cyclicity in all animals was examined by vaginal cytology throughout the study.

Uterine carcinogenicity study: All female pups at 11

Table 1. Reproductive Ability and Body Weights of Offspring

	Group			
	0 mg/kg	0.1 mg/kg	10 mg/kg	100 mg/kg
No. of dams	10	10	10	7
Pregnant	10	10	10	7
At the termination of PND21	10	10	10	7
Pregnant period	21 ± 0.0 (#)	21.11 ± 0.33	21 ± 0.0	21 ± 0.0
No. of pups at birth (g)				
Female	5.6 ± 1.84	6.5 ± 1.84	6.3 ± 1.06	5.2 ± 2.66
Male	5.5 ± 1.96	5.9 ± 2.64	6.8 ± 1.75	6.5 ± 2.42
Total (a)	11.1 ± 2.13	12.4 ± 1.71	13.1 ± 1.20	12.0 ± 2.71
No. of dead pups during PNDs1–5				
Female	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Male	0 ± 0.00	0 ± 0.00	0.7 ± 1.89	0.3 ± 1.41
No. of implantation (b)	12.5 ± 1.35	13 ± 1.41	13.9 ± 0.99	13.0 ± 1.63
a/b	0.89 ± 0.15	0.95 ± 0.09	0.94 ± 0.07	0.9 ± 0.17

A ± B (#), Mean ± SD. PNDs, post natal days.

weeks of age were administered a single dose of 20 mg/kg N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG, Nacarai Tesque Inc., Tokyo Japan) into a uterine horn using a stainless steel catheter via the vagina, as reported previously³⁰. At 12 months of age, 5 or 6 animals per group were euthanized and examined histologically to evaluate development of uterine proliferative lesions. At the termination (15 months of age), all surviving animals underwent a histopathological examination. After complete necropsy, the reproductive and representative organs were fixed in 10% neutral buffered formalin, and then routinely processed. Animals found dead and killed when moribund were also examined similarly. Each uterus was cut into about 12–16 slices in cross-section for hematoxylin and eosin staining. Endometrial proliferative lesions were classified into three degrees of hyperplasia (slight, moderate or severe) and adenocarcinomas, according to our categories described previously³¹. In addition, adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae and tumors with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers³².

Serum and tissue concentration of NP in dams and their offspring

Serum and milk of dams were collected at weaning from offspring and at PNDs 10, respectively. The milk in the stomachs of the male and female pups was collected and pooled for each litter. In offspring, NP levels in the liver were sequentially measured at PNDs 21 and 28. The analysis was accomplished by gas chromatography mass spectrometry (QT-5050, Shimadzu, Kyoto, Japan) using the modified method reported previously⁸.

Fig. 1. Growth curve of female offspring up to the weaning. The body weights are comparable among the groups.

Statistical analysis

Values for incidences were statistically analyzed using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between NP-treated and control groups were made with the Dunnett's t-test. *p* Values less than 0.05 were considered to be statistically significant.

Results

The body weights of dams were comparable in the control and treated groups during the NP-treatment period (GD2 to PND21) and no treatment-related clinical signs were observed in any treated groups. Table 1 shows data of reproductive ability of dams. Examinations at birth and necropsy revealed no significant differences among the groups in the gestation period, the number of implantation sites, the average number of offspring per litter, and the body weights of offspring.

In female offspring, the growth curves were comparable among control and treated groups from prepuberty (Fig. 1) up to 15 months of age and no external abnormalities were detected in any offspring. Data of

Fig. 2. The incidence (%) of animals showed persistent estrus (PE) at their vaginal cytology. The number of the animals with PE is increased with advanced age and their incidence is not significantly different among the groups.

Table 2. Growth and Development of the Female Reproductive Organs in Offspring

Time of examined	Group			
	0 mg/kg	0.1 mg/kg	10 mg/kg	100 mg/kg
<i>Number of rats examined</i>				
PNDs10	4	3	4	2
PNDs14	4	3	4	3
PNDs21	4	4	4	4
PNDs28	4	4	4	4
17wks*	4	4	4	4
<i>Uterine weights (mg)</i>				
PNDs14	26.8 ± 6.0	28.3 ± 5.0	32.3 ± 4.3	21.0 ± 1.0
PNDs21	42.3 ± 5.6	41.0 ± 7.2	38.3 ± 7.7	46.3 ± 3.8
PNDs28	141.6 ± 34.2	187.3 ± 137.4	242.3 ± 145.2	290.8 ± 134.6
17wks	712.3 ± 117.7	653.3 ± 76.8	653.3 ± 76.8	720.8 ± 113.1
<i>Uterine gland-genesis (Number of gland per cross section)</i>				
PNDs10	0	0	0	0
PNDs14	4.07 ± 1.49	4.27 ± 1.74	4.29 ± 1.59	3.00 ± 0.87
PNDs21	4.57 ± 1.60	4.76 ± 1.15	5.31 ± 1.49	4.78 ± 2.46
PNDs28	6.00 ± 2.62	5.92 ± 1.26	5.73 ± 2.34	5.07 ± 1.77
<i>The time of vaginal opening (days)</i>				
	29.6 ± 1.8	30.1 ± 1.3	29.8 ± 1.1	29.0 ± 1.4
<i>Number of rats examined</i>				
	34	34	34	34
<i>Ovulation (Number of ova in the oviduct in the morning of estrus at 17wks of age)</i>				
	11.3 ± 1.5	11.3 ± 1.5	11.5 ± 1.0	12.3 ± 0.6

*; Examined in the morning at estrus stage.

females are shown in Table 2; the days of vaginal opening of offspring demonstrated no significant intergroup differences. Thereafter, precise 4-day cycles of estrous stages started in all animals. Persistent estrus, characterized by vaginal smears exhibiting nucleated epithelial and/or cornified cells, began to appear after 5 months of age in control animals and gradually increased with age in this group, all animals showing persistent estrus at 10 months of age, as shown in Fig. 2. In all NP-treated groups also, occurrence of persistent estrus demonstrated a similar profile. At PNDs 14, 21, 28, and 8 weeks of age, uterine

weights did not differ among the groups, and sequential changes in number of uterine glands in both treated and control animals were comparable (Table 2). No obvious changes in morphology, expression of estrogen receptor α or proliferative activity in the uterus were observed in any of the treated groups before puberty, compared to those in controls. Other endocrine tissues and representative organs showed no abnormalities in NP-treated and control groups. The numbers of ova at 8 weeks of age were not significantly different among the groups (Table 2).

Serum FSH and inhibin levels for NP-treated and

Fig. 3. Serum hormone profiles of follicle stimulating hormone, gonadotropin (A), and inhibin, a hormone secreted in the ovary (B) up to puberty. In all treated groups, FSH levels are comparable to that in controls. Inhibin levels were anti-related with levels of FSH.

Table 3. Incidence of Uterine Proliferative Lesions

Group	No. of rats examined	No. of rats without lesions	No. of rats with lesions			
			Hyperplasia			Adenocarcinoma
			Slight	Moderate	Severe	
0 mg/kg	24	3 (13)*	3 (13)	8 (33)	4 (17)	6 (25)
0.1 mg/kg	24	2 (8)	3 (13)	9 (38)	1 (4)	9 (38)
10 mg/kg	23	1 (4)	1 (4)	10 (43)	3 (13)	8 (35)
100 mg/kg	22	1 (5)	2 (9)	5 (23)	4 (18)	10 (45)

*( ); Percentage.

control rats at prepuberty are shown in Fig. 3. Up to PND 28, serum FSH and its linked inhibin levels were comparable among NP-treated and control groups, and the gonadotropins demonstrated no tendency for lowering with the treatment.

The incidences of uterine preneoplastic and neoplastic lesions are shown in Table 3. There were no significant differences and/or treatment-related tendencies among the groups. Sub-classification of adenocarcinomas by differentiation and invasion also demonstrated no variation. Most of the ovaries in all groups were atrophic with small cystic follicles and lacking corpus lutea. Various non-neoplastic and neoplastic lesions were observed in the representative organs and other endocrine tissues such as the liver, kidneys, adrenals, pituitary, and thyroids in all groups, although there were no significant differences among the NP-treated and control groups. Necropsy of animals found dead or euthanized when moribund did not reveal any treatment-related changes.

Serum and tissue concentrations of NP are summarized in Table 4. In dams, NP was detected in milk at PND 14 with dose dependence in the 10 and 100 mg/kg groups, but not serum. NP was not found to have accumulated in the livers

of offspring in any of the treated groups at PNDs 21 and 28, the latter being 7 days after the final treatment.

Discussion

Inappropriate exposure to estrogens or EDCs with estrogenic activity in the fetal and/or newborn period is well known to exert irreversible influence on the female reproductive system due to disruption of the hypothalamic-pituitary-ovary controlled system. Typically 'androgenized' influences appear in prepuberty, characterized by lowering of gonadotropin levels, anovulation, hypoplastic ovary, persistent estrus status immediately after early vaginal opening, and abnormal uterine development, with inhibition of gland-genesis and anomalous ER α expression^{10,14,15,33}. In addition to the typical 'androgenized' effects described above, perinatal exposure may also cause 'delayed' effects including the 'anovulatory syndrome'^{15,23,24}. For example, high-dose exposure to *p*-tert-octylphenol, an alkylphenolic compound with weak estrogenic activity, during the first 5 days after birth in female Donryu rats exerted a delayed influence, detected as ovarian atrophy with polycystic

Table 4. Tissue and Serum Concentration of Nonylphenol

	Group			
	0 mg/kg	0.1 mg/kg	10 mg/kg	100 mg/kg
<i>Dam</i>				
Serum level at PNDs21	<0.1 ppm*	<0.1 ppm	<0.1 ppm	<0.1 ppm
Number of dam pooled	5	5	5	5
Milk at PNDs14	<0.1 ppm	<0.1 ppm	0.4 ppm	1.6 ppm
Number of pup stomach collected	8	8	8	6
<i>Offspring</i>				
Liver at PNDs21	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm
Number of pup pooled	4	4	4	6
Liver at PNDs28	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm
Number of pup pooled	4	4	4	4

*, Under the detectable limit.

Table 5. Comparison of Effects on the Female Reproductive System in the Present Study with Previous Studies Showing Typical Androgenized- or Delayed Androgenized-effects

	Nonylphenol by gavage (present study)			Octylphenol (previous studies)	
	0.1 mg/kg	10 mg/kg	100 mg/kg	Typical androgenization (a) 100 mg/kg subcutaneous	Delayed-androgenization (b) 100 mg/kg subcutaneous
<i>Examinations at prepuberty</i>					
Growth	N (c)	N	N	N	N
Uterine weight	N	N	N	N	N
Uterine gland-genesis	N	N	N	↓(d)	N
Expression of estrogen receptor in the uteri	N	N	N	Abnormal	N
Time of vaginal opening	N	N	N	Early opening	N
Gonadotropin secretion	N	N	N	↓	N
<i>Examinations after maturation</i>					
Ovulation	N	N	N	Anovulation	N
Estrous cyclicity	N	N	N	PE (e)	Normal after vaginal opening but earlier occurrence of PE
Uterine carcinogenesis	N	N	N	Enhanced	Enhanced

(a); Exposure of octylphenol during first 15 days after the birth referred by Katsuda *et al.*, 2000A(14) and Yoshida *et al.*, 2002A(33).

(b); Exposure of octylphenol during first 5 days after the birth Referred by Yoshida *et al.*, 2002B(15).

(c); Normal or comparable data compared with those in the control animals.

(d); Decreased.

(e); PE, Persistent estrus at vaginal cytology.

follicles resulting in early persistent estrus compared to aged-matched control animals, although no apparent abnormalities were found up to maturation¹⁵. In the present study, however, NP-treated animals showed no abnormalities in gonadotropin and associated ovarian hormone secretion, or in uterine growth and development. Thus uterine gland-genesis and ER α expression as well as the time of vaginal opening and subsequent sexual maturation were comparable to those in controls. The treatment also did not exert any effects on ovulation and estrous cyclicity throughout the study, as compared to the age-matched control animals or our control data for the Donryu strain rat. The results clearly demonstrated that maternal treatment with 0.1 – 100 mg/kg NP did not exert any influence on the female reproductive system of offspring

at prepuberty, and delayed modulation of the system after sexual maturation appeared lacking.

The most striking examples of the effects caused by EDCs on the female reproductive system are induction of vaginal or uterine cancers in humans and rodents^{34–36}. Recently many studies of induction of uterine endometrial adenocarcinomas in rodents by perinatal treatment with estrogenic compounds or EDCs have been conducted^{12,15,35,37}. The uterine endometrial adenocarcinoma is one of the most common malignant tumors in women and has increased in number in recent years, although some epidemiological aspects remain unclear^{38,39}. The Donryu strain rat is a high-incidence strain for spontaneous endometrial adenocarcinomas and the tumors have morphological and biological similarities to those found in

women^{40–42}. Similar to the human case, ovarian hormonal imbalance is a crucial factor. In particular, the model features early occurrence of ovarian atrophy with cystic atresia follicles and lack of corpus lutea, associated with prolonged elevation of the serum estrogen/progesterone ratio and persistent estrus in vaginal cytology⁴³. In the present study, maternal treatment of NP did not exert any influence on estrous cyclicity and uterine carcinogenesis. To determine ovarian function in rats, examination of estrous cyclicity might be the most useful indicator, as previously reported⁴⁴.

For assessment of effects of EDCs on offspring with maternal treatment, biotransfer from dams is crucial because the effects on the target organs are fundamentally related to serum EDCs levels⁸. However, data for transfer of test compound via the placenta or milk to offspring and toxicokinetics of low-dose EDCs were very limited⁴⁵. In the present study, NP levels in the milk of the 10 and 100 mg/kg groups were elevated, but the compound was not detected in the serum of dams and the liver of offspring.

A summary of the present study results in comparison with androgenized- or delayed androgenized-effects reported previously is given in Table 5. We can conclude that transplacental and lactational exposure to various doses NP does not appreciably influence the growth and development of the female reproductive system or sensitivity to uterine carcinogenesis. No accumulation of the compound was found in the offspring livers, although NP was detectable in the milk at 10 and 100 mg/kg.

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