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Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice

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Abstract

In rodents, steroid hormones are thought to be transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses may have higher serum levels of estradiol, and lower serum levels of testosterone, relative to siblings of the same sex that develop between two male fetuses. The consequence in the variation of postnatal growth, development, and function in the intrauterine position, using various parameters such as anogenital distance, preputial separation and vaginal opening, estrous cycle, locomotor activity, and growth of reproductive organs, were examined in Sprague–Dawley rats. ICR mice were treated with 17 β -estradiol before copulation and during pregnancy to address the interaction with endogenous estradiol during pregnancy. In rats, no evidence of effects of prior intrauterine position was observed for any of the parameters examined. Mouse fetal exposure via the mother to low-dose 17 β -estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. The results of this study suggested that the intrauterine position of the embryos/fetuses did not affect the postnatal growth of the reproductive organs, sexual maturation, or behavior in rats and mice.

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1. Introduction

The development of sexually differentiated phenotypes depends upon the hormonal environment during a critical period of growth [1]. Testosterone secretion by the fetal testis causes a longer anogenital distance (AGD), seen in neonatal males, relative to females. The AGD of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position of the animals [1–4]. A longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus. Females with a male fetus on only one side are immediate [4].

In all litter-bearing species that have been examined to date, the intrauterine position that a fetus occupies relative to fetuses of the same or opposite sex has profound effects on its reproductive, behavioral, and morphological traits measured during adult life [4–7]. Gerbil males and females that

developed in utero between two female fetuses or two male fetuses, respectively, did not differ in relative hippocampal size [8].

The effects of intrauterine position are apparently not the result of the position itself, but rather of the movement of steroid hormones between the fetuses, and variations in the hormonal environment relative to the proximity of an individual fetus to other fetuses of the same or opposite sex [9]. Male rats located between two females had elevated serum estradiol and larger prostates than males located between two males, which had elevated serum testosterone and larger seminal vesicles [10]. The effect of intrauterine position in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity [11,12].

Recently, intrauterine position has been the focus of discussions in the toxicology community because of its potential to alter the susceptibility of fetuses to endogenous hormones and endocrine disrupting chemicals [13,14]. In this regard, failure to account for intrauterine position in endocrine disrupting chemical toxicology studies could lead to false negative results, especially when adverse alterations

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are produced by low doses in fetuses from only one intrauterine position [14,15]. This possibility has been raised because of investigations into estrogenic compounds in mice. In rats, consistent effects due to intrauterine position on testosterone concentrations, and therefore potential interactions with endocrine disrupting chemicals, have not been found. Howdeshell and vom Saal [16] demonstrated that the greatest response to the estrogenic chemical, bisphenol A, occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A within the range of human exposure, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development. It has been demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin interacted with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses [17].

The objectives of this study were to determine the effects of intrauterine position, under normal physiological conditions, on the development of rat offspring, as well as sexual maturation, estrous cycle, behavior, and reproductive organ development. Another objective of this study was to determine whether the intrauterine position of mouse fetuses, which is related to background levels of estradiol and testosterone, would influence the response of the postnatal growth of gonads, including sexual maturation, to low dose 17 β -estradiol.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Crj:CD, IGS), and ICR mice (Crj:CD-1) were purchased from Charles River, Laboratories, Inc. (Atsugi, Japan). Twenty-seven male rats (9 weeks of age), 84 female rats (8 weeks of age), 130 male mice (9 weeks of age), and 130 female mice (8 weeks of age), were used. The rats and mice arrived with mean weights of 301.1 ± 7.9 g for males and 216.2 ± 8.1 g for females, and 37.2 ± 1.2 g for males and 29.1 ± 0.9 g for females (mean \pm S.D.), respectively. The animals were acclimated to the laboratory for 7–14 days prior to the start of the experiments to evaluate weight gain and any gross signs of disease or injury. The animals were housed individually in stainless steel, wire-mesh cages in a room with controlled temperature (22–25 °C) and humidity (50–65%), with lights on from 07:00 to 19:00 h daily. The animals were given access to food (NIH-07-PLD; phytoestrogen low diet, Oriental Yeast Co., Japan) and tap water through metal pipes (distilled water, Wako Pure Chem., Japan) ad libitum. In a few instances, the temperature and humidity were outside the standard ranges, but the magnitude and duration of these incidents were minimal and judged to be of no consequence. The contents of genistein and daidzein in the diet

and wood bedding (ALPHA-dri, Shepherd Specialty Paper, USA) used in the present study were determined. Neither genistein nor daidzein were not detected in the diet or wood bedding (detection limit: 0.5 mg/100 g in each individual phytoestrogen, by HPLC).

Animal care and use conformed to published guidelines [18].

2.2. Experiment I (examination of intrauterine position effect on postnatal growth in rats)

2.2.1. Cesarean delivery and fostering

Estrous female rats at 10–11 weeks of age were cohabited overnight with a single male to obtain 66 pregnant females within 4 days. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Thirty-three pregnant females were killed by CO₂ asphyxiation and cervical dislocation, and subjected to cesarean sectioning on day 21 of gestation. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed. Anogenital distance (AGD) was measured with a digital micrometer (reproductive precision of 0.01 mm, Digimatic caliper CD-15C, Mitutoyo Co., Kanagawa, Japan) under an Olympus dissecting microscope for each fetus, and the average was taken. The subject was held steady and in the same position during measurement. Measurements were made without knowledge of intrauterine position by one person. The AGD was measured from the center of the phallus to the center of the anus. The fetuses obtained by cesarean delivery were fostered to 33 dams that had just given birth naturally (one litter to each female). The original littermates remained together when cross-fostered. The litter sizes were similar for each cross-fostered dam. The day of cesarean section was considered as postnatal day (PND) 0. Pup body weights were recorded on PND 21 (day of weaning). Following weaning, and until 10 weeks of age, offspring were weighed once a week.

Neonates from 33 pregnant females were categorized as occupying six different intrauterine positions: 2M (male fetus located between two male fetuses; number of pups and litters on PND 0 = 36 and 19); 1M (male fetus that located between a male fetus and a female fetus; $n = 73$ and 27); 0M (male fetus located between two female fetuses; $n = 45$ and 24); 2F (female fetus located between two female fetuses; $n = 38$ and 18); 1F (female fetus located between a female fetus and a male fetus; $n = 83$ and 29); 0F (female fetus located between two male fetuses; $n = 41$ and 27). Fetuses adjacent to dead embryos (resorptions or macerated fetuses), and fetuses that were closest to each ovary or the cervix, were discarded from further analyses.

2.2.2. Observations of postnatal growth

2.2.2.1. Measurement of AGD and reproductive organ weights, and evaluation of sexual maturation. On PND

4, the AGD was measured for pups in each group using calipers with a reproductive precision of 0.01 mm. On PND 21, all pups were weaned and half of the pups in each group (2M = 13, 1M = 37, 0M = 11, 2F = 14, 1F = 43, 0F = 12) were subjected to necropsy, and the testes, epididymides, and prostates with seminal vesicles (fluid was not removed and all lobes were included) in males, and uteri and ovaries in females, were weighed. For the remaining male and female pups in each litter (2M = 21, 1M = 32, 0M = 30, 2F = 23, 1F = 36, 0F = 26), as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 28), and preputial separation for males (beginning on PND 35), were assessed, and each rat was weighed when these criteria were achieved.

2.2.2.2. Postweaning tests of behavior, evaluation of estrous cycle, and histological observation of reproductive organs.

One male and one female were randomly selected from each litter in each group (number of rats examined: 2M = 18; 1M = 27; 0M = 25; 2F = 17; 1F = 27; 0F = 25), and were subjected to an open field test and wheel cage activity test to assess the emotionality and regulatory running activity, respectively. At 4 weeks of age, the rats were placed into a circular area (140 cm in diameter) surrounded by a wall (40 cm in height). The light and noise levels averaged 500 lx and 50 dB, respectively, at the center of the circular area. Rearing, grooming, defecation, and urination were counted, and ambulation was recorded automatically on a computer (Unicom, Inc., Japan), during a 3-min trial between 13:00 and 16:00 h on one day. At 7 weeks of age, the rats were placed into a wheel cage (Nippon Cage, Inc., Japan), 32 cm in diameter and 10 cm in width, as a measure of spontaneous activity. Each rat was kept within the wheel for 24 h with free access to food (NIH-07-PLD) and distilled water in the same animal room. The number of revolutions was automatically recorded with a 20-channel digital counter (Seiko Denki, Inc., Japan).

Each morning (9:00–10:00 h), from 6 to 10 weeks of age, all females in each group were subjected to vaginal lavage. The lavage fluid was applied to a glass slide, air-dried, and stained with Wright–Giemsa stain. Cytology was evaluated and the stage of the estrous cycle was determined using the method of Everett [19].

At 10 weeks of age, 3–5 males in each group were weighed, and anesthetized. Transcardial perfusions were carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the prostate gland was sampled, rinsed three times in phosphate buffer, postfixed for 2 h at 4 °C in 2% osmium tetroxide, and dehydrated in alcohol; the prostate gland was embedded in epoxy resin. Ultrathin sections of the prostates were stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7100, Hitachi, Japan). The remaining males in each group (2M = 18, 1M = 27, 0M = 25) were weighed and subjected to necropsy, and the testes, epididymides, ventral prostate, and

dorsal prostates with seminal vesicles, were weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. All females (2F = 17, 1F = 27, 0F = 25) were weighed and subjected to necropsy when the stage of the estrous cycle was diestrus. The ovaries and uteri were then weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. These reproductive organs were embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.3. Experiment II (examination of low-dose in utero effects of 17 β -estradiol in mice)

The objective of this experiment was to determine whether the intrauterine position of male fetuses, which is related to background levels of estradiol (elevated in males located between two female fetuses) and testosterone (elevated in males located between two male fetuses), would influence the response of the developing prostate to low dose 17 β -estradiol. In addition, we examined whether the intrauterine position of male and female fetuses would affect the postnatal growth of other reproductive organs and sexual maturation.

2.3.1. Administration, cesarean delivery and fostering

Thirty female mice at 9 weeks of age were administered 17 β -estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μ g/kg per day for 7 days before mating, during a mating period of 7 days at the longest, and on day 0 through 17 of gestation. In a preliminary study, the offspring of the ICR pregnant females exposed to 17 β -estradiol at 0.05 μ g/kg per day on day 0 through 17 of gestation showed no changes in weight and histological morphology of reproductive organs in adulthood. However, the offspring of dams exposed to 17 β -estradiol at 0.1 μ g/kg per day on these gestational days showed changes in the parameters in adulthood (data not shown). In the present study, 30 control females were administered corn oil (Nacalai Tesque, Co., Tokyo). After the administration for 7 days before mating, female mice were caged with untreated males overnight and examined for a vaginal plug the next morning. The day on which a plug was found was termed day 0 of gestation. In this study, 30 female mice in the 17 β -estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO₂ asphyxiation, and subjected to cesarean sectioning. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed, and then the AGD was measured. The fetuses obtained by cesarean delivery were fostered to 60 dams that had just given birth naturally (one litter to each female). The day of cesarean section was considered as PND 0. Pup body weights were recorded on PND 21 (day of weaning), and at 5, 7, and 10 weeks of age.

Neonates from 30 pregnant females exposed to corn oil and 30 pregnant females exposed to 17 β -estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17β -estradiol exposed group and the control group: 38 and 41) and 0M (33 and 32), and 2F (41 and 32) and 0F (28 and 37). Fetuses adjacent to dead embryos, and fetuses that were closest to each ovary or the cervix, were discarded. In this experiment, fetuses of two intrauterine positions, 1M and 1F, were also discarded.

2.3.2. Observations of postnatal growth

2.3.2.1. Evaluation of sexual maturation. On PND 21, all male and female pups (2M, 0M, 2F, 0F) in each litter were weaned. For all male and female mice in each litter, as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 25), and preputial separation for males (beginning on PND 30), were assessed, and each pup was weighed when these criteria were achieved.

At 10 weeks of age, five males in each group were weighed and processed to the transcatheter perfusion to observe the histological alteration of the prostate by electron microscope. The remaining males in each group were weighed and subjected to necropsy, and the testes, epididymides and seminal vesicles, were weighed. All females were weighed and subjected to necropsy. The ovaries were then weighed. These reproductive organs including prostates and uteri were fixed in 0.1 M phosphate-buffered 10% formalin solution and embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.4. Data analyses

Statistical analysis of the data for the offspring (AGD, body weight and organ weight, organ/body weight ratios, timing of vaginal opening and preputial separation) was per-

formed using the litter as the unit [20,21]. The AGD, body weight and organ weight, organ/body weight ratios (relative organ weight), timing of vaginal opening and preputial separation, were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect the significances among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, or there was a group whose variance was zero, Kruskal–Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. Comparisons between groups were made using $P \leq 0.05$ as the level of significance.

3. Results

3.1. Experiment I

3.1.1. AGD and body weights of fetuses at cesarean section and pups at PND 4

Table 1 shows the AGD, body weight, AGD/body weight (AGDI: anogenital distance index), and AGD/ $\sqrt[3]{\text{body weight}}$ of fetuses at various intrauterine positions and pups at PND 4. It is reasonable to anticipate that the AGD might vary with body weight of fetus or pup. It has been proposed that the relationship between AGD and body weight should be more properly evaluated using the cube root of the body weight [22–25]. If it is desirable to normalize AGD to body weight, the AGD/ $\sqrt[3]{\text{body weight}}$ seems to provide a more appropriate adjustment.

There were no statistically significant differences in any parameter evaluated at cesarean section (PND 0) or PND 4

Table 1
Effects of prior intrauterine position on anogenital distance in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
AGD of fetuses at cesarean section						
No. of litters	19	27	24	18	29	27
No. of pups	36	73	43	38	83	41
Body weight (g)	5.6 ± 0.4 ^a	5.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4
AGD	2.43 ± 0.22	2.42 ± 0.22	2.42 ± 0.28	1.21 ± 0.20	1.23 ± 0.19	1.22 ± 0.24
AGD/body weight	0.43 ± 0.04	0.42 ± 0.04	0.42 ± 0.05	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
AGD/ $\sqrt[3]{\text{body weight}}$	1.36 ± 0.12	1.36 ± 0.14	1.35 ± 0.18	0.69 ± 0.08	0.70 ± 0.07	0.70 ± 0.08
AGD of pups on PND 4						
No. of litters	19	27	24	18	29	27
No. of pups	34	69	41	37	79	38
Body weight (g)	10.9 ± 1.5	11.2 ± 1.5	10.8 ± 1.1	10.4 ± 1.4	10.3 ± 1.1	10.4 ± 1.3
AGD	4.57 ± 0.54	4.41 ± 0.48	4.43 ± 0.51	2.00 ± 0.22	1.99 ± 0.19	2.00 ± 0.21
AGD/body weight	0.42 ± 0.06	0.40 ± 0.05	0.41 ± 0.03	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.04
AGD/ $\sqrt[3]{\text{body weight}}$	2.06 ± 0.22	1.99 ± 0.19	1.99 ± 0.19	0.92 ± 0.12	0.91 ± 0.11	0.92 ± 0.13

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

Table 2
Effects of prior intrauterine position on reproductive organs before maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight on PND 21						
No. of litters	18	27	24	17	27	25
No. of offspring	13	37	11	14	43	12
Body weight (g)	40.9 ± 6.3 ^a	40.5 ± 6.5	40.2 ± 9.6	38.8 ± 6.3	38.2 ± 7.0	40.7 ± 7.0
Testes (mg) ^b	169.3 ± 27.5	172.2 ± 22.2	164.9 ± 26.1			
Testes ^c	416.3 ± 48.4	429.2 ± 40.3	418.2 ± 46.7			
Epididymides (mg) ^b	23.3 ± 3.1	23.5 ± 4.8	21.9 ± 4.4			
Epididymides ^c	60.5 ± 10.8	58.2 ± 7.8	55.2 ± 7.0			
Prostate + SV (mg) ^{b,d}	47.2 ± 9.9	46.7 ± 10.3	45.9 ± 7.9			
Prostate + SV ^{c,d}	115.6 ± 18.0	115.7 ± 19.0	117.2 ± 19.5			
Ovaries (mg) ^b				24.3 ± 4.0	22.9 ± 3.9	24.8 ± 3.6
Ovaries ^c				63.5 ± 10.1	60.8 ± 9.5	61.5 ± 7.3
Uterus (mg) ^b				10.2 ± 2.0	11.2 ± 3.7	11.8 ± 2.9
Uterus ^c				26.4 ± 4.5	28.8 ± 6.8	29.1 ± 6.0

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

^b Absolute weight.

^c Relative weight (g or mg per 100 g body weight).

^d Seminal vesicle.

between groups 2M, 1M and 0M in males, or groups 2F, 1F and 0F in females.

No significant differences in viability of fetuses at cesarean section (PND 0), or that from PND 0 to PND 4 (the number of pups died; 2M = 2, 1M = 4, 0M = 2, 2F = 1, 1F = 4, 0F = 3), were detected between the groups. In addition, there were no statistically significant differences in body weight at PND 0 and 4.

3.1.2. Body weight and reproductive organ weight of offspring at PND 21

The absolute and relative weights of testes, epididymides, and prostates with seminal vesicles in males, and ovaries and uteri in females, as well as body weight of offspring at PND 21 are shown in Table 2. Irrespective of the intrauterine position, no significant differences were

detected between the groups in absolute or relative reproductive organ weights, or body weights of male and female weanlings, suggesting that the intrauterine position did not affect postnatal growth before weaning in rats.

3.1.3. Sexual maturation and estrous cycle of offspring

Table 3 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups. The estrous cycle of female offspring from 6 to 10 weeks of age is shown in Table 4. No significant differences were detected between the groups in mean estrous cycle length, or the frequency of females showing each stage of estrous cycle.

Table 3
Effects of prior intrauterine position on sexual maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
No. of litters	18	27	24	17	27	25
No. of offspring	21	32	30	23	36	26
Day of preputial separation	43.3 ± 1.3 ^a	43.4 ± 1.2	44.0 ± 1.8			
Body weight (g) ^b	211.8 ± 5.5	212.1 ± 4.3	212.9 ± 5.2			
Day of vaginal opening				33.8 ± 2.2	33.8 ± 1.8	34.1 ± 1.7
Body weight (g) ^b				125.6 ± 4.1	124.6 ± 4.4	126.1 ± 3.9

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

^b Body weight when the criterion was achieved.

Table 4
Effects of prior intrauterine position on estrous cycle in Sprague–Dawley rats

	Group		
	2F	1F	0F
No. of litters	17	27	25
No. of female offspring	23	36	26
Mean estrous cycle length (day)	4.16 ± 0.29 ^a	4.08 ± 0.30	4.20 ± 0.42
No. of females showing Regular cycle (%)	18 (78.3)	28 (77.8)	21 (80.8)
No. of females showing Irregular cycle (%)	5 (21.7)	8 (22.2)	5 (19.2)

2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses. No significant differences were observed between groups.

^a Mean ± S.D.

3.1.4. Behavior and locomotor activity of offspring

Table 5 shows the results of an open field test at 4 weeks of age, and spontaneous activity within the wheel for 24 h at 7 weeks of age, for male and female offspring. There were no significant differences between groups 2M, 1M and 0M in latency, ambulation, rearing, grooming, defecation and urination, or number of revolutions for 24 h in a wheel cage. In the females, urination in group 0F was significantly increased as compared with that in group 2F, whereas other behavioral parameters, including the number of revolutions in a wheel cage were comparable between groups 2F, 1F and 0F.

3.1.5. Weights and histology of reproductive organs of offspring in adulthood

Table 6 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed

in any of the reproductive organs, including the prostates, of the offspring.

3.2. Experiment II

3.2.1. AGD and body weights of fetuses at cesarean section

Table 7 shows the body weight, AGD, AGD/body weight, and AGD/ $\sqrt[3]{\text{body weight}}$, of embryonic day 18 (PND 0) fetuses exposed to corn oil or 17 β -estradiol. There were no significant differences in any of the parameters between the groups. No significant differences in viability of fetuses at cesarean section, or that from PND 0 to PND 21 were detected between the groups (the number of pups died from PND 0 to PND 21: see Tables 7 and 8).

3.2.2. Sexual maturation of offspring

Table 8 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups.

Table 5
Effects of prior intrauterine position on postnatal behavior in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Open field						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Latency (s)	20.4 ± 40.8 ^a	17.9 ± 16.9	15.3 ± 16.2	12.0 ± 9.4	13.8 ± 12.3	16.9 ± 36.1
Ambulation (cm)	676.3 ± 411.3	627.1 ± 417.2	659.0 ± 501.9	940.6 ± 538.1	1039.8 ± 436.3	970.7 ± 449.8
No. of rearing	2.3 ± 3.1	3.0 ± 3.2	1.5 ± 1.4	3.5 ± 2.1	4.5 ± 3.4	3.8 ± 2.3
No. of grooming	0.6 ± 0.9	0.7 ± 0.7	1.1 ± 1.2	0.8 ± 0.9	0.4 ± 0.5	0.8 ± 0.8
No. of defecation	2.8 ± 1.9	2.1 ± 1.5	3.3 ± 2.2	1.9 ± 1.9	1.7 ± 1.8	1.8 ± 1.9
No. of urination	0.4 ± 0.6	0.4 ± 0.6	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.7 ± 0.6**
Spontaneous activity						
Count/24 h	1547 ± 467	1789 ± 697	1559 ± 638	4107 ± 1140	4429 ± 1501	4746 ± 1831

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

**Significantly different from group 2F, $P < 0.01$ (by multiple comparison and Student t -test).

^a Mean ± S.D.

Table 6
Effects of prior intrauterine position on reproductive organs after maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight at 10 weeks old						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Body weight (g)	417.2 ± 31.6 ^a	416.1 ± 34.4	413.6 ± 36.9	270.0 ± 23.2	271.8 ± 28.9	273.6 ± 29.1
Testes (mg) ^b	3.00 ± 0.20	2.98 ± 0.15	3.00 ± 0.17			
Testes ^c	0.72 ± 0.05	0.72 ± 0.06	0.73 ± 0.07			
Epididymides (mg) ^b	0.77 ± 0.05	0.78 ± 0.07	0.76 ± 0.06			
Epididymides ^c	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.02			
Ventral prostate (g) ^b	0.46 ± 0.08	0.44 ± 0.08	0.43 ± 0.10			
Ventral prostate ^c	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.03			
Dorsal prostate (g) + SV ^{b,d}	1.53 ± 0.28	1.56 ± 0.24	1.52 ± 0.27			
Dorsal prostate + SV ^{c,d}	0.37 ± 0.07	0.38 ± 0.05	0.37 ± 0.07			
Ovaries (mg) ^b				92.6 ± 13.3	91.8 ± 13.7	95.4 ± 16.9
Ovaries ^c				34.3 ± 3.6	33.8 ± 3.4	35.0 ± 5.8
Uterus (g) ^b				0.36 ± 0.06	0.38 ± 0.06	0.38 ± 0.05
Uterus ^c				0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.02

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

^b Absolute weight.

^c Relative weight (g or mg per 100 g body weight).

^d Seminal vesicle.

Table 7
Effects of prior intrauterine position on anogenital distance in ICR mice exposed to 17 β -estradiol

Treatment and intrauterine position	Corn oil				17 β -Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	41	32	32	37	38	33	41	28
Body weight (g)	1.41 ± 0.08 ^a	1.42 ± 0.05	1.32 ± 0.05	1.33 ± 0.04	1.42 ± 0.09	1.41 ± 0.10	1.32 ± 0.07	1.30 ± 0.11
AGD	1.92 ± 0.07	1.90 ± 0.06	0.95 ± 0.02	0.95 ± 0.03	1.92 ± 0.08	1.93 ± 0.06	0.93 ± 0.09	0.95 ± 0.05
AGD/body weight	1.36 ± 0.09	1.35 ± 0.10	0.75 ± 0.03	0.73 ± 0.05	1.40 ± 0.09	1.38 ± 0.10	0.75 ± 0.05	0.74 ± 0.07
AGD/ $\sqrt[3]{\text{body weight}}$	1.71 ± 0.07	1.70 ± 0.10	0.88 ± 0.03	0.89 ± 0.05	1.70 ± 0.09	1.72 ± 0.11	0.89 ± 0.07	0.88 ± 0.08

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

Table 8
Effects of prior intrauterine position on sexual maturation in ICR mice exposed to 17 β -estradiol

Treatment and intrauterine position	Corn oil				17 β -Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	39	30	31	35	37	31	39	28
Day of preputial separation	27.2 ± 1.5 ^a	27.3 ± 1.3			27.0 ± 1.8	26.9 ± 2.0		
Body weight (g)	30.3 ± 1.9	31.1 ± 1.5			30.0 ± 2.1	31.3 ± 1.8		
Day of vaginal opening			24.5 ± 1.6	25.1 ± 1.5			24.4 ± 1.7	24.9 ± 1.6
Body weight (g)			21.5 ± 0.9	21.6 ± 1.2			21.6 ± 1.1	22.0 ± 1.5

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.

No significant differences were observed between groups.

^a Mean ± S.D.

Table 9
Effects of prior intrauterine position on reproductive organs after maturation in ICR mice exposed to 17 β -estradiol

Treatment and intrauterine position	Corn oil				17 β -Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	34	25	26	30	32	26	34	23
Terminal body weight (g)	51.5 \pm 4.2 ^a	53.6 \pm 4.4	40.3 \pm 2.7	41.2 \pm 3.4	55.1 \pm 5.1	53.1 \pm 6.2	41.8 \pm 2.1	42.1 \pm 3.1
Testes (g)	257.9 \pm 28.4 ^b	266.5 \pm 23.5			259.3 \pm 25.5	260.3 \pm 19.9		
	486.3 \pm 76.3 ^c	484.2 \pm 71.3			488.3 \pm 62.3	479.1 \pm 60.9		
Epididymides (mg)	89.5 \pm 8.7	92.2 \pm 9.3			94.6 \pm 7.1	93.1 \pm 7.1		
	175.2 \pm 21.5	161.9 \pm 16.9			161.2 \pm 13.6	173.6 \pm 4.9		
Seminal vesicle (mg)	413.9 \pm 30.6	452.2 \pm 13.9			431.3 \pm 18.2	454.8 \pm 21.0		
	812.8 \pm 56.9	802.6 \pm 44.2			811.5 \pm 42.3	809.3 \pm 33.5		
Ovary (mg)			15.3 \pm 3.3	14.6 \pm 4.2			15.5 \pm 3.9	15.3 \pm 4.5
			35.6 \pm 7.5	33.2 \pm 4.6			34.1 \pm 6.9	33.6 \pm 5.1

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.

Five males in each group were processed to the transcardial perfusion. Male pups shown here were subjected to necropsy.

No significant differences were observed between groups.

^a Mean \pm S.D.

^b Absolute weight.

^c Relative weight (mg per 100 g body weight).

3.2.3. Weights and histology of reproductive organs of offspring in adulthood

Table 9 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed.

4. Discussion

4.1. Anogenital distance

The AGD of newborn rats and mice is longer in males than in females, and it has been demonstrated that the AGD varies as a function of the intrauterine position of the animals [1–4]. The AGD is commonly regarded as a hormonally sensitive developmental measure in rodents [26], and it has been reported that a longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus [27,28]. Evidence supports the hypothesis that exposure to testosterone and estrogen in utero are critical components of the intrauterine position effect [29]. Female mouse fetuses located between two males have significantly higher serum testosterone levels and lower estradiol levels than their sisters that were located between two females. Male mice located between two females have significantly higher levels of estradiol and lower levels of testosterone than males located between two

males [4,12]. The mechanism for these intrauterine position effects can be traced to amniotic fluid transport between adjacent fetuses in uterus [30,31]. However, our data were not consistent with previous reports showing a significant effect of intrauterine position on AGD in rats and mice [2,32–34].

A failure to replicate the effects of intrauterine position on AGD may have potentially arisen for a number of methodological reasons. A set of potential problems revolves around possible errors in the measurement of the AGD. One possibility was that our calipers were not accurate enough to detect small mean differences between females located in various positions in the uterus, found by other investigators [2,32–34]. However, as the calipers could be read to an accuracy of 0.01 mm, they were clearly accurate enough to detect differences of this magnitude. Another possibility is that of human error. Given the short distances being measured, it was absolutely essential that all fetuses or pups be oriented in exactly the same fashion, as even a slight arching of the animal's back could significantly distort the AGD measurements. Two attempts were made to minimize these sorts of errors: (i) efforts were made to orient all fetuses or pups in exactly the same fashion when measuring, and (ii) two independent measurements were taken for each fetus or pup and averaged to obtain the value used. In most cases, the different measurements were highly similar for the same animal.

Simon and Cologer-Clifford [35] reported an absence of an intrauterine position effect on AGD in CF-1 mice. Their finding is only the second study to examine AGD in CF-1 mice, and the original report was more than 10 years old [2]. Therefore, it is possible that either genetic drift, or differences in the source of the CF-1 breeding stock, may

underlie the discrepant findings. In this context, Jubilan and Nyby [6] also found no effect of intrauterine position on the AGD/body weight (AGDI) in CF-1 offspring, using stock from the same supplier employed by the Simon and Cologer-Clifford [35] report.

4.2. Sexual maturation and estrous cycle

Since prenatal exposure of females to testosterone delays vaginal opening [36,37], it was predicted that females situated proximate to males in utero would display vaginal opening later than females not proximate to males during gestation. However, in the present study in rats and mice, there were no significant differences in days of vaginal opening or preputial separation between the groups (see Tables 3 and 8), suggesting that intrauterine position did not influence the sexual maturation in males and females. vom Saal [4] reported that 2F and 0F mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than 0F (see categorization of the different intrauterine positions shown in Section 2).

Female mouse fetuses occupying an intrauterine position between male fetuses exhibit longer estrous cycles in adulthood than females formerly residing in utero next to other female fetuses [11,27]. Prior intrauterine position is therefore a source of individual variation in the production of, and sensitivity to, cues that modulate the timing of puberty and the length of subsequent estrous cycles in female mice, suggesting that prenatally androgenized females occupying an intrauterine position between male fetuses may have a reproductive advantage over other females at high population densities [4]. In the present study of rats, however, 0F and 2F did not differ significantly in the estrous cycle length, although the estrous cycle length of 2F (4.16 ± 0.29) tended to be shorter than that of 0F (4.20 ± 0.42). Prior studies have shown that, in the absence of males, vaginal estrus does not correlate with ovulation in peripubertal CF-1 female mice [38,39]. Further studies in which ovulation is confirmed by the presence of corpora lutea and tubal ova is thus required.

4.3. Behavior

Kinsley et al. [40] demonstrated that female mice located in utero between two female fetuses exhibited higher levels of regulatory running activity (locomotor activity) in adulthood than females located between two male fetuses. Male mice, which were less active than females, were also influenced by intrauterine contiguity, indicating that intrauterine position influences the behaviors involved in the maintenance of metabolic homeostasis. Previous work has shown that female rats and mice display higher levels of regulatory running activity than males, and that perinatal testosterone is responsible for this sex difference [41–43]. The present study also showed female rats displayed higher levels of running activity than males.

In the present study of rats, however, there were no significant differences in spontaneous activity in the wheel cage, or in ambulation in the circular area, as well as the frequency of rearing, grooming and defecation between the groups in both sexes, suggesting no intrauterine position effects on locomotor activity in rats. Interestingly, the frequency of urination for females that developed in utero between male fetuses was significantly increased, more than in females that developed in utero between female fetuses (see Table 5). Females that were located between female fetuses in utero were found to urine mark at higher rates than females that were located between male fetuses, in adulthood in CF-1 mice [2]. The frequency of urination in the circular area, observed in the present study, would relate to the emotionality of the animals when placed in a novel environment, and differ from urine marking. Female urine marking may play an important role in communication between female mice, as well as in inter-sexual communication. It has been suggested that in natural populations of mice, females urine mark to advertise their dominant breeding status to other females; urine-marking appears to be dependent on female social/reproductive status [44]. Taken together, these observations suggest that the intrauterine position did not affect behavior as evaluated by the open field test and the wheel cage.

4.4. Prostate development

Growth and differentiation of the prostate is primarily under the control of androgen. Expression of the androgen metabolizing enzyme, 5α -reductase, within prostatic mesenchyme cells is also necessary for normal development of the prostate [45]. The possibility that estrogen might be involved in modulating the effects of androgen on prostatic development during early life has been the subject of speculation for over 60 years [46–49]. Timms et al. [50] demonstrated that development of the urogenital system in male and female rat fetuses is influenced by their intrauterine proximity to fetuses of the same or opposite sex, and suggested that exposure to supplemental estradiol (due to being positioned between two female fetuses) induces prostatic bud development in females, and enhances the growth of prostatic buds in both males and females. An enlarged prostate in males located between two female fetuses was hypothesized to be mediated by an elevated level of serum estradiol, relative to males located between two males, due to the transport of estradiol from adjacent female fetuses [4,30]. This hypothesis was confirmed in a study in which estradiol was experimentally elevated by 50% in male mouse fetuses (via maternal administration), and the estrogen-treated males showed both a significant increase in prostatic glandular buds and significantly larger buds during fetal life, as well as enlarged prostates in adulthood [46].

In the present study, however, the weights of the prostates (with seminal vesicles) of the rats at PND 21, and the ventral and dorsal prostates (with seminal vesicles) of the rats at 10

weeks of age, were not significantly different between the groups. In addition, morphological observation of prostates in the rats and mice, in weanlings or adulthood, by light and electron microscope revealed no alteration in males located in any uterine position.

4.5. Developmental exposure to 17 β -estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17 β -estradiol administration to pregnant mice on the early development of the prostate in male mouse fetuses, with attention being paid to the intrauterine position of the males. Timms et al. [17] reported that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly reduced serum estradiol in males located between two females, but not males located between two males, and also significantly interfered with initial budding and subsequent growth of the prostate in males located between two females or two males. In sharp contrast, the seminal vesicles were larger in the control males located between two males than in control males located between two females, similar to prior findings in mice [29], and TCDD only decreased the size of the seminal vesicles in males located between two males. Taken together, the findings of Timms et al. [17] demonstrate that in utero exposure to TCDD disrupts the development of the prostate, but this disruption depends on an interaction with background levels of estradiol. Howdeshell and vom Saal [16] reported that fetal mouse exposure via the mother to an estrogen-mimicking chemical, bisphenol A, increased the rate of postnatal growth in males and females, and also advanced the timing of puberty in females. They also demonstrated that the greatest response to bisphenol A occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A treatment, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development.

In the present study, however, mouse fetal exposure via the mother to low-dose 17 β -estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. Therefore, we concluded that exposure to low-dose estrogenic endocrine disrupting chemicals during fetal life does not contribute to the intrauterine position.

5. General discussion

We are at a loss to explain why we were unable to replicate the effects of intrauterine position on AGD, or to find intrauterine position effects upon sexual maturation, and the estrous cycle. However, we know the difficulty in demonstrating intrauterine position effects upon morphology and

behavior [35]. In addition, in contrast to earlier work [11] which examined blood androgen titers in mouse fetuses, Baum et al. [51] reported that whole-body androgen levels in female rat fetuses did not vary as a function of intrauterine position, and suggested that intrauterine position effects upon rodent morphology and behavior may not have the robust generality that is generally assumed.

Howdeshell and vom Saal [16] demonstrated that one source of variability in the response of both male and female mouse fetuses to an estrogen-mimicking chemical, bisphenol A, is their background levels of endogenous sex hormones. They suggested that a very small increase in the level of endogenous estradiol may substantially increase the susceptibility of fetuses to endocrine disrupting chemicals consumed or absorbed through the skin or lungs by pregnant animals and humans.

Contiguous [1,52], caudal [53,54], and no effect [51,55–57], due to intrauterine position, have been reported. Hotchkiss et al. [55] in a study with Sprague–Dawley rats examined the effect of intrauterine position on concentrations of testosterone in several different tissues. No effect of either contiguous or caudal intrauterine position on testosterone concentration was detected in fetal carcasses, reproductive tracts, or amniotic fluid. Furthermore, no correlation was found between masculinization due to intrauterine position and increasing anogenital distance. It is unclear at this time why there is such a discrepancy between the previous findings and the present results in rats and mice. However, varied strains of rats and mice, multiple uncontrolled variables, and different criteria for defining the effects of intrauterine positioning, may all contribute to this uncertainty. In addition, the discrepancies in the data may be attributed to such factors as the dietary influences (such as background levels of phytoestrogens and caloric intake), caging (steel versus polycarbonate), bedding, housing (group versus individual), and seasonal variation, as well as differences among the studies in control body and prostate weights [58,59].

The results of the present study clearly showed that intrauterine position of embryos/fetuses did not influence postnatal development, including sexual maturation and behavior.

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Review

A New Hypothesis for Uterine Carcinogenesis: A Pathway Driven by Modulation of Estrogen Metabolism through Cytochrome P450 Induction in the Rat Liver

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Abstract: Estrogen dependence is generally accepted as a cue for mammary and uterine carcinogenesis, but recently estrogen metabolite or catechol-estrogen driven pathways have also come under consideration. Endometrial adenocarcinoma is a leading cause of cancer death in women. Although the cancer is rare in most strains of rodents, it develops spontaneously and can be readily induced in the Donryu rat strain, with many morphological, endocrinological and molecular similarities to the human case. The goal of this review is to weigh the hypothesis for a new pathway for endometrial carcinogenesis driven by modulation of estrogen metabolism through cytochrome P450 enzyme induction using data from the Donryu rat model. To test our hypothesis, indole-3-carbinol (I3C), an active ingredient of cruciferous vegetable, was selected, since it induces cytochrome P450 enzymes in the liver which impact on hydroxylation of estrogens. In uterotrophic assays using ovariectomized rats, neither 500 ppm nor 2000 ppm of I3C in the diet caused any estrogenic or anti-estrogenic activity. However, in our 2-stage rat uterine cancer model, dietary I3C and subcutaneous injection of 4-hydroxyestradiol (4HE), one of hydroxylation metabolites of 17 β -estradiol (E2), elevated both incidences of uterine adenocarcinomas and multiplicities of uterine proliferative lesions. I3C treatment increased mRNAs for 1A1, 1A2 and 1B1 in the liver, reflecting the distribution of corresponding enzymes immunohistochemically demonstrated. In the uterus, only CYP 1A1 mRNA was increased by the treatment, without reflecting protein expression. In the liver, I3C consistently elevated estradiol 2 and 4 hydroxylation. These results indicate that modulation of estrogen metabolism, particularly to increase 4HE through induction of CYP 1 in the liver, plays a crucial role in promoting effects of dietary I3C on endometrial adenocarcinoma development, providing support for our hypothesis of a new pathway for endometrial carcinogenesis in the rat. (*J Toxicol Pathol* 2006; 19: 57–67)

Key words: uterine carcinogenesis, estrogen, metabolism, CYPs, liver, rat

Introduction

The main target of toxicology research is to detect adverse effects of chemicals on living organisms, while generating an understanding of underlying mechanisms to provide a rational basis for: 1) interpreting descriptive toxicity data; 2) estimating the probability that a chemical will cause harmful effects; 3) establishing procedures to prevent or antagonize toxic effects, 4) designing drugs and industrial chemicals that are less hazardous; and 5)

developing pesticides that are more selectively toxic for their target organisms.

The liver is the primary target organ for most chemicals since its task is to maintain the body's metabolic balance. Bioactivation and detoxification, that comprise the major outcome of chemical metabolism, are usually carried out in hepatocytes having high constitutive activities of many phase I and II enzymes, with frequent enhancement of these activities by repeated chemical exposure, as morphologically manifested by hepatocellular hypertrophy. In general, the ratio between phase I and phase II reactions governs whether a compound causes liver cell injury or is safely detoxified.

Recent work has provided evidence that certain metabolic enzyme inducers in the liver exert indirect effects on other organs. For example, phenobarbital (PB), which is a prototype hepatic microsomal inducer that targets a spectrum of cytochrome P-450 isoenzymes (CYPs), also increases activity of uridine diphosphate

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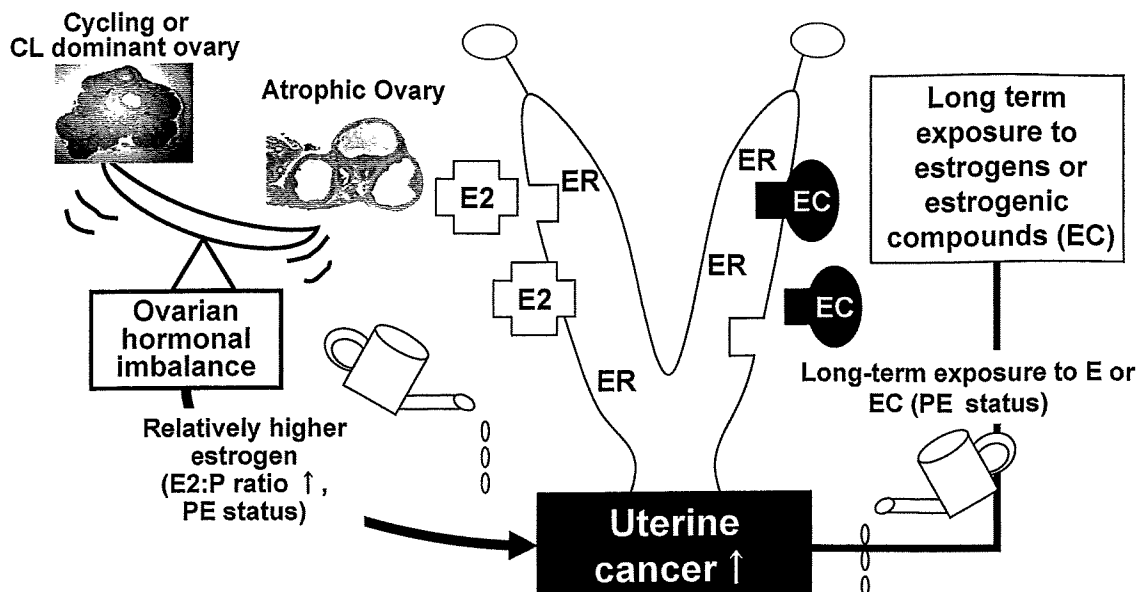


Fig. 1. Uterine carcinogenesis in the Donryu rat model with long-term exposure to a relatively high E2 status or estrogenic metabolites. CL, Corpora lutea; PE, persistent estrus determined by vaginal cytology; E2, 17 β -estradiol; EC, estrogenic compounds; ER, estrogen receptors.

glucuronosyltransferase (UDP-GT), the rate-limiting enzyme in T4 metabolism in the rat^{1,2}, and thus leads to a decrease in blood T4^{1,2}. Consequently, activation of the hypothalamo-pituitary-thyroid control system is activated with resultant elevation in the output of thyroid stimulating hormone (TSH). In 2-year or lifetime chronic toxicity/carcinogenicity studies, such long-term stimulation by PB and compensatory elevation of serum TSH levels finally results in thyroid follicular cell tumors^{3,4}. Additionally, recent studies have proved that the decrease in serum thyroxine level by PB in rats is not necessarily dependent on increase in hepatic UDP-GT⁵.

Induction of CYPs by non-genotoxic carcinogens is not thought to directly cause tumors, but many of the inducible forms are common to those responsible for metabolism of estrogen or other steroids, such as hydroxylation^{6,7}. A number of chemicals such as environmental pollutants and dietary supplements⁸⁻¹⁵ are inducers of such CYPs, especially of the CYP 1 or 3 family. Recently much attention has been paid to 4-hydroxyestradiol (4HE), a hydroxylation metabolite of 17 β -Estradiol (E2) that have been reported to show stronger carcinogenic potential than E2, its parent compound, with reference to production of DNA damage in humans and animals¹⁶⁻¹⁹. This may be of particular importance for endometrial adenocarcinomas in the uterine corpus²⁰, which are generally increasing in women residing in developed countries and are now a leading cause of cancer death. While naturally occurring endometrial adenocarcinomas are generally very rare in rats²¹, Maekawa *et al.* found a high-incidence of spontaneous lesions in aged Donryu rats with close similarities to human tumors^{22,23}, and have subsequently established a rat model of

uterine carcinogenesis²⁴. Elevated binding of E2 or other estrogenic compounds to estrogen receptor (ER) α is also a trigger for uterine carcinogenesis process of rodents²⁵⁻²⁸ (Fig. 1), although precise mechanisms remain to be determined.

The hypothesis assessed in the present review is that chemicals exerting no estrogenic activity themselves but inducing CYPs might modify estrogen-dependent tumor development, and that a new pathway driven by modulation of estrogen metabolism via CYPs inducer might contribute to uterine carcinogenesis in rats. For this purpose I briefly introduce features of endometrial adenocarcinomas in Donryu rats, and present recent supportive data regarding induction of uterine cancers by CYP inducers. Whereas the liver is the main target of estrogen metabolism, in situ modulation of metabolite formation by CYPs in the mammary gland or uterus has also been recently reported¹⁶. Therefore a discussion of the possibility of in situ induction of CYPs in the uterus is included.

Morphological and Endocrinological Features of Endometrial Adenocarcinomas in Donryu Rats

Endometrial adenocarcinomas morphologically develop through multiple stages in rats, as in the human case^{20,22,23}. In middle-aged Donryu female rats (approximately 10 months of age, and equivalent to the menopausal phase in women) atypical hyperplasias characterized by endometrial epithelial cell hyperplasias with slight cellular atypia begin to develop in the endometrium as localized foci of proliferation, then increasing in number and size, demonstrating cellular atypia,

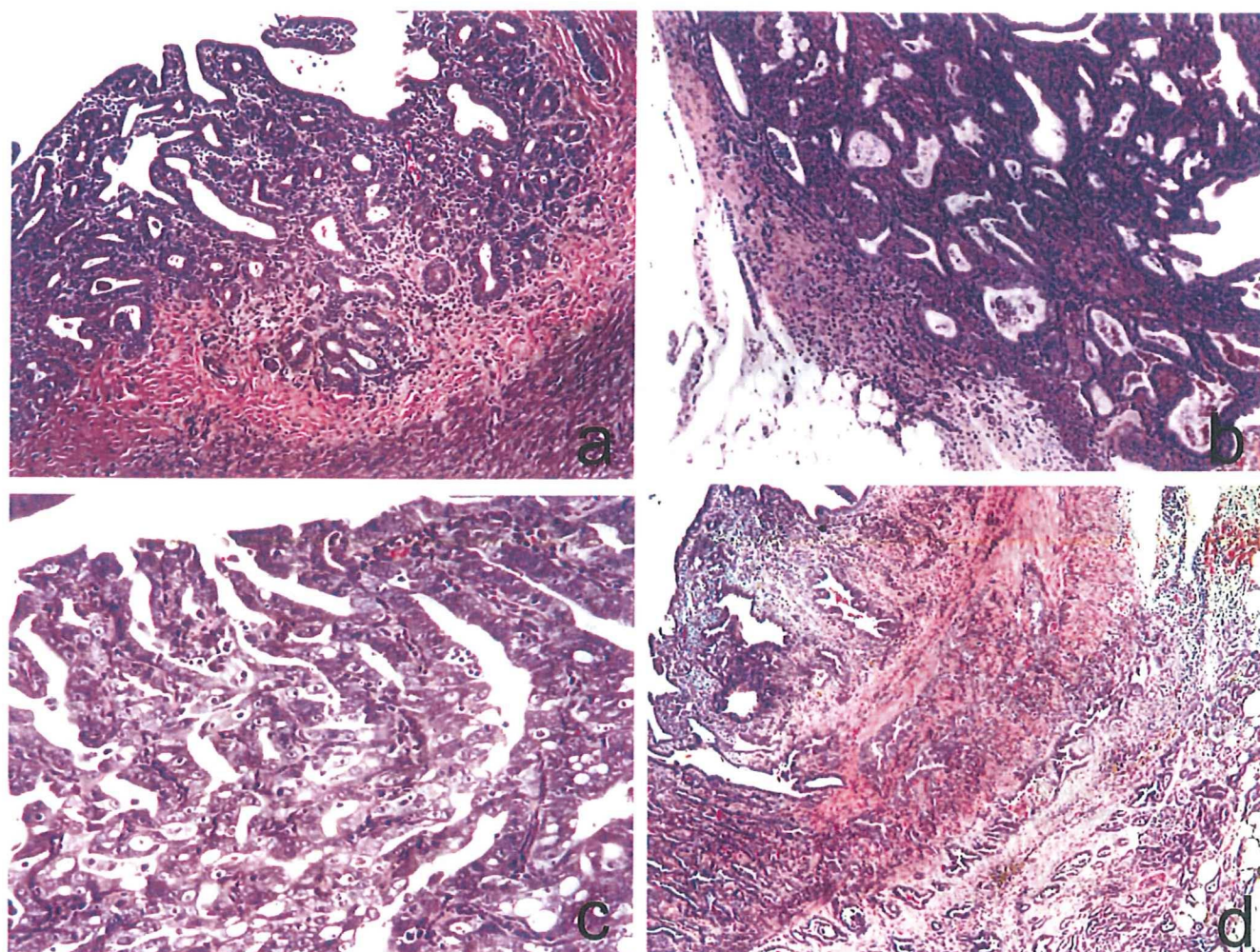


Fig. 2. Histopathological features of atypical endometrial hyperplasia (a), and well-differentiated (b, c) and moderately-differentiated (d) endometrial adenocarcinomas in Donryu rats occurred spontaneously (a, c) and induced by ENNG treatment (b, d). a, $\times 100$; b, $\times 40$; c, $\times 200$. H-E.

with aging. In the next step, well-differentiated endometrial adenocarcinomas with morphological similarities to severe atypical hyperplasias but featuring invasion into the muscle layer or spread to the serosa in the uteri and/or the abdominal cavity, can be found. The incidence of such adenocarcinomas finally rises to 30–50% at 24 months of age. Furthermore, some adenocarcinomas demonstrate progression to moderately or poorly differentiated forms, increasing their malignancy. Based on the sequential development, the atypical hyperplasias are recognized as precursors of endometrial adenocarcinomas (Fig. 2).

A characteristic feature strongly linked to uterine carcinogenesis in this strain of rats is ovarian hormonal imbalance leading to elevation of serum E2 levels relative to progesterone (P), in line with uterine proliferating lesion development²⁷. This imbalance leads to anovulation, morphologically detectable as atrophic ovaries with polycystic atretic follicles and loss of corpora lutea, eventually resulting in persistent estrus (PE) confirmable by vaginal cytology. PE first appears from 4 months of age in

Donryu rats and affects most animals at 11 months, when other rat strains are still capable of reproduction. It should be noted that polycystic ovary is a high risk factor for uterine cancer in women^{29–31}.

Establishment of a Uterine Endometrial Adenocarcinoma Model Using Donryu Rats

Based on the morphological and endocrinological similarities of uterine cancers in Donryu rats to those in women, a 2-stage uterine carcinogenesis model was established by Maekawa's group³² to detect promoting or preventive effects of test-chemicals^{24,27,33–35}. Briefly, in this model female Donryu rats at 10 or 11 weeks of age are initiated with *N-ethyl-N'-nitro-N-nitrosoguanidine* (ENNG) at the concentration of 20 mg/kg dissolved in polyethylene glycol and introduced into a unilateral uterine horn via vagina using a stainless steel catheter for initiation. Then the rats are exposed to test materials for 12 months and at 15 months of age are sacrificed to determine incidences or

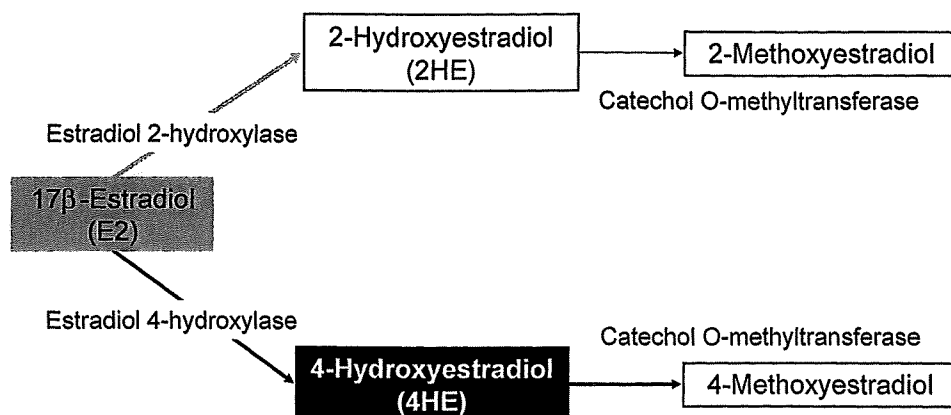


Fig. 3. Major pathway of catechol estrogen metabolism.

Table 1. Incidences (%) of Uterine Proliferative Lesions and Their Multiplicities at 15 Months of Age [Modified from ref. 52]

	None	Atypical hyperplasia			Adenocarcinoma	Multiplicity(a)
		Slight	Moderate	Severe		
Control	16.7	8.3	20.8	29.2	25.0	1.04 ± 0.62
I3C 500 ppm	3.3	6.8	10.0	23.2	56.7*	1.50 ± 0.63*
Control	11.1	11.1	38.9	16.7	22.2	1.17 ± 0.62
I3C 2000 ppm	5.6	11.1	27.8	11.1	44.4	1.78 ± 0.73**
E2 1 µg/kg	0	18.8	12.4	18.8	50.0	1.50 ± 0.52
4HE 5 µg/kg	0	0	31.2	6.3	62.5*	1.69 ± 0.60**

(a) Multiplicities are average numbers of uterine proliferative lesions per rat, mean ± SD.

*, **, Significantly different from the relevant control group ($p < 0.05$ and 0.01 , respectively). I3C, indole-3-carbinol.

multiplicities of uterine neoplastic lesions for comparison with controls (ENNG initiation only). The intrauterine treatment with a single dose of ENNG yields higher and earlier development of uterine neoplastic lesions than is the case with spontaneous development³² and their morphologic features are extremely similarities between spontaneous and induced cases. In addition, the ENNG treatment is specific to the uteri, not affecting ovarian function and other organ tumorigenesis.

Estrogen Metabolite or Catechol Estrogen Driven Uterine Carcinogenesis

In the rat liver, E2 is metabolized by estradiol 2- and 4-hydroxylases into the catechol estrogens, 2-hydroxyestradiol (2HE) and 4-hydroxyestradiol (4HE), respectively^{7,36} (Fig. 3). 2-Hydroxylation of estradiol is the dominant pathway for catechol estrogen formation^{7,36}, and 2HE can bind to ER α , but with a markedly reduced affinity. This metabolite thus possesses much weaker hormonal potential than the parent hormone^{37,38}, and there is evidence that it is not a carcinogenic agent³⁹. In contrast, 4HE, produced only in small amounts in the liver compared to 2HE, is hormonally active and can stimulate uterine growth with strong binding to estrogen receptors when injected into animals^{7,36,40,41}. In

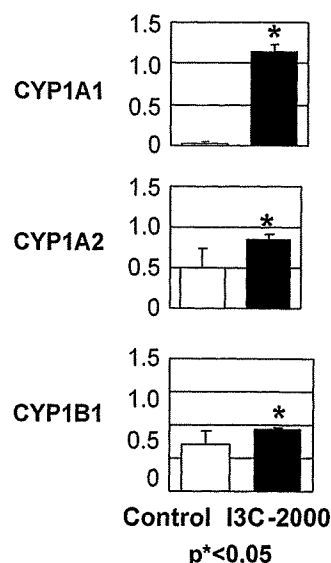


Fig. 4. Levels of expression of cytochrome P450s 1A1, 1A2 and 1B1 mRNA relative to GAPDH mRNA in the liver (calculated as %) in the rat at 15 months of age. Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000 ppm in basal diet. [Modified from ref. 52]

addition, this catechol estrogen has been reported to be a stronger carcinogen than the parent E2 due to production of DNA damage^{6,20}, and causes tumor development in the kidney in hamsters⁴², and possibly uterine and mammary gland neoplasia in human beings^{18,19}.

Recently, a number of dietary supplements extracted from vegetables have been produced, some of which are known to induce CYPs related to estrogen metabolism in the liver and estrogen dependent organs^{18,40,43}. Since most of these products exert no direct estrogenic activity in target organs, they can only indirectly impact on estrogen dependent organ carcinogenesis through induction of CYPs and consequent modulation of estrogen metabolism. Indole-3-carbinol (I3C), an active ingredient of cruciferous vegetable, is reported to induce the CYP1 family enzymes in the liver^{44,45}, to thereby influence hydroxylation of estrogens and to suppress^{39,46-49} or promote^{50,51} carcinogenesis depending on the animal model. Regarding preventive effects, I3C acts as an anti-estrogen and can induce apoptosis, but precise mechanisms remain to be determined.

To determine estrogenic or anti-estrogenic activity of I3C in rat uteri, female Donryu rats were ovariectomized (OVX) at 9 weeks of age, and starting 2 weeks thereafter were assigned to nine groups receiving: only ovariectomy (controls); daily administration of 500 or 2000 ppm I3C in basal diet (I3C 500 or I3C 2000); 1 $\mu\text{g}/\text{kg}$ E2 plus I3C 500 or I3C 2000; daily subcutaneous treatment with E2 at a dose of 1 $\mu\text{g}/\text{kg}$, and E2 metabolites such as 4HE at 5 $\mu\text{g}/\text{kg}$; 2HE at

5 $\mu\text{g}/\text{kg}$ and 16 α estradiol at 1 $\mu\text{g}/\text{kg}$ ⁵². After 2 weeks treatment, neither dose of I3C was found to have affected uterine weight or the height of the luminal epithelium in ovariectomized rats, with or without E2, and the estrogenic activity of 5 $\mu\text{g}/\text{kg}$ 4HE was comparable to 1 $\mu\text{g}/\text{kg}$ E2 treatment, while 2HE treatment had no activity. In addition, long-term treatment with dietary I3C at 500 or 2000 ppm did not disturb estrous cyclicity in Donryu rats, clearly demonstrating a lack of any estrogenic or anti-estrogenic activity, in line with results of uterotrophic assays in OVX rats⁵².

To clarify the effects of I3C on uterine adenocarcinoma development, 500 or 2000 ppm doses were administered to Donryu rats after ENNG initiation. The incidences and/or the multiplicities of lesions were significantly increased compared with those of the control group, as with 4-HE (Table 1)⁵². All uterine adenocarcinomas were of well-differentiated type, and morphological or biological malignancy was not influenced by the I3C treatment.

Histologically, hypertrophy of centrilobular hepatocytes was found in I3C treated groups, along with significantly increased CYP1A1, and to a lesser extent CYP1A2 and 1B1 mRNA expression compared to the control group (Figs. 4, 5). Immunohistochemically, CYP 1A1 and 1A2 were clearly demonstrable in the hepatocytes of centrilobular areas in the liver in both I3C-treated groups (Fig. 6), while mRNA and /or immunohistochemical findings for other cytochrome P450s such as 2B1, 3A1/2 or

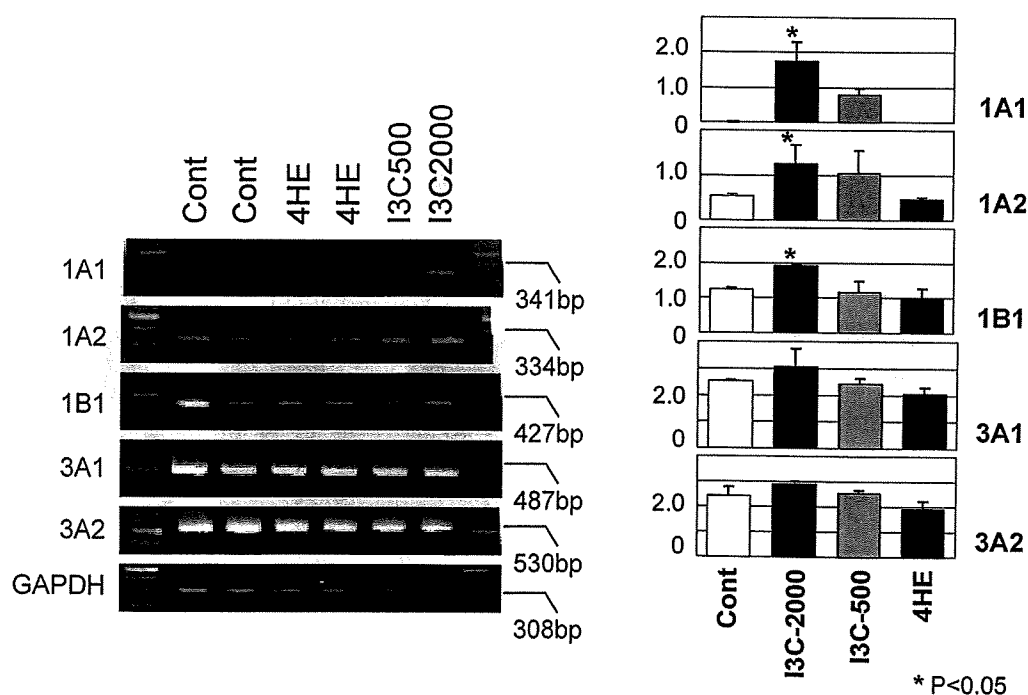


Fig. 5. mRNA Expression of cytochrome P450s 1A1, 1A2 1B1, 3A1 and 3A2 and GAPDH (Left figure), and levels relative to GAPDH mRNA in the liver (calculated as % values) in ovariectomized rats (Right one). Control, control group given basal diet only; 4HE, daily subcutaneous injection of 5 $\mu\text{g}/\text{kg}$ 4-hydroxyestradiol for 2 weeks; I3C-500 and I3C-2000, dietary indole-3-carbinol at 500 or 2000 ppm for 2 weeks, respectively; and I3C-2000+E2, concurrent treatment with I3C-2000 and daily subcutaneous injections of 1 $\mu\text{g}/\text{kg}$ E2 for 2 weeks.

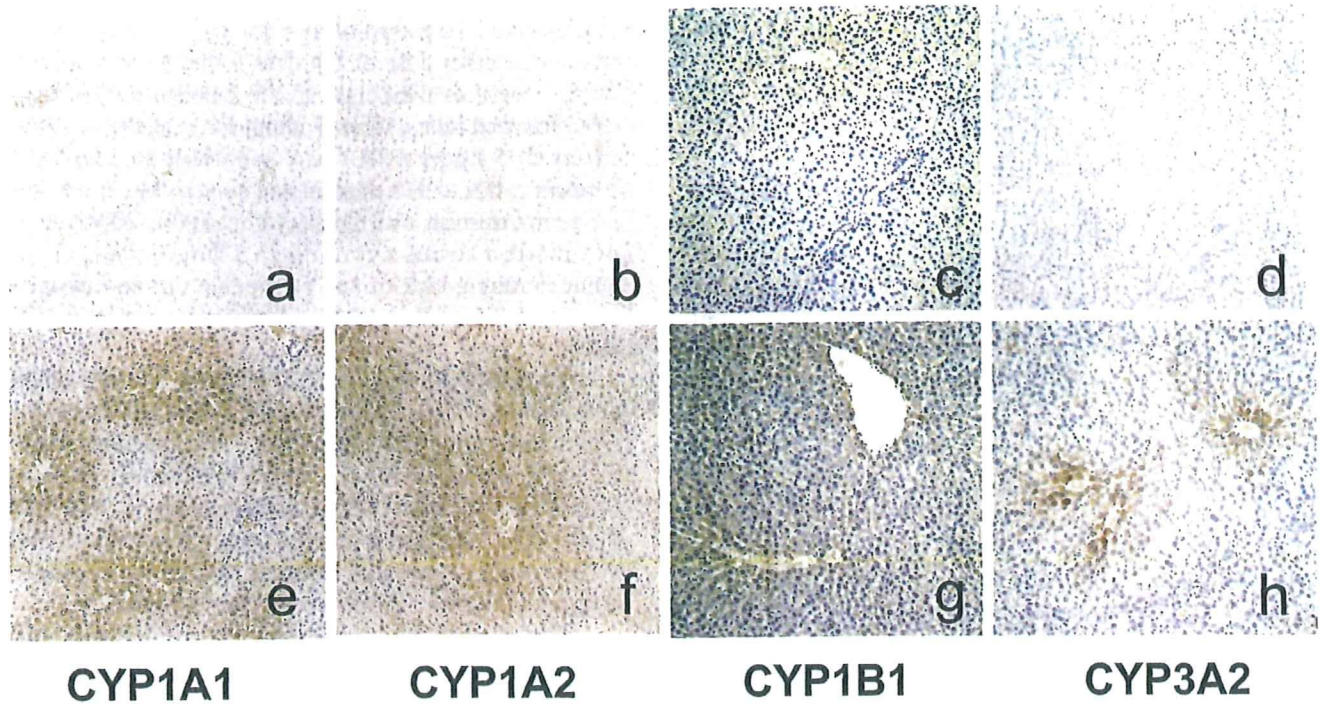


Fig. 6. Immunohistochemical localization of CYP1A1, 1A2, 1B1 and 3A2 in the livers of ovariectomized rats. a–d, Control group. e–h, dietary 2000 ppm I3C treated group. Note that centrilobular hepatocytes are strongly positive to CYP1A1 and 1A2, and slightly positive for 3A2, but negative for 1B1. Positive areas are visualized by diaminobenzidine, and counterstaining is with hematoxylin. $\times 100$.

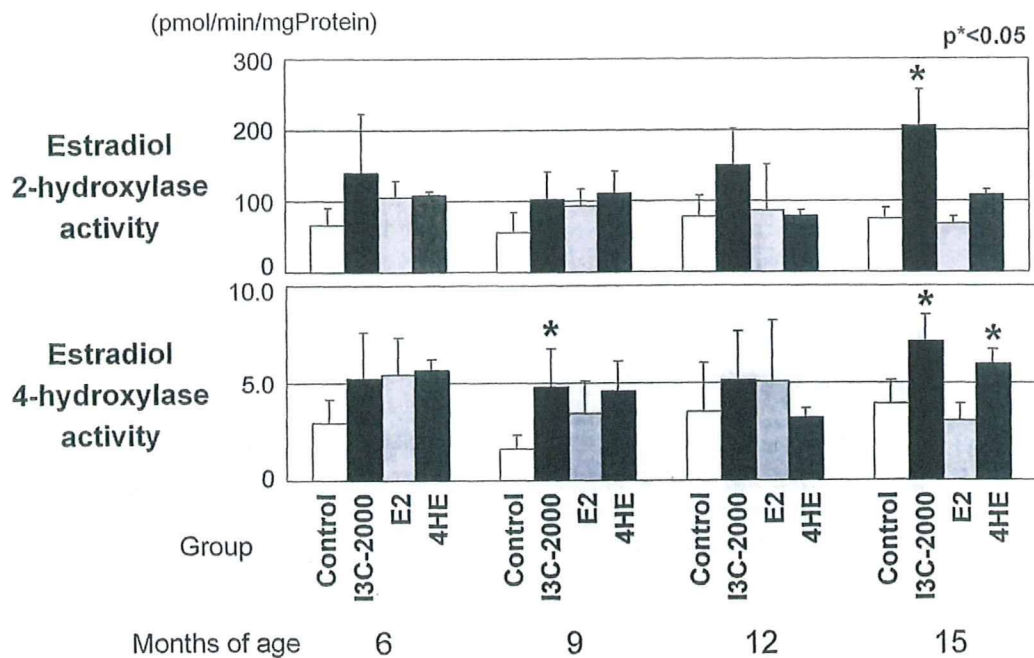


Fig. 7. Sequential changes in enzyme activity related to estrogen metabolism in the liver. Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000 ppm in basal diet; E2, subcutaneous injection of 17β -estradiol at $1 \mu\text{g}/\text{kg}$; 4HE, subcutaneous injection of 4-hydroxyestradiol at $5 \mu\text{g}/\text{kg}$.

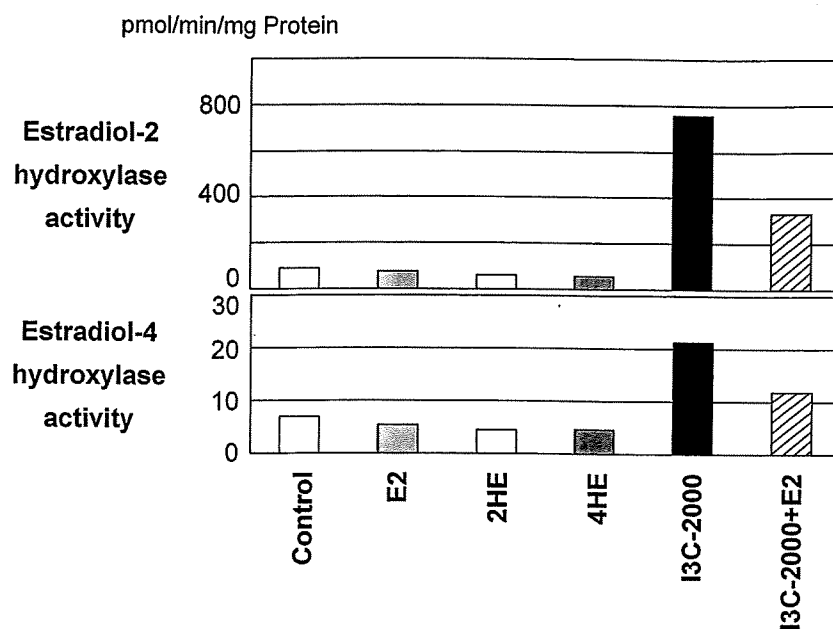


Fig. 8. Sequential changes in enzyme activity related to estrogen metabolism in the livers of ovariectomized rats. Control, control group given basal diet only; E2, subcutaneous injection of 1 $\mu\text{g}/\text{kg}$ 17 β -estradiol for 2 weeks; 2HE, subcutaneous injection of 5 $\mu\text{g}/\text{kg}$ 2-hydroxyestradiol for 2 weeks; 4HE, subcutaneous injection of 5 $\mu\text{g}/\text{kg}$ 4-hydroxyestradiol for 2 weeks; I3C-2000, indole-3-carbinol at 2000 ppm in basal diet for 2 weeks and I3C-2000+E2, concurrent treatment with I3C2000 ppm and E2.

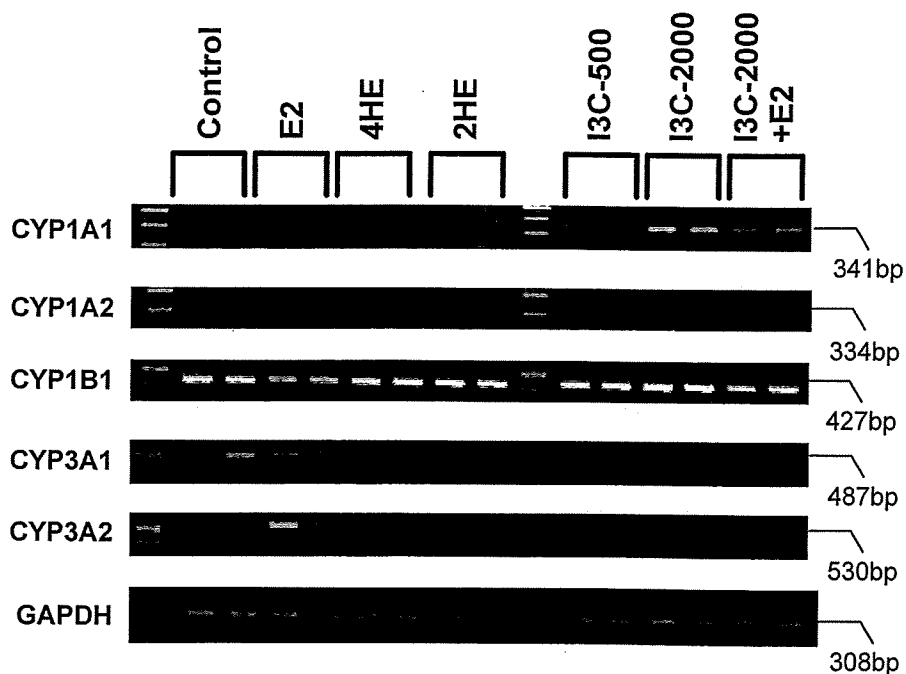


Fig. 9. mRNA Expression of cytochrome P450s 1A1, 1A2, 1B1, 3A1 and 3A2 and GAPDH in the uteri of ovariectomized rats. Control, control group given basal diet only; E2, Daily subcutaneous injection of 1 $\mu\text{g}/\text{kg}$ 17 β -estradiol for 2 weeks 4HE, daily subcutaneous injection of 5 $\mu\text{g}/\text{kg}$ 4-hydroxyestradiol for 2 weeks; 2HE, daily subcutaneous injection of 5 $\mu\text{g}/\text{kg}$ 2-hydroxyestradiol for 2 weeks; I3C-500 and I3C-2000, dietary indole-3-carbinol at 500 or 2000 ppm for 2 weeks, respectively; and I3C-2000+E2, concurrent treatment with I3C-2000 and E2.

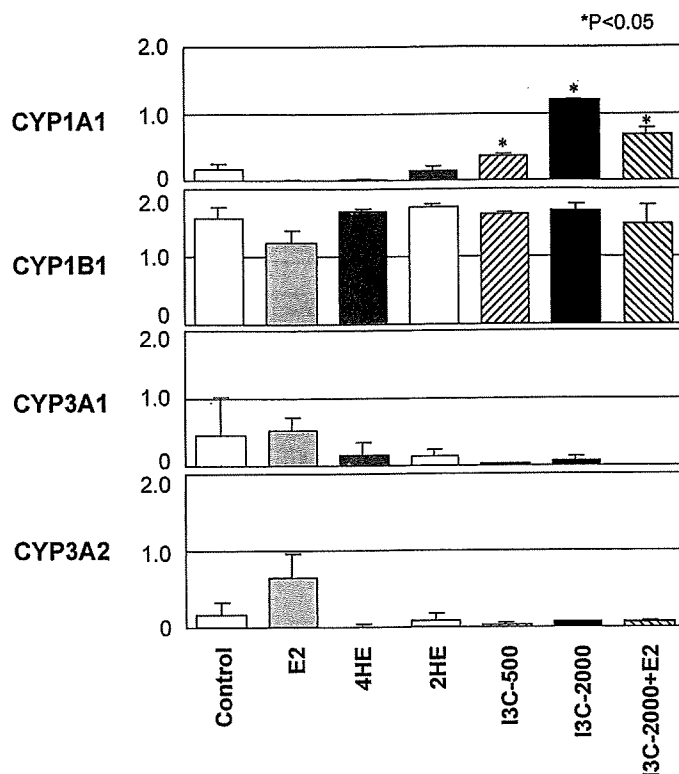


Fig. 10. mRNA Levels of the genes expressed in the Fig. 8 relative to GAPDH mRNA (calculated as %).

2C6 in the livers were comparable to the control case⁵². Measurement also demonstrated increase for estradiol 2- and 4- but not 16 α -hydroxylase activities related to estrogen metabolism (Figs. 7 and 8)⁵². In both of studies, 4-hydroxylation was particularly significantly increased.

In Situ Expression of CYPs in the Uterus

Recently, extrahepatic CYP induction related to estrogen metabolism has been a focus of attention regarding the relationship to tumorigenesis in hamster kidney and human uterine tissue or myoma development^{18,19,42}. In the hamster kidney, estrogen 2-hydroxylation is catalyzed by members of both CYP 1A and 3A families, the latter known to be related with steroid metabolism. CYP 1B1, which catalyzes E2 to 4-hydroxylation of estradiol, is a candidate key enzyme for development of human myomas. We have investigated CYP 1A1/2, 1B1, and 3A1/2-mRNA expression in the uterus of ovariectomized Donryu rats treated with dietary I3C at 2000 ppm for 2 weeks⁵³, but no alteration in CYP 1A2, 1B1, 3A1 or 3A2 was observed (Figs. 9, 10). CYP 1A1 mRNA, but not its protein, was slightly up-regulated (Fig. 11). Additional studies of estradiol 4-hydroxylase activity or DNA adduct formation in the uterus need to be performed for confirmation, but the results do strongly suggest that I3C treatment does not affect in situ expression of CYPs in the rat uterus.

Exploration of a New Pathway for Human Uterine Carcinogenesis

Induction of CYPs is not considered a direct trigger for promoting effects of non-genotoxic carcinogens. While we have no direct proof that the new pathway described in this review indeed contributes to human uterine carcinogenesis, CYP 1A1, 1A2 and 1B1 show high homology in their functions and control systems among mammals. In addition, a number of studies have indicated that CYP1B1 is a key enzyme for 4HE production through metabolism of E2 in both humans⁵⁴ and animals^{8,15}. We have yet to determine which CYP enzyme is the most important for modulation of estrogen metabolism but further investigation of this area would appear to be clearly warranted.

In conclusion, our finding that I3C treatment promotes uterine endometrial adenocarcinoma development in the Donryu rat provides support for a new hypothesis of uterine carcinogenesis driven by modulation of estrogen metabolism in the liver due to CYP 1 family inducers which exert no estrogenic activity by themselves. The promoting effects are likely due to acceleration of 4-hydroxylation of E2, resulting in 4HE binding to ERs to afford a driving force for carcinogenic responses (Fig. 12).

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