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Effects of Neonatal Administration of Diethylstilbestrol on Aberrant Crypt Foci Induced by 7,12-Dimethylbenz[α]anthracene in Rats

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Abstract. *Gastrointestinal carcinoma is affected environmental factors, however, it remains to be determined whether neonatal administration of an estrogenic endocrine disruptor, such as diethylstilbestrol (DES), affects gastrointestinal carcinogenesis. The effects of neonatally administered DES on gastrointestinal tumorigenesis induced by 7,12-dimethylbenz[α]anthracene (DMBA) were investigated in male and female rats. Male and female rats in group I were daily administered oil alone from 0-14 days after birth. Male and female rats in groups II and III were daily administered DES at 1 and 10 μg/rat, respectively. The administration periods of DES in subgroups a (IIa and IIIa), b (IIb and IIIb) and c (IIc and IIIc) were from 0-14, 0-5 and 6-14 days after birth, respectively. At 28, 42 and 56 days after birth, all male rats were given 10 mg of DMBA. At 50 days after birth, all female rats were given 10 mg of DMBA. In the digestive tracts of male rats, forestomach masses (FMs) in all groups (13-58%), small intestine masses in group IIIa (17%), and colon masses (CMs) in groups IIIa (8%) and IIIb (33%) were observed, although there were no significant changes in the incidence and number. In the digestive tracts of female rats, FMs in groups I (10%), IIa (13%), IIb (33%),IIc (25%) and IIIc (33%), CMs in groups IIa (6%) and IIIa (6%) were seen, although there were no significant changes in the incidence. Aberrant crypt foci (ACF) in the colon and rectum were seen in all male and female rats. The neonatal administration of DES in male rats increased the number of ACF while that in female rats did not. These results suggest that neonatal administration of DES may affect male colorectal carcinogenesis.*

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The incidences of colon and mammary cancer have increased with the spread of Western dietary habits in Japan (1). Gastrointestinal cancer is more common in men than women and is affected by environmental (*e.g.* high salt and fat diets, alcohol drinking and smoking), host and genetic factors (2).

In rat models of gastrointestinal carcinoma using chemical carcinogens, *N*-nitrosomethylbenzylamine (NMBA) in esophageal squamous cell carcinoma (3), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) in stomach carcinoma (4), azoxymethane (AOM) (5) and 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) (6) in colorectal cancer have been widely studied. Colon carcinogenesis in a two-stage model in female rats initiated with 7,12-dimethylbenz[α]anthracene (DMBA) and 1,2-dimethylhydrazine (DMH) has been reported (7), however, no reports have addressed colon carcinogenesis induced by exposure to only DMBA.

It is known that the frequency of vaginal clear cell adenocarcinoma increased in young women whose mothers used diethylstilbestrol (DES), a synthetic estrogen with strong estrogenic activity and a known endocrine disruptor (8), to prevent or lower the risk of abortion (9). Recently, it was reported that daily administration of 1 and 10 μg DES from 0-14 days after birth induced persistent estrus (PE) and an absence of any corpus luteum (CL) in female rats due to disturbance of the gonadotropin-secreting system in the hypothalamus resulting in inhibition of the mammary tumorigenesis induced by DMBA (10, 11). Alternatively, it was reported that diethylhexyl phthalate, a known endocrine disruptor, was related to multidrug-resistance gene expression in colon cancer LS174T cells, while no reports addressed estrogenic endocrine disruptors in colon carcinogenesis (12).

Hamster progeny exposed to DES prenatally and DMBA postnatally developed higher rates of forestomach tumor than hamsters given DMBA alone postnatally (13). Estrogen may be involved in the proliferation and differentiation of the limiting ridge and anorectal junction of rats (14), however, the effects of neonatally administered DES on gastrointestinal tumorigenesis in rats have not yet been reported. Therefore, it remains to be determined whether neonatal administration of an estrogenic endocrine disruptor, such as DES, affects gastrointestinal carcinogenesis. In the present study, the effects of neonatally administered DES on gastrointestinal tumorigenesis induced by DMBA were investigated in male and female rats.

Materials and Methods

Animals. The animals were inbred Sprague-Dawley (SD) male and female rats, maintained in a filtered air laminar flow room at the Institute of Laboratory Animal Sciences, Frontier Science Research Center, Kagoshima University. The animals were given a commercial diet (CE-2; CLEA Inc., Tokyo, Japan) and tap water *ad libitum*. The room temperature was maintained at 25˚C ± 2˚C and relative humidity at 55% ± 10%, with a 12 h light/dark cycle. At 21 days after birth, all rats were weaned. The use of animals in this research complied with all relevant guidelines set by Kagoshima University.

Study designs. Experiment A (Figure 1): Male rats in group I (n=8) were subcutaneously administered 0.05 ml sesame oil once daily from 0-14 days after birth. The rats in groups II and III were subcutaneously administered DES (Sigma Chemical Co., St. Louis, Mo. USA) dissolved in 0.05 ml sesame oil once daily at 1 and 10 μg/rat, respectively. The periods of DES administration in subgroups a (IIa: n=4, IIIa: n=12), b (IIb: n=8, IIIb: n=3) and c (IIc: n=11, IIIc: n=8) were from 0-14, 0-5 and 6-14 days after birth, respectively. The rats in subgroups b and c were given 0.05 ml sesame oil alone once daily from 6-14 and 0-5 days after birth, respectively, to provide an overall treatment period comparable to that of Group I (control). At 21 days after birth, all rats were weaned. At 28, 42 and 56 days after birth, all groups were given 10 mg of DMBA (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in 1 ml sesame oil once a day by gastric intubation. At 272 days after birth, all surviving animals underwent necropsy (Figure 1). The body, testes, epididymides, accessory gonad (including seminal vesicle and prostate), pituitary and adrenal glands were weighed.

Experiment B (Figure 2): Female rats in group I (n=10) were subcutaneously administered 0.05 ml sesame oil once daily from 0-14 days after birth. Female rats in groups II and III were subcutaneously administered DES dissolved in 0.05 ml sesame oil once daily at 1 and 10 μg/rat, respectively. The periods of DES administration in subgroups a (II a: n=16, III a: n=16), b (II b: n=21, III b: n=8) and c (II c: n=12, III c: n=9) were from 0-14, 0-5 and 6-14 days after birth, respectively. The rats in subgroups b and c were given 0.05 ml sesame oil alone once daily from 6-14 and 0-5 days after birth, respectively, to provide an overall treatment period comparable to that of the controls. At 21 days after birth, all rats were weaned. At 50 days after birth, all groups were given 10 mg of DMBA dissolved in 1 ml sesame oil once a day by gastric intubation. At 368 days after birth, all surviving animals underwent necropsy. These female rats were part of a long-term study of mammary tumorigenesis (10, 11).

Histopathological examination. All tumors and testes in Experiment A were fixed in 10% phosphate-buffered formalin, dehydrated, and embedded in paraffin. The widest cut surface of tumors and the cross-cut surface of testes were sectioned to 5 μm, stained routinely with hematoxylin and eosin (H&E), and examined histopathologically. All colons and rectums were fixed in 10% phosphate-buffered formalin, stained with 0.05% methylene blue (MB) and all areas of the colon and rectum were examined in males (approximately 17.5-19.5 cm) and females (approximately 17-19 cm) using a stereoscopic microscope.

Statistics. The mean differences were evaluated by Student's *t*-test. Data are shown as the mean ± standard deviation (SD). The incidences (percentages) were tested using a four-fold contingency table (chi-square test).

Results

Experiment A (male rats). The day when testes dropped in groups IIa-c and IIIa-c was significantly later than in the controls (all $p<0.01$) (Table I). In histopathological examination of the testes, the incidence of an absence of sperm of rats in groups IIa, IIIa and IIIb (all $p<0.05$) and of tubule atrophy in groups IIa, IIc, and IIIa-c (all $p<0.01$) were significantly higher than in controls.

At necropsy, the body weight (BW) of rats in groups IIa ($p<0.01$), IIb ($p<0.01$), IIc ($p<0.01$), IIIa ($p<0.01$), IIIb ($p<0.05$) and IIIc ($p<0.05$) was significantly lower than that in the controls (Table II). The absolute weights (AW) of the testes in groups IIa ($p<0.01$), IIc ($p<0.01$), IIIa ($p<0.01$), IIIb ($p<0.05$) and IIIc ($p<0.01$), the AW/BW of the testes in groups IIa ($p<0.05$), IIc ($p<0.05$), IIIa ($p<0.05$), IIIb ($p<0.05$) and IIIc ($p<0.01$), the AW of the epididymides in groups IIa-c, IIIa and IIIc (all $p<0.01$), the AW/BW of the epididymides in groups IIa ($p<0.01$), IIIa ($p<0.01$) and IIIc ($p<0.05$), the AW and the AW/BW of the accessory gonad, which included the prostate and seminal vesicle, in groups IIa-c and IIIa-c (all $p<0.01$), the AW of the pituitary in groups IIa ($p<0.05$), IIb ($p<0.01$), IIc ($p<0.05$), IIIa ($p<0.01$), IIIb ($p<0.05$) and IIIc ($p<0.01$), the AW of the adrenal glands in groups IIa and IIIa (both $p<0.05$) were significantly lower than those in the controls. The AW/BW of the adrenal glands in groups IIIa and IIIc was significantly higher than that in the controls (both $p<0.05$).

In the digestive tract, forestomach masses (FMs) in all groups (13-58%), small intestine masses (SIMs) in group IIIa (17%) and a colon mass (CM) in groups IIIa (8%) and IIIb (33%) were seen, while no tumors were seen in the esophagus or cecum in any rat (Table III). There were no significant changes in the incidence of these masses and number of these masses per rat. FMs were localized in the forestomach and limiting ridge, and were diagnosed as squamous cell papilloma. SIMs and CMs were diagnosed as adenoma.

Study Design of Experiment A (male rats)

Group	Dose of DES	Treatment period (days after birth)
I	0	None
IIa	1 μg	0-14
IIb		0-5
IIc		6-14
IIIa	10 μg	0-14
IIIb		0-5
IIIc		6-14

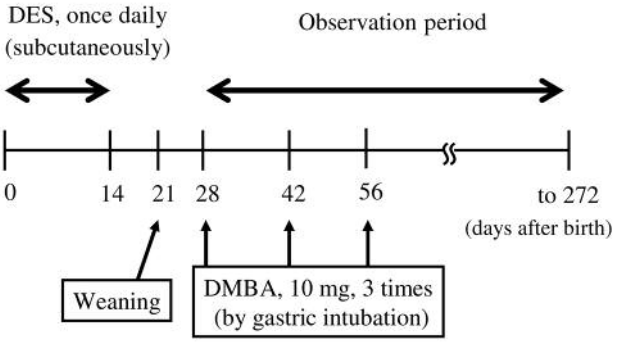

Figure 1. *Summarized protocol of experiment A used in this study.*

Study Design of Experiment B (female rats)

Group	Dose of DES	Treatment period (days after birth)
I	0	None
IIa	1 μg	0-14
IIb		0-5
IIc		6-14
IIIa	10 μg	0-14
IIIb		0-5
IIIc		6-14

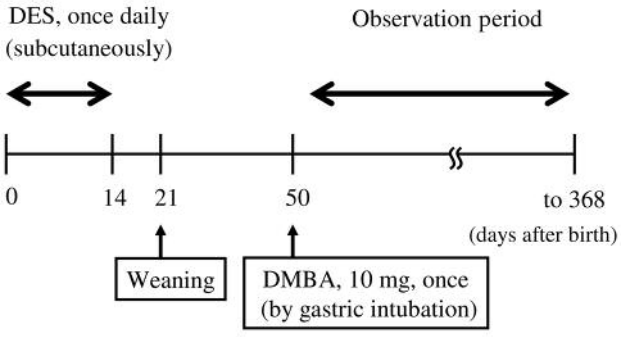

Figure 2. *Summarized protocol of experiment B used in this study.*

Table I. *Effects of neonatal administration of DES on the testes of male rats (Experiment A).*

Group	Treatment		Number of rats	Dropped testes (days after birth)	Number of rats with absence of sperm in testes at necropsy (%)	Number of rats with tubule atrophy in testes at necropsy (%)
	Dose of DES (μg)	Period#				
I	None	0-14	8	19.4±0.5	0 (0)	0 (0)
II a	1	0-14	4	80.5±4.0**	2 (50.0)*	4 (100)**
II b	1	0-5	8	30.3±3.2**	2 (25.0)	3 (37.5)
II c	1	6-14	11	25.5±0.7**	3 (27.3)	9 (81.8)**
III a	10	0-14	12	79.3±3.4**	6 (50.0)*	12 (100)**
III b	10	0-5	3	33.7±1.2**	2 (66.7)*	3 (100)**
III c	10	6-14	8	27.9±1.8**	3 (37.5)	7 (87.5)**

DES: Diethylstilbestrol. #Periods show days after birth. *$p<0.05$, **$p<0.01$: significantly different from Group I.

In the colon and rectum stained with MB, aberrant crypt foci (ACF) were seen in all rats (Figures 3-5). The number of ACF per rat in groups IIa ($p<0.05$), IIc ($p<0.05$), IIIa ($p<0.01$), IIIb ($p<0.05$) and IIIc ($p<0.05$) was higher than in the controls (Table IV).

Experiment B (female rats). In the digestive tract, FMs in groups I (10%), IIa (13%), IIb (33%), IIc (25%) and IIIc (33%), CMs in groups IIa (6%) and IIIa (6%) were seen, while no tumors were seen in the esophagus, small intestine or cecum in any rat (Table V). There were no significant changes in the incidence of these masses and number of these masses per rat. FMs were localized in the forestomach and limiting ridge, and were diagnosed as squamous cell papilloma as those in male rats. SIMs and CMs were diagnosed as adenoma as these in male rats.

In the colon and rectum stained with MB, ACF were seen in all rats while the number of ACF per rat in Experiment B was slightly lower than in Experiment A. No significant changes were noted in the number of ACF per rat (Table VI).

Table II. *Effects of neonatal administration of DES on the body, genital and endocrine organ weights in male rats at necropsy (Experiment A).*

Group	Treatment				AW (g) AW/BW (mg/g)			AW (mg) AW/BW (mg/g)	
	Dose of DES (μg)	Period[#]	Number of rats	BW (g)	Testes	Epididymides	Accessory gonad[$]	Pituitary	Adrenal glands
I	None	0-14	8	452.8±33.4	1.54±0.40 3.41±0.89	0.55±0.05 1.21±0.13	2.12±0.23 4.72±0.70	12.0±1.2 0.027±0.002	29.1±4.8 0.064±0.009
II a	1	0-14	4	361.5±38.2**	0.83±0.19** 2.29±0.34*	0.28±0.04** 0.77±0.14**	0.26±0.04** 0.73±0.07**	9.5±1.3* 0.026±0.001	24.3±1.0* 0.068±0.006
II b	1	0-5	8	382.5±43.4**	1.10±0.47 2.84±1.15	0.42±0.09** 1.10±0.20	0.58±0.35** 1.50±0.86**	9.6±1.7** 0.025±0.003	27.4±4.7 0.072±0.09
II c	1	6-14	11	388.7±43.3**	0.93±0.12** 2.40±0.21*	0.43±0.06** 1.10±0.10	0.98±0.31** 2.48±0.61**	10.6±1.0* 0.028±0.003	28.2±3.4 0.073±0.014
III a	10	0-14	12	324.4±46.5**	0.76±0.18** 2.36±0.59*	0.25±0.05** 0.78±0.14**	0.28±0.11** 0.85±0.26**	8.3±1.2** 0.026±0.002	24.7±3.3* 0.078±0.019*
III b	10	0-5	3	386.7±26.6*	0.70±0.35* 1.81±0.92*	0.46±0.17 1.19±0.39	0.52±0.29** 1.33±0.68**	9.7±1.5* 0.025±0.005	26.7±3.2 0.070±0.012
III c	10	6-14	8	377.0±69.6*	0.82±0.10** 2.21±0.29**	0.37±0.06** 0.99±0.16*	0.76±0.19** 2.00±0.23**	10.3±1.0** 0.028±0.003	30.1±3.0 0.083±0.019*

DES: Diethylstilbestrol; BW: body weight; AW: absolute weight. [#]Periods show days after birth. [$]Accessory gonad includes seminal vesicle and prostate. *$p<0.05$, **$p<0.01$: significantly different from Group I.

Table III. *Effects of neonatal administration of DES on gastrointestinal tumorigenesis induced by DMBA in male rats (Experiment A).*

Group	Treatment			FM		SIM		CM	
	Dose of DES (μg)	Period[#]	Number of rats	Number of rats with FM (%)	Number of FMs per rat	Number of rats with SIM (%)	Number of SIMs per rat	Number of rats with CM (%)	Number of CMs per rat
I	None	0-14	8	3 (37.5%)	1.13±1.81	0 (0)	-	0 (0)	-
II a	1	0-14	4	1 (25.0%)	0.25±0.50	0 (0)	-	0 (0)	-
II b	1	0-5	8	1 (12.5%)	0.50±1.41	0 (0)	-	0 (0)	-
II c	1	6-14	11	3 (27.3%)	0.55±1.04	0 (0)	-	0 (0)	-
III a	10	0-14	12	7 (58.3%)	1.58±1.73	2 (16.7)	0.17±0.39	1 (8.3)	0.08±0.29
III b	10	0-5	3	1 (33.3%)	0.67±1.15	0 (0)	-	1 (33.3)	0.33±0.58
III c	10	6-14	8	1 (12.5%)	0.13±0.35	0 (0)	-	0 (0)	-

DES: Diethylstilbestrol; DMBA: 7,12-dimethylbenz[α]anthracene; FM: forestomach mass; SIM: small intestine mass; CM: colon mass. [#]Periods show days after birth.

Discussion

The daily administration of DES (1 or 10 μg) from 0-14, 0-5 and 6-14 days after birth in female rats induced disturbance of the gonadotropin-secreting system, resulting in rats with early opening of the vagina, PE and animals lacking CL (10, 11). Inappropriate exposure to estrogens in the neonatal period disrupts the hypothalamus–pituitary control system, resulting in impaired development of male reproductive organs (15). It was considered that the daily administration of DES (1 or 10 μg) from 0-14 days after birth in male rats also induced disturbance of the gonadotropin-secreting system, resulting in rats with late dropping of testes, absence of sperm, tubule atrophy and decreased weights of testes and the accessory gonad, while the

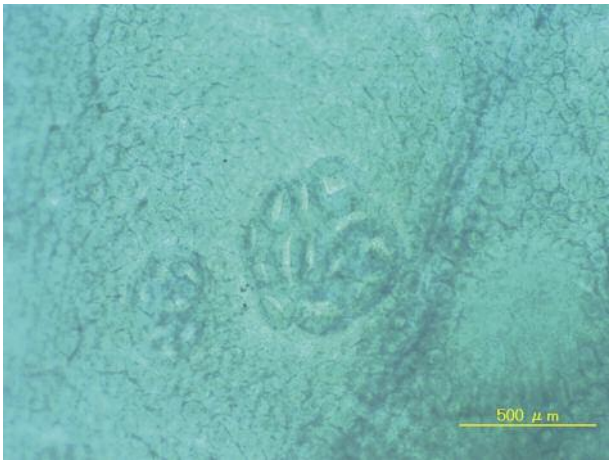

Figure 3. *ACF in the colon and rectum of a male rat in group IIIa. MB stain.*

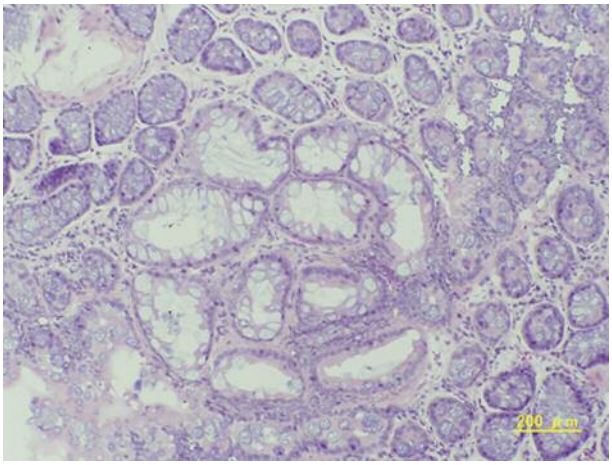

Figure 4. *ACF in the colon and rectum of a male rat in group IIIa. Low magnification. H&E stain.*

Table IV. *Effects of neonatal administration of DES on the number (incidence) of ACF induced by DMBA in colon of male rats (Experiment* A).

Group	Treatment				
	Dose of DES (μg)	Period#	Number of rats	Number of rats with ACF (%)	Number of ACF per rat
I	None	0-14	8	8 (100%)	15.1±8.2
II a	1	0-14	4	4 (100%)	32.0±18.3*
II b	1	0-5	8	8 (100%)	16.1±7.0
II c	1	6-14	11	11 (100%)	26.9±9.0*
III a	10	0-14	12	12 (100%)	39.4±11.1**
III b	10	0-5	3	3 (100%)	32.3±11.7*
III c	10	6-14	8	8 (100%)	24.9±9.7*

DES: Diethylstilbestrol; ACF: aberrant crypt foci; DMBA: 7,12-dimethylbenz[α]anthracene. #Periods show days after birth. ACF were examined in colon and rectum stained with methylene blue (MB) using a stereoscopic microscope. *p<0.05, **p<0.01: significantly different from Group I.

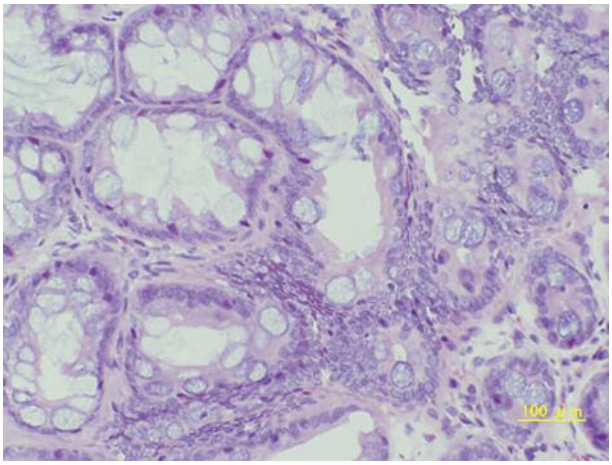

Figure 5. *ACF in the colon and rectum of a male rat in group IIIa. High magnification. H&E stain.*

possibility of direct estrogenic action of DES cannot be ruled out. Moreover, it was speculated that the critical periods and dose of DES in neonatal male rats were different from those in neonatal female rats because there were no significant changes in the number of rats with an absence of sperm and tubule atrophy in male rats daily administered 1 μg DES from 0-5 days after birth. Additional experiments with different DES administration periods and doses may be necessary.

ACF were reported as precancerous lesions of the colon (6). Experiments A and B showed that ACF were induced in all male and female rats administered DMBA alone. The present study added a new finding that single and triple administrations of 10 mg DMBA induced ACF in all female and male animals, respectively. Moreover, female rats may be more sensitive to the induction of colorectal cancer induced by DMBA than male rats since the induction of ACF in male rats required a higher dose of DMBA. In male rats, the number of ACF/cm in the colon was approximately 71 at 210 days after two administrations of azoxymethane (AOM) (15 mg/kg body weight) (16) and the incidence of ACF and carcinoma in the colon was 66% and 45% at 308 days after two administrations of AOM (20 mg/kg body weight) (17). In female rats, the number of ACF was approximately 86 at 30 days after administration of AOM (20 mg/kg body weight) (18). The potential effect of DMBA on colon carcinogenesis seems to be lower than that of AOM in both male and female rats.

Table V. *Effects of neonatal administration of DES on gastrointestinal tumorigenesis induced by DMBA in female rats (Experiment B).*

Group	Treatment		Number of rats	FM		CM	
	Dose of DES (μg)	Period[#]		Number of rats with FM (%)	Number of FMs per rat	Number of rats with CM (%)	Number of CMs per rat
I	None	0-14	10	1 (10.0)	0.70±2.21	0 (0)	-
II a	1	0-14	16	2 (12.5)	0.25±0.68	1 (6.3)	0.06±0.25
II b	1	0-5	21	7 (33.3)	0.43±0.68	0 (0)	-
II c	1	6-14	12	3 (25.0)	0.25±0.45	0 (0)	-
III a	10	0-14	16	0 (0)	-	1 (6.3)	0.06±0.25
III b	10	0-5	8	0 (0)	-	0 (0)	-
III c	10	6-14	9	3 (33.3)	1.67±3.94	0 (0)	-

DES: Diethylstilbestrol; DMBA: 7,12-dimethylbenz[α]anthracene; FM: forestomach mass; CM: colon mass. [#]Periods show days after birth.

Lower androgen levels increase the risk of human colon cancer (19). Neonatal administration of DES reduces the serum testosterone level in rats (20). The precise mechanisms are unknown, but the increase of ACF in male rats neonatally administered DES may be due to serum androgen levels. It may be necessary to further investigate serum hormone levels, such as testosterone, in male rats neonatally administered DES.

Estrogen inhibits the development of colon cancer by the inhibition of cell proliferation and the induction of apoptosis (21). The decrease in female hormone levels from the menopause increases the risk of human colon cancer (22). The daily administration of 1 μg DES from 0-14 and 0-5 days after birth (10), and 10 μg DES from 0-14, 0-5 and 6-14 days after birth (11), reduced serum 17β-estradiol (E_2) and progesterone (P) levels in female rats. However, the neonatal administration of DES in female rats did not significantly increase the number of ACF induced by DMBA in the present study. The sexual differences of DES in the induction of ACF induced by DMBA are very interesting. Moreover, the decrease of serum P levels in female rats neonatally administered DES was stronger than that of E_2, probably because the lack of CL resulted in rats with a relative excess of estrogen (10, 11). The precise mechanisms are unknown, but the reason why the neonatal administration of DES does not increase in female rats may be responsible for the relative excess of estrogen. It may also be necessary to further investigate serum levels of hormones such as testosterone in female rats.

To conclude, these results suggest that neonatal administration of DES may affect male colorectal carcinogenesis.

Acknowledgements

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Table VI. *Effects of neonatal administration of DES on the number (incidence) of ACF induced by DMBA in colon in female rats (Experiment B).*

Group	Treatment		Number of rats	Number of rats with ACF (%)	Number of ACF per rat
	Dose of DES (μg)	Period[#]			
I	None	0-14	10	10 (100)	12.6±6.6
II a	1	0-14	16	16 (100)	9.6±7.0
II b	1	0-5	21	21 (100)	12.7±7.1
II c	1	6-14	12	12 (100)	10.5±5.6
III a	10	0-14	16	16 (100)	11.5±8.8
III b	10	0-5	8	8 (100)	7.8±4.4
III c	10	6-14	9	9 (100)	12.4±4.2

DES: Diethylstilbestrol; ACF: aberrant crypt foci; DMBA: 7,12-dimethylbenz[α]anthracene. [#]Periods show days after birth. ACF were examined in colon and rectum stained with methylene blue (MB) using a stereoscopic microscope.

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