The Journal of Toxicological Sciences,
Vol.30, No.3, 175-194, 2005

INFLUENCE OF DI-(2-ETHYLHEXYL)PHTHALATE ON FETAL TESTICULAR DEVELOPMENT BY ORAL ADMINISTRATION TO PREGNANT RATS

Mariko SHIROTA[1], Yoshiaki SAITO[1], Kiyoshi IMAI[1*], Shinji HORIUCHI[1], Shinsuke YOSHIMURA[1], Masako SATO[1**], Tetsuji NAGAO[1***], Hiroshi ONO[1] and Masanobu KATOH[2]

[1] *Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan*
[2] *Mitsubishi Chemical Safety Institute Ltd, 2-1-30 Shiba, Minato-ku Tokyo 105-0014, Japan*

(Received January 18, 2005; Accepted April 8, 2005)

ABSTRACT — Influence of di-(2-ethylhexyl)phthalate (DEHP) on testicular development was studied by oral administration of DEHP at doses of 500 and 1000 mg/kg/day to pregnant rats on gestational days (G) 7 to 18. Ethinyl estradiol (EE) at dose levels of 0.25 and 0.5 mg/kg/day was used as a reference substance. Each 5-6 pregnant rats were sacrificed and their fetuses were examined on G12, 14, 16, 18 and 20. Fetal deaths averaging 20-36% were observed at every examination in the group receiving 1000 mg/kg of DEHP. Increases of fetal deaths over 50% were also observed in the reference group that received 0.5 mg/kg of EE. Microscopic examination of the fetal testis in groups treated with DEHP revealed degeneration of germ cells in G16 fetuses and localized proliferation or hyperplasia of interstitial cells in G18 and 20 fetuses. Germ cells having more than two nuclei were observed in a few cases including the control testes of G14 fetuses. These multinucleated cells were observed frequently in G20 fetuses treated with DEHP. Examination of testes of naturally delivered offspring of dams treated with 1000 mg/kg of DEHP at 7 weeks of age revealed scattered atrophy or dilatation of seminiferous tubules.

Another experiment was carried out to confirm the dose of DEHP affecting testicular development and spermatogenesis. DEHP was given to pregnant rats at doses of 125, 250 and 500 mg/kg/day during G7-18. Similar histopathological changes were observed in fetal testes of the group exposed to 500 and 250 mg/kg of DEHP, but not in those exposed to 125 mg/kg. In postnatal examinations, however, no abnormality was found in the testes at 5 and 10 weeks after birth in any of the treated groups. Furthermore, no abnormal findings were observed in the function of sperm, sperm counts and sperm morphology in the offspring of the group treated with DEHP during the fetal period at 10 weeks of age. Thus, 125 mg/kg/day is considered the no-observed-effect-level of DEHP on testicular development of rats by exposure *in utero* during the period of organogenesis.

KEY WORDS: Phthalic acid ester, Developmental toxicity, Testicular toxicity, Sertoli cells, Sperm function, Rats

INTRODUCTION

It has been shown that high doses of phthalic acid esters exert testicular toxicity in animals (Calley *et al.*, 1966; Gangolli, 1982). Toxic effects on the testis were similarly observed with a variety of phthalate esters such as di-(2-ethylhexyl) phthalate (DEHP) (Gray *et al.*, 1977), dibutylphthalate, (Cater *et al.*, 1977) and di-n-pentylphthalate (Creasy *et al.*, 1983, 1987). Among a variety of phthalate esters, DEHP has been investigated most frequently as a representative substance of phthalic acid esters. The mechanism of the testicular toxicity of phthalates is not yet wholly clear, although the damaging effect on Sertoli cells and blood-testis

Correspondence: Hiroshi ONO
Present address: * Biosafety Research Center, Foods, Drugs and Pesticides, 582-9 Shioshinden, Fukude-cho, Iwata-gun, Shizuoka 437-1213, Japan
** Syngenta Japan, 1-8-10 Harumi, Chuo-ku, Tokyo 104-6021, Japan
*** Department of Life Sciences, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 557-8502, Japan

barrier has been considered (Gray and Butterworth, 1980). We have conducted a series of experiments on testicular toxicity of DEHP in rats, and have clarified that ultrastructural changes were induced in seminiferous tubules at stages from IX to I of the spermatogenic cycle in 8 week-old Sprague-Dawley rats, 3 to 18 hr after single-dose administration of 2,800 mg/kg of DEHP (Saitoh *et al.*, 1997). Noteworthy changes were degeneration of spermatocytes, dilatation of rough-surfaced endoplasmic reticulum, especially those in the vicinity of the tight junction of ectoplasmic specialization of Sertoli cells, and disintegration of the intercellular junction between Sertoli cells. In a study utilizing electron microscopic autoradiography, we have demonstrated the distribution of phthalic acid into the testis, especially to Sertoli cells (Ono *et al.*, 2004). We have also observed that clear structural changes of testes were induced with single oral dose of 1400 mg/kg, and that the non-toxic dose level of DEHP on testes was 700 mg/kg in mature rats. Furthermore, we have employed a lanthanum trace method to examine the effects of DEHP on Sertoli cell function, especially on the condition of blood-testis barrier in rats (Saitoh *et al.*, 1997). In this study, lanthanum particles were observed 6 hr after administration at the tight junction between Sertoli cells, which showed that the function of Sertoli cells to maintain the blood-testis-barrier was affected with DEHP as early as 6 hr after oral administration, but had recovered by 24 hr. The fetal stage is known to be vulnerable to chemical exposure, and the effects on gonadal and endocrine systems are of special concern. In this context, de Kretser and Kerr (1994) described that the blood-testis barrier in rats was established during 16~19 days of postnatal life. In the present study, influence of *in utero* exposure to DEHP on development of testes in rats was examined. Ethinyl estradiol was used as a reference substance for estrogenic activity of DEHP, if any.

MATERIALS AND METHODS

Materials

Di-(2-ethylhexyl)phthalate (DEHP) was purchased from Wako Pure Chemical Industries Ltd. and was diluted with corn oil (Nacalai Tesque Inc.) to a concentration appropriate for administration at the constant volume of 5 mL/kg. Ethinyl estradiol (EE, Wako Pure Chemical) was suspended in corn oil on the same principle and used as the reference compound.

Animals

Adult rats of Sprague-Dawley strain (Crj: CD IGS) were purchased from Charles River Japan Inc., and were kept for a week to acclimatize them to the laboratory condition. The animals were reared individually in a metallic cage sized 220×270×190 mm, in a room with conditioned temperature at 24~26°C and relative humidity within 50~65%. Lighting was alternated at 12 hr intervals (lights on 7:00~19:00). Appropriate bedding material such as White flake (Charles River) was provided for pregnant and nursing rats. The animals were fed with pellet food (CE-2, CLEA Japan Inc.) *ad libitum* and were supplied with tap water.

A female rat was mated with a male and a vaginal smear specimen was examined every morning. The day when a vaginal plug or sperm in the specimen was confirmed was defined as gestational day (G) 0. The pregnant animals were allocated to groups in a random fashion stratified by body weight on the day of administration (G7).

Dosage and administration

Preliminary dose-finding study showed that administration of 2000 mg/kg/day DEHP to pregnant rats from G7 to G18 caused high incidence of absorption of embryos and fetal deaths. Similar administration of 1000 mg/kg/day of DEHP caused a few fetal deaths and some pathological findings in the testis. Thus the doses of DEHP were decided on 1000 mg/kg for the highest and 500 mg/kg for the lowest in the first experiment. The doses of DEHP in Experiment 2 were selected to be 500, 250 and 125 mg/kg, considering the results of the first experiment. The doses of EE were set at 0.5 and 0.25 mg/kg referring to the study by Yasuda *et al.* (1985) in mice. Oral administration by gavage was started on G7 and continued till G18.

Experimental design

The study was designed in two phases; observation of the histopathological changes of testicular development by intra-uterine exposure to DEHP was made in Experiment 1, including the dose finding, and in Experiment 2 a search for the no-effect level was attempted, together with confirmation of the findings in Experiment 1.

In Experiment 1, 28-30 dams per group were given oral administration of DEHP, EE or the vehicle from G7 to G18. Each 6 of these dams were killed by ether inhalation on G12, 14, 16, 18 and 20 to examine their fetuses. In addition, each 5 dams of groups given 500 and 1000 mg/kg of DEHP were allowed to deliver

spontaneously to examine postnatal changes in the testis and epididymis of their offspring. The male offspring were reared and kept until examination at 7 weeks of age.

In Experiment 2, each 11-12 pregnant females were given oral administration of DEHP or vehicle. Each 3 dams of the groups were submitted to Caesarean section on G20 to examine their fetuses. Other dams were allowed to deliver spontaneously and male offspring chosen for examination at 5 and 10 weeks of age. The day of delivery was defined as Day 0 of lactation.

Observations of dams

Dams were examined daily for general conditions in all experiments and body weight was measured occasionally. Delivery and nursing conditions were observed and the numbers of fetuses delivered and live offspring were determined. From these data and the number of implantations counted at the necropsy, viability of the offspring, namely, delivery index (fetuses delivered/implantation sites, %), birth index (live offspring at birth/implantation sites, %), viability index (live offspring on day 4 of lactation/live offspring at birth, %) and weaning index (live offspring on day 21 of lactation/live offspring on day 4 of lactation, %), were determined.

Examination of the fetuses and offspring

In Experiment 1, fetuses on G12 were collected only for histopathological examination. Live fetuses collected on G14, 16, 18 and 20 were weighed and the external appearances examined. Whole bodies and testes from these live fetuses were submitted for histopathological or electron microscopic examination. The testes and epididymides of male offspring of the DEHP-treated groups were collected at 7 weeks of age for histopathological examination.

For histopathological examination, the specimens were fixated in Bouin's solution and then immersed in a buffered neutral formalin solution. The fixed tissues were embedded in paraffin and cut in 4 μm slices. These sections were stained with hematoxylin and eosin (HE) and were examined under light microscopy.

For electron microscopic examination, the tissues were immersed in an ice-cold mixture of 2% paraformaldehyde buffered with 0.1 M s-collidine and 1.25% glutaraldehyde for 3 hr. The fixed tissues were cut into small pieces and post-fixed with 2% osmium tetraoxide buffered with 0.1 M s-collidine. The post-fixed tissues were dehydrated in ethanol and embedded in epoxy resin (Quetol-651, Nissin EM, Tokyo). Semithin sections (1 μm) were stained with toluidine blue and observed under a light microscope. Representative areas were selected from the testis preparations and ultra-thin sections were prepared and stained with uranyl acetate and lead citrate, and then examined with an electron microscope (H-7100, Hitachi, Tokyo).

In Experiment 2, all of the live fetuses examined on G20 were weighed by sex and examined for their external appearance, and then testes were dissected from live male fetuses for histopathological examination as described in Experiment 1, and for staining of androgen receptors. The offspring were weighed and reared until examination. Each 4 male offspring from each group were killed at 5 and 10 weeks of age, and testes with epididymides were dissected and HE-stained thin sections prepared as described above. For electron microscopic examination, each 2 male offspring were used and fixation was performed by a systemic perfusion of a mixture solution of 2% paraformaldehyde buffered with 0.1 M s-collidine and 2.5% glutaraldehyde from the aorta to the body with a perfusion pump under sodium pentobarbital anesthesia. The testes were submitted to electron microscopic observation. The other 4 offspring of each group were killed by ether inhalation at 10 weeks of age to obtain their testes and epididymides for sperm examination.

Immunohistochemistry of androgen receptors

In addition, in order to confirm the development of hormone receptors, expression of androgen receptors in the testis was observed by an immunohistochemical method (Dalgaard *et al.*, 2001), using a rabbit polyclonal antibody for N-terminal of the androgen receptor (AR-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Examination of spermatogenesis

In Experiment 2 the seminiferous epithelium cycle was examined on testis sections stained with HE obtained at 5 weeks of age, and the spermatogenic stage was determined according to the simplified method described by Matsui *et al.* (1996). Briefly, seminiferous tubules on a specimen were classified into four groups by spermatogenic stages I-VI, VII-VIII, IX-XI and XII-XIV (Dym and Clermont, 1970). One corresponding section of the testis was stained with periodic acid Schiff (PAS) to confirm acrosomes of spermatogonia. Each 5 seminiferous tubules belonging to 4 groups were chosen and the numbers of germ

cells and Sertoli cells in a tubule determined to calculate a ratio of germ cells to Sertoli cells in each group.

Analysis of morphology and function of sperm

Sperm were collected through micropuncture of the cauda epididymis of rats at 10 weeks of age, and were examined as previously described (Sato *et al.*, 2000). Sperm motility was measured using a computer-assisted sperm motion analysis system (HTM-IVOS ver 10.6, Hamilton-Thorne Research, Beverly, MA, USA) and for morphological analysis of spermatozoa as described previously (Sato *et al.*, 2002a). After the collection of sperm for motility analysis, the cauda epididymis was dissected at the transition point to the vas deferens and at the middle of the cauda and body of epididymis, weighed and then stored at –20°C. The frozen cauda epididymis was thawed to room temperature and homogenized in distilled water. The sperm heads in the homogenate were counted with HTM-IVOS as previously described (Sato *et al.*, 2002b).

Statistical analysis

When uniformity of variance was confirmed among the groups by the method of Bartlett, data obtained were analyzed by ANOVA (Yoshimura, 1986). When the uniformity was not confirmed, Kruskal-Wallis's rank-sum test was applied instead (Yoshimura, 1986). When significant differences between groups were observed in either of the analyses, Dunnett test was applied for a comparison between the control and treated groups of either DEHP or EE (Yoshimura, 1986). A p value less than 0.05 was considered statistically significant.

RESULTS

Effects of DEHP treatment on dams

Daily oral administration of DEHP at a level of 1000 mg/kg and EE at levels of 0.25 and 0.5 mg/kg slightly suppressed body weight gain of pregnant rats during the treatment period. Administration of the lower dose levels of DEHP did not affect maternal body weight (Tables 1 and 2).

Effects of maternal treatment with DEHP on fetuses and offspring

Reproductive performance data, including fetal weights on G14, 16, 18 and 20 in Experiment 1, are summarized in Table 1. Oral DEHP treatment at 1000 mg/kg reduced fetal body weights at G14 and 18 significantly ($p<0.01$) as compared with those of the control group. Furthermore, 1000 mg/kg of DEHP treatment increased intrauterine mortality to 20-36%. DEHP treatment at 500 mg/kg did not cause increase in fetal deaths and changes in fetal body weight significantly. Treatment with 0.5 mg/kg of EE also increased intrauterine mortality of fetuses, even to more than 50% on G16 and 20.

External observation of fetuses on G20 revealed various malformations in treated groups. Two fetuses with branchyury from a single dam given 500 mg/kg DEHP were observed and each one fetus with general edema, club foot or anal atresia and 3 fetuses with kinked tail from a single dam given 1000 mg/kg of DEHP were observed. In the group treated with 0.5 mg/kg of EE, one fetus with kinked tail was observed. Two out of 5 dams given 500 mg/kg DEHP did not deliver any offspring because of early embryonic loss. However, 1000 mg/kg of DEHP did not cause any abnormality in delivery.

In Experiment 2, DEHP-treatment up to 500 mg/kg did not show any marked effect on fetuses (Table 2). External malformations observed in the 500 mg/kg group in Experiment 1 were not reproduced in Experiment 2. Birth weights of the offspring were significantly higher in the groups exposed to DEHP at 250 and 500 mg/kg than control. Viability and growth rate of the offspring are summarized in Table 3. Differences of body weight among the groups were insignificant on the 4th day of lactation.

Histopathological findings of fetuses and offspring

Histopathological findings of fetal testes in Experiment 1 are summarized in Table 4. Representative photographs are shown in Photos 1~3. The testis was not distinguishable in the fetuses on G12, when a few round germ cells with clear cytoplasm were scattered in mesenchyma around mesonephros. The testis was distinguished morphologically on G14, when the germinal ridge was formed and a few germ cells, some showing mitosis, were seen in the gonadal cord. On G16, the testicular cord became prominent, containing many round nucleated germ cells and Sertoli cells on its margin (Photo 1a). On G18, the interstitium was widened in the center of the gonad containing rich interstitial cells (Photo 2a), when the density of germ cells in the reproductive tract was increased. On G20, the testicular cord developed further, although the tubular structure was not yet formed (Photo 3a).

No abnormalities were observed in any group on G14. On G16, degeneration of germ cells was noted in

Table 1. Viability and development of fetuses exposed to di-(2-ethylhexyl)phthalate (DEHP) or ethinyl estradiol (EE) during gestational days 7-18 (Experiment 1).

	DEHP (mg/kg)			EE (mg/kg)	
	0 [a]	500	1000	0.25	0.5
Gestational day 14	(6)	(6)	(6)	(5)	(5)
Maternal body weight (g)	336.9 ± 17.4	326.5 ± 31.0	320.2 ± 14.9**	295.7 ± 14.7**	278.4 ± 22.7**
Implantations	17.0 ± 1.4	15.0 ± 1.7	16.2 ± 1.2	15.6 ± 1.1	13.6 ± 4.7
Intrauterine mortality (%)	7.1 ± 5.9	3.0 ± 5.0	20.3 ± 18.4	3.8 ± 5.6	6.9 ± 9.4
Live fetuses	15.8 ± 2.1	14.5 ± 1.2	12.8 ± 2.9	15.0 ± 1.4	12.6 ± 4.5
Mean fetal weight (g)	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.02**	0.15 ± 0.01	0.15 ± 0.02
Gestational day 16	(5)	(6)	(6)	(0)	(5)
Maternal body weight (g)	344.9 ± 4.9	344.3 ± 21.3	311.4 ± 20.0**		285.7 ± 30.4**
Implantations	15.4 ± 1.3	16.0 ± 1.3	15.3 ± 1.6		13.6 ± 6.2
Intrauterine mortality (%)	1.3 ± 2.8	12.4 ± 7.6	33.1 ± 31.3		72.0 ± 36.9
Live fetuses	15.2 ± 1.3	14.0 ± 1.4	10.2 ± 4.8		4.2 ± 5.4
Mean fetal weight (g)	0.44 ± 0.02	0.43 ± 0.02	0.37 ± 0.04		0.42 ± 0.02 [b]
Gestational day 18	(6)	(6)	(6)	(4)	(5)
Maternal body weight (g)	370.7 ± 36.5	360.0 ± 36.6	335.5 ± 20.2*	327.6 ± 42.4*	321.7 ± 16.3**
Implantations	14.5 ± 1.4	15.2 ± 2.6	14.8 ± 1.6	15.3 ± 2.2	14.4 ± 2.4
Intrauterine mortality (%)	3.6 ± 6.3	1.0 ± 2.4	35.6 ± 36.5	5.7 ± 7.9	1.3 ± 3.0
Live fetuses	14.0 ± 1.8	15.0 ± 2.5	9.5 ± 5.6	14.3 ± 1.0	14.2 ± 2.4
Mean fetal weight (g)	1.35 ± 0.07	1.32 ± 0.06	1.03 ± 0.13**	1.33 ± 0.05	1.25 ± 0.10
Gestational day 20	(6)	(6)	(6)	(0)	(2)
Maternal body weight (g)	404.2 ± 6.5	410.8 ± 30.2	365.0 ± 25.4**		322.8
Implantations	14.7 ± 1.6	14.8 ± 2.6	14.5 ± 1.5		15.5
Intrauterine mortality (%)	0.0 ± 0.0	2.7 ± 4.4	36.4 ± 26.5		50.8
Live fetuses	14.7 ± 1.6	14.5 ± 2.9	9.0 ± 3.5		7.5
Mean fetal weight (g)	3.68 ± 0.20	3.52 ± 0.14	2.82 ± 0.11		2.90
External malformations	0.0 ± 0.0	2.22 ± 5.44	6.25 ± 15.31		7.14

[a] Vehicle control (corn oil, 5 mL/kg). [b] Data from 3 dams having live fetuses.

* Significantly different from control ($p<0.05$). ** Significantly different from control ($p<0.01$).

Table 2. Reproductive parameters on gestational day 20 in rats treated with di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Gestational day 20				
Dams examined	3	3	3	3
Maternal body weight (g)	408.3 ± 32.6	428.8 ± 42.5	399.3 ± 43.4	427.9 ± 50.8
Implantations	14.7 ± 0.6	15.0 ± 2.6	14.0 ± 1.7	16.0 ± 1.7
Intrauterine mortality (%)	2.2 ± 3.9	0	2.8 ± 4.8	4.1 ± 3.6
Live fetuses	14.3 ± 0.6	15.0 ± 2.6	13.7 ± 2.3	15.3 ± 1.5
Males	5.3 ± 1.2	7.0 ± 3.5	6.3 ± 2.1	9.3 ± 2.1
Females	9.0 ± 1.0	8.0 ± 1.0	7.3 ± 0.6	6.0 ± 1.0
Sex ratio (%)	37.2 ± 7.6	44.7 ± 17.2	45.5 ± 8.5	60.5 ± 9.1
Fetal body weight (g)	14.0 ± 1.8	15.0 ± 2.5	9.5 ± 5.6	14.2 ± 2.4
Males	3.77 ± 0.13	3.86 ± 0.40	4.02 ± 0.13	3.57 ± 0.14
Females	3.51 ± 0.14	3.67 ± 0.34	3.77 ± 0.16	3.40 ± 0.03
External malformations	0	0	0	0

Values represent mean ± S.D.

[a] Vehicle control (corn oil, 5 mL/kg).

one of 12 examined fetuses of the 1000 mg/kg DEHP group (Photos 1b, 1c). No such findings were noted in other fetuses of the group exposed to DEHP at 1000 mg/kg and also at 500 mg/kg. On G18, interstitial cells were increased in number and aggregated topically in the 500 mg/kg DEHP group (Photo 2b), and the hyperplasia of interstitial cells was intensified in the 1000 mg/kg DEHP group (Photo 2c), while such findings were not noted in any testes of fetuses exposed to EE. Testicular size was smaller in the groups of 1000 mg/kg DEHP and 0.5 mg/kg EE on G18 and G20. On G20, germ cells having more than two nuclei were noted and thickened seminiferous cords containing rich germ cells were seldom observed in the 500 mg/kg DEHP group. In fetal testes of the 1000 mg/kg DEHP group hyperplasia of interstitial cells, multinucleated germ cells were also seen (Photos 3b, 3c). Topically thickened seminiferous cords due to aggregation of germ cells were observed frequently in this group.

Table 5 summarizes histopathological findings in the testis of the offspring in Experiment 1. Representative pictures are shown in Photos 4~6. In the offspring at 7 weeks after birth prenatally exposed to DEHP at a level of 500 mg/kg, no obvious abnormalities were found except for multinucleated giant cells in the seminiferous tubules and cell debris in the epididymal lumens (Photos 4a, 4b). In the 1000 mg/kg-exposed group, however, most of the animals had developed abnormalities, such as branched seminiferous tubules with atrophy and/or dilatation, multinucleated giant cells and dilatation of rete testis (Photos 4c, 5a, 5b). In addition to these findings, testes from several animals in this group showed hyperplasia of the interstitial cells (Photo 4c), necrosis and/or mineralization of testes, foreign body giant cells, focal loss of seminiferous tubules and malformed seminiferous tubules (Photos

Table 3. Reproductive data and development of the offspring treated with di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Dams examined	8	9	8	8
Gestation length (days)	21.8 ± 0.5	22.0 ± 0.0	22.0 ± 0.0	22.0 ± 0.0
Implantation sites	15.4 ± 1.2	15.6 ± 2.4	15.4 ± 1.1	14.9 ± 1.2
At birth (Day 0 of lactation)				
Live offspring	14.0 ± 2.1	14.6 ± 2.6	14.4 ± 1.7	14.1 ± 1.2
Birth index (%) [b]	90.8 ± 9.0	93.2 ± 6.3	93.5 ± 8.8	95.1 ± 5.7
Sex ratio (%)	42.0 ± 10.4	45.7 ± 8.9	42.9 ± 12.3	50.5 ± 12.3
Body weight, males (g)	6.5 ± 0.3	6.7 ± 0.5	7.0 ± 0.5	7.1 ± 0.3*
Body weight, females (g)	6.1 ± 0.3	6.3 ± 0.5	6.7 ± 0.6*	6.7 ± 0.3*
Day 4 of lactation				
Live offspring	13.9 ± 2.2	14.3 ± 2.5	14.4 ± 1.7	14.0 ± 1.3
Viability (%)	99.0 ± 2.7	98.6 ± 2.8	100.0 ± 0.0	99.1 ± 2.5
Sex ratio (%)	42.3 ± 10.1	46.3 ± 8.5	42.9 ± 12.3	50.9 ± 11.4
Body weight, males (g)	10.3 ± 1.1	10.4 ± 1.0	10.7 ± 0.7	10.5 ± 1.3
Body weight, females (g)	9.8 ± 1.2	9.7 ± 1.0	10.3 ± 0.8	10.0 ± 1.3
Body weight, preserved males (g) [c]	10.4 ± 0.9	10.7 ± 0.7	11.0 ± 0.5	10.7 ± 0.1
Day 7 of lactation				
Body weight, preserved males (g)	17.1 ± 2.3	16.9 ± 1.0	18.2 ± 0.7	17.2 ± 0.2
Day 14 of lactation				
Body weight, preserved males (g)	36.1 ± 2.8	34.5 ± 1.5	37.5 ± 0.8	37.6 ± 1.3
At weaning (Day 21 of lactation)				
Body weight, preserved males (g)	58.7 ± 4.7	57.1 ± 4.1	62.2 ± 1.5	60.5 ± 3.3
Weaning index (%)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Values represent mean ± S.D. * Significantly different from control ($p<0.05$).

[a] Vehicle control (corn oil, 5 mL/kg). [b] Live offspring/implantation sites.

[c] Each 2~3 male offspring from dams were preserved.

Table 4. Histopathological findings of testes of fetuses exposed to di-(2-ethylhexyl) phthalate (DEHP) or ethinyl estradiol (EE) during gestational days 7-18 (Experiment 1).

Group	DEHP 0 mg/kg					DEHP 500 mg/kg					DEHP 1000 mg/kg					EE 0.25 mg/kg					EE 0.5 mg/kg				
Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Gestational day 12	(12)					(10)					(10)										(10)				
Degeneration of fetal tissue	12	0	0	0	0	10	0	0	0	0	8	0	0	2	0						2	0	0	8	0
Gestational day 14	(9)					(9)					(9)					(9)					(9)				
Multinucleated germ cells	8	1	0	0	0	9	0	0	0	0	8	1	0	0	0	9	0	0	0	0	8	1	0	0	0
Gestational day 16	(10)					(10)					(12)										(4)				
Degeneration of germ cells	10	0	0	0	0	10	0	0	0	0	11	0	0	1	0						4	0	0	0	0
Multinucleated germ cells	10	0	0	0	0	9	1	0	0	0	10	2	0	0	0						4	0	0	0	0
Gestational day 18	(20)					(20)					(10)					(11)					(16)				
Multinucleated germ cells	20	0	0	0	0	18	2	0	0	0	10	0	0	0	0	11	0	0	0	0	14	2	0	0	0
Increased germ cells in a cord	20	0	0	0	0	20	0	0	0	0	8	2	0	0	0	11	0	0	0	0	16	0	0	0	0
Hyperplasia of interstitial cells	20	0	0	0	0	8	12	0	0	0	0	3	7	0	0	11	0	0	0	0	16	0	0	0	0
Decrease in testicular size	20	0	0	0	0	20	0	0	0	0	4	0	6	0	0	11	0	0	0	0	9	7	0	0	0
Gestational day 20	(17)					(17)					(18)										(8)				
Multinucleated germ cells	16	1	0	0	0	0	0	10	7	0	0	3	13	2	0						4	4	0	0	0
Increased germ cells in a cord	17	0	0	0	0	14	3	0	0	0	1	12	5	0	0						5	3	0	0	0
Hyperplasia of interstitial cells	17	0	0	0	0	0	0	14	0	0	0	1	17	0	0						8	0	0	0	0
Decrease in testicular size	17	0	0	0	0	17	0	0	0	0	5	8	5	0	0						5	3	0	0	0

− : negative, ± : very slight, + : slight, ++ : moderate, +++ : severe.
Figures in parentheses show number of dams examined.

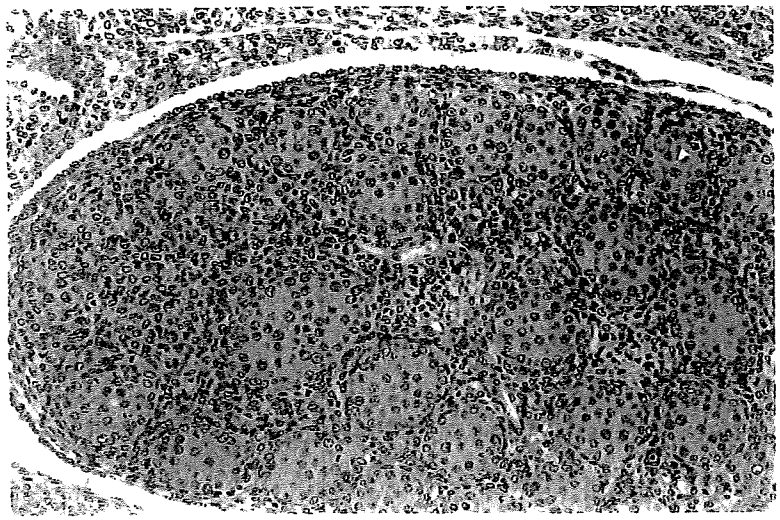

Photo 1-a. Transverse section of a fetus from the control group on G16 showing the genital ridge. HE stain, ×160.

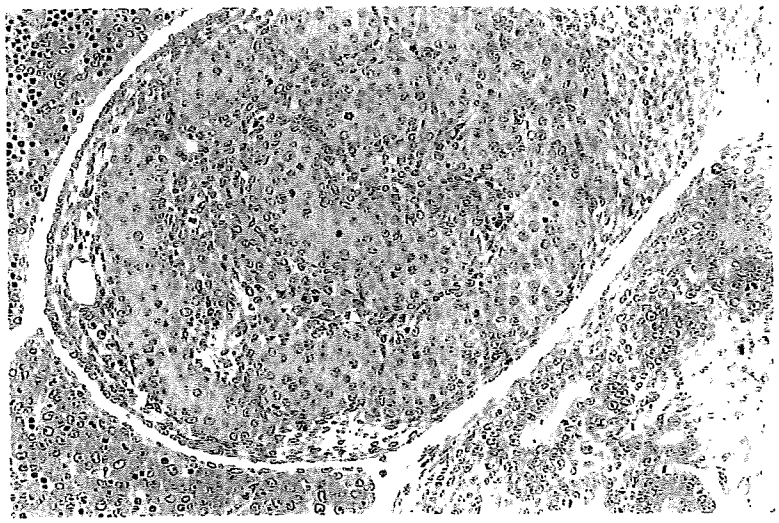

Photo 1-b. Transverse section of a fetus from the 1000 mg/kg DEHP group on G16 showing no abnormality in the genital ridge. HE stain, ×160.

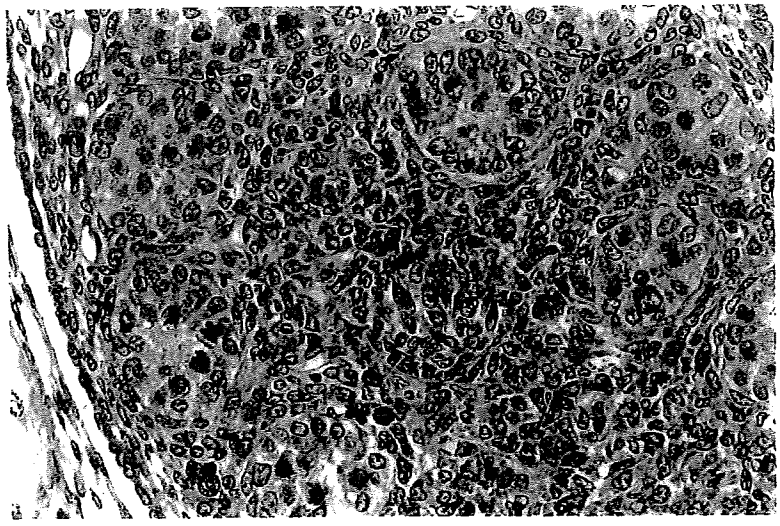

Photo 1-c. Transverse section of a fetus from the 1000 mg/kg DEHP group on G16 showing the genital ridge. Many germ cells are degenerated and densely stained. HE stain, ×310.

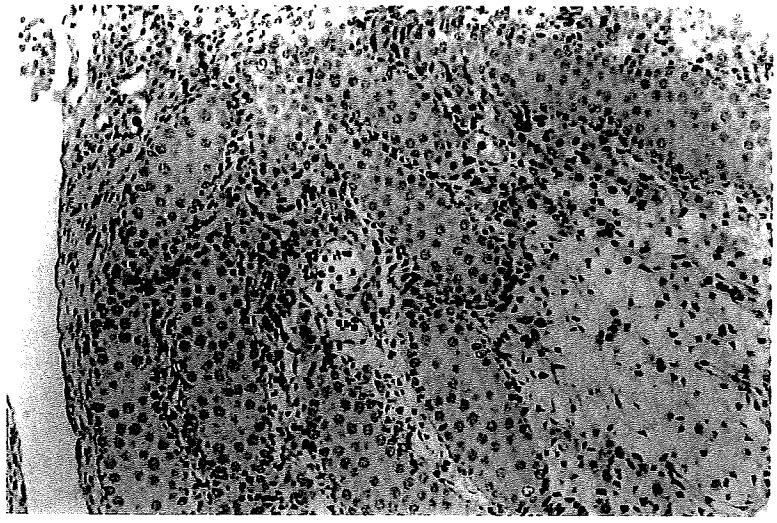

Photo 2-a. Testis of a G18 rat fetus from the control group. HE stain, ×160.

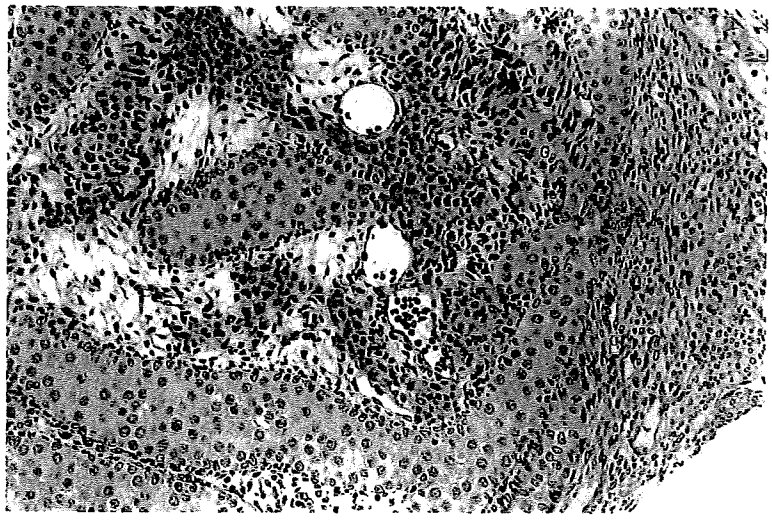

Photo 2-b. Testis of a G18 fetus from a rat treated with 500 mg/kg of DEHP showing hyperplasia of interstitial cells. HE stain, ×160.

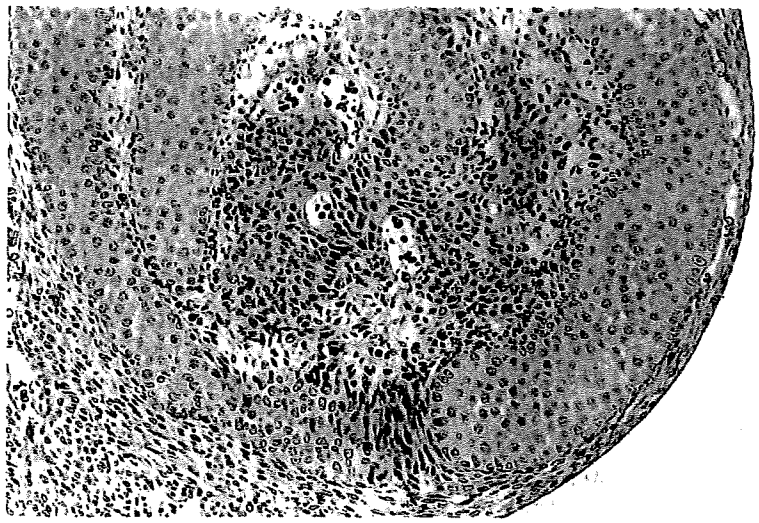

Photo 2-c. Testis of a G18 fetus from a rat treated with 1000 mg/kg DEHP showing hyperplasia of interstitial cells. HE stain, ×160.

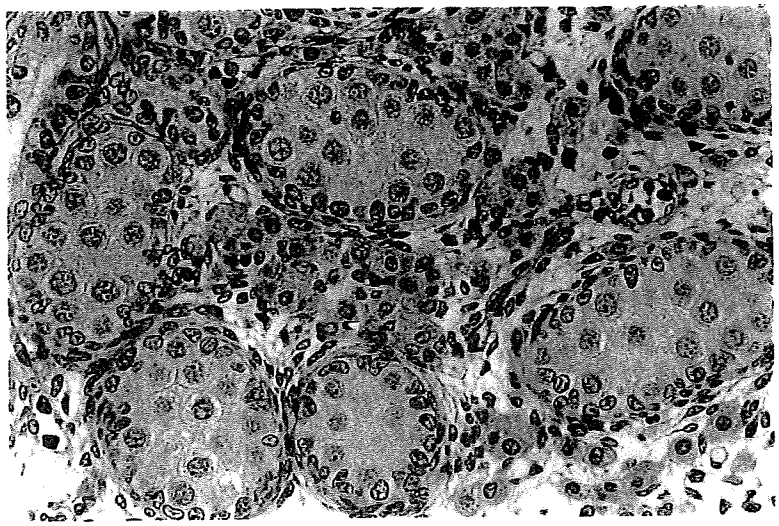

Photo 3-a. Testis of a G20 fetus from the control group showing the seminiferous cords and interstitial cells. HE stain, ×310.

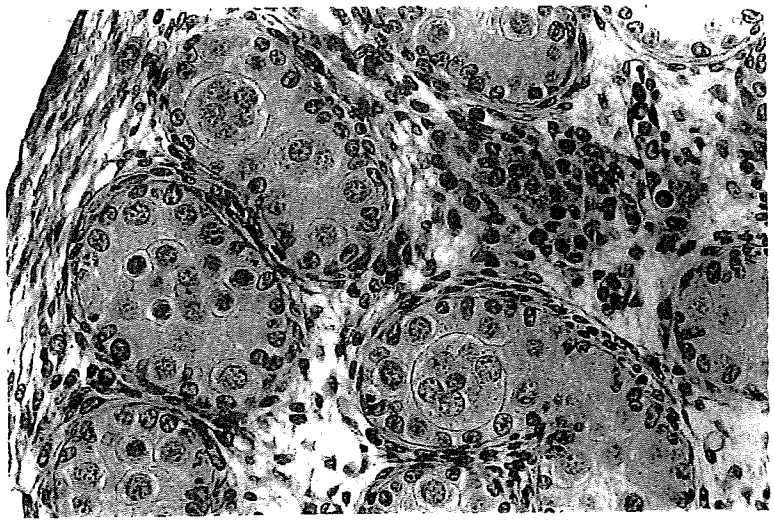

Photo 3-b. Testis of a G20 fetus from the group treated with 500 mg/kg of DEHP showing multinucleated germ cells in seminiferous cords. HE stain, ×310.

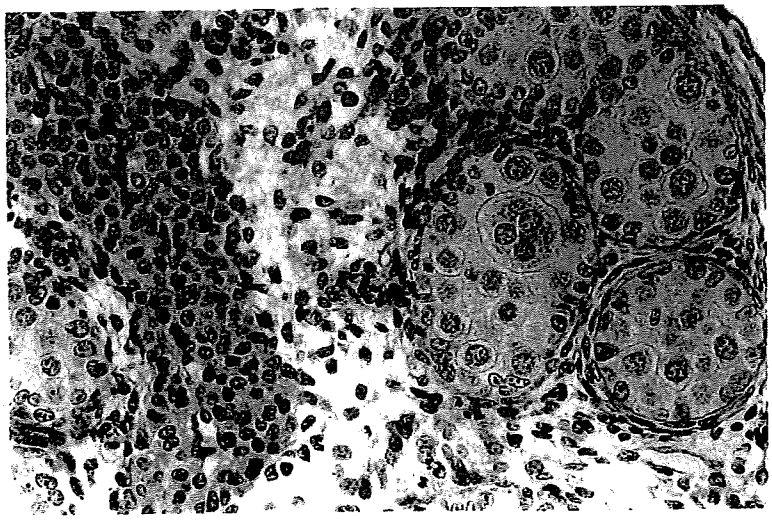

Photo 3-c. Testis of a G20 fetus from the group treated with 1000 mg/kg of DEHP showing multinucleated germ cells in seminiferous cords, and hyperplastic smaller-sized interstitial cells. HE stain, ×310.

5a, 5b). In their epididymides, atrophy was found in all of the animals and cell debris in the epididymal lumen was also found (Photos 6a, 6b).

Effects of the lower doses of DEHP on testicular development were examined in Experiment 2. Table 6 summarizes histopathological findings of fetal testes on G20 and testes of offspring at 5 and 10 weeks of age in Experiment 2. Multinucleated germ cells were found in the fetal testes of all the groups exposed to DEHP, although its incidence was very low in the 125 mg/kg group. In the groups exposed to 250 mg/kg and 500 mg/kg of DEHP, partly thickened germinal cords due to aggregation of increased number of germ cells and hyperplasia of the interstitial cells were observed. Degenerated germ cells and apoptosis were observed in a few animals in the group exposed to 500 mg/kg of DEHP. These findings are comparable to those in DEHP-exposed testes at the same dose in Experiment 1.

In contrast to the findings of the fetal testes, no abnormalities were found in testes of the offspring at 5 and 10 weeks of age in any group in histopathological examination. Furthermore, the seminiferous cycles in the testis determined at 5 weeks of age were comparable between control and DEHP-exposed groups (Table 7).

Electron microscopic findings of fetuses

Electron microscopic examination of fetal testes was performed in Experiment 1. In the fetal testis of the groups exposed to DEHP at 500 and 1000 mg/kg, degenerated germ cells were found in the testicular cord on G16 (Photo 7), and smaller-sized interstitial cells containing fewer lipid droplets were noted on G18 (Photo 8a). These changes of the interstitial cells became more obvious on G20 (Photo 8b).

In the fetal testis from the group exposed to EE at 0.5 mg/kg, degeneration of germ cells was found only on G14. No abnormalities such as those observed with DEHP treatment were found on G16, 18 and 20. Slightly swollen mitochondria and hyperplastic smooth endoplasmic reticulum were noted in interstitial cells on G18 and 20. Furthermore, degeneration of interstitial cells surrounded by neutrophils infiltration were observed on G20.

In examination of offspring at 5 and 10 weeks after birth in Experiment 2, ultrastructural changes were not observed in the testis and epididymides of any

Table 5. Histopathological findings in the testis and epididymis of offspring exposed to DEHP during gestational days 7-18 (Experiment 1) 7 weeks after birth.

Group	DEHP 500 mg/kg (6)					DEHP 1000 mg/kg (12)				
Grade	−	±	+	++	+++	−	±	+	++	+++
Testis										
Atrophy of seminiferous tubules	6	0	0	0	0	2	2	4	3	1
Multinucleated giant cells	5	1	0	0	0	0	3	5	4	0
Dilatation of seminiferous tubules	6	0	0	0	0	2	0	4	6	0
Dilatation of rete testis	6	0	0	0	0	8	2	1	1	0
Hyperplasia of interstitial cells	6	0	0	0	0	10	1	1	0	0
Necrosis	6	0	0	0	0	11	0	0	0	1
Mineralization	6	0	0	0	0	10	0	1	1	0
Foreign body giant cells	6	0	0	0	0	10	0	1	1	0
Focal loss of seminiferous tubules	6	0	0	0	0	11	0	1	0	0
Malformation of seminiferous tubules	6	0	0	0	0	11	0	1	0	0
Epididymis										
Atrophy	6	0	0	0	0	0	1	2	2	7
Cell debris in lumens	0	0	6	0	0	3	3	6	0	0
Dilatation of lumens	6	0	0	0	0	8	0	3	1	0
Infiltration of lymphocytes	6	0	0	0	0	8	2	2	0	0
Granuloma	6	0	0	0	0	11	0	0	1	0

Figures in parentheses indicate number of offspring examined.
− : not observed, ± : very slight, + : slight, ++ : moderate, +++ : severe.

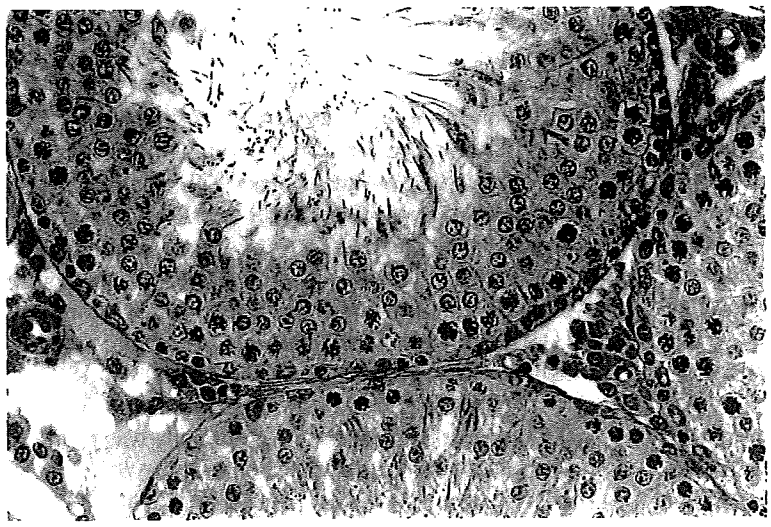

Photo 4-a. Testis of a 7-week-old rat treated with 500 mg/kg of DEHP *in utero* showing no abnormalities in the seminiferous tubules and interstitial cells. HE stain, ×310.

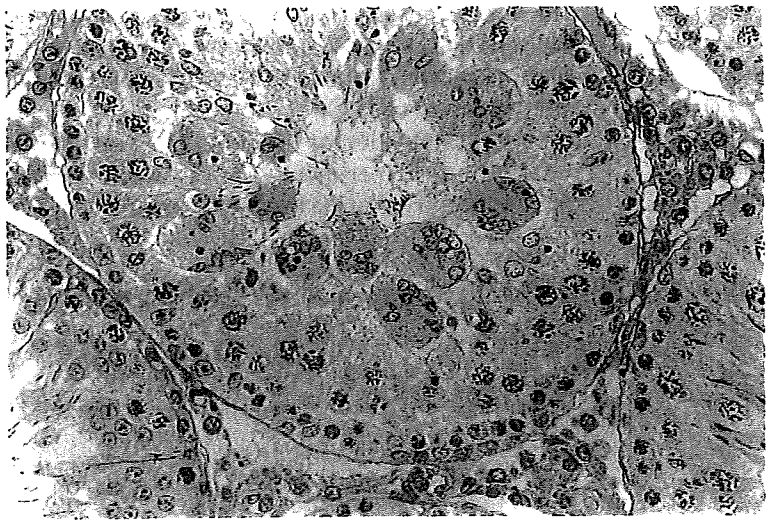

Photo 4-b. Testis of a rat of the same group as Photo 4-a, showing multinucleated giant cells in a seminiferous tubule. HE stain, ×310.

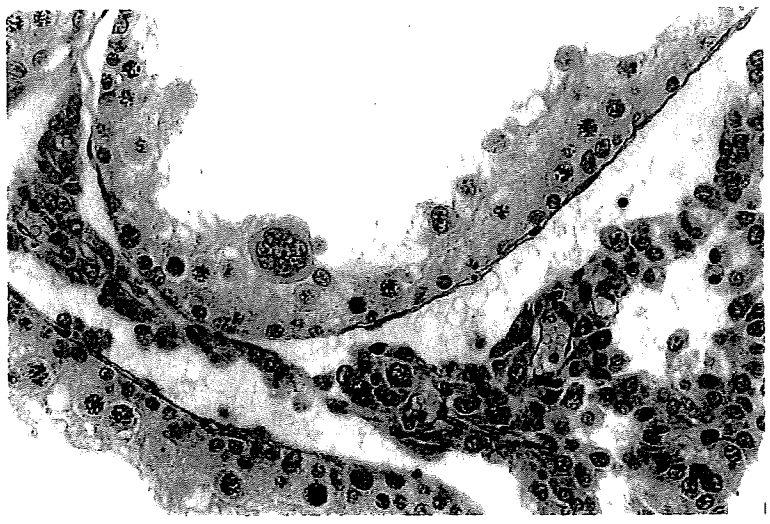

Photo 4-c. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing atrophy of seminiferous tubule epithelia with multinucleated giant cell in the lumen, and also hyperplasia of interstitial cells. HE stain, ×310.

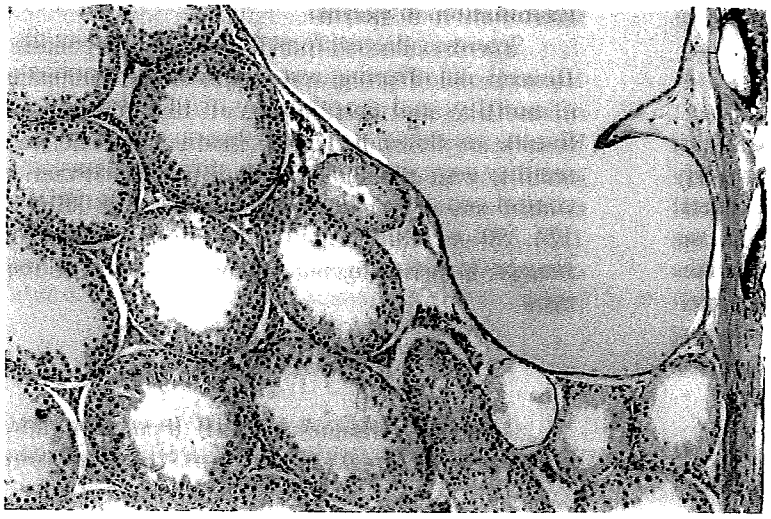

Photo 5-a. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing dilatation of seminiferous tubules and of rete testis. HE stain, ×80.

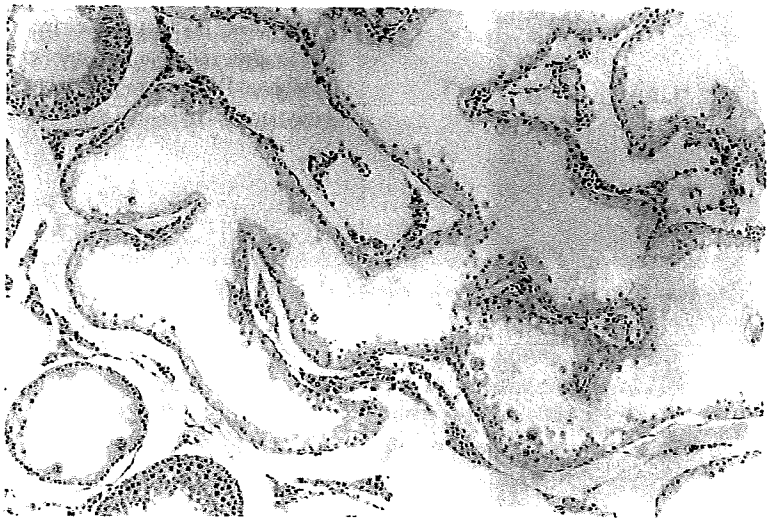

Photo 5-b. Testis from a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing branching of atrophic seminiferous tubules. HE stain, ×80.

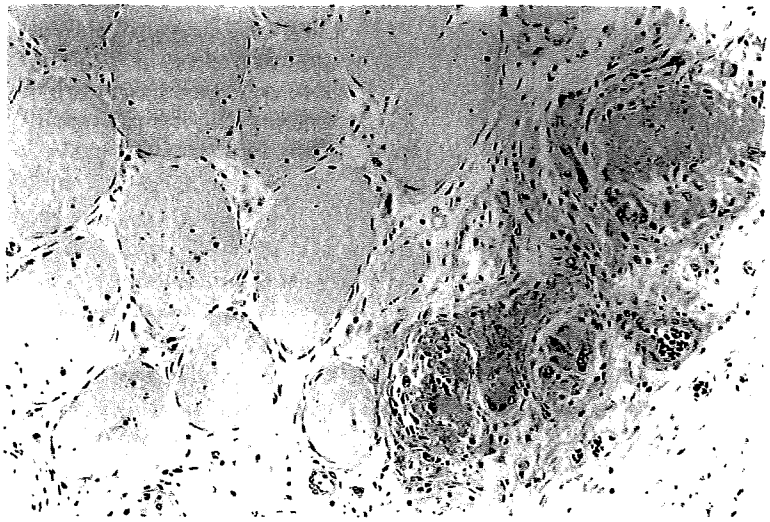

Photo 5-c. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing extensive necrosis and foreign body giant cells. HE stain, ×160.

of the offspring in any groups (control and 500 mg/kg DEHP groups).

Expression of androgen receptors

Immunohistochemical staining revealed an increase of androgen receptor-positive cells, namely hyperplasia of Leidig cells, in the interstitium of fetal testes at G20 in the 500 mg/kg group (Photo 9). In the offspring at 5 and at 10 weeks after birth, however, the expression of androgen receptors observed in Sertoli cells, myoid cells and interstitial cells was not different among the control and DEHP treated groups (data not shown).

Examination of sperms

Sperms collected from the cauda epididymidis of 10-week old offspring were subjected to examination of motility and morphology of the spermatozoa. Results are shown in Table 8. Sperm count and sperm motility were not significantly different between the control and any of the groups treated with DEHP at 125, 250 or 500 mg/kg. There were no remarkable changes in spermatogenic parameters related to treatment.

DISCUSSION

Oral administration of DEHP to pregnant rats at doses up to 1000 mg/kg from G7 to G18, which corre-

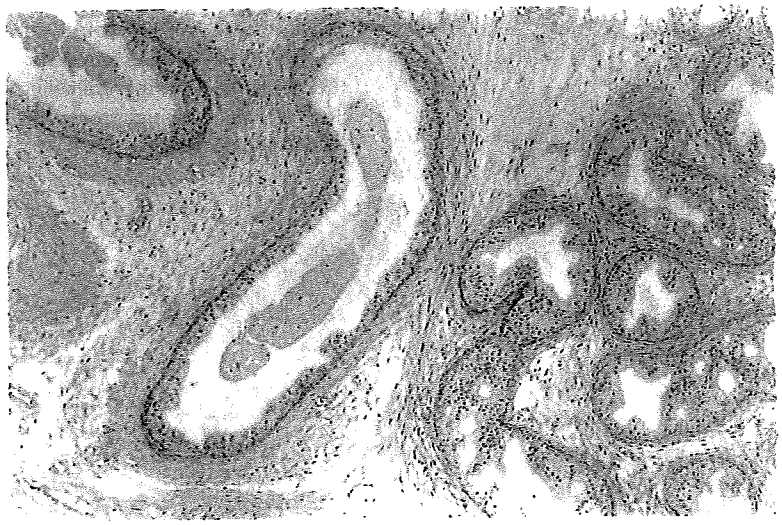

Photo 6-a. Epididymis of a 7-week old rat treated with 1000 mg/kg of DEHP *in utero*, showing atrophy of epididymal ducts and cell debris in the lumen. HE stain, ×80.

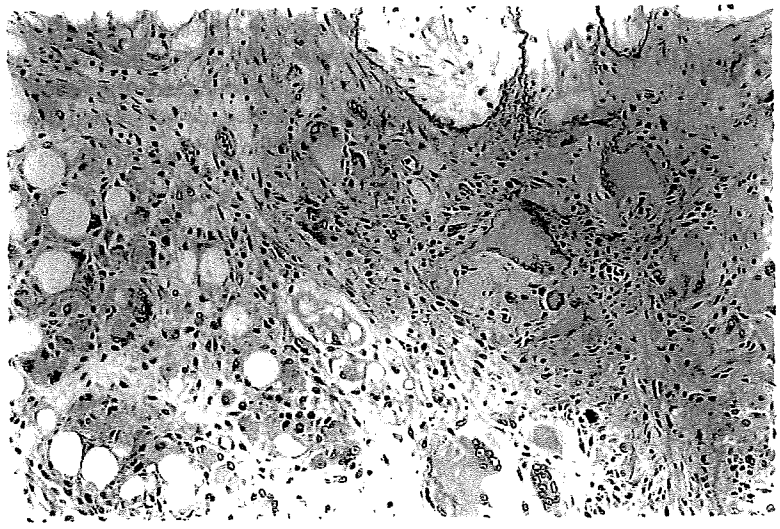

Photo 6-b. Granuloma formed in the epididymis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, accompanied by numerous foreign body giant cells and fibrosis. HE stain, ×160.

sponded to the organogenetic period of a rat fetus, induced fetal damage such as increase in fetal mortality, inhibition of fetal weight gain, and some malformations in the highest dose. Histopathological studies revealed degeneration of germ cells and hyperplasia of interstitial cells in the fetal testis in the groups treated with DEHP at doses of 500 mg/kg and above. Similar changes were also observed in slight degree in the 250 mg/kg group but not in the 125 mg/kg group. Electron microscopic examination of these testes of affected groups revealed smaller-sized interstitial cells in which lipid droplets were depleted. Testicular toxicity of a phthalate ester by *in utero* exposure in rats have been described by Mylchreest *et al.* (2000) using di-(*n*-

Table 6. Histopathological findings of testes of offspring exposed to di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

Group	DEHP 0 mg/kg [a]					DEHP 125 mg/kg					DEHP 250 mg/kg					DEHP 500 mg/kg				
Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Gestational day 20	(15)					(21)					(19)					(28)				
Multinucleated germ cells	15	0	0	0	0	16	5	0	0	0	4	15	0	0	0	2	25	1	0	0
Increase of germ cells in a cord	15	0	0	0	0	21	0	0	0	0	16	3	0	0	0	1	21	6	0	0
Hyperplasia of interstitial cells	15	0	0	0	0	21	0	0	0	0	6	12	1	0	0	6	5	17	0	0
Degeneration of germ cells	15	0	0	0	0	21	0	0	0	0	19	0	0	0	0	26	2	0	0	0
Apoptosis of germ cells	15	0	0	0	0	21	0	0	0	0	19	0	0	0	0	27	1	0	0	0
5 weeks after birth	(4)					(4)					(4)					(4)				
Abnormalities	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0
10 weeks after birth	(4)					(4)					(4)					(4)				
Abnormalities	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0

[a] Vehicle control (corn oil, 5 mL/kg). Figures in parentheses indicate number of fetuses or offspring examined.
− : not observed, ± : very slight, + : slight, ++ : moderate, +++ : severe.

Table 7. Morphometric analysis of spermatogenesis of the offspring exposed to 500 mg/kg of di-(2-ethylhexyl)phthalate (DEHP) during gestational days 7-18 5 weeks after birth (Experiment 2).

	DEHP 0 mg/kg [a]	DEHP 500 mg/kg
(Number of offspring examined)	(4)	(4)
Group 1 (Stage I~VI)		
Count of germ cells in a seminif. tubule	1098.5 ± 43.4	1150.8 ± 110.9
Count of Sertoli cells in a seminif. tubule	133.8 ± 7.9	130.8 ± 4.6
Germ cells/Sertoli cells	8.2 ± 0.7	8.8 ± 1.1
Group 2 (Stage VII~VIII)		
Count of germ cells in a seminif. tubule	1026.5 ± 84.3	1039.3 ± 24.4
Count of Sertoli cells in a seminif. tubule	137.0 ± 7.4	120.5 ± 9.0
Germ cells/Sertoli cells	7.5 ± 0.9	8.7 ± 0.7
Group 3 (Stage IX~XI)		
Count of germ cells in a seminif. tubule	933.8 ± 66.5	938.3 ± 20.9
Count of Sertoli cells in a seminif. tubule	135.3 ± 3.0	125.0 ± 8.2
Germ cells/Sertoli cells	6.9 ± 0.6	7.5 ± 0.4
Group 4 (Stage XII~XIV)		
Count of germ cells in a seminif. tubule	768.5 ± 28.9	738.8 ± 62.9
Count of Sertoli cells in a seminif. tubule	130.8 ± 7.0	127.0 ± 9.7
Germ cells/Sertoli cells	5.9 ± 0.5	5.8 ± 0.2

Values represent mean ± S.D.
[a] Vehicle control (corn oil, 5 mL/kg).

butyl)phthalate (DBP). They made oral administration of DBP at doses of 0.5, 5, 50, 100 and 500 mg/kg to pregnant rats from G12 to 21, and observed histopathological changes in fetal testes such as degeneration of seminiferous tubules, focal interstitial cell hyperplasia and adenoma at 500 mg/kg, but not at 100 mg/kg. Parks *et al.* (2000) treated maternal rats with 750 mg/kg of DEHP from G14 to postnatal day 3 and observed the appearance of multinucleated genocytes and hyperplasia of interstitial cells in the testis of G20 fetuses and in offspring at Day 3 of lactation. Thus, the present study has confirmed the characteristics of phthalate toxicity on testicular development in rats, which seems to occur in spite of differences in esterifying alcohol and administration protocol. The no-observed effect-level of DEHP on the testicular development of rats by *in utero* exposure during the period of organogenesis was 125 mg/kg. Target cells of the testicular toxicity of phthalates are the germ cells in the fetal rat, while they are the Sertoli cells in the adult rat when the blood-tes-

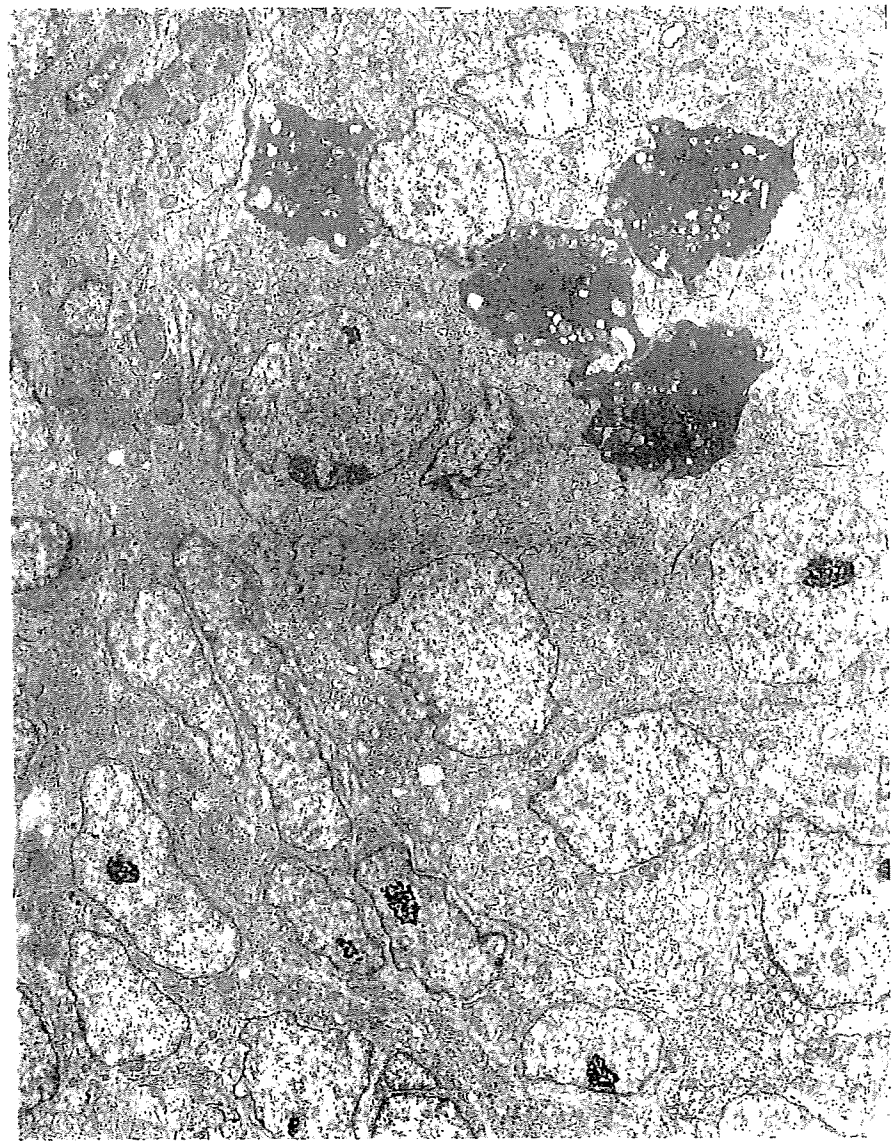

Photo 7. An electron micrograph of genital ridge of a rat fetus on gestation day 16 treated with 1000 mg/kg of DEHP, showing degenerated germ cells. ×2830.

tis barrier is established (Creasy *et al.*, 1983, Saitoh *et al.*, 1997, de Kretser and Kerr, 1994).

In the present study, EE was used as a reference compound, considering some interventions of estrogenic activity of DEHP for its toxicity on the testis. The result was negative for this consideration, although some relation may have existed to the increase in embryonic mortality. Estrogenic activity of various phthalate esters was investigated by Zacharewski *et al.* (1998). They observed weak estrogen receptor affinity *in vitro* for some phthalate esters other than DEHP, but no estrogenic activity *in vivo* for any of the phthalate esters by rat uterotropic assay. On the other hand, antiandrogenic activity has been suggested as one of the mechanisms of testicular toxicity of phthalate esters (Mylchreest *et al.*, 1998). Mylchreest *et al.* (1999) observed disturbances in male reproductive development with 500 mg/kg of DBP comparable to 100 mg/

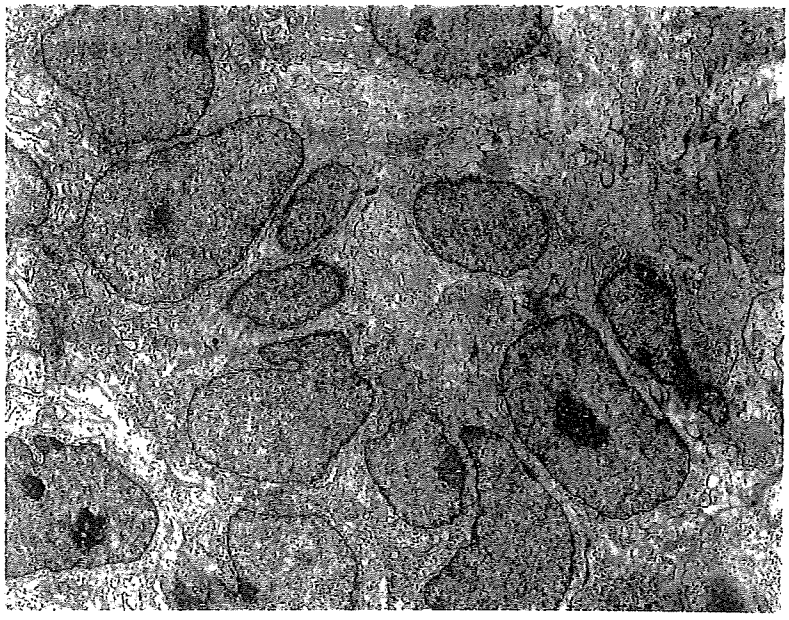

Photo 8-a. An electron micrograph of testis of a rat fetus on gestation day 18 treated with 1000 mg/kg of DEHP, showing decreased number of lipid droplets in small-sized interstitial cells. ×3140.

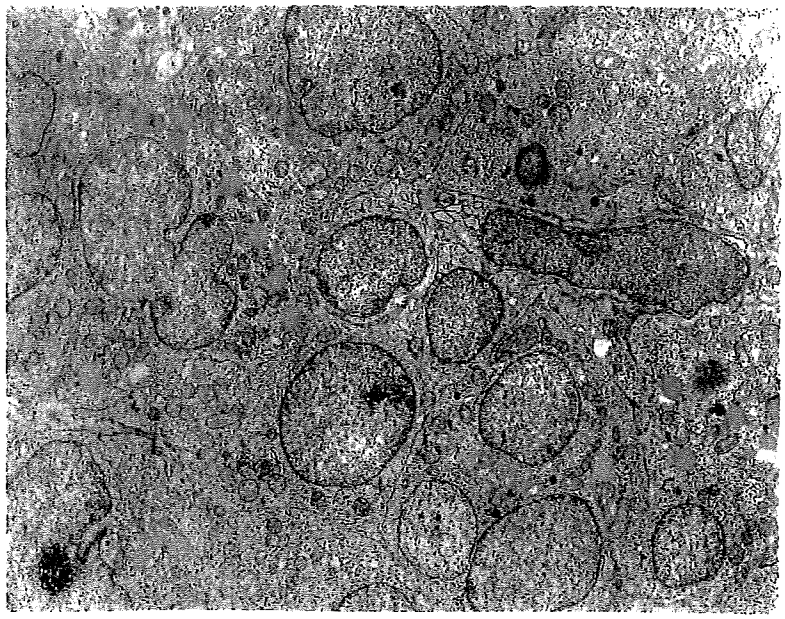

Photo 8-b. An electron micrograph of testis of a rat fetus on gestation day 20 treated with 1000 mg/kg of DEHP, showing decreased number of lipid droplets in small-sized interstitial cells. ×3140.

kg of flutamide, a known anti-androgen, but they could not confirm any interaction of phthalate with androgen receptor *in vitro*. They explained that DBP exerted its anti-androgenic activity by indirectly interfering with androgen signaling pathways (Mylchreest and Foster, 2000). Parks *et al.* (2000) observed inhibition of testosterone production of fetal testis (G17-20) with DEHP (750 mg/kg) in the experiment cited above. In the present study, increase of androgen receptor-positive interstitial cells was observed in G20 fetal testis in the groups treated with DEHP at 250 mg/kg and above. It is conceivable that interstitial cells and androgen receptors are increased by compensatory responses to reduced testosterone levels. Thus, anti-androgenic activity of DEHP is suggested from the observation of the present study, although malformations of male genital organs typical of anti-androgens such as flutamide (Mylchreest *et al.*, 1998, 1999) were not observed with DEHP up to 1000 mg/kg in the present study.

The present study has demonstrated that testicular damage in fetal rats produced by DEHP at 500 mg/kg (but not at 1000 mg/kg) had been repaired by 7 weeks of age. This was confirmed in the second experiment at 5 and 10 weeks of age. Expression of androgen receptors in testicular cells was normal in these stages of rats. Moreover, examination of sperm in off-

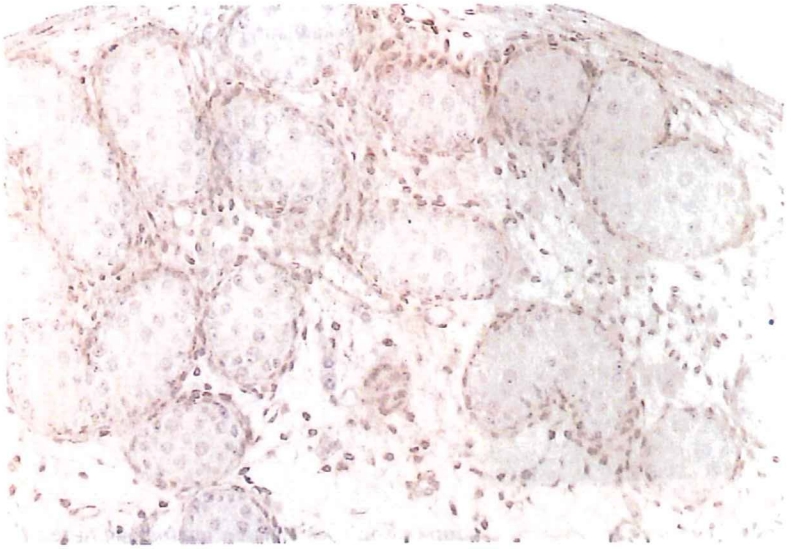

Photo 9-a. Immunohistochemical staining of androgen receptors in testis on rat fetus on G20 from the control group. Positive signals are observed on peritubular myoid cells and interstitial cells. ×175.

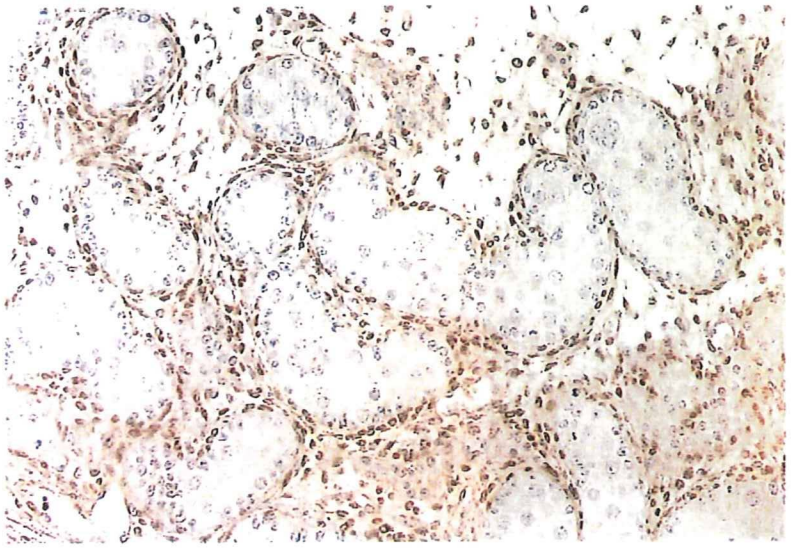

Photo 9-b. Immunohistochemical staining of androgen receptors in testis of rat fetus on G20 from the group treated with 500 mg/kg of DEHP. Interstitial cells with positive androgen-receptor signals are increased. ×175.

spring of DEHP-treated rats at 10 weeks of age showed no abnormal features of sperm function and morphology.

REFERENCES

Calley, D., Autian, J. and Guess, W.L. (1966): Toxicology of a series of phthalate esters. J. Pharm. Sci., **55**, 158-162.

Cater, B.R., Cook, M.W., Gangolli, S.D. and Grasso, P. (1977): Studies on dibutyl phthalate-induced testicular atrophy in the rat: effect on zinc metabolism. Toxicol. Appl. Pharmacol., **41**, 609-618.

Creasy, D.M., Beech, L.M., Gray, T.J.B. and Butler, W.H. (1987): The ultrastructural effect of di-*n*-pentyl phthalate on the testis of the mature rats. Exp. Mol. Pathol., **46**, 357-371.

Creasy, D.M., Foster, J.R. and Foster, P.M.D. (1983): The morphological development of di-*n*-pentyl phthalate induced testicular atrophy in the rat. J. Pathol., **139**, 309-321.

Dalgaard, M., Nellemann, C., Lam, H.R., Sorensen, I.K. and Ladefoged, O. (2001): The acute effects of mono(2-ethylhexyl)phthalate (MEHP) on testes of prepubertal Wistar rats. Toxicol. Lett., **122**, 69-79.

de Kretser, D.M. and Kerr, J.B. (1994): The cytology of the testis. In "Physiology of Reproduction", 2nd ed., (Knobil, E. and Neill, J.D., eds.), pp. 1177-1290, Raven Press, New York.

Dym, M. and Clermont, Y. (1970) Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. Am. J. Anat., **128**, 265-282.

Gangolli, S.D. (1982): Testicular effects of phthalate esters. Environ. Health Perspect., 45, 77-84.

Gray, T.J. and Butterworth, K.R. (1980): Testicular atrophy produced by phthalate esters. Arch. Toxicol. Suppl., **4**, 452-455.

Table 8. Examination of epididymal spermatozoa at 10 weeks after birth in the offspring exposed to di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Animals examined	4	4	4	4
Sperm counts (per cauda epididymis) [b]	176.7 ± 55.7	142.9 ± 51.2	149.9 ± 48.9	175.0 ± 49.8
Sperm counts/cauda epididymis weight (g) [b]	1015.2 ± 241.0	878.0 ± 305.6	872.9 ± 198.7	992.1 ± 335.2
Sperm motility				
Rate of motile sperm (%) [b]	98.1 ± 1.2	96.6 ± 1.0	98.4 ± 1.1	97.2 ± 1.8
Rate of progressive sperm (%) [b]	84.4 ± 5.2	85.5 ± 1.8	88.7 ± 2.0	88.4 ± 3.2
Sperm morphology				
Sperms examined	800	800	800	800
Sperms with abnormalities	34	49	45	44
Abnormality rate (%) [b]	4.3 ± 1.7	6.1 ± 1.7	5.6 ± 5.5	5.5 ± 1.8
Types and incidence (%) of abnormal sperms				
Pin head	0	0	0.3	0.3
Amorphous head	0	0	0.1	0
Short head	0.1	0	0	0.1
Banana head	0	0.1	0	0
Reduced hock	0.1	0.5	0.4	0.3
No hock	0	0.1	0.1	0.1
Excessive hock	0	0	0.1	0
Bent flagellum	0.1	0.1	0	0
Broken flagellum	0.1	0.5	0.1	0.4
Bent neck	0.4	0.6	0.1	0.6
Isolated head	3.3	4.1	4.4	3.8
Two heads, one tail	0.1	0	0	0

[a] Vehicle control (corn oil, 5 mL/kg). [b] Values represent mean ± S.D.

Gray, T.J., Butterworth, K.R., Gaunt, I.F., Grasso, G.P. and Gangolli, S.D. (1977): Short-term toxicity study of di-(2-ethylhexyl) phthalate in rats. Food Cosmet. Toxicol., **15**, 389-399.

Gray, T.J. and Gangolli, S.D. (1986): Aspects of the testicular toxicity of phthalate esters. Environ. Health Perspect., **65**, 229-235.

Matsui, H., Toyoda, K., Kawanishi, T., Mitsumori, K. and Takahashi, M. (1996): Direct toxic effects of ethylene-1,2-dimethanesulfonate(EDS) on the rat epididymis. J. Toxicol. Pathol., **9**, 65-72.

Mylchreest, E., Cattley, R.C. and Foster, P.M.D. (1998): Male reproductive tract malformations in rats following gestational and lactational exposure to di(*n*-butyl) phthalate: An antiandrogenic mechanism? Toxicol. Sci., **43**, 47-60.

Mylchreest, E. and Foster, P.M.D. (2000): DBP exerts its antiandrogenic activity by indirectly interfering with androgen signaling pathways. Toxicol. Appl. Pharmacol., **168**, 174-175.

Mylchreest, E., Sar, M., Cattley, R.C. and Foster, P.M.D. (1999): Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. Toxicol. Appl. Pharmacol., **156**, 81-95.

Mylchreest, E., Wallace, D.G., Cattley, R.C. and Foster, P.M.D. (2000): Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di-(*n*-butyl) phthalate during late gestation. Toxicol. Sci., **55**, 143-151.

Ono, H., Saito, Y., Imai, K. and Kato, M. (2004): Subcellular distribution of di-(2-ethylhexyl) phthalate in rat testis. J. Toxicol. Sci., **29**, 113-124.

Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J. and Gray, L.E.Jr. (2000): The plasticizer diethylhexyl phthalate induces malformation by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol. Sci., **58**, 339-349.

Saitoh, Y., Usumi, K., Nagata, T., Marumo, H., Imai, K. and Katoh, M. (1997): Early changes in the rat testes induced by di-(2-ethylhexyl) phthalate and 2,5-hexandione – Ultrastructure and lanthanum trace study. J. Toxicol. Pathol., **10**, 51-57.

Sato, M., Ohta, R., Kojima, K. and Shirota, M. (2002a): Strain differences in the spontaneous incidence of sperm morphological abnormalities in Hatano rats. J. Vet. Med. Sci., **64**, 389-390.

Sato, M., Ohta, R., Kojima, K., Shirota, M., Koibuchi, H., Asai, S., Watanabe, G. and Taya, K. (2002b): A comparative study of puberty, and plasma gonadotropin and testicular hormone levels in two inbred strains of Hatano rats. J. Reprod. Dev., **48**, 111-119.

Sato, M., Ohta, R., Wada, K., Marumo, H., Shirota, M. and Nagao, T. (2000): Utilization of a computer-assisted sperm motion analysis system to examine effects of dinoseb on rat sperm. J. Reprod. Dev., **46**, 279-286.

Yasuda, M., Kihara, T. and Tanimura, T. (1985): Effect of ethynyl estradiol on the differentiation of mouse fetal testis. Teratology, **32**, 113-118.

Yoshimura, I. (1986): Statistical Analysis of Toxicological and Pharmacological Data. (in Japanese) Scientists (Publisher), Tokyo.

Zacharewski, T.R., Meek, M.D., Clemons, J.H., Wu, Z.F., Fielden, M.R. and Mattews, J.B. (1998): Examination of the *in vivo* estrogenic activities of eight commercial phthalate esters. Toxicol. Sci., **46**, 282-293.