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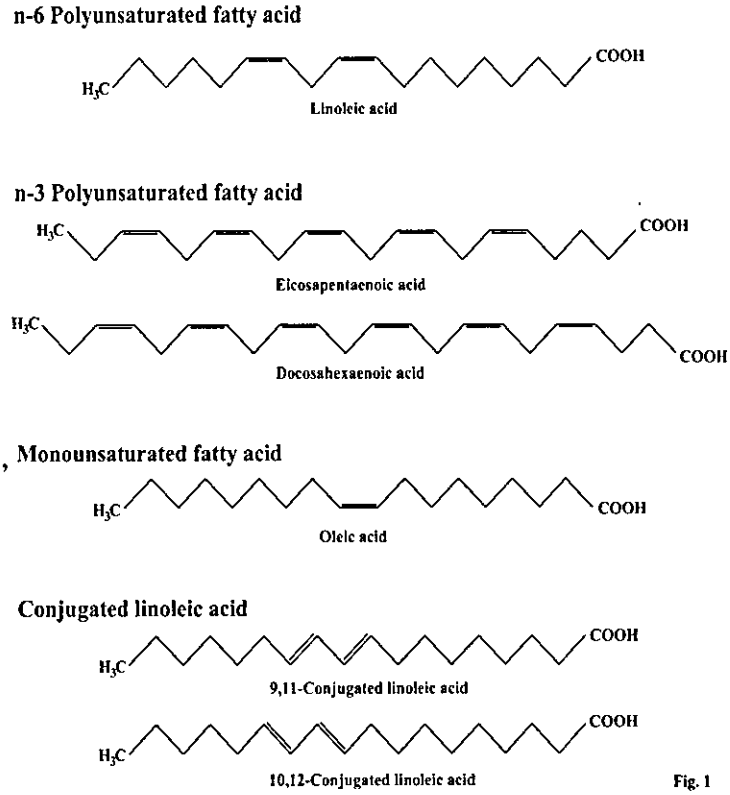

Fig. 1 Structures of n-6 and n-3 polyunsaturated fatty acids, monosaturated fatty acid, and conjugated linoleic acids that affect mammary carcinogenesis. The terms "n-6" and "n-3" describe the position of the double bond nearest to the terminal methyl group, and the terms "polyunsaturated" and "monosaturated" indicate the number of double bonds. The two double bonds in conjugated linoleic acid are at positions 9 and 11, or 10 and 12 (compare with parent linoleic acid), and each of the double bonds can be in the cis or trans configuration. The most common isomer in the diet is cis-9, trans-11.

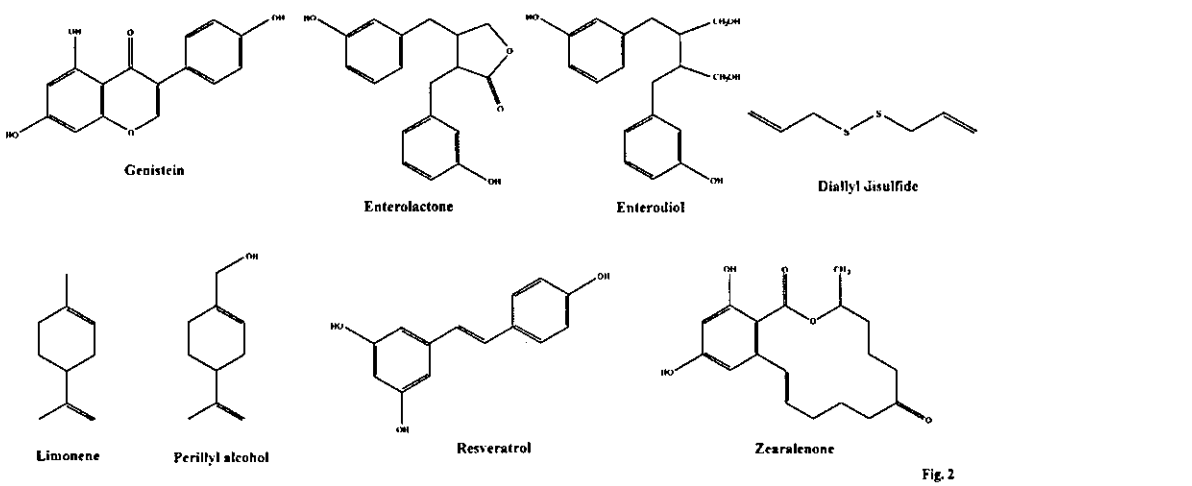

Fig. 2 Structures of chemicals derived from plants or produced by molds, that affect mammary carcinogenesis.

Table I. The 50% inhibitory concentration values of food chemicals with which breast-derived cell lines were incubated for 72 hours

Chemical	Cell line	Cell type	Estrogen receptor	IC_{50} (μM)	References
Eicosapentaenoic acid	MCF-7	Human breast carcinoma	+	562	26
	T-47D	Human breast carcinoma	+	234	
	KPL-1	Human breast carcinoma	+	669	
	MDA-MB-231	Human breast carcinoma	-	209	
	MKL-F	Human breast carcinoma	-	434	
Docosahexaenoic acid	KPL-1	Human breast carcinoma	+	270	28
Conjugated docosahexaenoic acid	KPL-1	Human breast carcinoma	+	97	28
Genistein	DD-762	Murine mammary tumor	-	7	40
	Sm-MT	Insectivora mammary tumor	-	86	
	MCF-7	Human breast carcinoma	+	274	
	MDA-MB-231	Human breast carcinoma	-	131	
	HBL-100	Human breast epithelial cell	-	100	
Enterolactone	MCF-7	Human breast carcinoma	+	82*	52
	MDA-MB-231	Human breast carcinoma	-	>100*	
Diallyl disulfide	MCF-7	Human breast carcinoma	+	4	64
	KPL-1	Human breast carcinoma	+	12	
	MDA-MB-231	Human breast carcinoma	-	2	
	MKL-F	Human breast carcinoma	-	18	
Perillyl alcohol	KPL-1	Human breast carcinoma	+	720	72
	MKL-F	Human breast carcinoma	-	993	
Resveratrol	MCF-7	Human breast carcinoma	+	137	81
	KPL-1	Human breast carcinoma	+	149	
	MKL-F	Human breast carcinoma	-	105	

*IC_{50} for 24 hours

Effects of Prepubertal Exposure to Xenoestrogen on Development of Estrogen Target Organs in Female CD-1 Mice

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Key words: Genistein, Resveratrol, Zearalenone, Zeranol, Bisphenol A, Diethylstilbestrol, CD-1 mouse, Prepubertal

Running title: Prepubertal xenoestrogen in mice

Abstract. *Background*: There have been no previous reports comparing the effects of prepubertal xenoestrogen exposure on development of the reproductive tract and mammary glands in female mice. Effects of genistein (GEN), resveratrol (RES), zearalenone (ZEA), zeranol (ZER), bisphenol A (BPA) and diethylstilbestrol (DES) were examined. *Materials and Methods*: Beginning at 15 days of age, female CD-1 mice were administered 4 daily subcutaneous injections of 10 mg/kg/day of GEN, RES, ZEA, ZER or BPA, or 10 μg/kg/day of DES dissolved in dimethylsulfoxide (DMSO), or DMSO vehicle. Vaginal opening was checked; estrous cyclicity was monitored from 5, 9 or 21 weeks of age for 21 consecutive days; and 6 animals per group were autopsied at 4, 8 and 24 weeks of age. *Results*: Prepubertal exposure to GEN, ZEA, ZER and DES (but not RES or BPA) accelerated puberty onset (vaginal opening). Vaginal smears indicated that all xenoestrogen-treated mice were cycling, but ZEA-, ZER- and DES-treated mice spent more time in estrus. At 4 weeks of age, absence of corpora lutea (anovulatory ovary) was observed in untreated controls (33%, 2/6) and the GEN (50%, 3/6) and RES (50%, 3/6), ZEA (100%, 6/6), ZER (100%, 6/6), BPA (83%, 5/6) and DES groups (100%, 6/6). At 8 weeks of age, absence of corpora lutea was observed in the ZEA (33%, 2/6) group. Corpora lutea were present in all mice sacrificed at 24 weeks of age. Groups that received prepubertal xenoestrogen injections exhibited no morphological abnormalities of the uterus and vagina, and exhibited mammary gland growth similar to that of the untreated controls at all time points. *Conclusions*: GEN, ZEA, ZER and DES (but not RES or BPA) caused early vaginal opening; mice exposed to ZEA, ZER or DES spent more time in estrus phase; and ZEA-treated mice had a longer period of anovulatory ovary than other xenoestrogen-treated mice; however, none of the xenoestrogens tested altered uterine or vaginal morphology or mammary gland growth.

Introduction

Xenoestrogens (chemicals with estrogenic activity) are endocrine-disrupting chemicals that include naturally occurring substances produced by plants (phytoestrogens) and molds (mycoestrogens) and man-made chemicals released into the environment (1). They may be ingested directly in plant material, in the tissues of animals that ingest xenoestrogen-producing plants or plants infected by xenoestrogen-producing molds, or in foodstuffs contaminated by release of xenoestrogens from polycarbonate plastic plates. Exposure to xenoestrogens during critical stages of growth can interfere with the development and differentiation of estrogen target organs (2). These effects can be severe, particularly in

prepubertal children, whose endogenous estrogen concentration is low (3). There is evidence that exposure of humans to the xenoestrogen diethylstilbestrol (DES; (E)-3,4-bis (4-hydroxyphenyl)-3-hexene) alters development of estrogen target organs. *In utero* exposure to DES as an antiabortive has been found to induce clear cell adenocarcinoma of the vagina in daughters after puberty (4). Although the DES-exposed daughters in that study had low risk of development of clear cell adenocarcinoma (<1%), DES is associated with increased frequency of benign reproductive tract dysfunction and structural abnormality. Many experiments using rodent models have shown strikingly similar abnormalities after exposure to DES in early life (5); mouse models have proven to be effective for examination of abnormalities in estrogen target organs.

Naturally occurring and man-made chemicals that exhibit estrogenic biologic activity are widely distributed in the environment (1). Among the chemicals that exhibit such activity are genistein (GEN), resveratrol (RES), zearalenone (ZEA), zeranol (α-zearalanol; ZER) and bisphenol A (BPA). GEN (4', 5, 7-trihydroxy isoflavone) is a major component of soy-based foods. It is estimated that infants who consume a diet of soy-based formulas are exposed to 6 to 9 mg/kg/day of soy isoflavones, with GEN comprising more than 65% of these isoflavones by weight (6). It has been estimated that infants fed soy infant formula are exposed to GEN at a dose level of 4 mg/kg/day (7). The main sources of RES (trans-3, 4', 5-trihydroxystilbene) include grapes and red wine (8). A person who drinks one glass of red wine per day consumes ~0.02 mg/kg/day of RES (9). ZEA (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resocylic acid-lactone) is a mycotoxin synthesized by *Fusarium* mold, and is present as a natural contaminant in food as a result of infection of grain by *Fusarium* species. Human exposure to ZEA in the United States is ~0.1 mg/kg/day (10). ZER (6-6, 10-dihydroxyundecyl-β-resorcylic acid lactone) is a natural metabolic product of ZEA (11). In the United States, ZER and ZEA have been widely used to promote growth of livestock, due to their potent anabolic effects (12). BPA (4,4'-isopropylidenediphenol), an industrial chemical that exhibits estrogenic action, is a monomer used in the manufacture of many chemical products including the interior lining of food and beverage cans, dental sealants, and polycarbonate plastic products including baby bottles. Human exposure to environmental BPA is ~0.25 mg/kg/day (13). Developmental exposure to estrogenic chemicals induces morphological and functional abnormalities in estrogen target organs. Adverse effects of estrogenic chemicals on reproductive organs and mammary glands at crucial stages of development are a matter of concern. A recent study examined effects of prenatal exposure of mice to xenoestrogens at doses comparable to typical human exposure and doses 20 times greater than typical human exposure (14). However, effects of prepubertal exposure to these xenoestrogens have not been examined. There is a need to evaluate the effects of xenoestrogens at different stages of development, to clarify their effects on reproductive organs and mammary glands.

In the present study, to compare effects of early exposure to various xenoestrogens, prepubertal female mice were injected subcutaneously (sc) with 1 of several xenoestrogens once daily at a dose of 10 mg/kg/day for 4 consecutive days, beginning at 15 days of age. DES, a model xenoestrogen, was used as a positive estrogenic control, and was administered at doses approximately 1/1000 of those of the other xenoestrogens (10 μg/kg/day). The present doses of test chemicals were based on our previous findings (14). The aim of the present study was to evaluate effects of prepubertal exposure to xenoestrogens on development of the female reproductive system and mammary glands in CD-1 mice. We found that prepubertal exposure to any of several estrogenic chemicals induces early vaginal opening and disrupts the estrous cycle. However, permanent morphological alteration of reproductive organs was not observed, and mammary gland development was not affected.

Materials and Methods

Test chemicals. GEN was purchased from Fujicco (Kobe, Japan); ZER was purchased from Wako Pure Chemical (Osaka, Japan); and RES, ZEA, BPA and DES were obtained from Sigma (St. Louis, MO). The purity of all test chemicals was ≥99%. All chemicals arrived in powder form, and were kept at 0°C in the dark. Immediately before use, each chemical was dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto), and stored at 4°C.

Animals. Fourteen-day-old outbred Crj:CD-1 (ICR) female mice (10 pups per nursing mother) were purchased from Charles River Japan (Atsugi). The room in which the animals were housed was kept at 22±2°C and 60±10% humidity, with a 12 hr light/dark cycle. To avoid exposure to endocrine-disrupting chemicals, mice were housed in standard mouse polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River, Yokohama) as bedding. To avoid exposure to dietary phytoestrogens, mice were fed a low-phytoestrogen diet (NIH-07 PLD; Oriental Yeast, Chiba, Japan); NIH standard dietary pellets (NIH-07 open formula) contain phytoestrogens from soy products and alfalfa (15). Water was supplied in polycarbonate bottles with rubber stoppers. Thus, exposure to known environmental endocrine-disrupting agents was minimized.

Experimental procedures. Beginning at 15 days of age, female mice were given 4 daily sc injections of 10 mg/kg/day of GEN, RES, ZEA, ZER or BPA, 10 μg/kg/day of DES, or the DMSO vehicle alone (untreated control). Doses were adjusted daily according to body weight, to provide constant dose levels. The mice were weaned at 21 days of age. Timing of vaginal opening was recorded; vaginal smears were taken for 21 consecutive days beginning at 5, 9 and 21 weeks of age; and the estrous cycle was monitored. In each group, body weight was recorded every week. At 4, 8, 12 and 24 weeks of age, 6 randomly selected mice from each group were weighed, anesthetized, sacrificed by cervical dislocation, and autopsied. At sacrifice, the ovaries, uterus, vagina and the inguinal mammary glands from one side were fixed in 10% neutral buffered formalin. Mid-uterine transverse segments, vaginal transverse segments, the center of each ovary, and inguinal mammary glands were sectioned (thickness, 4 μm) and stained with hematoxilin and eosin (HE). Ovaries were analyzed histologically for the presence or absence of the corpus luteum and polyovular follicles (16). The inguinal mammary glands from the remaining side were processed for whole-mount preparation, and the degree of growth and differentiation was evaluated. The degree of differentiation of inguinal mammary glands was assigned a score ranging from 1 to 4, using previously reported criteria (14), as follows: Score 1, little differentiation, terminal end buds (TEBs) in the periphery with lateral buds but no alveolar development; Score 2, small number of alveoli in poorly developed ductal tree; Score 3, intermediate development of alveolar structure; Score 4, high degree of development, and lobulo-alveolar formation in the gland. Our experimental protocol was approved by the Animal Experimentation Committee, Kansai Medical University.

Statistical analysis. All data were expressed as mean±S.E. After assurance of homogeneity of variance, analysis was performed using the non-repeated measure ANOVA parametric test or Kruskal-Wallis non-parametric test. If the p value of these pre-tests was <0.05, post-hoc analysis was performed using Fisher's protected least significant difference test. Differences between groups were considered significant if the p value was <0.05.

Results

Body weight gain in female CD-1 mice. Prepubertal exposure to test chemicals did not influence body weight gain, compared with untreated controls (Fig. 1). At 24 weeks of age, body weights of all groups were comparable.

Vaginal opening. The GEN, ZER, ZEA, and DES groups exhibited accelerated timing of vaginal opening, compared with untreated controls (Fig. 2). Vaginal opening was accelerated

by 3 to 7 days in the GEN, ZER, ZEA and DES groups ($p<0.01$, respectively) (Table I). In contrast, the RES and BPA groups were similar to the untreated group.

Estrous cycle. All untreated controls exhibited a regular cycle during 5-8, 9-12 and 21-24 weeks of age (Table II). Although vaginal cycling was observed in all xenoestrogen-treated mice, the time spent in the estrus phase was significantly longer in the ZEA, ZER and DES groups than in untreated controls.

Reproductive tract structure. Ovarian histology of mice sacrificed at 4 weeks of age revealed absence of corpora lutea in untreated controls (2/6) and the GEN, RES, ZEA, ZER, BPA and DES groups (3/6, 3/6, 6/6, 6/6, 5/6 and 6/6, respectively). At 8 weeks of age, absence of corpora lutea was only observed in the ZEA group (2/6) (Fig. 3). Corpora lutea were present in all mice sacrificed at 24 weeks of age. However, polyovular follicles were not observed in xenoestrogen-treated mice or untreated controls. The test chemicals produced no morphological abnormalities in the uterus or vaginal epithelium.

Mammary gland development. In untreated control mice, growth of the mammary ductal tree progressed normally with increasing age. In untreated controls at 4 weeks of age, TEBs were observed at the periphery, but alveolar differentiation was unclear (Fig. 4a; Score 1). At 8 and 24 weeks of age, alveoli and lobuli were observed in the untreated controls, but the degree of development varied somewhat among the animals. In some animals, the mammary glands were relatively poorly differentiated, with few alveoli (Fig. 4b; Score 2); others exhibited somewhat greater alveolar development (Fig. 4c; Score 3); and some exhibited complete lobulo-alveolar development (Fig. 4d; Score 4). At 4, 8 and 24 weeks of age, none of the xenoestrogen-treated mice exhibited adverse effects on growth or differentiation of mammary glands. Scores of mammary gland differentiation after prepubertal xenoestrogen exposure are summarized in Fig. 5.

Discussion

In the present study, prepubertal exposure to xenoestrogens (phytoestrogens, mycoestrogens and industrial chemicals) produced various degrees of functional and structural alteration in the reproductive tract of female CD-1 mice; mammary gland growth and differentiation were unaffected. In our previous studies, prenatal exposure to GEN, RES, ZEA, BPA or DES accelerated body weight gain at 16 weeks of age (14). In the present study, all xenoestrogen-treated groups exhibited body weight gain that was comparable to that of the untreated controls.

Prepubertal exposure of adult rats to estrogen has previously been shown to accelerate vaginal opening and disrupt estrous cyclicity (17). In rats, prepubertal exposure to 30 mg/kg GEN, 100 mg/kg RES, 10 mg/kg ZEA or 0.1 mg/kg ZER causes earlier vaginal opening (18-21). Neonatal exposure to 0.4 mg/kg GEN or 0.04 mg/kg ZEA causes earlier vaginal opening in mice (22). In the present study, prepubertal exposure of mice to GEN, ZEA, ZER or DES caused significantly earlier vaginal opening (which is consistent with previous xenoestrogen studies), whereas RES and BPA had no such effect. Thus, RES and BPA appear to be less estrogenic than GEN, ZEA, ZER and DES.

In a previous study, exposure of female mice to DES during their first 5 days of life decreased the frequency of presence of corpora lutea at 13 months of age (23). In another study, prenatal exposure of female mice to 10 mg/kg BPA significantly reduced the frequency of presence of corpora lutea at 30 days of age, but 91% of the BPA-treated mice exhibited a normal estrous cycle at 41 to 70 days of age, and all were fertile at 90 days of age; suggesting transient delay of ovulation (24). In the present study, at 4 weeks of age, absence of corpora lutea was observed in several xenoestrogen-treated mice and untreated controls. However, at 8 weeks of age, corpora lutea were present in all groups other than the ZEA-treated mice (2/6); at 24 weeks of age, corpora lutea were present in all groups. ZER has previously been

found to have greater estrogenic potential than ZEA (25). However, in the present study, injections of ZER produced a longer duration of absence of corpora lutea (anovulatory ovary) than ZEA treatment. In a previous study, among mice exposed to ZEA prenatally, ovaries without corpora lutea were observed at 4, 8, 12 and 16 weeks of age (83%, 5/6; 100%, 6/6; 83%, 5/6; 33%, 2/6, respectively), with the frequency of absence of corpora lutea decreasing with increasing age (14). In humans, absence of corpora lutea is the most frequent cause of female infertility (26). Polyovular follicles have previously been observed in mice exposed neonatally to DES or genistein (27, 28). However, in the present study, polyovular follicles were not observed in any of the xenoestrogen-treated mice.

Neonatal administration of 40 mg/kg GEN has previously been shown to induce permanent estrus in female mouse pups (7); 4 mg/kg GEN caused no such effects. Perinatal or prepubertal exposure of rats to RES causes estrous cycle abnormalities (29), and causes an increase in the percentage of time spent in the estrus phase (19). Prenatal or neonatal administration of ZEA to rats and mice induces abnormal vaginal cyclicity by prolonging estrus (30, 31); irregular estrous cycle with prolonged estrus precedes persistent estrus (32). Prenatal and neonatal administration of estrogenic chemicals to female animals can abolish luteinizing hormone (LH) surges (33). The persistent estrus induced in animals exposed to ZEA may be the result of an inability to produce an LH surge. Exposure of mice to the estrogenic chemical coumestrol throughout their entire lactation period produces an acyclic condition in early adulthood, resembling premature anovulatory syndrome (34). In the present study, ZEA, ZER and DES increased the length of the estrous cycle by prolonging estrus phase.

Neonatal exposure of mice to DES causes squamous metaplasia of the uterine gland later in life (23). Treatment of mice with DES (1 μg/kg/day) or GEN (50 mg/kg/day) at 1 to 5 days of age has been shown to cause considerable numbers of uterine adenocarcinomas at 18 months of age (35). In the present study, morphological changes in the uterus and vagina were not observed in all xenoestrogen-treated mice; carcinogenic response was not observed in reproductive organs during the present 24-week observation period. Longer observation may be necessary to accurately assess carcinogenecity of xenoestrogens in reproductive organs.

Neonatal treatment of mice with DES decreases the frequency of presence of corpora lutea, and induces a castrate-like morphology in mammary glands (23). Prenatal treatment of mice with ZEA has been shown to induce retardation of mammary gland growth and absence of corpora lutea (14); the mammary glands consisted only of the major duct system with dilated ducts that exhibited a beaded appearance and were filled with secreted fluid. In contrast, gestational exposure to BPA or ZEA accelerates mammary gland growth in female mice with intact ovaries (14). In one study, prenatal and neonatal exposure to GEN at levels comparable to or greater than typical human exposure had no effect on mammary gland morphology in pubertal female mice (36). In the present study, prepubertal exposure to xenoestrogen produced transient anovulatory ovaries (at 4 and 8 weeks of age), but mammary gland growth later in life was not affected.

In conclusion, the present data show that prepubertal exposure to xenoestrogens at doses greater than typical human exposure induced various degrees of functional alteration and structural changes in the estrogen target organs. Further study is needed to clarify the consequences for humans of possible side-effects of xenoestrogens contained in food, and to determine the dose levels that produce harmful effects.

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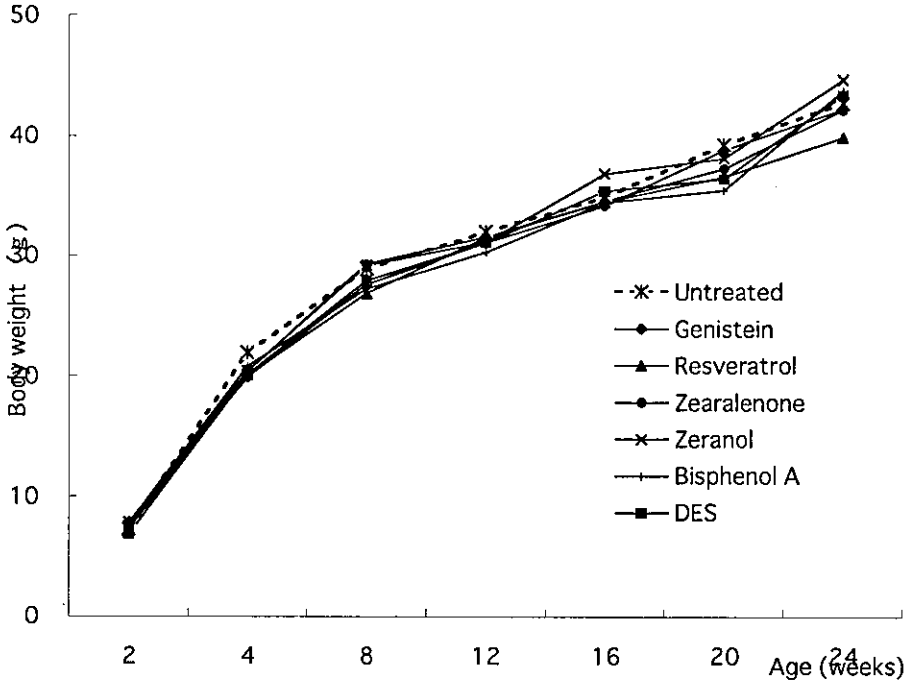

Figure 1. Body weight gain in female CD-1 mice administered 4 daily injections of xenoestrogen beginning at 15 days of age.

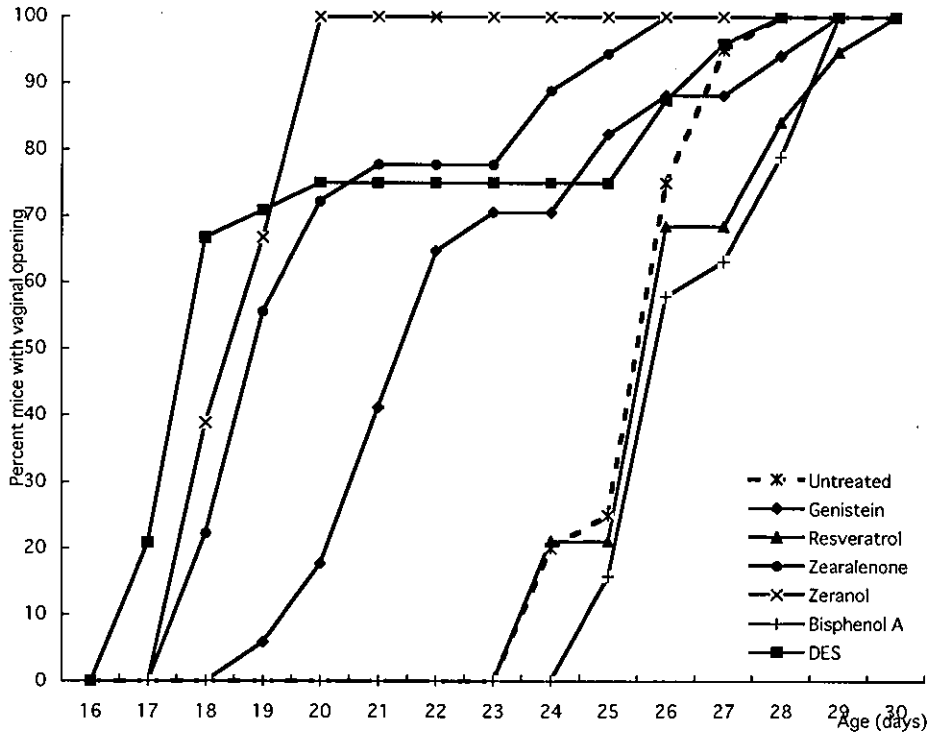

Figure 2. Vaginal opening in xenoestrogen-treated mice and untreated controls. Mice treated prepubertally with GEN, ZEA, ZER or DES exhibited earlier vaginal opening.

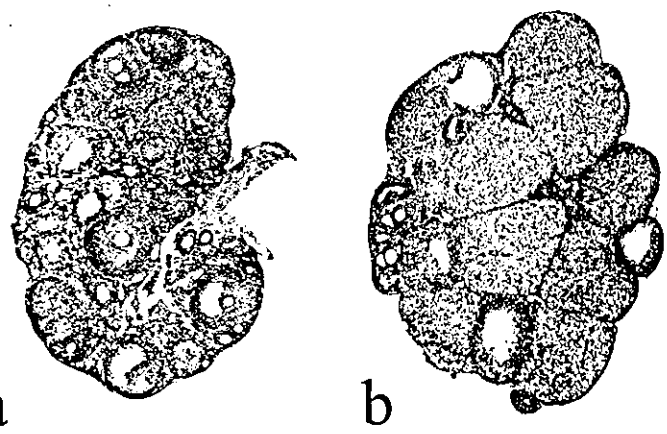

Figure 3. Ovaries from 8-week-old CD-1 mice exposed prepubertally to zearalenone. a.Mouse without corpora lutea. b. Mouse with corpora lutea.

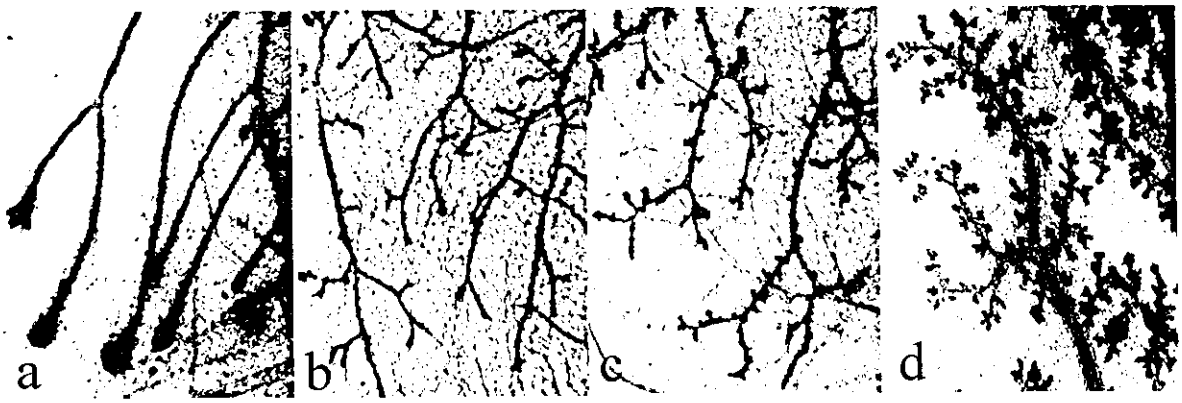

Figure 4. Mammary glands from untreated control mice. a. Note terminal end buds at the periphery and the lack of alveolar differentiation (Score 1). b. Note small number of alveoli within poorly developed duct (Score 2). c. Note more advanced alveolar development, compared with b (Score 3). d. Note prominent lobulo-alveolar development (Score 4).

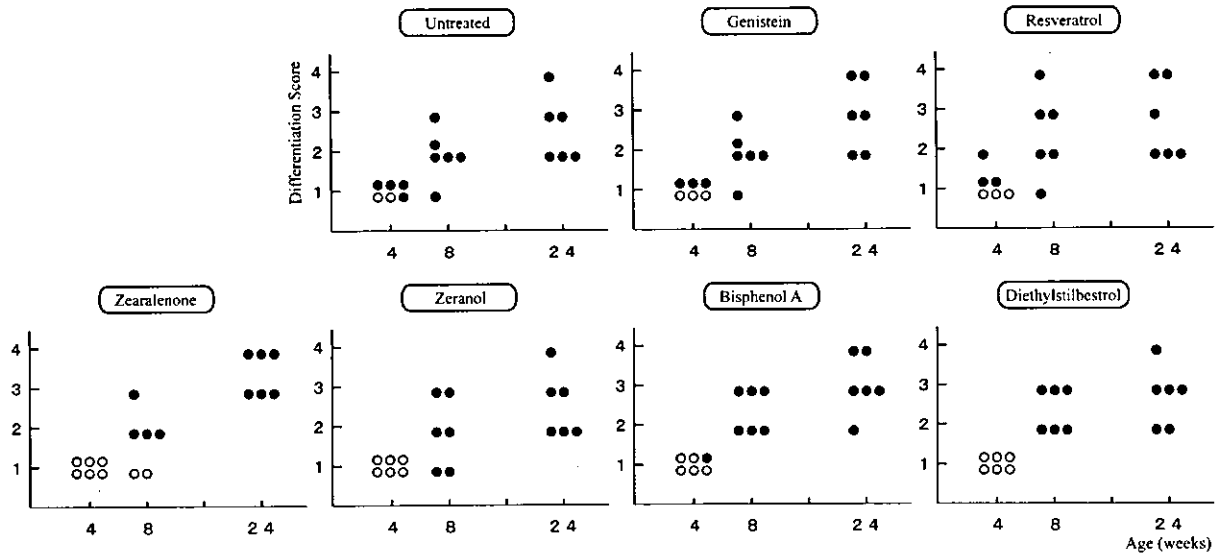

Figure 5. Degree of mammary gland development in female CD-1 mice administered 4 daily injections of xenoestrogen beginning at 15 days of age (● , mouse with corpora lutea; ○ , mouse without corpora lutea).

Table I. Mean age at vaginal opening in female CD-1 mice exposed to xenoestrogen (4 daily injections, beginning at 15 days of age)

Test chemical	Dose	Vaginal opening (days)
Untreated	-	25.9 ± 0.3
Genistein	10 mg/kg x4	22.8 ± 0.7*
Resveratrol	10 mg/kg x4	26.4 ± 0.4
Zearalenone	10 mg/kg x4	20.3 ± 0.6*
Zeranol	10 mg/kg x4	18.9 ± 0.2*
Bisphenol A	10 mg/kg x4	26.8 ± 0.3
Diethylstilbestrol	10 μg/kg x4	20.1 ± 0.8*

Values represent mean ± SE.
Each group consists of 17-24 mice.
*p<0.01, compared with untreated controls.

Table II. Estrous cycle alteration in female CD-1 mice exposed to xenoestrogen (4 daily injections, beginning at 15 days of age)

Chemical	Dose	Age 5-8		9-12		21-24 (weeks)	
		One cycle length	Day spent in estrus	One cycle length	Day spent in estrus	One cycle length	Day spent in estrus
Untreated	-	5.4 ± 0.3	1.0 ± 0.0	5.9 ± 0.4	1.0 ± 0.1	6.0 ± 0.4	1.1 ± 0.1
Genistein	10 mg/kg x4	5.2 ± 0.3	1.1 ± 0.0	6.0 ± 0.3	1.2 ± 0.1	6.3 ± 0.3	1.1 ± 0.1
Resveratrol	10 mg/kg x4	6.0 ± 0.3	1.0 ± 0.0	5.7 ± 0.2	1.0 ± 0.1	6.1 ± 0.4	1.2 ± 0.1
Zearalenone	10 mg/kg x4	4.8 ± 0.4	2.8 ± 0.4**	6.5 ± 0.4	2.9 ± 0.3**	6.7 ± 0.4	2.9 ± 0.4**
Zeranol	10 mg/kg x4	5.8 ± 0.3	3.2 ± 0.4**	6.8 ± 0.5	2.0 ± 0.2**	6.2 ± 0.5	1.2 ± 0.1
Bisphenol A	10 mg/kg x4	5.7 ± 0.3	1.1 ± 0.0	5.3 ± 0.2	1.0 ± 0.0	5.2 ± 0.2	1.0 ± 0.0
Diethylstilbestrol	10 μg/kg x4	6.1 ± 0.3	1.9 ± 0.2*	6.1 ± 0.3	2.1 ± 0.2**	6.0 ± 0.2	1.7 ± 0.2*

Values represent mean ± SE (days) of ≥6 mice.
*p<0.05, **p<0.01 compared with untreated controls.

周生期Genistein暴露による化学発癌剤誘発ラット乳癌の抑制ならびにその作用機序

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要 旨

植物エストロゲンであるGenisteinの出生前(妊娠15-19日)あるいは思春期前(15-19日齢)の少量(1.5mg/kg／日：アジア人の1日消費量に相当)あるいは大量(30mg/kg／日)5日間連日皮下投与の28日齢*N*-methyl-*N*-nitrosourea(MNU)処置Sprague-Dawley雌ラットにおける乳癌発生におよぼす影響、ならびにその作用機序につき、28日齢の正常乳腺を検索対象に加えて検討した。MNU処置ラットは≧1cm乳腺腫瘍を確認した時点あるいは26週齢で屠殺し、実験は終了した。MNU処置時(28日齢)の正常乳腺の形態像に違いは認めなかったが、Genistein暴露ラットでは有意に終末乳腺芽(TEB)におけるERα、PgR、p63、PCNA陽性細胞数の減少を見た。乳癌多発率や≧1cm乳癌採取時期に差はみなかったが、Genistein暴露により≧1cm乳癌発生率は減少傾向をみとめ、特に思春期前少量Genistein投与により、有意の減少をみた。いずれの群においても≧91％の乳癌はホルモン依存性であったことより、GenisteinによるMNU誘発乳癌の抑制機序は、発癌の標的とされるTEBにおける乳癌の前駆細胞と考えられるERα、PgR陽性数の減少、腫瘍細胞の更新にかかわるp63陽性乳腺幹細胞の減少、ならびに細胞増殖に関与するPCNA陽性細胞の減少と考えられた。

はじめに

乳癌の発生率を地理病理学的に俯瞰すると、好発国と嫌発国が存在する。これには人種・民族素因の関与もあろうが、環境因子とりわけ食餌要因の重要性が示唆されている[1),2)]。そのひとつとして、アジアの女性に乳癌が少ない理由に、多量の植物エストロゲンを含有する大豆あるいは大豆製品の大量摂取が論じられている[3)-5)]。大豆成分のなかでGenisteinは注目されており、これは大豆に含まれる主たる植物エストロゲンである。個体の発達過程をみると、未だ体内エストロゲンが低値な周生期では、外来性エストロゲン様化学物質に最も高感受性である。早期満期産や出生早期のエストロゲン様化学物質の暴露は乳癌を抑制する[6)]。一方、母体が妊娠中大量のエストロゲンに暴露されると、生まれてきた女児の乳癌の危険性が増す[7,8)]。妊娠期あるいは出生早期のエストロゲンによる乳腺の反応性は複雑である。

実験病理学的にGenisteinを新生仔期あるいは思春期前のラットに与えると乳癌が抑制される[9)-11)]。一方、Genisteinを妊娠ラットに投与すると出生雌乳仔の乳癌を促進する[12)]。これらは化学発癌剤の単回投与によるラット乳癌誘発モデルを用いた検討結果であるが、いずれも常法により、発癌剤によるイニシエーションは50日齢に行っている。発癌剤刺激による乳腺上皮の形質転換は、乳腺の分化と密に関連しており、終末乳腺芽(TEB)が発癌の標的とされている[13)]。しかし、新生仔期あるいは思春期前のGenistein

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投与による乳癌抑制機序として、Genisteinによる乳腺分化の促進による50日齢時におけるTEBの減少がその理由と結論づけられている。逆に胎仔期Genistein暴露による乳癌促進機序として、発癌剤暴露時の有意なTEBの存続がその理由とされている[14]。ヒトにおける発癌のイニシエーション時期は不明であり、乳癌のGenisteinによる抑制あるいは促進を実験的に評価する場合、発癌剤処置は乳腺の分化が均一な時期に行う必要がある。さらに、発癌剤投与時のラットの発情周期も乳癌誘発に影響をおよぼすとされている[15),16)]。代謝を要しない直接発癌剤である*N*-methyl-*N*-nitrosourea（MNU）は、未成熟ラットに投与しても乳腺発癌を惹起する[17)]。よって今回、周生期Genistein暴露ラットに対し、思春期発来前、未だGenisteinによる形態変化が惹起されていない時期にMNUを処置し、乳腺発癌に対する影響を検討した。

材料と方法

動物：妊娠14日齢のSprague-Dawleyラットを日本チャールス・リバー（日野）より購入した。内分泌かく乱物質を極力排除すべく、植物エストロゲンを除いたNIH-07 PLD食（オリエンタル酵母、千葉）[18)]を給餌し、22±2℃、湿度60±10％、12時間照明の環境下で飼育した。

化学物質：Genistein（純度＞99％）はフジッコ（神戸）より購入し、0℃に保存し、使用直前にDMSO（ナカライテスク、京都）に溶解し、4℃に貯蔵した。MNU（ナカライテスク）は暗所、-20℃に保存し、使用直前に0.05％酢酸加生理食塩水に溶解した。

実験手技：妊娠15日から19日にかけて連日被験物質を母体の皮下に投与した。1群：DMSO（無処置対照群）、2群：1.5mg/kg／日Genistein、3群：30mg/kg／日Genisteinの3群を作製し、各群出生した雌乳仔を実験に供した（1群：26匹、2群：30匹、3群：30匹）。また、妊娠中Genistein無処置ラットより生まれた雌乳仔に対し、15日齢から19日齢にかけて連日Genisteinを皮下投与した、4群：1.5mg/kg／日Genistein（30匹）と5群：30mg/kg／日Genistein（30匹）を作製した。なお、1.5mg/kg Genisteinは、ほぼアジア人の1日消費量に相当する[19)]。各群のラットは21日齢で離乳し、膣開口を連日観察し、未だ膣開口をみない28日齢で各群任意に6匹を屠殺し、乳腺のホールマウント標本と組織標本を作製し、Genistein投与による乳腺発育に対する影響を比較した。残余のラットに対しては同日（28日齢時）に、50mg/kg MNUを単回腹腔内投与し、乳腺発癌を促した。26週齢で実験を終了したが、途中最大乳腺腫瘍径が1.0cmに達したラットは順次屠殺した。

乳腺発癌：全例剖検を行ったが、肉眼的に認めた乳腺腫瘍とともに、すべての正常乳腺も10％中性緩衝ホルマリン固定・パラフィン包埋し、HE標本を作製し、組織的に認めえた微小乳腺腫瘍を同定した。乳腺発癌の指標として、腫瘍発生率（≧1.0cm乳腺腫瘍を有するラット／有効ラット匹数）、腫瘍多発率（組織的に確認しえた全乳腺腫瘍／ラット）、潜伏期（MNU投与から≧1.0cm乳腺腫瘍の出現までの期間）を算出した。なお、乳腺腫瘍の組織診断はRussoらの分類[20)]に則った。

免疫組織化学：各群28日齢の左側鼠蹊乳腺と、≧1.0cm乳腺腫瘍を有するラットの最大乳腺腫瘍につき、エストロゲン受容体（ERα：6F11、ノボカストラ）、プロゲステロン受容体（PgR：PR 10A9、インムノテック）、p63（4A4、ネオマーカー）、PCNA（PC10、ノボカストラ）の発現を、LSAB染色キット（ダコ）を用いて、マイクロウェーブによる抗原賦活を行い、免疫組織化学的に同定し、各の陽性率を算出した。なお正常乳腺については発癌の標的とされるTEBでの陽性率を計算した。また、乳癌のホルモン依存性の基準として、乳癌の80％以上の細胞がERαあるいはPgR陽性細胞より構成されているものをホルモン依存性とした[21)]。

統計処理：すべてのデータは平均±SEで表現した。腫瘍発生率はMantel-Cox Logrank試験、ホルモン依存性はχ^2検定で行い、他の事項については、まず正規性の検定を行い、ANOVAあるいはKruskal-Wallis検定にて<0.05であれば、FisherのPLSDあるいはBonferroni/Dunn

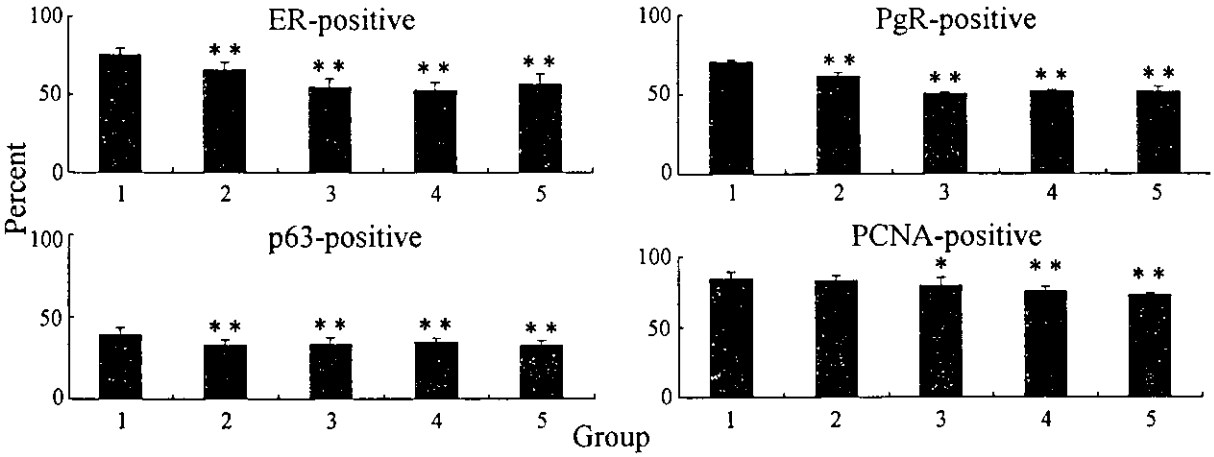

図1　出生前あるいは思春期前Genistein暴露による28日齢終末乳腺芽（TEB）におけるERα、PgR、p63、PCNA陽性率の比較（1. 無処置対照；2. 出生前1.5mg/kg Genistein；3. 出生前30mg/kg Genistein；4. 思春期前1.5mg/kg Genistein；5. 思春期前30mg/kg Genistein）1.と比較して*p＜0.05、**p＜0.01

の試験にかけ、p＜0.05を有意と判定した。

結　果

28日齢における乳腺の発育ならびに機能分化：ホールマウントならびに組織標本における定性的評価では、いずれの群も均等な数のTEBを乳腺の辺縁部にみとめ、出生前あるいは思春期前Genistein投与による形態学的変化は認めなかった。なお、この時期全例膣開口は認めていない。一方、Genistein投与は、TEBにおけるERα、PgR、p63とPCNA陽性率を有意に減少させた（図1）。よって、乳腺の発育は、形態的には差は明かではなかったが、発癌の標的とされるTEBの細胞形質に変化を惹起した。

乳腺発癌：発癌実験の経過中、3群と5群の各1匹は体重減少により死亡したため、統計からは除外した。また、乳腺腫瘍の大半は組織的に乳癌と診断され、少数の乳腺々腫（1群：1個、3群：1個、5群：2個）をみたが、群間の比較は乳癌に限定して行った（図2）。いずれのGenistein投与群においても、乳癌発生率（≧1.0cm乳癌を有するラットの頻度）を減少させる傾向がみられ、特に思春期前の少量（4群：1.5mg/kg Genistein）投与群では、Genistein非投与群（1群）に比して有意の減少をみた。Genistein投与により、乳癌多発率も2、4、

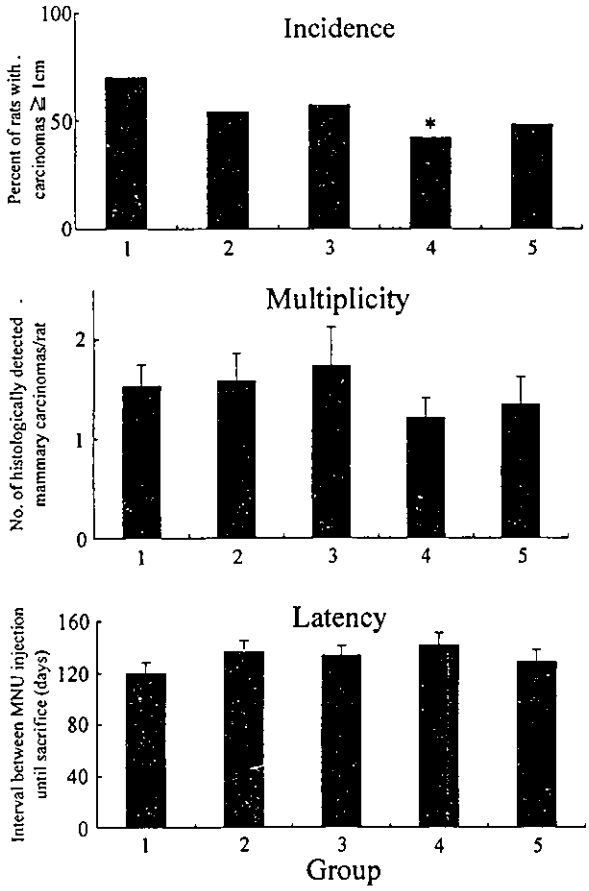

図2　MNU誘発乳癌発生におよぼすGenisteinの影響（1. 無処置対照；2. 出生前1.5mg/kg Genistein；3. 出生前30mg/kg Genistein；4. 思春期前1.5mg/kg Genistein；5. 思春期前30mg/kg Genistein）1.と比較して*p＜0.05

5群では減少傾向にあり、潜伏期も若干延長する傾向がみられたが有意には至らなかった。なお、いずれの群でも≧91％の誘発乳癌はホルモン依存性であった（図3）。

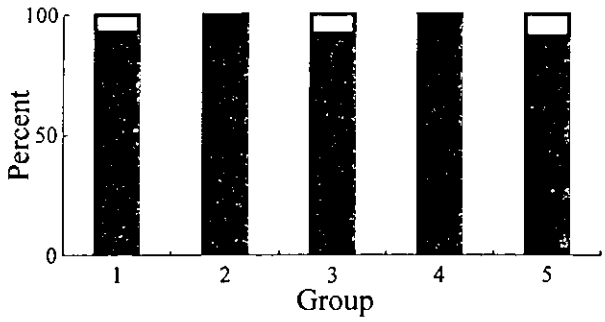

図3 MNU誘発乳癌のホルモン依存性。■、ホルモン依存性乳癌；□、ホルモン非依存性乳癌（1. 無処置対照；2. 出生前1.5mg/kg Genistein；3. 出生前30mg/kg Genistein；4. 思春期前1.5mg/kg Genistein；5. 思春期前30 mg/kg Genistein）

考 察

発癌剤を処置した28日齢では全例膣開口は未だみられず、乳腺はGenistein投与により形態変化をみとめなかった。よって形態的に近似した分化を呈する乳腺が、思春期発来前に同一の発癌刺激に暴露されたことになる。誘発乳癌は、出生前あるいは思春期前に大量あるいは少量のGenistein投与により、非投与群に比して抑制される傾向にあり、特に思春期前の少量（生理的容量）のGenistein投与は有意に≧1.0cm乳癌の発生率を抑制した。ラットの出生早期におけるGenistein暴露による乳癌抑制は乳腺の分化の促進によるTEB数の減少[22]、逆に胎仔期のGenistein暴露による乳癌促進は発癌剤暴露時の有意なTEBの存続がその理由とされている[14]。しかし、今回の出生前・思春期前Genistein暴露による乳癌抑制機序は乳腺の分化（TEBの多寡）の観点からは説明不能である。

早期の満期妊娠は乳癌を抑制し[6]、この現象はラットにも存在する[21]。その機序として妊娠により正常乳腺におけるERα、PgR陽性細胞数が減少し、細胞増殖能（PCNA陽性細胞）が減少することが示されている[21]。今回の結果では、出生前・思春期前のGenistein投与により、発癌剤投与時の乳腺の発育形態に違いは認めなかったが、発癌の標的とされるTEBにおいて、ERα、PgR陽性細胞数が減少し、PCNA標識率の低下を認めた。加うるに、TEBにおいて乳腺の幹細胞を標識するとされるp63陽性細胞[23]の減少もみた。大半の誘発乳癌がホルモン依存性であることを勘案すると、Genisteinの作用機構は、ホルモン依存性乳癌の前駆細胞であるERα、PgR陽性細胞の減少、腫瘍細胞の更新にかかわる幹細胞の減少、さらに細胞増殖率の低下と考えられる。アジア人女性は成人して欧米に移住しても乳癌の発生は低率にとどまる[24]。これは、女性の出生早期におけるGenisteinの乳癌抑制に対する有用性を示唆している。今回の検討ではアジア人のGenisteinの1日消費量に相応する量（1.5mg/kg/日）あるいはその20倍量で検討を加えたが、特に思春期前期の短期（5日）間の生理的容量の暴露で、有意の乳癌抑制をみた。また、胎仔期の暴露においても乳癌抑制傾向をみとめ、乳癌の増悪をみないことは、周生期Genistein暴露は、たとえ生理的容量の20倍量でも安全と考えられた。我々の結果から、ヒト新生児での豆乳摂取は推奨されよう。

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