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.

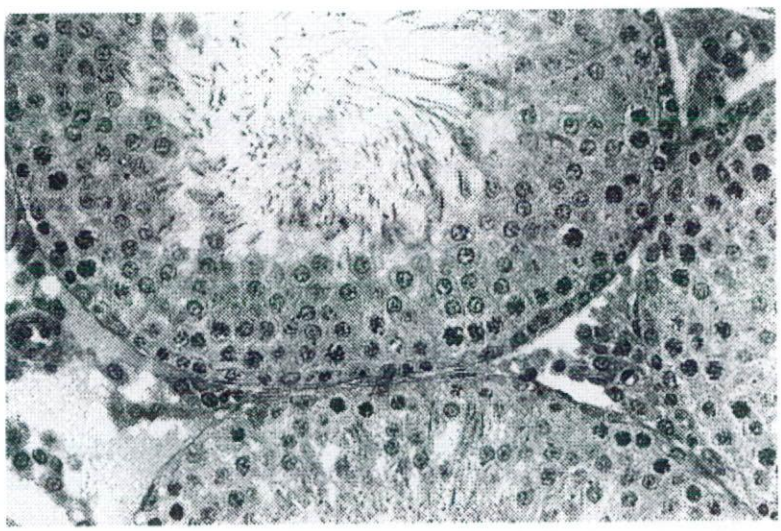

Photo 4-a. Testis of a 7-week-old rat treated with 500 mg/kg of DEHP *in utero* showing no abnormalities in the seminiferous tubules and interstitial cells. HE stain, ×310.

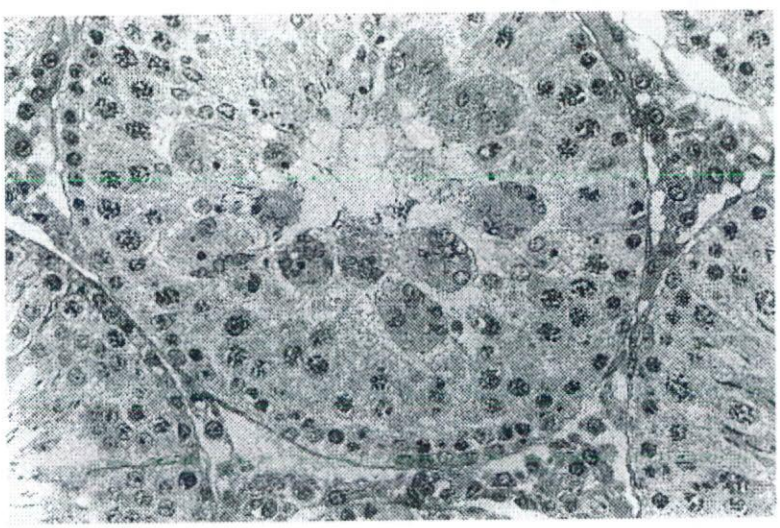

Photo 4-b. Testis of a rat of the same group as Photo 4-a, showing multinucleated giant cells in a seminiferous tubule. HE stain, ×310.

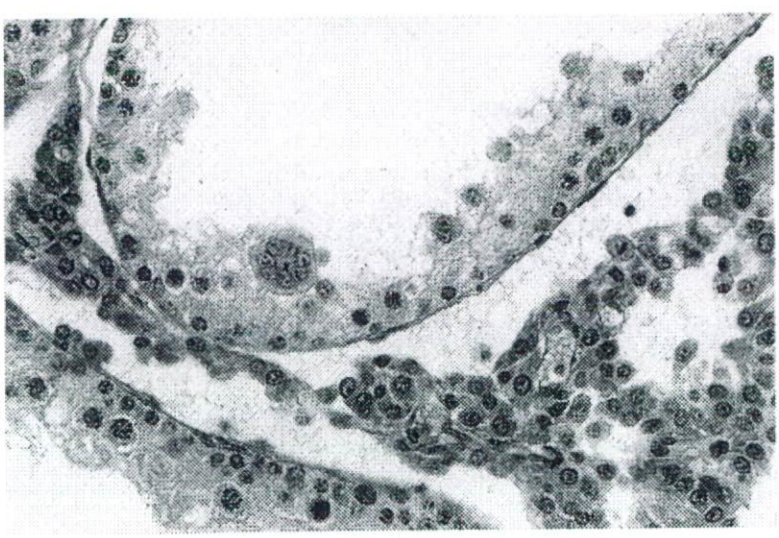

Photo 4-c. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing atrophy of seminiferous tubule epithelia with multinucleated giant cell in the lumen, and also hyperplasia of interstitial cells. HE stain, ×310.

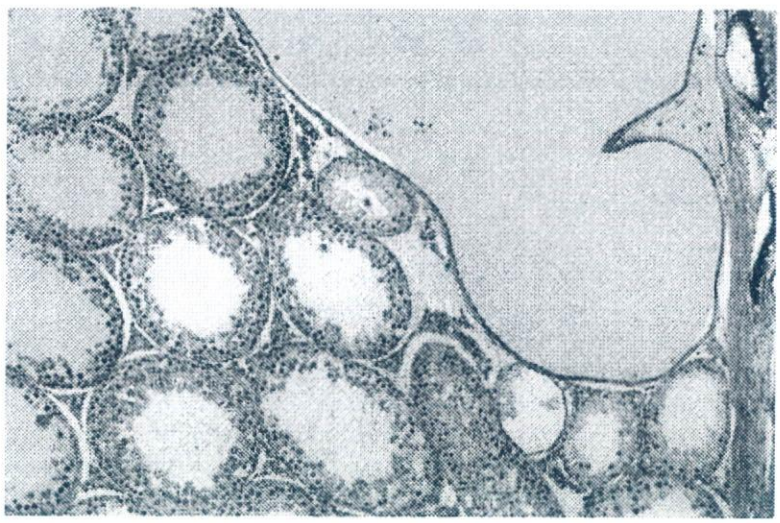

Photo 5-a. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing dilatation of seminiferous tubules and of rete testis. HE stain, ×80.

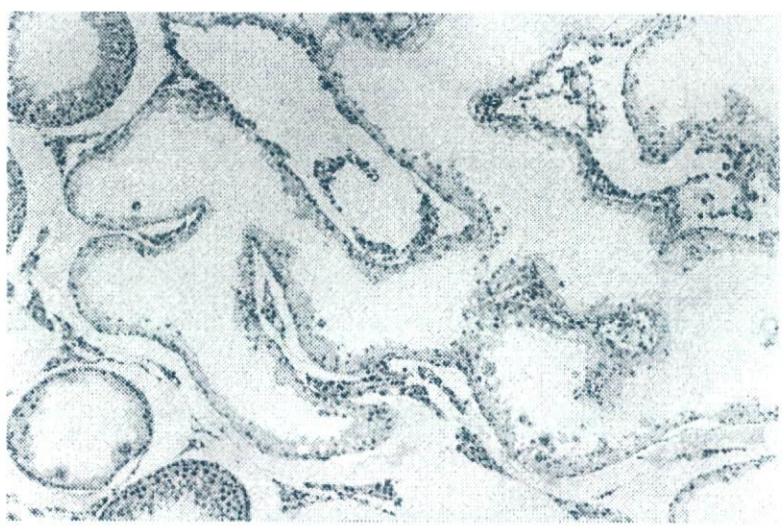

Photo 5-b. Testis from a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing branching of atrophic seminiferous tubules. HE stain, ×80.

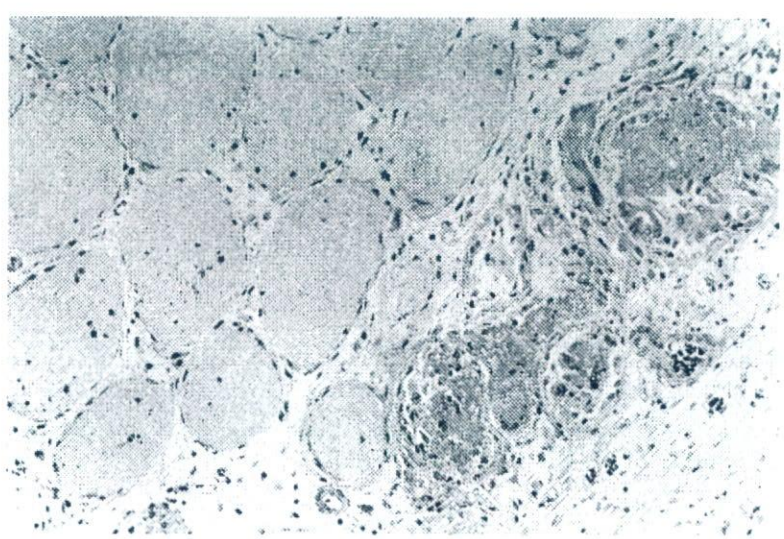

Photo 5-c. Testis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, showing extensive necrosis and foreign body giant cells. HE stain, ×160.

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-

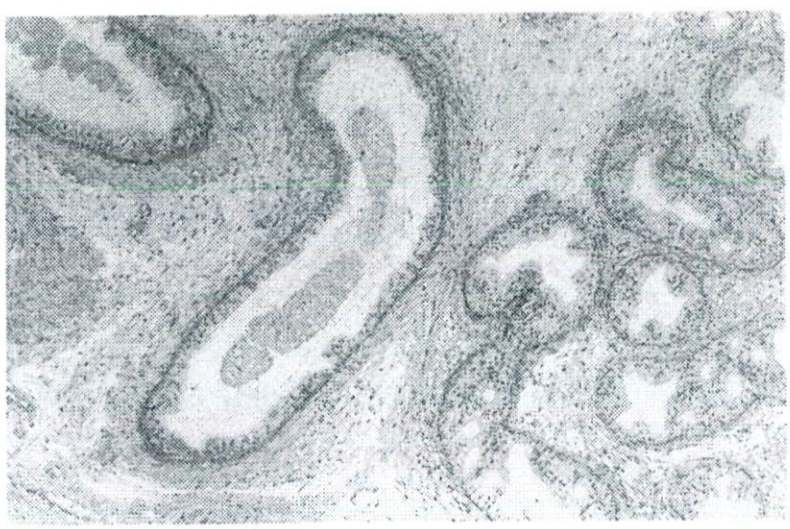

Photo 6-a. Epididymis of a 7-week old rat treated with 1000 mg/kg of DEHP *in utero*, showing atrophy of epididymal ducts and cell debris in the lumen. HE stain, ×80.

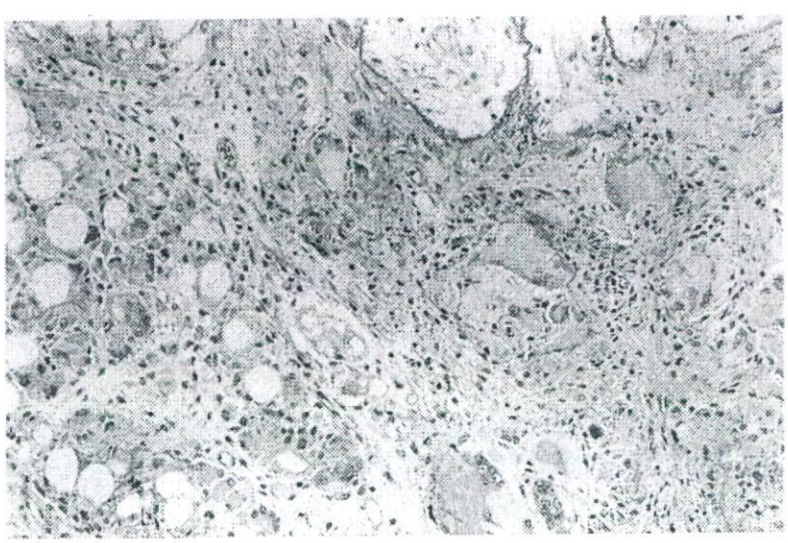

Photo 6-b. Granuloma formed in the epididymis of a 7-week-old rat treated with 1000 mg/kg of DEHP *in utero*, accompanied by numerous foreign body giant cells and fibrosis. HE stain, ×160.

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-

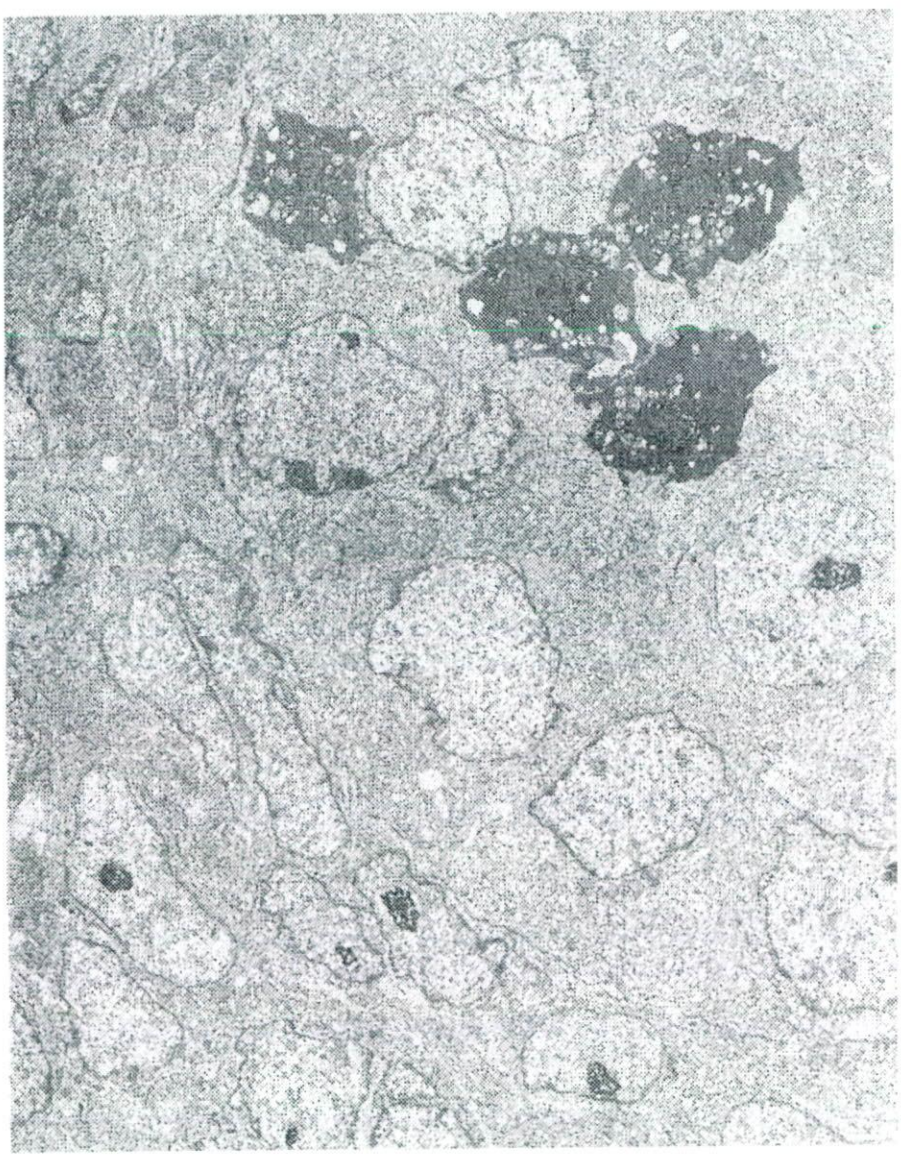

Photo 7. An electron micrograph of genital ridge of a rat fetus on gestation day 16 treated with 1000 mg/kg of DEHP, showing degenerated germ cells. ×2830.

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/

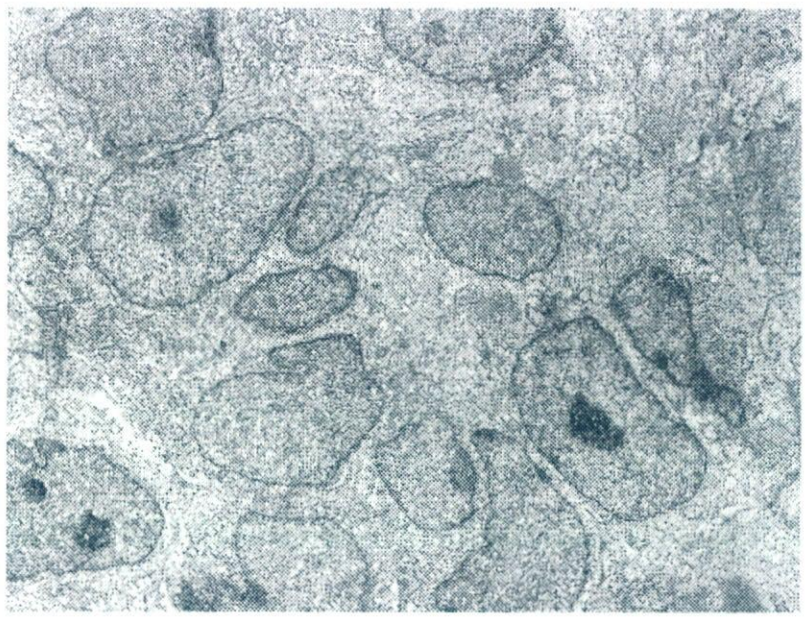

Photo 8-a. An electron micrograph of testis of a rat fetus on gestation day 18 treated with 1000 mg/kg of DEHP, showing decreased number of lipid droplets in small-sized interstitial cells. ×3140.

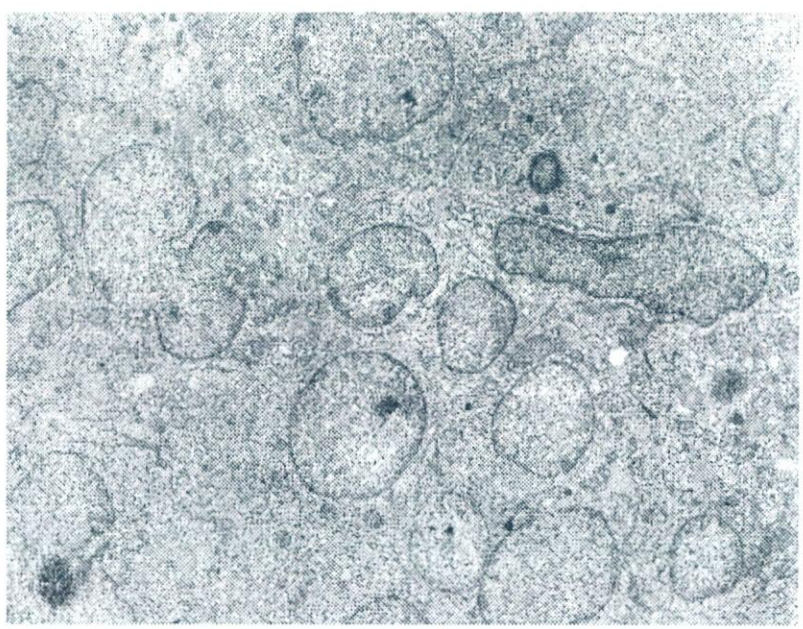

Photo 8-b. An electron micrograph of testis of a rat fetus on gestation day 20 treated with 1000 mg/kg of DEHP, showing decreased number of lipid droplets in small-sized interstitial cells. ×3140.

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-

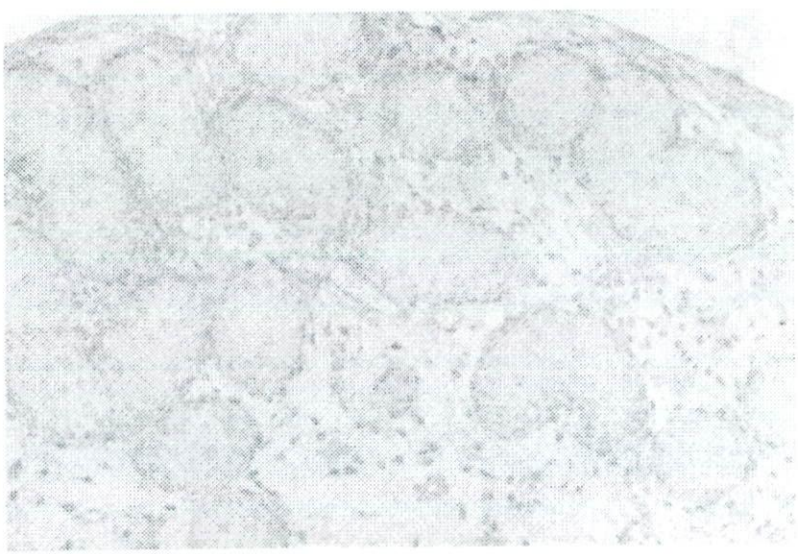

Photo 9-a. Immunohistochemical staining of androgen receptors in testis on rat fetus on G20 from the control group. Positive signals are observed on peritubular myoid cells and interstitial cells. ×175.

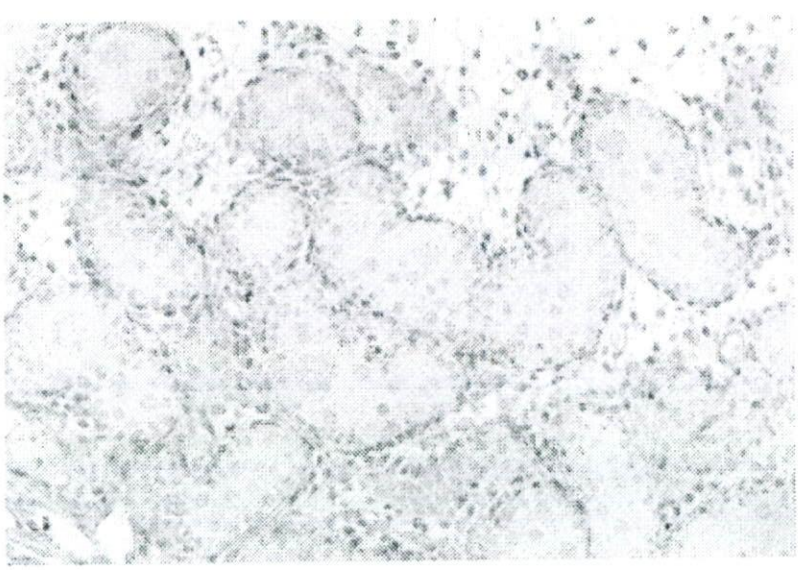

Photo 9-b. Immunohistochemical staining of androgen receptors in testis of rat fetus on G20 from the group treated with 500 mg/kg of DEHP. Interstitial cells with positive androgen-receptor signals are increased. ×175.

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.

内分泌攪乱性確定試験としてのラット一生涯試験の試み

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Rat One-lifespan Test as a Definitive Test for Endocrine Disruptors

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内分泌攪乱性確定試験としてのラット一生涯試験の試み

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Rat One-lifespan Test as a Definitive Test for Endocrine Disruptors

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In order to establish a definitive test protocol for endocrine disruptors, a one-lifespan test was performed using rats and the aging process of reproductive function was observed. Neonates of Sprague-Dawley rats received forced oral administration of diethylstilbestrol (DES) at doses of 0 (vehicle), 0.05, 0.5 and 5 μg/kg for 5 days after birth. Sexual maturation (vaginal opening and preputial separation), estrous cycles (from 8 to 49 weeks of age), mating (at 12, 23, 34, 56 and 68 weeks) and litter size (of the 1st to 3rd parturitions) were observed. Each half of the males were examined for sperm counts and organ weights at 26 and 52 weeks of age. In half of the females, hCG induced ovulation and organ weights were examined at 54 weeks of age. Then the observation of remaining animals was terminated at 101 weeks and survival rate were determined.

Vaginal opening in the group received DES at 5 μg/kg was significantly earlier than the vehicle control group. Normal estrous cycles were observed in no animals of 5 μg/kg DES group throughout the study, and in less than 10% of 0.5 μg/kg DES group at 28 weeks and on. Fertility rate of 12 week-old females of the 5 μg/kg DES group was 0%, and that of 23 week-old females of the 0.5 μg/kg group was 33.3%. Mating rate of 0.05 μg/kg females of this age was reduced to 60%. Influence of neonatal DES exposure was not observed in the first delivery in any group, but in the second parturitions litter size was reduced significantly in the 0.5 μg/kg group. Organ weights of 54 week-old females showed dose-related significant increase of pituitary weight in the 0.05 to 5 μg/kg groups. Adrenal weight was increased in the 0.5 and 5 μg/kg groups. Weight of ovaries was lowered significantly in the 0.5 and 5 μg/kg groups. Testing of induced ovulation with hCG revealed lack of influence of DES on number of shed oocytes. No effects of neonatal DES exposure in males were observed on preputial separation, fertility, sperm counts and organ weights. The lower survival rate was observed in the 5 μg/kg group females.

These results showed that early life exposure of low doses of DES potentially cause precocious sexual maturation, and decreases in reproductive function such as estrous cyclicity, fertility or litter size in female rats. These effects were considered to cause through disruption of hypothalamo-pituitary system, not through direct disturbance on ovarian function. The effects of DES observed in this study indicate the usefulness of one-lifespan test as a definitive test protocol for endocrine disruptors.

緒言

現在，内分泌攪乱化学物質（環境ホルモン，EDC）研究の焦点は，化学物質の内分泌攪乱性を確定する試験法の開発にある．ホルモン活性を有する化学物質が環境中にも存在することは既知の事実で，化学物質のホルモン活性の有無を検討する方法はEDCのスクリーニング試験となり得る．しかし，ホルモン活性を有する化学物質が，生体に有害な影響すなわち内分泌攪乱性を示すか否かを判定する試験法は確立されていない．実

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際，これら外因性ホルモン活性物質よりはるかに強力な受容体結合性を持つ内因性ホルモンの影響が，従来の生殖発生毒性試験（多世代繁殖試験）では確認されないにもかかわらず，ジエチルスチルベストロール（DES）のような物質では内分泌系など高次調節系の遅発性の異常が臨床的に起っている．つまりEDCは実際に存在し，それを試験する方法が求められている．このような理由から，現在，EDCの確定試験として従来の多世代繁殖試験に代る「一生涯試験」が考案された．

本研究ではエストロゲン活性を有するDESをSprague-Dawley（SD）ラットの新生児期に投与し，児の発達，成熟および老化に至る各段階において生殖器系機能の変化を検索する「ラット一生涯試験」を試みた．本研究では，新生児期DES投与が引き起こす遅発性の生殖機能異常を検索するために，雌は8週齢から49週齢まで性周期を観察し，12，23および34週齢で交配実験を行った．雄については，26および52週齢で精子検査を行い，12，23，34，56および68週齢で交配実験を行った．

材料および方法

試験には，日本チャールス・リバーから8週齢で入手したCrl:CD（SD）雌雄ラットを使用した．SD系ラットは，毒性試験において一般的に用いられている系統であり，生殖毒性に関する背景データが豊富で，Wistar系ラットに比べて性周期の加齢性変化が早期に起り易いことが知られている[1]．11週齢時に交配し，交尾が確認された雌を1群12匹以上からなる4群に振分けた．動物は温度22～25℃，湿度50～65％，照明12時間（7時～19時点灯）に調節された飼育室で，固型飼料（CE-2，日本クレア）と水道水を自由摂取させて飼育した．妊娠雌は，紙パルプ製チップを入れた金属製ケージに1匹ずつ収容した．全ての実験操作は，「財団法人食品薬品安全センター秦野研究所 動物実験に関する指針」に基づいて実施した．

EDCには子宮内あるいは新生児期の曝露での影響が指摘されていることから，DESの投与経路は新生児への強制経口投与を選択した．投与量は，内分泌攪乱化学物質に対する厚生労働省の試験スキーム[2]を考慮し，子宮肥大試験の結果をもとに設定した．すなわち，0.05～15 μg/kg/dayのDESを卵巣摘出マウスに3日間反復経口投与し，最終投与の約24時間後に子宮重量を測定した結果，5 μg/kg/day以上を投与した群で子宮重量が有意に増加したことから，5 μg/kg/dayを確実影響量として一生涯試験の最高用量に設定し，無影響量と考えられる0.5および0.05 μg/kg/dayをそれぞれ中用量および低用量に設定した．投与液は，DES（Sigma-Aldrich, St. Louis, MO）20 mgを1 mLのエタノールに溶解し，コーン油で段階希釈して調製した．

新生児は，生後1日（分娩日を生後0日とする）に性別および外表奇形の有無を検査し，異常のない雌雄各5匹を1腹毎に選抜し，四肢の皮下に墨汁を注入して個体識別した．投与は生後1日から生後5日まで1日1回，マイクロシリンジおよび新生児用カテーテル[3]を用いて行い，投与液量は10 mL/kgとした．投与終了後は同腹児数を雌雄各4匹に調整し，生後21日に離乳させた．離乳後は，金属製金網床ケージに2匹ずつ収容した．体重は，生後0～5日（毎日），7，14および21日に測定し，離乳後は週1回，10週齢以降は隔週1回，26週齢以降は4週間毎に測定した．

雌は生後25日から腟開口を，雄は生後35日から陰茎包皮分離[4]を性成熟の指標として毎日観察した．各腹の雌2匹は，8週齢から49週齢まで2週間間隔で連日2週間，腟垢を採取し，性周期を観察した．腟垢像は発情前期，発情期，発情休止期に分類し，渡辺らの報告[1]と同様に性周期の型を分類した．各腹の雌雄各2匹は，12，23および34週齢から2週間を限度に，兄妹交配を避けて1：1で群内交配させた．群内交配で交尾が確認されなかった場合，雄は無処置雌と，雌は交尾が確認された同群の雄と，いずれも2週間を限度に再交配させた．雄は，さらに56および68週齢から2週間を限度に無処置雌と交配させた．群内交配で交尾が確認された雌は自然分娩させ，妊娠日数および産児数を確認し，哺育0および4日の哺育児体重を測定した．無処置雌は妊娠13日以降に帝王切開し，妊娠の有無を確認した．

雄は26，52および101週齢時，雌は54および

101週齢時にペントバルビタールナトリウム麻酔下で採血し，剖検した．雌雄とも101週齢以外の剖検時には，脳，下垂体，甲状腺，肝臓，脾臓，腎臓，副腎，精巣，精巣上体，前立腺（腹葉），精嚢（凝固腺を含む），卵巣，子宮の重量を測定した．

26および52週齢の剖検時に雄から採取し，凍結保存した精巣上体尾部および精巣を用いて精子数および精子頭部数を測定した．精巣上体尾部および精巣は，解凍後，ホモジナイズした精子懸濁液をModified IDENT STAIN Kit（Hamilton-Thorne）により染色し，HTM-IVOSにより，精巣上体尾部および精巣重量当たりの精子数および精子頭部数を求めた[5]．

各腹とも雌1匹は，54週齢時に排卵可能な卵胞の有無を確認するため，剖検16～17時間前にヒト絨毛性性腺刺激ホルモン（hCG，Sigma）を10 IU尾静脈内投与し，剖検時に卵管内の誘起排卵数を数えた[1]．

離乳前の児に関するデータは腹単位，離乳以降のデータは個体を標本単位として解析した．体重，器官重量，産児数および精子数のデータは，一元配置型の分散分析を行い，群間に有意差が認められた場合はDunnett法による多重比較検定を行った．性成熟，妊娠日数および生存率のデータは，Kruskal-Wallisの順位検定を行い，群間に有意差が認められた場合には，順位化した値を用いてDunnett法による多重比較を行った．交尾率および受胎率の差は，Fisherの直接確率法による検定を行った．有意水準は5%および1%とした．

結果

体重：生後0日から離乳まで，および離乳後から26週齢までの体重は，各群とも順調に増加し，雌雄ともDES投与の影響は認められなかった．また，26週齢以降の体重推移についても，46週齢から50週齢にかけて対照群の雌の体重が低下した以外に異常は認められなかった．

性成熟：雌の腟開口時期（平均±S.D.，日）は，5 μg/kg投与群（29.8 ± 2.2）で対照群（32.9 ± 1.7）より有意に早まったが，雄の陰茎包皮分離時期にはDES投与の影響はみられなかった．腟開口時期が早まった5 μg/kg投与群では，雌の全例で尿道開口部の過剰開裂[6]が観察された．

性周期：正常な性周期を示した雌の割合を図1に示した．5 μg/kg投与群では，観察を開始した8週齢から正常な性周期を示す雌は認められなかった．0.5 μg/kg投与群では8週齢から13週齢にかけては80%以上の雌が正常な性周期を示したが，20週齢から25週齢時には約50%，28週齢以降は10%未満となった．0.05 μg/kg投与群は対照

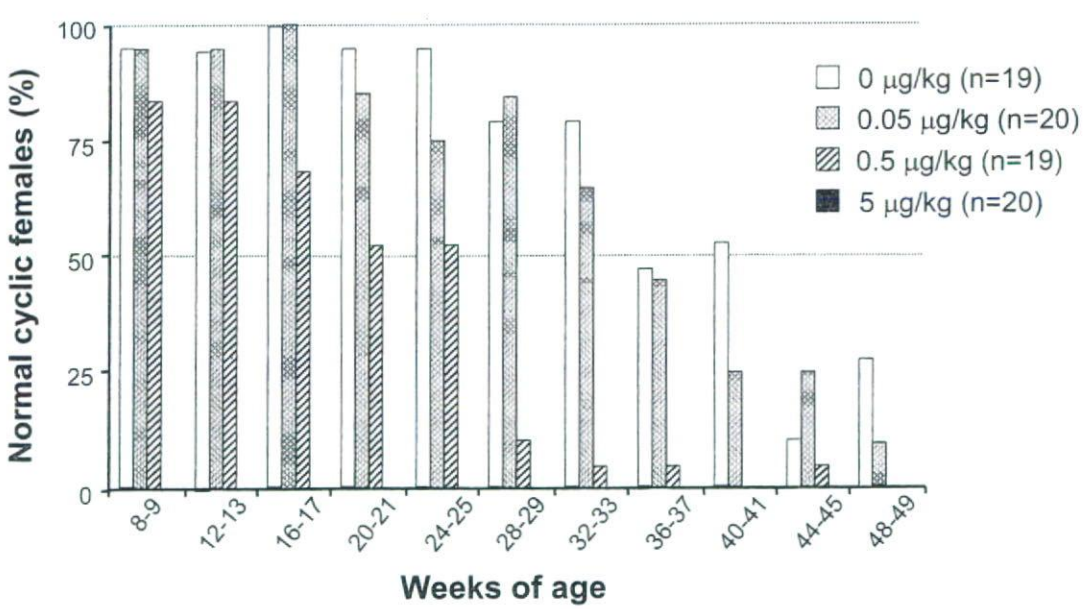

図1　新生児期にDESを投与したSD系雌ラットの性周期（正常性周期の割合の推移）

群とほぼ同様に推移し，正常な性周期を示す雌が29週齢までは80％以上，36週齢以降は50％未満となった．性周期を型別（図2）に見ると，5 μg/kg投与群で早期にみられた異常周期の型は連続発情であったのに対し，0.5 μg/kg以下の用量で加齢に伴って増加した異常周期の型は不規則周期や無発情であった．

交尾率・受胎率：交配結果を表1に示した．雄は12，23および34週齢のいずれの交配時期においても，交尾率および受胎率にDES投与の影響は認められなかった．また，56および68週齢の無処置雌との交配においても，DES投与の影響を示唆する変化は認められなかった．雌は12週齢の交配では，5 μg/kg投与群の交尾率は90％であったが，受胎率は0％となったため同群雌の23週齢以降の交配は中止した．23週齢の交配では，0.05 μg/kg投与群の交尾率が60％に低下し，0.5 μg/kg投与群の受胎率が33.3％に低下した．34週齢の交配では，対照群を含む各投与群の交尾率および受胎率が低下した．

分娩・哺育：分娩した雌の哺育成績を表2に示した．初回分娩では5 μg/kg投与群で産児が得られなかった以外にDES投与の影響は認められなかった．2産目では，0.5 μg/kg投与群の産児数が対照群より有意に減少し，0.05 μg/kg投与群の妊娠日数が対照群より延長する傾向にあった．3産目については，対照群を含む各投与群で受胎率が低下したことから，産児数の評価はできなかった．

雄の精子数および器官重量：26週齢および52週齢で精子数と器官重量を調べたが，いずれの時期においても，精巣上体尾部の精子数，精巣重量当りの精子頭部数，ならびに生殖器を含むいずれの器官重量にもDES投与の影響を示唆する変化は認められなかった．

雌の器官重量：54週齢の雌の器官重量を図3に示した．雌では，全てのDES投与群で下垂体重量が対照群より有意に増加し，5および0.5 μg/kg投与群で副腎重量が，5 μg/kg投与群で甲状腺重量が有意に増加した．また，5および0.5 μg/kg投与群で卵巣重量が対照群より有意に低下した．剖検時には，皮下に乳汁が貯留している例が0.05 μg/kg投与群で20例中2例，0.5 μg/kg投与群で18例中3例，5 μg/kg投与群で19例中9例みられた．その他，血中ホルモン濃度の測定では，0.05 μg/kg以上の投与群でプロラクチン濃度の上昇が，0.5 μg/kg以上の投与群でLH濃度の上昇

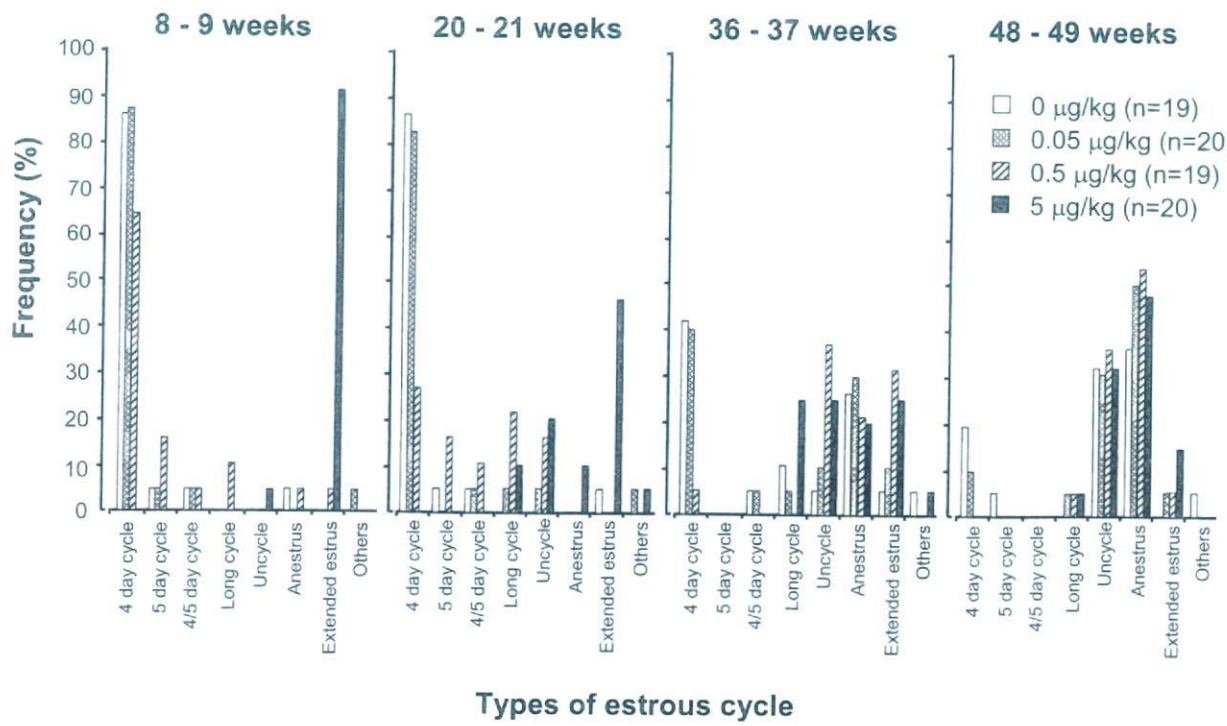

図2　新生児期にDESを投与したSD系雌ラットの性周期（性周期の型別の推移）

表1　新生児期にDESを投与したSD系ラットの交配成績

DES (μg/kg)	Males 0	0.05	0.5	5	Females 0	0.05	0.5	5
At 12 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)
Fertility index (%) (No. pregnant/no. copulated)	100.0 (20/20)	84.2 (16/19)	95.0 (19/20)	100.0 (18/18)	90.0 (18/20)	81.3 (13/16)	80.0 (16/20)	0.0 ** (0/18)
At 23 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (17/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	60.0 ** (12/20)	90.0 (18/20)	
Fertility index (%) (No. pregnant/no. copulated)	89.5 (17/19)	100.0 (17/17)	100.0 (20/20)	83.3 (15/18)	80.0 (16/20)	58.3 (7/12)	33.3 ** (6/18)	
At 34 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	55.0 (11/20)	25.0 (5/20)	20.0 * (4/20)	
Fertility index (%) (No. pregnant/no. copulated)	95.0 (19/20)	72.2 (13/18)	90.0 (18/20)	84.2 (16/19)	54.5 (6/11)	60.0 (3/5)	25.0 (1/4)	
At 56 weeks of age								
Copulation index (%) (No. copulated/no. mated)	90.0 (18/20)	60.0 (12/20)	60.0 (12/20)	79.8 (15/20)				
Fertility index (%) (No. pregnant/no. copulated)	72.2 (13/18)	66.7 (8/12)	83.3 (10/12)	66.7 (10/15)				
At 68 weeks of age								
Copulation index (%) (No. copulated/no. mated)	57.9 (11/19)	47.4 (9/19)	55.6 (10/18)	55.6 (10/18)				
Fertility index (%) (No. pregnant/no. copulated)	72.7 (8/11)	66.7 (6/ 9)	80.0 (8/10)	50.0 (5/10)				

*, ** は対照群と比較して有意差（5%および1%）があることを示す．
データには無処置雌との交配結果も含まれる．

表2　新生児期にDESを投与したSD系母ラットの哺育成績

DES (μg/kg)	0	0.05	0.5	5
At the 1st parturition				
Number of dams	18	13	16	0
Gestation length in days	22.1 ± 0.3	22.2 ± 0.7	22.1 ± 0.5	
Number of newborns	13.9 ± 3.4	12.9 ± 3.9	14.1 ± 3.6	
Pup weight (g) Male	6.9 ± 0.3	6.8 ± 0.6	6.7 ± 0.7	
Female	6.5 ± 0.3	6.4 ± 0.6	6.3 ± 0.7	
Viability index on PND 4	99.7 ± 1.5	98.5 ± 3.0	99.0 ± 3.0	
At the 2nd parturition				
Number of dams	16	7	8	0
Gestation length in days	22.3 ± 0.5	22.9 ± 0.4 *	22.5 ± 0.5	
Number of newborns	12.8 ± 3.9	12.7 ± 3.7	7.6 ± 5.8 *	
Pup weight (g) Male	7.2 ± 0.8	6.9 ± 0.7	7.4 ± 0.9	
Female	6.7 ± 0.7	6.8 ± 0.7	6.6 ± 0.6	
Viability index on PND 4	93.3 ± 25.1	100.0 ± 0.0	100.0 ± 0.0	
At the 3rd parturition				
Number of dams	6	3	1	0
Gestation length in days	22.4 ± 0.5	22.7 ± 0.6	22.0	
Number of newborns	12.0 ± 4.3	11.0 ± 6.6	14.0	
Pup weight (g) Male	7.1 ± 0.5	7.2 ± 1.2	6.8	
Female	6.6 ± 0.5	6.8 ± 1.4	6.7	
Viability index on PND 4	96.5 ± 5.9	100.0 ± 0.0	100.0	

* は対照群と比較して有意差（5%）があることを示す．各値は平均±標準偏差を示す．

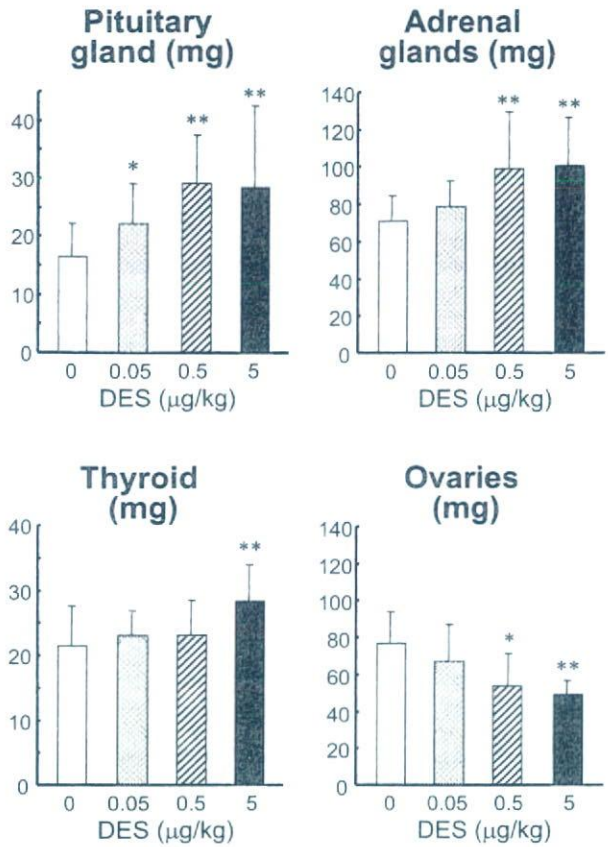

図3　新生児期にDESを投与したSD系雌ラットの54週齢における器官重量

*, ** は対照群と比較して有意差（5%および1%）があることを示す.

対照群（n=16），0.05 μg/kg群（n=20），0.5 μg/kg群（n=19），5 μg/kg群（n=19）

が確認されたが，T_3，T_4およびFSH濃度には，群間の差は認められなかった.

排卵検査：54週齢の排卵検査では，hCG投与により排卵した雌が対照群で8例中7例，0.05 μg/kg投与群で10例中6例，0.5 μg/kg投与群で10例中9例，5 μg/kg投与群で10例中8例みられ，誘起排卵数に群間の差は認められなかった.

生存曲線：雌雄の生存曲線を図4に示した．5 μg/kg投与群の雌では，生存日数が短縮したが，雄の生存日数にDES投与の影響はみられなかった.

考察

性成熟の観察では，5 μg/kg投与群で腟開口時期の早期化がみられ，同群では雌の尿道開口部の過剰開裂も認められた．性成熟の早期化[7]や尿道開口部の過剰開裂[6]を内分泌攪乱物質の生体に及ぼす有害影響と断定するには，さらに慎重に検討すべきであるが，一生涯試験の中では早期に検査できる項目であることから，後に得られる結果とあわせて内分泌攪乱性を判断する材料の一つになると考えられる．陰茎包皮分離時期に関しては，5 μg/kg投与群においても投与の影響はみられなかった．吉村ら[8]は，DESを出生後1～5日に投与したSD系ラットのうち，100 μg/kg以上の投与群で陰茎包皮分離時期の遅延を報告している．したがって，本研究で用いたDESの投与量では，雄の性成熟に影響を及ぼさないと判断される.

5 μg/kg投与群では8週齢から正常な性周期を示す雌動物は認められなかった．この結果から，同群ではDES投与により性成熟前の性腺刺激ホルモンが低下し，androgenizationを起し，腟開口後も排卵はなかったと推察される．一方，0.5 μg/kg投与群でも，16週齢以降に正常な性周期を示す動物の割合が減少した．TCDDの性成熟前投与[9]やビスフェノールAの胎生期投与[10]でも性周期の異常は対照群よりも早く起こることが示されている．これらのことは，内分泌攪乱化学物質の検索において，性周期を長期にわたって観察することの重要性を示している．本研究でみられた異常周期の型は，5 μg/kg投与群では主に連続発情であったのに対し，0.5 μg/kg投与群では不規則周期や無発情などが主であった．このことから，5 μg/kg投与群でみられた異常周期は無排卵に起因したのに対し，0.5 μg/kg投与群でみられた異常は，対照群にみられる加齢性変化が早期に誘発されたものと推察される.

12週齢の交配では，5 μg/kg投与群の雌で交尾が確認されたものの，受胎率は0%であった．これは，前述の排卵を伴わない連続発情を反映した結果と考えられる．一方，0.5 μg/kg投与群の雌では，23週齢の交配で受胎率が低下し，2産目の産児数が減少した．この変化も，同群で早期に加齢性変化，すなわち性周期の乱れを来したことと一致すると考えられ，2産目の産児数の低下は，エストロゲン分泌の低下に起因した排卵数の減少が原因と推定される[11]．なお，23週齢の交配で0.05 μg/kg投与群にみられた交尾率低下の原因は

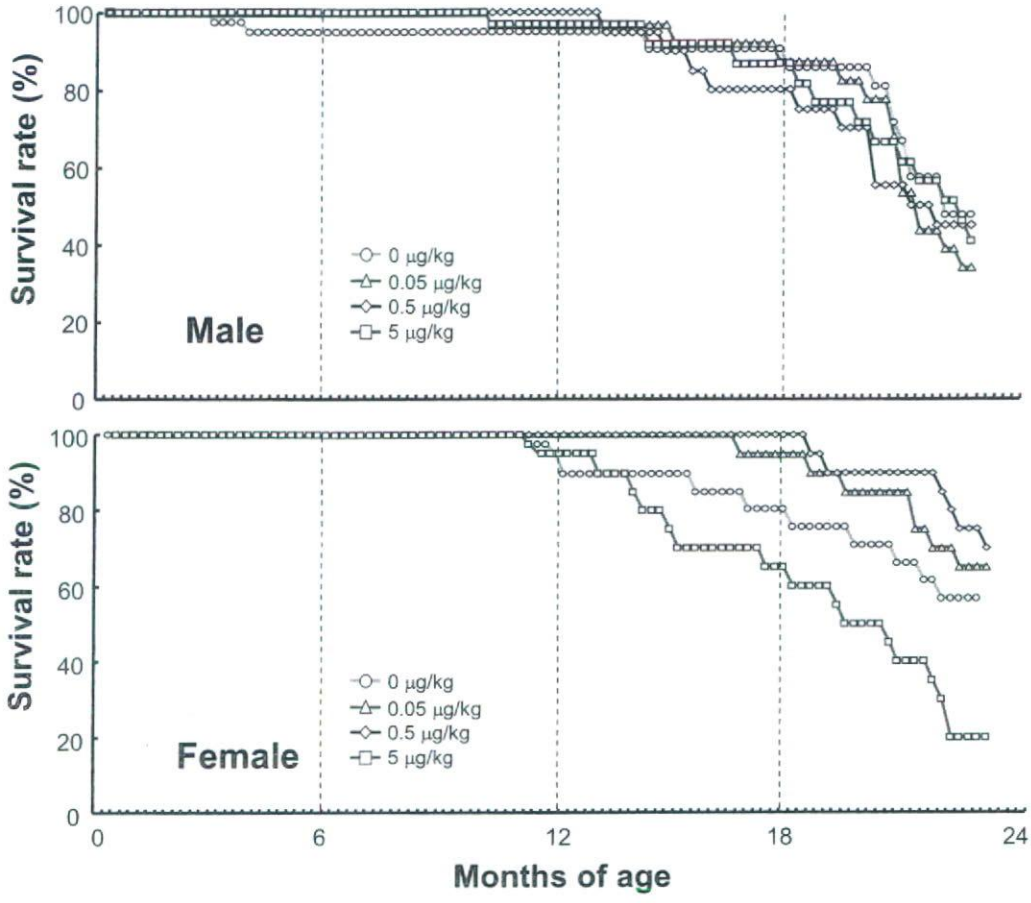

図4　新生児期にDESを投与したSD系ラットの生存曲線

不明である．

雄では，いずれの時期の交配においても，DES投与の影響は認められなかった．また，26週齢および52週齢では，ともに精子数および生殖器重量にDES投与の影響を示唆する変化も認められなかった．新生児期のDES投与では，雄動物の生殖能力への影響[12,13]が報告されているが，いずれも高用量（0.1 mg/pup/day以上）での報告であり，今回用いた5 μg/kgまでのDESは，新生児期投与で雄の生殖機能に影響を及ぼさないと考えられる．しかしながら，Vom Saalら[14]は，胎生期に0.02 μg/kgのDESを投与したマウスで前立腺重量の増加を報告していることから，同程度の投与量を用いて胎生期曝露を追試することが必要であろう．

54週齢の雌では，下垂体および副腎重量が用量に依存して増加し，血中プロラクチンとLH濃度の上昇が確認された．剖検時に乳汁貯留も観察されていることから，高プロラクチン血症が疑われる．一方，5 μg/kg投与群では甲状腺重量の増加が認められたが，血中T_3およびT_4濃度に変化はなかった．卵巣重量は，0.5 μg/kg以上の投与群で低下したが，hCG投与による誘起排卵がすべての投与群で確認されたことから，新生時期のDES投与は，卵巣機能には直接影響を及ぼさず，下垂体からの刺激の低下，すなわち視床下部－下垂体系の内分泌攪乱作用に起因したものと推定される．

結論

低用量DESは雌の性成熟を早めるだけでなく，雌の老化過程における性周期，受胎率，交尾率あるいは産児数に影響を与える可能性が示唆された．また，それらの変化は視床下部－下垂体系の内分泌攪乱作用に起因したものと推定され，卵巣機能への直接的な影響ではないと考えられた．本研究から，内分泌攪乱性を確定する上で一生涯試

験は有効であることが示されたが，雄の生殖機能異常を検出することは出来なかった．

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文　献

1) 渡辺千朗，代田眞理子，長尾哲二：SD系雌ラットの性周期の加齢性変化に関する研究．秦野研究所年報 **17**: 37-40 (1994)
2) 厚生労働省 医薬食品局審査管理課 化学物質安全対策室：内分泌かく乱化学物質ホームページ．http://www.nihs.go.jp/edc/torikumi/scheme.htm
3) Watanabe, C., Kuwagata, M., Yoshimura, S., Azegami, J., Kojima, K., Ono, H., Nagao, T.: An improved technique for repeated gavage administration to rat neonates. Congenit. Anom. **43**: 177-179 (2003)
4) 和田和義：AGD測定および包皮分離観察ならびにそれらの問題点．秦野研究所年報 **25**: 109-114 (2002)
5) Sato, M., Ohta, R., Kojima, K., Shirota, M., Koibuchi, H., Asai, S., Watanabe, G., Taya, K.: 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 (2002)
6) Sawaki, M., Noda, S., Muroi, T., Mitoma, H., Takakura, S., Sakamoto, S., Yamasaki, K.: *In utero* through lactational exposure to ethinyl estradiol induces cleft phallus and delayed ovarian dysfunction in the offspring. Toxicol. Sci. **75**: 402-411 (2003)
7) Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G., vom Saal, F. S.: Exposure to bisphenol A advances puberty. Nature **401**: 763-764 (1999)
8) Yoshimura, S., Yamaguchi, H., Konno, K., Ohsawa, N., Noguchi, S., Chisaka, A.: Observation of preputial separation is a useful tool for evaluating endocrine active chemicals. J. Toxicol. Pathol. **18**: 141-157 (2005)
9) Franczak, A., Nynca, A., Valdez, K. E., Mizinga, K. M., Petroff, B. K.: Effects of acute and chronic exposure to the aryl hydrocarbon receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the transition to reproductive senescence in female Sprague-Dawley rats. Biol. Reprod. **74**: 125-130 (2006)
10) Rubin, B. S., Murray, M. K., Damassa, D. A., King, J. C., Soto, A. M.: Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ. Health Perspect. **109**: 675-680 (2001).
11) vom Saal, F. S., Moyer, C. L.: Prenatal effects on reproductive capacity during aging in female mice. Biol. Reprod. **32**: 1116-1126 (1985)
12) Atanassova, N., Mckinnell, C., Walker, M., Turner, K. J., Fisher, J. S., Morley, M., Millar, M. R., Groome, N. P., Sharpe, R. M.: Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. Endocrinology **140**: 5364-5373 (1999)
13) Warita, K., Sugawara, T., Yue, Z. P., Tsukahara, S., Mutoh, K., Hasegawa, Y., Kitagawa, H., Mori, C., Hoshi, N.: Progression of the dose-related effects of estrogenic endocrine disruptors, an important factor in declining fertility, differs between the hypothalamo-pituitary axis and reproductive organs of male mice. J. Vet. Med. Sci. **68**: 1257-1267 (2006)
14) vom Saal, F. S., Timms, B. G., Montano, M., Palanza, P., Thayer, K. A., Nagel, S. C., Dhar, M. D., Ganjam, V. K., Parmigiani, S., Welshons, W. V.: Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc. Natl. Acad. Sci. U. S. A. **94**: 2056-2061 (1997)

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OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals

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Abstract

The Organization for Economic Co-operation and Development (OECD) has initiated the development of new guidelines for the screening and testing of potential endocrine disrupters. The Hershberger assay is one of the assays selected for validation based on the need for in vivo screening to detect androgen agonists or antagonists by measuring the response of five sex accessory organs and tissues of castrated juvenile male rats: the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis. The Phase 1 feasibility demonstration stage of the Hershberger validation program has been successfully completed with a single androgen agonist and a single antagonist as reference substances. The Phase 2 validation study was performed, employing a range of additional androgen agonists and antagonists. Recently, the Phase 3 validation study was conducted and performed in several International laboratories. Three Japanese laboratories have contributed to the blind study using coded materials of Phase 3 validation. Four coded test substances in the agonistic version and seven substances in the antagonistic version were orally administered by gavage for 10 consecutive days, respectively. In the antagonist version of the assay, 0.2 mg/kg/day of testosterone propionate (TP) was coadministered by subcutaneous injection. All five accessory sex reproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window in both versions. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Keywords: Blind study; Endocrine; Hershberger assay; OECD validation

1. Introduction

Certain reproductive and developmental toxicants may have the potential to interfere with normal sexual differentiation and development in animals and humans by modulating or interfering with the endocrine system (McLachlan, 1993; McLachlan and Korach, 1995). The Organization for Economic Co-operation and Development (OECD) has initiated an activity to revise existing guidelines and develop new screening and testing guidelines to aid in the identification and assessment of such toxicants (OECD, 1998, 2000, 2002).

One proposed assay, referred to as the Hershberger assay, uses the androgen sensitivity of several accessory sex organs and tissues of the male reproductive tract. The assay was originally developed in the 1930s by Korenchevsky and coworkers, and a number of accessory sex organs and tissues were shown to be use-

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