FULL PAPER *Pathology*

Effects of Fetal Exposure to Diethylstilbestrol on Mammary Tumorigenesis in Rats

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ABSTRACT. The aim of this study was to investigate the effect of fetal exposure to diethylstilbestrol (DES) on the induction of mammary tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female rats. Pregnant rats were fed only normal diet, diet mixed with 0.1 ppm DES throughout pregnancy period or diet mixed with 0.1, 1 or 10 ppm DES from day 13 of pregnancy till the end of pregnancy. Delivered pups were given 10 mg DMBA by gastric intubation at 50 days after birth and observed till 336 days after birth. Some rats exposed to DES throughout pregnancy and those from day 13 till the end of pregnancy showed endocrine disrupting effects such as absence of CL and active lactation in mammary glands at necropsy, while no abnormal estrus cycle such as persistent estrus was seen during the observation period until 88 days after birth. Fetal exposure to 0.1 ppm DES throughout pregnancy period, 0.1 and 1 ppm DES from day 13 of pregnancy increased the incidence and number of mammary carcinomas (MCs) at the earlier period while exposure to 0.1 ppm DES throughout pregnancy period enhanced the incidence and number of benign proliferative lesions (PLs) at the later period. MCs appeared earlier than benign PLs. These results suggest that exposure to DES throughout pregnancy and from day 13 of pregnancy could induce endocrine disrupting conditions and enhance the induction of MCs and that exposure to DES throughout pregnancy enhance PLs.

KEY WORDS: 7,12-dimethylbenz[a]anthracene (DMBA), diethylstilbestrol (DES), fetal, mammary tumor.

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There is evidence that many environmental endocrine disruptors act as sex hormones, particularly during the perinatal or neonatal period, directly or indirectly affecting reproduction in human and rats [13, 23, 27]. Endogenous hormone mimics are proposed for a number of adverse human health effects, including infertility, abnormal prenatal and childhood development, and reproductive cancers [18].

We have reported that the neonatal administration of diethylstilbestrol (DES) [12, 14, 33], 4-*n*-octylphenol [10], 17β-estradiol (E_2) [4] and testosterone propionate [9, 28–30] affected mammary tumorigenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female rats.

In humans, women exposed to diethylstilbestrol (DES), a synthetic estrogen with strong estrogenic activity, during pregnancy have a moderately increased risk of breast cancer [17, 27]. It is also known that the frequency of vaginal clear cell adenocarcinoma was increased in young women whose mothers used DES to prevent or lower the risk of abortion, while DES was ineffective to prevent miscarriages and premature births [3, 27].

Huggins *et al.* previously showed that a single administration of 20 mg DMBA at 50 days after birth induced visible mammary cancer in rats by 110 days after birth [6]. This animal has since been a useful model for studying the carcinogenesis of human breast cancer. DMBA is known to be a polycyclic hydrocarbon carcinogen forming DNA adducts [24]. The presence of estrogen and progesterone receptors in DMBA-induced mammary carcinomas (MCs) has been well documented in rats [31, 32]. DMBA-transformed cells (intraductal papillary proliferative lesions) could exist in unpalpable lesions long after the administration of DMBA under conditions in which estrogen was present but progesterone (P) was absent [28].

We have reported that rat mammary dysplasia (MD) induced by DMBA is morphologically similar to that in humans, and is characterized by gross cysts (GCs) and solid masses with microscopic features, including fibrotic adenosis (FA) and acinar adenosis (AA) [9, 11, 29, 30, 33]. MD, FA and AA are relevant to benign proliferative lesion (PL), fibroadenoma and lobular hyperplasia in Fisher rats, respectively [1, 12].

In turn, the mammary line stage is observed in 6–14 mm crown-rump length embryos between day 11 and 13 of pregnancy, and then hillocks following the mammary line stage are observed on day 13 of intrauterine life in 17–18 mm crown-rump length fetuses [19]. Mammary hillocks persist through the 16th day, and then become less evident, although they apparently occupy the position of the future nipples [19]. It was reported that injections of DES on day 15 and 18 of gestation increased incidence of mammary tumors (MTs) induced by DMBA in rats [2]; however, it remains to be determined how exposure to DES throughout pregnancy affects the induction of MTs.

Recently, we have also reported that DES affects pregnancy and that the exposure period (throughout pregnancy

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or during pregnancy after the development of mammary hillocks) and dose may result in sterility, abortion, poor fetal growth and reduced number of pups born in rats [8]. The aim of this study was to investigate how exposure to DES throughout pregnancy or during pregnancy after the development of mammary hillocks affects the induction of MTs induced by DMBA using female pups in early report [8] at detailed histopathological analysis.

MATERIALS AND METHODS

Animals: The animals were seventy inbred Sprague-Dawley female rats (15 weeks old, our own breeding animals). The animals were maintained in filtered air laminar flow at the Institute of Laboratory Animal Sciences, Frontier Science Research Center, Kagoshima University, and given a commercial diet (CE-2; CLEA Inc., Tokyo, Japan) and tap water *ad libitum*. CE-2 contains 4–5 ppb estradiol/kg, 25.1% crude protein and 4.8% crude fat; Crude protein derived from soybean meal, white fish meal and yeast, and crude fat derived from germ oil and soybean oil; Soybeans contain 0.072–0.249 isoflavones/100 g of dry weight [7]. The room temperature was maintained at 25°C ± 2°C and relative humidity at 55% ± 10%, with a 12 hr light/dark cycle. The use of animals in this research complied with all relevant guidelines set by Kagoshima University.

Experimental design: Rats in groups I, II and III were fed only CE-2, CE-2 mixed with DES (Sigma Chemical Co., St. Louis, MO. U.S.A.) during pregnancy, and CE-2 mixed with DES from day 13 of pregnancy, and DES in subgroups a, b, c and d was mixed into the diet at 0.1, 1, 10 and 100 ppm, respectively, and no alive pups were available from groups IIb-d, and IIId, as previously described (Fig. 1) [8]. The numbers of dams in groups I, IIa-d and IIIa-d were 7, 8, 8, 8, 10, 8, 7, 7, and 7, respectively. All surviving pups were weaned at 25 days after birth. At 50 days after birth, all pups were given 10 mg DMBA (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in 1 m*l* sesame oil by gastric intubation. All DMBA-administered animals, except those intermediately sacrificed, were examined once weekly by palpation to detect mammary tumors from 50–336 days after birth. The estrus cycles of all animals were examined once daily from the day of vagina opening to 88 days after birth by a vaginal smear test. At 336 days after birth, all surviving animals underwent necropsy. All palpable mammary masses were weighed, and the longest diameter and shortest diameter of the surface were measured, and the size (cm^2) calculated by multiplying their lengths. The body, ovaries, uterus, adrenal glands and pituitary gland were weighed.

Histopathological examination: All mammary tumors, residual mammary glands (L1-6 and R1-6), ovaries, adrenal glands, pituitary gland, uterus and vagina were fixed in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin. They were sectioned at 5 μm, stained routinely with hematoxylin and eosin (HE) and then examined histopathologically.

Statistics: Statistical analyses were performed using Dr. SPSS II program for Windows, as described previously [10, 14]. The mean differences were evaluated by Student's *t*-test. The data are shown as the mean ± standard deviation (SD). The incidences (percentages) were tested using a four-fold contingency table (chi-square test).

RESULTS

The mean food volume/day during the full pregnancy in groups IIa-d and from day 13 of pregnancy in groups IIIa-d, which was significantly lower than in group I (control), as described previously [8]. Delivery occurred in 100, 63, 100, 86 and 71% bred rats in groups I (controls), IIa, IIIa-c, respectively, while no delivery occurred in groups IIb-d and IIId as described previously [8]. The number of female pups/dam in group IIIc decreased: The number of female pups/dam in groups I, IIa and IIIa-c was 4.7 ± 2.0, 3.8 ± 0.8, 5.0 ± 2.0, 3.3 ± 1.2, 2.2 ± 1.3 (mean ± SD), respectively, as described previously [8].

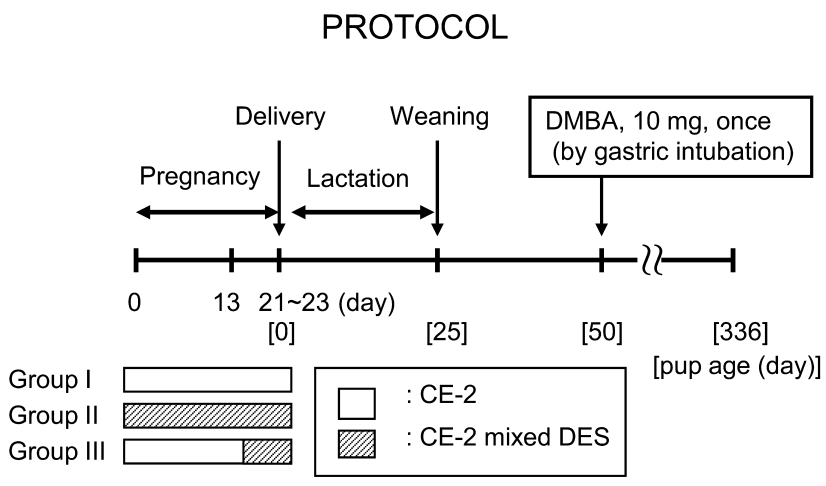

Fig. 1. Summarized protocol.

Table 1 shows the body weight (BW) of female pups in groups IIa, IIIa, IIIb and IIIc at birth were significantly lower than in controls and the number of female pups that survived at weaning compared with the number at birth in groups IIa, IIIb and IIIc was significantly lower than in the controls. BW in group IIIb at weaning and the administration of DMBA (50 days after birth) were significantly lower than in controls.

Table 2 shows that the day of eye opening in groups IIa and IIIb and the day of vagina opening in group IIIb were significantly longer than in controls. No animals with persistent estrus (PE) were seen, and the patterns were regular and successive estrus cycles of 4 or 5-day duration.

Table 3 shows that BW in group IIIb and the absolute weight (AW) of ovaries in groups IIa and IIIb at necropsy were significantly lower than in controls. AW/BW of the uterus in group IIIa and the pituitary gland in groups IIIa and IIIb were significantly higher than in controls.

Pathology of mammary tumors (MTs) induced by DMBA: Single or multiple mammary masses, diagnosed as MC

Table 1. The effects of diethylstilbestrol (DES) on the survived number and body weight (BW) of female pups

Group	Treatment		At birth		At weaning		At administration of DMBA (50 days after birth)	
	Dose of DES	Pregnancy periods (days)	Number of female pups	BW (g) (Mean ± SD)	Number of pups survived comparing with the number at birth (%)	BW (g) (Mean ± SD)	Number of pups survived comparing with the number at weaning (%)	BW (g) (Mean ± SD)
I	0	None	22	5.7 ± 0.6	22 (100%)	52.4 ± 6.1	22 (100%)	149.7 ± 6.4
IIa	0.1 ppm	0–21	12	4.7 ± 0.3**	9 (75.0%)*	50.8 ± 4.5	9 (100%)	146.0 ± 6.7
IIIa	0.1 ppm	13–21	18	5.0 ± 0.1**	18 (100%)	52.5 ± 5.0	18 (100%)	150.8 ± 10.6
IIIb	1 ppm	13-21	15	4.2 ± 0.5**	10 (66.7%)**	40.7 ± 9.3**	10 (100%)	139.3 ± 15.6*
IIIc	10 ppm	13–21	11	3.8 ± 0.1**	2 (18.2%)**	45.6 ± 3.8	2 (100%)	136.2 ± 7.4

* $p<0.05$, ** $p<0.01$: significantly different from Group I.

Table 2. The effects of diethylstilbestrol (DES) on the day of eye's and vaginal opening in female pups

Group	Treatment		Number of female pups	Eye's opening (days after birth)	Vaginal opening (days after birth)	Number of rats with persistent estrus
	Dose of DES	Pregnancy periods (days)				
I	0	None	22	15.0 ± 0.7	30.8 ± 2.9	0
IIa	0.1 ppm	0–21	9	15.9 ± 1.1*	31.6 ± 1.7	0
IIIa	0.1 ppm	13–21	18	15.0 ± 0.0	31.6 ± 1.7	0
IIIb	1 ppm	13–21	10	16.2 ± 0.4**	33.4 ± 2.8*	0
IIIc	10 ppm	13–21	2	16.0	32.5	0

* $p<0.05$, ** $p<0.01$: significantly different from Group I.

Table 3. The effects of diethylstilbestrol (DES) on the body and organ weights in female pups at necropsy

Group	Treatment		Number of female pups	BW (g)	Ovaries AW (mg) AW/BW (mg/g)	Uterus AW (g) AW/BW (mg/g)	Adrenal glands AW (mg) AW/BW (mg/g)	Pituitary gland AW (mg) AW/BW (mg/g)
	Dose of DES	Pregnancy periods (days)						
I	0	None	22	280.7 ± 22.6	74.5 ± 14.1 0.27 ± 0.06	0.60 ± 0.17 2.1 ± 0.6	34.7 ± 3.3 0.13 ± 0.02	12.1 ± 1.2 0.044 ± 0.006
IIa	0.1 ppm	0–21	9	283.3 ± 23.0	64.3 ± 10.6* 0.23 ± 0.05	0.85 ± 0.64 3.0 ± 2.1	33.1 ± 1.7 0.12 ± 0.01	12.7 ± 0.7 0.045 ± 0.004
IIIa	0.1 ppm	13–21	18	282.6 ± 28.6	65.4 ± 16.3 0.24 ± 0.07	0.74 ± 0.23 2.6 ± 1.0*	33.9 ± 4.0 0.12 ± 0.02	13.6 ± 2.7 0.048 ± 0.008*
IIIb	1 ppm	13–21	10	255.4 ± 23.1*	59.6 ± 18.4* 0.24 ± 0.09	0.59 ± 0.21 2.3 ± 0.8	33.9 ± 3.8 0.13 ± 0.01	13.4 ± 3.5 0.052 ± 0.011*
IIIc	10 ppm	13–21	2	288.9	66.5 0.23	0.38 1.3	31.5 0.11	14.0 0.049

BW: body weight; AW: absolute weight.
* $p<0.05$: significantly different from Group I.

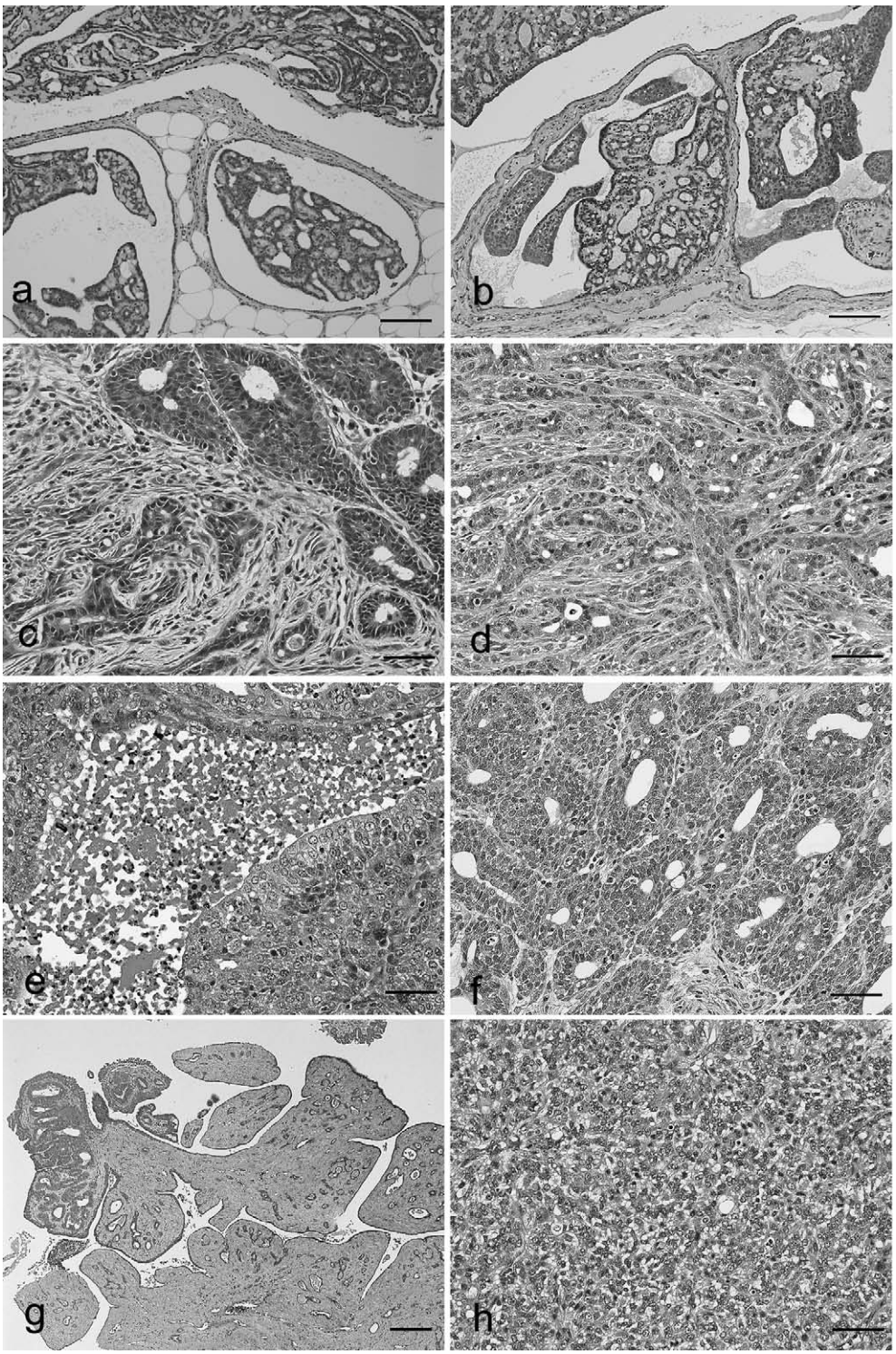

Fig. 2. Mammary carcinoma (adenocarcinoma). Papillary and tubular proliferations without interstitial invasion are seen in duct in groups I (a) and IIa (b). Interstitial invasion, tubular proliferation containing acidophilic secretions in lumens and multilayered epithelium are seen in groups I (c) and IIIa (d). In other subtypes of mammary carcinomas, secretory type in group IIa (e), ductal type (f) and phyllodes type (g) in group IIIa and solid type in group I (h) are seen. Bar=100 μm (a, b), 50 μm (c-f, h) and 200 μm (g). HE stain.

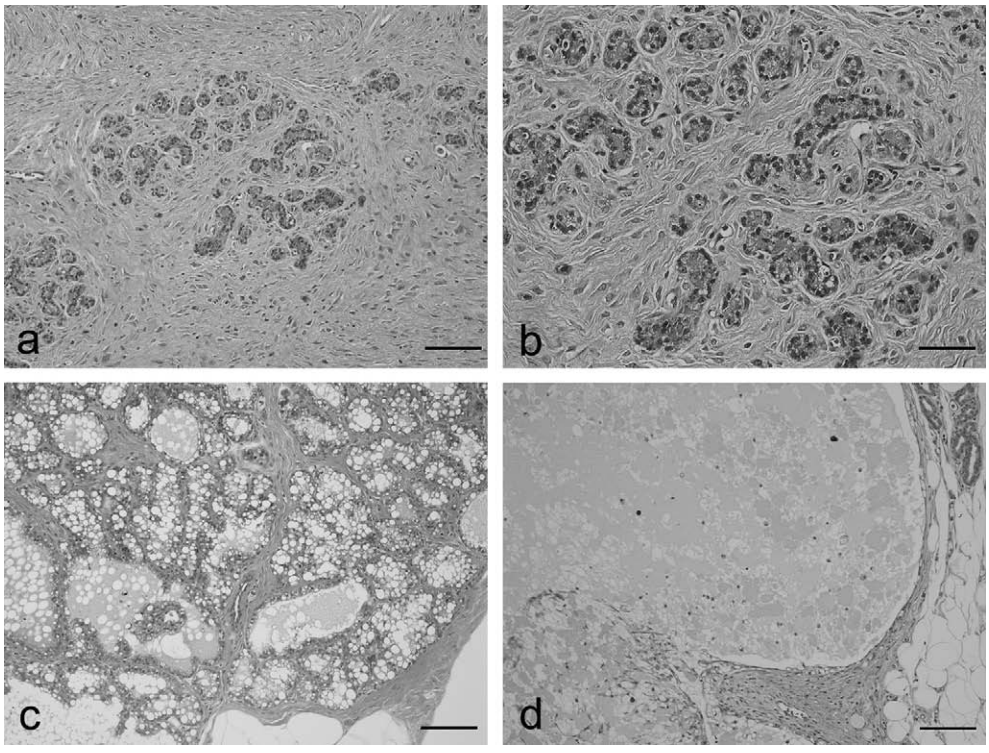

Fig. 3. Benign proliferative lesions. Fibroadenoma in group IIa consists of rich hyalinized stroma and developed acini (a, b). Lobular hyperplasia in group IIa consists of large lobular structures composed of acini and the lumens of the acini are dilated and filled with acidophilic secretions (c). Gross cyst in group IIIa is filled with milky fluid and lined with a single layer of flattened or cuboidal epithelium (d). Bar=100 μm (a, c, d) and 50 μm (b). HE stain.

(adenocarcinoma) or PL, were seen in rats, as described previously [10, 12]. No metastasis was recognized in any rats. These masses were localized in all areas (L1-6 and R1-6) of the mammary glands. MCs were briefly subclassified into papillary type, secretory type, ductal type, solid type or phyllodes type (like human phyllodes tumor) in this study, as described previously [5, 12, 16]. Phyllodes tumor is an uncommon biphasic breast tumor, with the ability to recur and metastasize, and it behaves biologically like a stromal neoplasm. Traditionally, phyllodes tumors are graded by the use of a set of histologic data into benign, borderline, and malignant [26]. Microscopically, phyllodes tumors show prominent stromal proliferation, so that the stroma abuts into the epithelial lined spaces, forming the slit-like spaces or leaflike pattern, hence the name phyllodes (leaf like) [26]. MCs proliferated in the duct without interstitial invasion and MCs with interstitial invasion were seen (Fig. 2). PLs (Fig. 3) consisted of solid masses, which included fibroadenoma and lobular hyperplasia, and GCs, as described previously [10, 12]. Fibroadenoma, by far the most common benign neoplasm of the rat mammary gland [1], consisted of rich hyalinized stroma and developed acini. Lobular hyperplasia consisted of large lobular structures composed of acini. The lumen of the acini were dilated and filled with acidophilic secretions. GCs were grossly soft, yellow, oval, and filled with milky fluid. Microscopically, GCs were lined with a single layer of flattened or cuboidal epithelium.

In MCs, Fig. 4 shows that the incidence in group IIIb at 91 and 105 days after birth, group IIIa at 105 and 119 days after birth, and group IIa at 119 days after birth was significantly higher than in controls and that the incidence in group IIa at 203 and 252 days after birth was significantly lower than in controls. The number of MCs/rat in groups IIIa and IIIb at 105 and 119 days after birth was significantly higher than in controls. Table 4 shows that the mean first day of detection (latent period) in groups IIa and IIIa was significantly longer than in controls. Table 5 shows there were no significant differences on the incidence of each subtypes and the number of subtypes/rat.

In benign PLs, Fig. 5 shows that the incidence in group IIa at 322 and 336 days after birth was significantly higher than in controls. The number of PLs/rat in group IIa at 322 and 336 days after birth was significantly higher than in controls (Table 6). Table 7 shows the highest incidence of each histological type of mammary gland lesions was fibroadenoma in all groups while there were no significant changes in the incidence of each histological type of mammary gland lesions.

Table 8 shows that the incidence of rats without corpus lutea (CL) in group IIIb and the incidence of rats with active

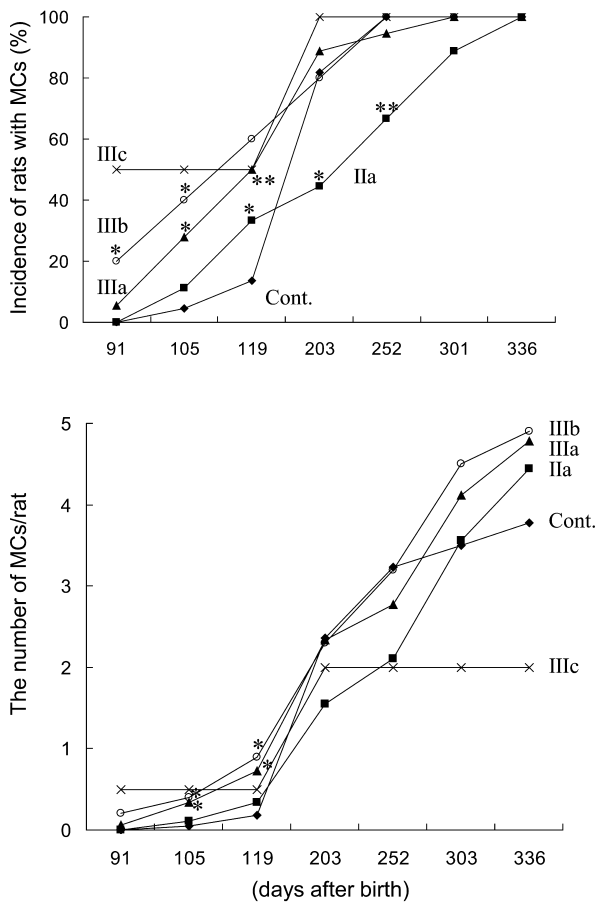

Fig. 4. Incidence of rats with mammary carcinomas (MCs) and number of MCs/rat. ◆ Group I (Cont.), ■ Group IIa, ▲ Group IIIa, ○ Group IIIb, × Group IIIc. * $p<0.05$, ** $p<0.01$: significantly different from Group I.

lactation in residual mammary glands in groups IIa, IIIa and IIIb were significantly higher than in controls. Other than the lack of CL, there were no typical ovarian changes suggestive of estrogenic effect, such as multiple follicular cysts, in rats exposed prenatally to DES.

Table 9 shows the observed benign and malignant mixed MTs. The latent period in group IIIa and IIIb was significantly longer than in controls. These benign and malignant mixed MTs showed various histological features (Fig. 6), such as a mass composed of adenocarcinoma and hyperplasia, another mass composed of adenocarcinoma, such as human phyllodes tumor, and fibroadenoma.

Histopathological changes in other endocrine-related organs: Table 10 shows hydrometra, neutrophil infiltration in the endometrium, vaginal keratosis were observed in some rats among control and DES-treated rats. No abnormal changes, such as atrophy, were seen in the endometrium and vaginal mucosa or in the adrenal glands, or such as prolactinoma in the pituitary gland.

DISCUSSION

Feeding DES during pregnancy decreased the BW of pups at birth, as previously described [8]. Prenatal exposure to endocrine disruptors has been hypothesized to affect birth size [25]. The present study revealed that the survival rates of pups exposed gestationally to DES decreased, while those after weaning did not change. Decreased survival rate of pups exposed gestationally to DES until weaning may also be related with continued DES ingestion through milk from DES administered dams during pregnancy in the same way to lactational transfer of DES that is known to induce apparent developmental effects in rats [15]. The delay of opening the eyes in pups with exposure to DES throughout pregnancy and from day 13 of pregnancy and vagina in pups with exposure to DES from day 13 of pregnancy contributed to the growth disturbance of infants.

We reported that a high dose of DES (100 μg) during the neonatal period induced PE and anovulatory ovaries with lack of ovulation in female rats due to disturbance of the gonadotropin-secreting system in the hypothalamus, and ovary weights without CL halved the weight compared with controls [14]. In this study, relative pituitary weight increase appeared to be dose-related. This change may be the endocrine disrupting effect of DES. In this study, no PE was seen in rats with exposure to DES throughout pregnancy and from day 13 of pregnancy; however, it was possible that some rats with exposure to DES from day 13 of pregnancy developed endocrine disrupting conditions because some showed an absence of CL (it was unknown whether they accompanied with PE or lack of ovulation) at

Table 4. Effects of diethylstilbestrol (DES) on induction of mammary carcinomas (MCs) in female pups

Group	Treatment		Number of female pups	Number of rats with MCs (%)	Number of MCs	Number of MCs per rat ± SD	Mean weight (g) of MCs per rat ± SD	Mean size (cm^2) of MCs per rat ± SD	Mean day at detection ± SD
	Dose of DES	Pregnancy periods (days)							
I	0	None	22	22 (100%)	83	3.77 ± 1.90	1.38 ± 2.86	1.90 ± 2.38	189.7 ± 59.2
IIa	0.1 ppm	0–21	9	9 (100%)	40	4.44 ± 3.54	1.07 ± 2.33	1.64 ± 2.22	233.0 ± 73.1**
IIIa	0.1 ppm	13–21	18	18 (100%)	86	4.77 ± 3.92	0.97 ± 2.17	1.53 ± 2.05	215.7 ± 79.5*
IIIb	1 ppm	13–21	10	10 (100%)	49	4.90 ± 2.88	1.40 ± 2.77	1.79 ± 2.44	209.7 ± 73.6
IIIc	10 ppm	13–21	2	2 (100%)	4	2.00	0.35	1.24	156.5

* $p<0.05$, ** $p<0.01$: significantly different from Group I.

Table 5. Effects of diethylstilbestrol (DES) on the number (incidence) of subtype of mammary carcinomas (MCs) in female pups

Group	Number of rats	Papillary type		Secretory type		Ductal type		Solid type		Phyllodes type	
		Number of rats with masses (%)	Number of masses/rat ± SD	Number of rats with masses (%)	Number of masses/rat ± SD	Number of rats with masses (%)	Number of masses/rat ± SD	Number of rats with cysts (%)	Number of cysts/rat ± SD	Number of rats with cysts (%)	Number of cysts/rat ± SD
I	22	19 (86.4%)	2.14 ± 1.55	15 (68.2%)	0.77 ± 0.69	9 (40.9%)	0.55 ± 0.74	2 (9.1%)	0.09 ± 0.29	4 (18.2%)	0.23 ± 0.53
IIa	9	8 (88.9%)	2.44 ± 2.01	8 (88.9%)	1.33 ± 1.41	4 (44.4%)	0.44 ± 0.53	0 (0%)	0.00	2 (22.2%)	0.22 ± 0.44
IIIa	18	15 (83.3%)	2.11 ± 2.25	13 (72.2%)	1.00 ± 0.84	11 (61.1%)	1.28 ± 1.93	0 (0%)	0.00	5 (27.8%)	0.39 ± 0.78
IIIb	10	8 (80.0%)	2.40 ± 2.17	8 (80.0%)	1.10 ± 0.74	5 (50.0%)	1.00 ± 1.25	1 (10.0%)	0.20 ± 0.63	1 (10.0%)	0.20 ± 0.63
IIIc	2	2 (100%)	1.00	0 (0%)	0.00	0 (0%)	0.00	0 (0%)	0.00	1 (50.0%)	1.00

MCs were briefly subclassified into papillary type, secretory type, ductal type, solid type or phyllodes type.

Table 6. Effects of diethylstilbestrol (DES) on induction of benign proliferative lesions (PLs) in female pups

Group	Treatment		Number of female pups	Number of rats with PLs (%)	Number of PLs	Number of PLs per rat ± SD	Mean weight (g) of PLs per rat ± SD	Mean size (cm^2) of PLs per rat ± SD	Mean day at detection ± SD
	Dose of DES	Pregnancy periods (days)							
I	0	None	22	8 (36.4%)	11	0.50 ± 0.67	0.54 ± 1.27	0.96 ± 1.62	280.0 ± 48.5
IIa	0.1 ppm	0–21	9	7 (77.8%)*	12	1.33 ± 1.22*	0.20 ± 0.50	0.63 ± 0.91	280.7 ± 32.6
IIIa	0.1 ppm	13–21	18	7 (38.9%)	32	1.78 ± 3.14	0.26 ± 0.79	0.64 ± 1.23	297.7 ± 37.8
IIIb	1 ppm	13–21	10	3 (30.0%)	4	0.40 ± 0.70	0.04 ± 0.02	0.29 ± 0.12	285.8 ± 38.6
IIIc	10 ppm	13–21	2	2 (100%)	4	2.00	0.18	0.71	332.0

* $p<0.05$: significantly different from Group I.

Table 7. Effects of diethylstilbestrol (DES) on the number (incidence) of solid masses and gross cysts in benign proliferative lesions (PLs) in female pups

Group	Treatment		Number of rats	Solid masses						Gross cyst		
				Fibroadenoma			Lobular hyperplasia					
	Dose of DES	Pregnancy periods (days)		Number of rats with masses (%)	Number of masses	Number of masses/rat ± SD	Number of rats with masses (%)	Number of masses	Number of masses/rat ± SD	Number of rats with cysts (%)	Number of cysts	Number of cysts/rat ± SD
I	0	None	22	9 (40.9%)	9	0.41 ± 0.50	1 (4.5%)	1	0.05 ± 0.21	1 (4.5%)	1	0.05 ± 0.21
IIa	0.1 ppm	0–21	9	6 (66.7%)	7	0.78 ± 0.67	2 (22.2%)	4	0.44 ± 1.01	1 (11.1%)	1	0.11 ± 0.33
IIIa	0.1 ppm	13–21	18	5 (27.8%)	21	1.17 ± 2.38	4 (22.2%)	6	0.33 ± 0.69	3 (16.7%)	5	0.28 ± 0.75
IIIb	1 ppm	13–21	10	2 (20.0%)	3	0.30 ± 0.48	0 (0%)	0	0.00	1 (10.0%)	33	0.10 ± 0.32
IIIc	10 ppm	13–21	2	2 (100%)	4	2.00	0 (0%)	0	0.00	0 (0%)	0	0.00

Benign proliferative lesions (PLs) consisted of solid masses, which included fibroadenoma and lobular hyperplasia, and gross cysts.

Table 8. The effects of diethylstilbestrol (DES) in the number (incidence) of rats with ovaries lacking development of corpus lutea and with active lactation in the residual mammary glands in female pups at necropsy

Group	Treatment		Number of female pups	Number of rats with ovaries lacking development of corpus lutea	Number of rats with active lactation in the residual mammary glands
	Dose of DES	Pregnancy periods (days)			
I	0	None	22	0 (0%)	0 (0%)
IIa	0.1 ppm	0–21	9	0 (0%)	2 (22.2%)*
IIIa	0.1 ppm	13–21	18	2 (11.1%)	6 (33.3%)**
IIIb	1 ppm	13–21	10	3 (30.0%)**	3 (30.0%)**
IIIc	10 ppm	13–21	2	0 (0%)	2 (100%)

* $p<0.05$, ** $p<0.01$: significantly different from Group I.

necropsy. In female rats with neonatal continuous administration of DES, anovulatory ovaries (progesterone deficiency) and a relative excess of estrogen were induced, and the mammary glands consisted of well-developed lobules with numerous acini showing active lactation [12, 33]. Active lactation in the residual mammary gland in this study contributed to the development of endocrine disrupting conditions, such as a relative excess of estrogen.

Table 9. Effects of diethylstilbestrol (DES) on induction of benign and malignant mixed mammary tumors (MTs) in female pups

Group	Treatment		Number of female pups	Number of rats with mixed MTs (%)	Number of mixed MTs	Number of mixed MTs per rat ± SD	Mean day at detection ± SD
	Dose of DES	Pregnancy periods (days)					
I	0	None	22	5 (22.7%)	5	0.23 ± 0.43	219.8 ± 34.9
IIa	0.1 ppm	0–21	9	2 (22.2%)	12	1.33 ± 2.83	231.3 ± 62.6
IIIa	0.1 ppm	13–21	18	5 (27.8%)	18	1.00 ± 2.28	270.3 ± 49.2*
IIIb	1 ppm	13–21	10	4 (40.0%)	9	0.90 ±1.60	284.1 ± 33.6*
IIIc	10 ppm	13–21	2	1 (50.0%)	2	1.00	199.0

* $p<0.05$: significantly different from Group I.

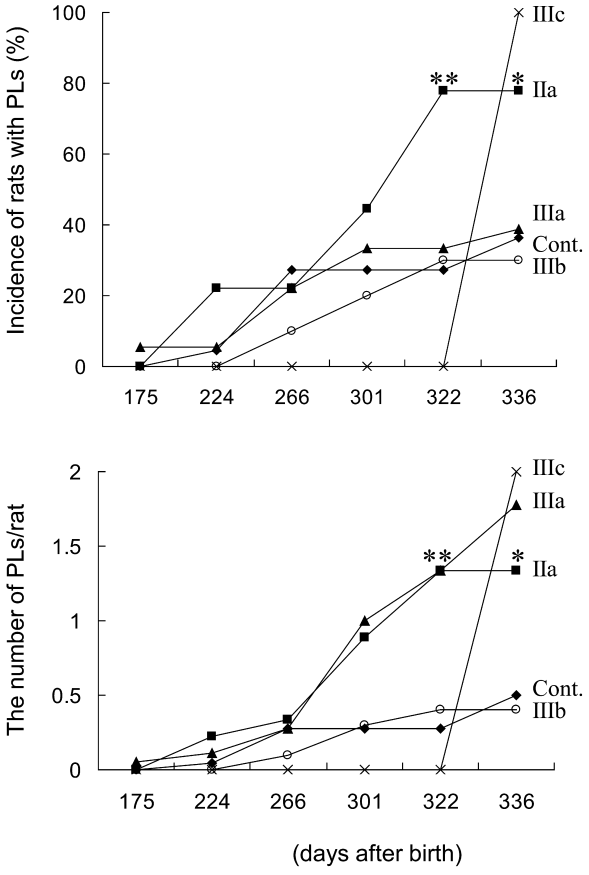

Fig. 5. Incidence of rats with proliferative lesions (PLs) and number of PLs/rat. ◆ Group I (Cont.), ■ Group IIa, ▲ Group IIIa, ○ Group IIIb, × Group IIIc. * $p<0.05$, ** $p<0.01$: significantly different from Group I.

Exposure to DES throughout pregnancy and from day 13 of pregnancy increased the incidence and number of MCs at earlier period in the present study. The incidence of rats with PE from 21–150 days after birth was 0 and 29% and the incidence of rats with anovulatory ovaries (until 300 days after birth) was 40 and 52% in neonatal administration of 0.1 and 1 μg DES, respectively [14]. Terminal end buds (TEBs) in mammary glands at 50 days after birth in rats with neonatal administration of 0.1 and 1 μg DES increased, resulting in a stimulatory effect on the initiation of MCs [14]. In this study, it is possible that exposure to DES throughout pregnancy and from day 13 of pregnancy increased TEBs at 50 days after birth (DMBA administration period), resulting in a stimulatory effect on the initiation of MCs, although it is necessary to further investigate whether fetal exposure to DES in our experimental conditions affects the development of TEBs.

Exposure to DES throughout pregnancy enhanced the incidence and number of PLs at the later period in the present study. Neonatal administration of DES induced disturbance of the gonadotropin-secreting system, resulting in rats with early opening of the vagina, persistent estrus and anovulatory ovaries [12, 15, 33]. Neonatal administration of DES also induced hormonal conditions with a relative excess of serum estrogen comparing to serum progesterone and active lactation in the residual mammary glands resulted in promotion of PLs [12, 33]. In the present study, it was speculated that exposure to DES throughout pregnancy promoted the induction of PLs, possibly due to hormonal conditions with the relative excess of serum estrogen because some rats with exposure to DES throughout pregnancy showed active lactation in the residual mammary glands.

Alternatively, TEBs differentiate to more mature structures, namely, alveolar buds (ABs) and lobules, which are less susceptible to carcinogens in rats [20, 21]. TEBs that had already differentiated into ABs before DMBA administration did not develop carcinomas, but remained unmodified or underwent dilatation, giving rise to hyperplastic lobules [22]; however, it is possible that dysplastic lobules may develop carcinomas because masses consisting of both MC and PL (mixed MTs) were found and had long latent periods. Alternatively, exposure to DES throughout pregnancy and from day 13 of pregnancy prolonged finally the latent period of MCs. The long latent period of mixed MTs may contribute to the long latent period of MCs because exposure to DES throughout pregnancy and from day 13 of pregnancy increased the number of mixed MTs.

In conclusion, these results suggest that exposure to DES throughout pregnancy and from day 13 of pregnancy could induce endocrine disrupting conditions and enhance the induction of MCs and that exposure to DES throughout pregnancy enhance PLs.

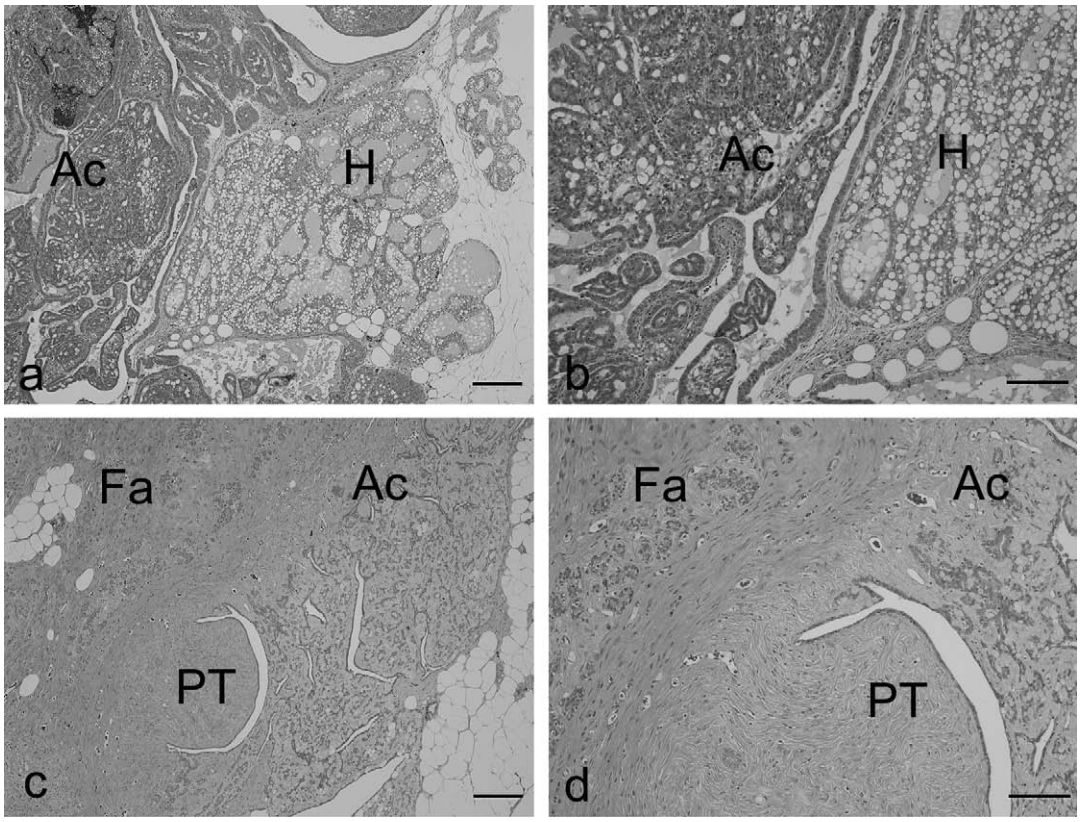

Fig. 6. Benign and malignant mixed mammary tumors in group IIa. A mass composed of adenocarcinoma (Ac) and hyperplasia (H) is seen (a, b). A mass composed of adenocarcinoma (Ac), such as human phyllodes tumor (PT), and fibroadenoma (Fa), is seen (c, d). Bar = 200 μm (a, c) and 100 μm (b, d). HE stain.

Table 10. Histopathological examination of the uterus and vagina

Group	Treatment		Number of bred rats	Uterus		Vagina
	Dose of DES	Pregnancy period (day)		Number of rats with hydrometra (%)	Number of rats with neurtrophils in endometrium (%)	Number of rats with keratosis (%)
I	0	None	22	4 (18.2%)	21 (95.5%)	6 (27.3%)
IIa	0.1 ppm	0–21	9	2 (22.2%)	8 (88.9%)	3 (33.3%)
IIIa	0.1 ppm	13–21	18	6 (33.3%)	16 (88.9%)	7 (38.9%)
IIIb	1 ppm	13–21	10	3 (30.0%)	8 (80.0%)	3 (30.0%)
IIIc	10 ppm	13–21	2	0 (0%)	1 (50.0%)	0 (0%)

* $p<0.05$, ** $p<0.01$: significantly different from group I.

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