Twenty-Eight-Day Repeated-Dose Toxicity Studies for Detection of Weak Endocrine Disrupting Effects of Nonylphenol and Atrazine in Female Rats

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Abstract: To assess the risk evaluation of endocrine disrupting chemicals with weak estrogenic or anti-estrogenic effects in mammals, the suitability of 28-day repeated dosing test and the strain difference were investigated in adult female rats. In the study, 60 and 250 (150) mg/kg/day of nonylphenol and 5 and 50 mg/kg/day of atrazine were dosed to female SD, F344, and Donryu rats for 28 days. Abnormal estrous cyclicity was observed in both groups of SD rats given nonylphenol, in the high-dose nonylphenol and atrazine F344 rats, and in both groups of Donryu rats receiving the test compounds. A slight tendency for increase in numbers of uterine BrdU-positive cells was observed in F344 rats given high-dose nonylphenol, and elevation was noted in one animal each which showed persistent estrus in the groups of Donryu rats given atrazine. Organ weight, necropsy and histopathological findings did not point to any effects of these chemicals on the endocrine or reproductive systems except for ovarian atrophy in the atrazine treated Donryu rats with persistent estrus. These results indicate that severe morphological effects might not be induced in female reproductive organs of adult rats by short-term exposure to these chemicals, unless very high doses are given. Vaginal smear may be the most sensitive parameter for detection of estrogenic or anti-estrogenic effects of chemicals, when normal cycling animals are used for the study. (*J Toxicol Pathol* 2000; 13: 13~20)

Key words: Nonylphenol, atrazine, endocrine disrupters, 28-day repeated study, rat

Introduction

Recently, a number of chemicals have been identified which disrupt normal endocrine functions and misgivings about their existence are rapidly rising worldwide, although the actual hazards posed to man and wildlife remain unclear^{1,2}. To detect the estrogenic or anti-estrogenic activity of environmental chemicals, the uterotrophic assay using immature or ovariectomized rats has been proposed as one of the screening test systems by OECD. On the other hand, for assessment of toxic effects of environmental chemicals, a short-term screening test protocol has been proposed as OECD Test Guideline (TG) 407: A 28-day repeated toxicity test protocol using adult rats as a rule. In addition, for endocrine disrupting chemicals, OECD has recently proposed a new screening test protocol, the enhanced OECD TG 407 protocol.

Nonylphenol, a surfactant used in the production of laboratory plasticware, and has been reported to have estrogenic activity^{3,4}. Atrazine is a chloro-s-triazine herbicide that has been widely employed for agricultural applications for many years and has anti-estrogenic activity⁵⁻⁷. However, the endocrine disrupting potential of these two compounds is relatively weak⁷⁻¹⁰, and their toxicity data with adult female rats with normal estrous cycle are very few.

On the other hand, estrogens are of essential importance for development of uterine endometrial adenocarcinomas and mammary carcinomas in mammals. In rats, however, spon-

taneous occurrence of endometrial adenocarcinomas is generally very rare, and estrogen alone can not induce carcinomas¹¹, although it acts as a complete carcinogen in mice^{11,12}. SD and F344 Fischer rats are the strains most widely used in toxicity study including carcinogenicity studies throughout the world. However, this may present problems because their spontaneous occurrence of endometrial adenocarcinomas is very low¹³. The Donryu rat, an inbred Japanese albino rat, is known to be a high incidence strain for spontaneous development of endometrial adenocarcinomas, associated with a hormonal imbalance¹⁴. Donryu rats are more sensitive to carcinogens/hormones than F344 rats with regard to uterine carcinogenesis¹¹. Strain-differences have been reported between F344 and SD rats for example concerning the induction incidence of mammary tumors by the endocrine disrupter, atrazine¹⁵. These strain differences might effect on the sensitivity of screening tests to detect endocrine disrupting activity of chemicals, because many toxicokinetic data revealed strain differences in the biological effects of chemicals in rats¹⁶.

To assess the risk evaluation of endocrine disruption chemicals with weak estrogenic or anti-estrogenic effects in mammals, 28-day repeated toxicity and strain difference of nonylphenol and atrazine were investigated in adult female rats.

Materials and Methods

Chemicals

Nonylphenol (mixed isomer, Lot no. JI208, purity: 99.6%) was provided by Mitsui Chemical Co. Ltd. (Tokyo), and atrazine (C₈H₁₄ClN₅, Lot no. MSF9593, purity: 98.7%)

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was purchased from Wako Pure Chemical Industries Ltd. (Osaka).

Animals and housing conditions

Ten-week-old female Crj : CD (SD) IGS, F344/DuCrj (Fischer), and Crj : Donryu rats were purchased from Charles River Japan, Inc. (Yokohama). After quarantine and acclimation for 2 weeks, only healthy animals showing favorable body weight gain, good general condition, and normal estrous cycles were used for the study. Groups of six animals were allocated using body weight-stratified randomization.

The animals were housed, one/cage in an animal room in which the temperature, relative humidity, and ventilation rate were set at $23 \pm 2^\circ\text{C}$, $55 \pm 10\%$, and 10–15 times/h, respectively, and allowed free access to pelleted diet (MF, Oriental Yeast Co., Ltd., Tokyo) and tap water. All experiments in the present study followed the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science¹⁷.

Experimental design

Nonylphenol was dissolved in olive oil, and atrazine was suspended in 0.5% sodium carboxy-methyl-cellulose. The rats of each strain were dosed at levels of 0, 60 or 250 mg/kg/day nonylphenol and 0, 5 or 50 mg/kg/day atrazine by stomach tube for 28 days or till the day on which their vaginal smear indicated diestrus after 28 days. The 250 mg/kg dose of nonylphenol was changed to 150 mg/kg at 5 days because the general condition of rats of all SD and F344 strains had markedly deteriorated.

The animals were observed for condition and behavior on a daily base during the dosing period. Body weights were measured at 1, 8, 15, 22, and 28 days of dosing and just before necropsy. All animals were examined for their estrous cycles every day during the dosing period, and necropsied on day 28 when their estrous cycle showed diestrus or the day showing diestrus after 28 days. One Donryu rat each in the 5 and 50 mg/kg of atrazine treated groups with vaginal smears indicating persistent estrus was sacrificed on day 44, these animals being given the chemical for 43 days. The animals which showed estrus for more than three days continuously were designated to be in persistent estrus. Wet organ weights of the hypophysis, liver, adrenals, kidneys, ovaries, and uterus were determined. In addition to the organs mentioned above, the vagina and mammary gland were fixed in neutralized buffered 10% formalin, processed routinely, and examined histopathologically. To examine the number of cells undergoing division, based on S-phase incorporation of marker, all animals were injected with a single shot of 5-bromo-2'-deoxyuridine (BrdU, 40 mg/kg of body weight, Sigma Chemical Co., USA) intraperitoneally 2 hr before necropsy, and sections of uterus were incubated with BrdU antibody (Dako Japan, Kyoto) using the ABC method, as described previously¹⁸. The numbers of BrdU-positive nuclei per 1,000 nuclei in the luminal epithelial cells were counted per animal.

Statistical analysis

With regard to body and organ weights, and numbers of S-phase cells, one-way analysis of variance was used for testing the significance of intergroup differences.

When significance was revealed in this analysis, the Dunnett's multiple comparison test¹⁹ was employed.

Results

General condition

In the nonylphenol treated SD rats of the 250 mg/kg group, decrease of spontaneous locomotion and respiratory rate, nasal bleeding, staining around the nose and mouth, lid closure, soft stools, moisture at the lower abdomen, and increased salivation were observed. Staining around the nose and mouth and moisture at the lower abdomen were also observed in the 60 mg/kg group. Decrease of spontaneous locomotion and respiratory rate, lid closure, soft stools, nasal bleeding, staining around the nose and mouth, moisture at the lower abdomen, decreased stool volume, unkempt hair, crouching position, reddish tears, and mucous stool were also observed in F344 rats of the 250 mg/kg nonylphenol group. These findings were almost recovered after 5 days when the 250 mg/kg dose was diminished to 150 mg/kg, although one SD rat in the 250 (150) mg/kg group died from an accident on day 9 of dosing. In contrast, in Donryu rats receiving the high-dose, only reddish tears and increased salivation were observed. After change of dosage, these changes detected in high dose groups of every three strain recovered. In the atrazine treated groups, no abnormalities of general condition were detected in SD or Donryu rats, although increased salivation was noted in F344 rats of the 50 mg/kg group.

Body weights

Body weights of animals given nonylphenol and atrazine are shown in Tables 1 and 2. In the nonylphenol treated groups, SD and F344 rats in the high-dose group demonstrated decreased body weights on day 8 of dosing, but then recovered. No effects on Donryu rat body weight gain were apparent.

In the atrazine treated groups, F344 and Donryu but not SD rats in the 50 mg/kg group had a slight tendency for decrease of body weights on days 22 and 28, compared to the control groups.

Estrous cycle

Data for estrous cycle abnormality in animals given nonylphenol and atrazine are summarized in Tables 3 and 4. One SD rat in the 60 mg/kg nonylphenol group showed an abnormal estrous cycle from days 6 to 9, although this then recovered. In the 250 (150) mg/kg group, 5 animals demonstrated abnormal cycles from days 5 or 7 of dosing, this recovering from day 9 in 2 cases but persisting in the remaining 3. In F344 rats, all animals in the high-dose group showed similar abnormal cycles from days 3 or 7, with recovery from days 9 to 15. Two of these animals showed persistent estrus continuing for 3 days. In the Donryu case, one 60 mg/kg animal showed an abnormal cycle from days 9 to 13, and two in the 250 (150) mg/kg group, one from days 5 to 15 and the other from day 26 onwards.

In the atrazine treated groups, there were no abnormalities of the estrous cycle in SD rats. One F344 rat in the 50 mg/kg group showed persistent estrus continuing for 4 days from day 18, but recovered after that. In Donryu rats, one animal each in the 5 and 50 mg/kg groups also demonstrated persistent estrus. In these animals, the estrous cycle stopped from day 31 or 33, persistent estrus continuing for 14 and 12 days, respectively.

Table 1. Body Weights of Rats Dosed with Nonylphenol for 28 Days

Strain	Doses (mg/kg/day)	Experimental days					
		-1	1	8	15	22	28
SD	0	239.4	238.4	244.6	247.1	251.3	258.1
		± 6.5	± 8.1	± 8.0	± 6.2	± 12.0	± 11.8
	60	239.0	237.3	238.4	238.8	242.9	250.3
		± 9.3	± 10.5	± 8.9	± 12.4	± 14.7	± 15.4
	250-150 ^{a)}	239.7	236.4	227.9	243.5	251.1	255.6
		± 5.9	± 6.3	± 25.2	± 8.3 (5) ^{b)}	± 11.2 (5)	± 9.6 (5)
F344	0	155.4	154.3	155.9	160.4	163.9	165.5
		± 7.8	± 6.4	± 8.9	± 11.1	± 10.4	± 11.3
	60	155.7	155.6	156.6	161.2	167.1	168.9
		± 4.7	± 4.0	± 4.3	± 4.9	± 6.4	± 7.9
	250-150 ^{a)}	156.0	154.8	133.7	158.9	169.9	174.1
		± 4.9	± 4.2	± 21.3	± 12.2	± 8.3	± 7.5
Donryu	0	264.6	264.8	266.9	269.3	273.7	281.4
		± 6.1	± 8.0	± 7.0	± 9.4	± 10.0	± 8.2
	60	264.4	264.9	263.0	267.8	268.8	272.5
		± 6.9	± 8.4	± 5.0	± 9.3	± 11.5	± 7.6
	250-150 ^{a)}	264.3	262.6	260.6	269.5	271.6	276.5
		± 5.5	± 8.1	± 9.5	± 12.8	± 10.9	± 9.5

Mean (g) ± S.D.

^{a)}Dosed at a level of 250 mg/kg from days 1 to 4 of dosing and at 150 mg/kg thereafter.^{b)}Values in parentheses are the numbers of animals used for the calculation.**Table 2.** Body Weights of Rats Dosed with Atrazine for 28 Days

Strain	Doses (mg/kg/day)	Experimental days							
		-1	1	8	15	22	28	36	43
SD	0	238.4	237.5	242.2	241.5	244.5	250.1		
		± 12.8	± 12.7	± 15.9	± 14.6	± 17.1	± 16.6		
	5	238.2	236.4	243.6	245.3	250.9	258.3		
		± 6.8	± 9.6	± 4.9	± 6.1	± 11.3	± 10.7		
	50	238.4	237.3	238.7	242.8	244.8	256.6		
		± 8.6	± 6.1	± 11.2	± 9.4	± 5.6	± 7.0		
F344	0	155.3	154.4	156.7	162.5	169.6	172.2		
		± 6.6	± 6.0	± 6.2	± 5.7	± 6.8	± 6.8		
	5	155.9	154.2	157.6	161.6	165.9	170.2		
		± 5.8	± 6.5	± 5.6	± 5.9	± 7.8	± 9.3		
	50	155.8	154.4	154.1	158.4	159.1	162.7		
		± 5.0	± 5.2	± 9.7	± 8.3	± 8.9	± 8.4		
Donryu	0	264.8	261.3	269.6	277.5	280.6	283.6		
		± 6.5	± 10.2	± 11.1	± 6.8	± 11.1	± 9.7		
	5	264.2	265.9	270.2	271.8	282.1	283.2	263.5 (1) ^{a)}	276.3 (1)
		± 6.0	± 8.9	± 5.8	± 9.6	± 9.8	± 9.7		
	50	264.4	260.7	270.5	276.4	272.1	277.8	273.7 (1)	288.3 (1)
		± 7.3	± 10.3	± 14.5	± 23.1	± 17.6	± 18.0		

Mean (g) ± S.D.

^{a)}Values in parentheses are the numbers of animals used for the calculation.

Organ weight

Data for relative organ weights are summarized in Tables 5 and 6. Absolute organ weights showed similar tendencies to relative organ weights (data not shown). In nonylphenol treated SD and Donryu rats, there were no abnormalities in organ weights of the uterus, adrenal, kidney,

ovary, or hypophysis, although liver weights were increased with the 250 (150) mg/kg dose. In F344 rats, liver, kidney, and adrenal weights were significantly increased in 250 (150) mg/kg group. In this rat strain, significant decrease of uterine weight was also observed in the 60 mg/kg group, although there was no dose-effect relation.

In the atrazine treated groups, there were no abnormal-

Table 3. Estrous Cycle Abnormality in Rats Dosed with Nonylphenol for 28 Days

Strain	Doses (mg/kg/day)	No. of animals with abnormal cycles
SD	0	0/6
	60	1/6
	250-150 ^{a)}	5/6 [#]
F344	0	0/6
	60	0/6
	250-150 ^{a)}	6/6 (2 : persistent estrus for 3 days)
Donryu	0	0/6
	60	1/6
	250-150 ^{a)}	2/6

^{a)}Dosed at a level of 250 mg/kg from days 1 to 4 of dosing and at 150 mg/kg thereafter.

[#] Including one animal which died.

Table 4. Estrous Cycle Abnormality in Rats Dosed with Atrazine for 28 Days

Strain	Doses (mg/kg/day)	No. of animals with abnormal cycle
SD	0	0/6
	5	0/6
	50	0/6
F344	0	0/6
	5	0/6
	50	1/6 (persistent estrus for 4 days)
Donryu	0	0/6
	5	1/6 (persistent estrus for 14 days)
	50	1/6 (persistent estrus for 12 days)

ities in organ weights of the uterus, adrenal, kidney, ovary, or hypophysis in any rat strain, although liver weights were increased in the 50 mg/kg groups of both SD and Donryu rats. In the latter, decrease of ovary weight was seen in one animal each showing persistent estrus in the 5 and 50 mg/kg groups (14.1 and 18.9 mg/100 g, respectively), although there were no significant differences in mean ovarian weights between the controls and the two treated groups.

Necropsy findings

In the nonylphenol treated groups of all rat strains, no abnormalities attributable to the test chemical were found.

In the atrazine treated groups, there were also no abnormalities in SD or F344 rats, although small ovaries were found in each one animal showing persistent estrus in the 5 and 50 mg/kg groups of Donryu rats.

Histopathological findings

In the nonylphenol treated groups of all rat strains, there were no abnormalities in the uterus and ovary, the height of endometrial epithelial cells being the same in the controls and treated rats, although swelling of hepatocytes was observed in F344 rats of the 250 (150) mg/kg group. There were also no

abnormalities in the vagina, mammary gland, hypophysis, or adrenals.

In the atrazine treated groups, there were no abnormalities in the female genital organs of SD or F344 rats. In Donryu rats, however, corpora lutea were not seen in the ovaries (Fig. 1) of one animal each in the 5 and 50 mg/kg groups, the ovaries being small in size. There were no abnormalities in the uterus or vagina of all treated rats including these two animals.

Mitotic cells in the uterine endothelium

In the nonylphenol treated groups, there were no differences in numbers of BrdU-positive cells in the uterine epithelium among groups of SD and Donryu rats given 250 (150) mg/kg. However, F344 rats in the high-dose group had tendencies for slightly increased numbers of S-phase cells but the standard deviation was very large (Table 7).

In the atrazine treated SD and F344 groups, there were no significant differences. In Donryu rats, increase of BrdU-positive cells was obvious in one animal each of the 5 and 50 mg/kg groups showing persistent estrus (89.69 and 79.32 respectively), but there was no difference at the group level, when numbers of S-phase cells were compared between the controls and both treated groups excluding one animal each showing persistent estrus (Table 8).

Discussion

In the uterotropic assay using immature or ovariectomized rats, uterine weight and luminal epithelial cell-height have been proposed to be good parameters for detection of estrogenic and anti-estrogenic effects of chemicals²⁰⁻²². It has been reported that nonylphenol exerts uterotrophic effects in immature female rats dosed intraperitoneally at levels of 20 and 50 mg/animal with a dose-dependent increase in uterine weights²³, also increases the mitotic index in endometrial epithelium of ovariectomized rats dosed at levels of 1.0, 2.0, and 5.0 mg/animal³. In the present study, there was no tendency for dose-dependent increase of uterine weight in any strains of rats given nonylphenol, and there were also no differences in the height of uterine epithelial cells between control and treated groups of nonylphenol. In the present study, abnormal estrous cyclicity was observed in the high-dose groups of SD, F344, and Donryu rats given nonylphenol, although the incidence was low in Donryu rats. This might have been due to direct effects of this chemical, because alteration in general condition and body weight was limited, at least after cessation of the 250 mg/kg dose, and abnormal cyclicity was also seen in the low-dose groups of SD and Donryu rats in which no or only slight changes of general condition were shown. However, these abnormal cyclicities in some animals of SD and F344 rats given the high-dose of nonylphenol were recovered after cessation of the 250 mg/kg dose. Thus, the possibility that abnormal cyclicity observed in these cases is due to indirect causes, can not be denied, because it is well known that stress induces abnormal cyclicity in rodent²⁴. In the present study, one SD rat in the 250 (150) mg/kg group died on day 9. In this animal, broad necrosis and calcification of the cardiac muscle were histologically observed, and these findings were considered to be the cause of death. However, no similar lesion was observed in any of other rats given the same dose in this study, and also in the previous report²⁵ by Okazaki in which dosage and treatment period of nonylphenol were almost the

Table 5. Relative Organ Weights of Rats Dosed with Nonylphenol for 28 Days

Strain			Doses (mg/kg/day)		
			0	60	250-150 ^{a)}
SD	Liver	(g/100 g)	3.10±0.19	2.96±0.09	3.57±0.21**
	Kidney	(g/100 g)	0.61±0.03	0.63±0.04	0.63±0.06
	Uterus	(g/100 g)	0.21±0.05	0.23±0.08	0.19±0.07
	Adrenal	(mg/100 g)	24.6±1.6	26.1±3.1	26.4±2.0
	Ovary	(mg/100 g)	30.4±4.7	31.5±4.6	31.4±4.2
	Hypophysis	(mg/100 g)	6.0±1.1	5.7±0.9	5.9±0.7
F344	Liver	(g/100 g)	2.70±0.07	2.97±0.08	3.54±0.20**
	Kidney	(g/100 g)	0.66±0.02	0.64±0.03	0.76±0.03**
	Uterus	(g/100 g)	0.28±0.07	0.20±0.02**	0.23±0.03
	Adrenal	(mg/100 g)	27.3±3.5	28.1±3.3	33.7±1.1**
	Ovary	(mg/100 g)	32.5±3.9	30.5±2.4	28.5±3.1
	Hypophysis	(mg/100 g)	6.0±1.3	6.0±0.8	5.9±1.2
Donryu	Liver	(g/100 g)	3.37±0.11	3.46±0.29	3.99±0.24**
	Kidney	(g/100 g)	0.60±0.04	0.62±0.04	0.61±0.05
	Uterus	(g/100 g)	0.22±0.04	0.23±0.02	0.23±0.07
	Adrenal	(mg/100 g)	25.8±2.4	27.0±1.1	28.8±4.9
	Ovary	(mg/100 g)	29.2±4.7	33.4±3.6	29.2±3.6
	Hypophysis	(mg/100 g)	4.3±0.5	4.1±0.3	4.8±1.2

Mean±S.D.

^{a)}Dosed at a level of 250 mg/kg from days 1 to 4 and at 150 mg/kg thereafter.**Significantly different from the control at $p<0.01$.**Table 6.** Relative Organ Weights of Rats Dosed with Atrazine for 28 Days

Strain			Doses (mg/kg/day)		
			0	5	50
SD	Liver	(g/100 g)	3.01±0.07	3.16±0.23	3.36±0.18**
	Kidney	(g/100 g)	0.65±0.07	0.65±0.05	0.71±0.03
	Uterus	(g/100 g)	0.21±0.04	0.19±0.04	0.17±0.04
	Adrenal	(mg/100 g)	25.8±4.6	24.3±4.2	24.3±2.0
	Ovary	(mg/100 g)	30.4±5.8	34.8±6.2	36.2±2.6
	Hypophysis	(mg/100 g)	5.5±0.5	5.3±1.2	6.1±0.9
F344	Liver	(g/100 g)	2.88±0.07	2.97±0.16	3.02±0.15
	Kidney	(g/100 g)	0.66±0.02	0.70±0.04	0.72±0.04
	Uterus	(g/100 g)	0.31±0.10	0.29±0.11	0.33±0.11
	Adrenal	(mg/100 g)	26.6±3.0	29.4±2.8	30.5±3.1
	Ovary	(mg/100 g)	33.6±3.8	35.7±4.2	38.6±4.6
	Hypophysis	(mg/100 g)	6.3±1.2	6.6±0.7	6.4±0.5
Donryu	Liver	(g/100 g)	3.57±0.17	3.74±0.16	4.04±0.15**
	Kidney	(g/100 g)	0.67±0.03	0.63±0.03	0.68±0.04
	Uterus	(g/100 g)	0.21±0.03	0.22±0.04	0.20±0.03
	Adrenal	(mg/100 g)	24.1±2.0	24.6±3.6	25.9±2.2
	Ovary	(mg/100 g)	33.7±3.3	29.3±9.7	32.5±7.7
	Hypophysis	(mg/100 g)	5.1±1.0	4.9±1.0	5.7±1.5

Mean±S.D.

**Significantly different from the control at $p<0.01$.

Fig. 1. A : A Donryu rat given 0 mg/kg atrazine. Corpora lutea (arrow) are clearly observed. HE, $\times 30.0$. B : A Donryu rat showing persistent estrus in the 50 mg/kg atrazine group. Cystic enlargement of the follicles is predominant and corpora lutea are not observed. HE, $\times 30.0$.

Table 7. BrdU Labeled Cells in the Endometrial Epithelium of Rats Dosed with Nonylphenol for 28 Days

Strain	Doses (mg/kg/day)	No. of animals examined	No. of labeled cells/1,000 cells
SD	0	6	25.45 ± 16.66
	60	6	19.51 ± 12.58
	250-150 ^{a)}	5	23.80 ± 23.08
F344	0	6	30.14 ± 32.21
	60	6	17.35 ± 21.10
	250-150 ^{a)}	6	68.65 ± 54.75
Donryu	0	6	19.77 ± 12.96
	60	6	12.94 ± 16.26
	250-150 ^{a)}	6	23.17 ± 13.10

Mean $\pm$ S.D.

^{a)}Dosed at a level of 250 mg/kg from days 1 to 4 of dosing and at 150 mg/kg thereafter.

Table 8. BrdU Labeled Cells in the Endometrial Epithelium of Rats Dosed with Atrazine for 28 Days

Strain	Doses (mg/kg/day)	No. of animals examined	No. of labeled cells/1,000 cells
SD	0	6	16.29 ± 20.53
	5	6	17.72 ± 13.60
	50	6	11.06 ± 11.05
F344	0	6	17.30 ± 18.50
	5	6	15.47 ± 11.16
	50	6	28.10 ± 27.69
Donryu	0	6	15.79 ± 14.60
	5	5 [#]	11.29 ± 15.96
	50	5 [#]	15.94 ± 17.51

Mean $\pm$ S.D.

[#]Excluding one animal each showing persistent estrus.

same, and the rat strain used was also the same. Thus the lesion was considered to be accidental. Although only slight tendencies for increase in numbers of BrdU-positive cells, but not significant, were observed, it was limited to the high-dose nonylphenol group of F344 rats, and it is uncertain whether the result is meaningful or not. These results indicate that adult rats are less sensitive than immature or ovariectomized animals, since almost the same or higher dosage levels as for the studies using immature animals or ovariectomized animals were used in the nonylphenol treated groups of the present study in line with the previous reports^{3,23}. Uterine weight and epithelial cell-height may be less sensitive than vaginal smear for evaluation of estrogenic

activity of chemicals, when adult animals are used for the study. In the previous 28-day toxicity study of nonylphenol²⁵, no endocrine disrupting effects were detected because estrous cycle was not checked. Thus, examination of estrous cycle by vaginal smear is considered to be very useful for evaluation of estrogenic activity of chemicals in 28-day toxicity study. Further studies with other chemicals should be needed in these points of view. In the present study, nonylphenol was given orally, because the main exposure route in humans is oral. However, oral application might be less sensitive than intraperitoneal or subcutaneous ones because the latter eliminates direct effects of metabolism of the chemicals during first passage through the liver.

Contrary to expectation, however, Donryu rats was not so sensitive, compared with F344 and SD rats, for detection of week estrogenic activity.

In the atrazine treated groups, persistent estrus was observed in F344 and Donryu. Although many toxicokinetic data revealed strain difference in the biological effects of chemicals in rats¹⁶, strain difference was not observed in the present study, further studies with other chemicals being needed for rats. This might have also been considered to be effects of atrazine treatment, because it has been reported that atrazine causes disruption of estrous cyclicity in laboratory rats, increasing the day in estrus²⁶, and there are no animals with persistent estrus in our background data in which these strains of animals were examined within 3 months of age. In addition, in Donryu rats, ovarian atrophy was observed in animals showing persistent estrus. In this strain of rats, the ovary weight decrease with age, and after 12 months of age it shows one-fourth the relative value observed at 1.5 months of age, although the relative weights remain almost constant in F344 rats after 6 months of age²⁷. Atrazine is known to show anti-estrogenic effects via the pituitarygonadal axis²⁸. These results indicate that atrazine might progress the development of age-dependent ovarian atrophy in Donryu rats. It is very interesting that increase of BrdU-positive cells in the uterine endometrial epithelium was obvious in rats with ovarian atrophy, because atrazine is reported to induce endometrial adenocarcinomas in rats²⁹. However, the possibility that these findings are not related to atrazine-treatment, may remain, since persistent estrus and ovarian atrophy were seen only in each one animal of both 5 and 50 mg/kg groups, and there were also no dose-effect relations in ovarian weights and BrdU positive cells of these animals. Further studies using large numbers of animals and many doses of the chemicals should be needed to confirm the effect of atrazine.

In the present study, liver weights were affected in any strain of rat given nonylphenol or atrazine, along with kidney weights in F344 rats given nonylphenol. This is in line with the previous reports that two compounds induce hepato- and/or renal toxicity in rats^{25,30,31}.

These results indicate that severe morphological effects might not be induced in female reproductive organs of adult rats by short-term exposure to these chemicals, because of homeostasis in animals, unless very high doses are given. Vaginal smear may be the most sensitive parameter for detection of estrogenic or anti-estrogenic effects of chemicals, when normal cycling animals are used for the study. On the other hand, putative endocrine disrupters with estrogenic or anti-estrogenic activities exist in very low concentrations in the environment, and mammals including humans might be exposed to these doses for long periods. However, the relation between exposed levels or periods and effects of these chemicals such as nonylphenol and atrazine is still ongoing debate.

In addition, fetus and newborn animals are considered to be more sensitive than adult ones. Thus, further long-term studies using low doses and/or immature animals are needed for risk evaluation of these chemicals in mammals including humans.

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