In addition, expression homogeneous in proliferative lesions. In clear contrast, no antibody binding is detected in any of the poorly-differentiated adenocarcinomas. Thus, the ERα expression in normal endometrial epithelium in the aged uteri, uterine atypical hyperplasias and well-differentiated adenocarcinomas suggests that up-regulation is not necessary for stimulation by estrogens. The loss of ERα in poorly-differentiated adenocarcinomas is linked with estrogen-independent growth of implanted tumors, as has been established for human endometrial adenocarcinomas, with no effects of hormone therapy on advanced malignancies [12, 13].

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 Makeawa and his co-workers to detect promoting or preventive effects of test-chemicals [14]. As the first step of this model (Figure 2), female Donryu rats at 10 or 11 weeks of age are treated with *N-ethyl-N'-nitro-N-nistroguanidine* (ENNG) at the concentration of 20mg/kg dissolved in polyethylene glycol, 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. At 15 months of age, the animals are sacrificed to determine incidences or multiplicities of uterine neoplastic lesions for comparison with control (ENNG initiation only) data. The intrauterine treatment with a single dose of ENNG results in earlier development and higher yields of endometrial adenocarcinomas, as well as precursor lesions, compared with intact Donryu females not receiving carcinogen (Table 4) [14]. The tumorigenesis is specific to the uterus and no other organs are affected and this 2-stage uterine carcinogenesis model has proven to be useful for detection of promotive or preventive effects of test materials [2, 15-17]. In an attempt to enhance uterine carcinogenesis in this model, ENNG at 10mg/kg body weight

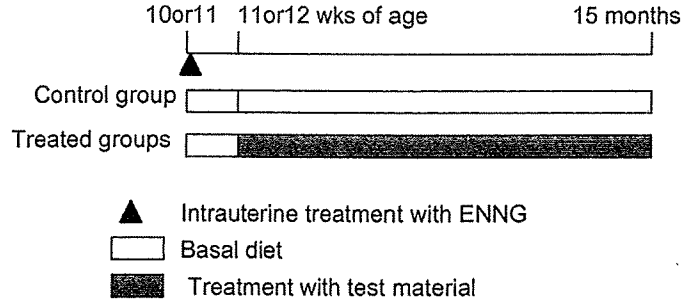

Figure 2. Protocol for the 2-stage uterine carcinogenesis model using Donryu rat.

was administered to Donryu rats once a week 4 times. The repeated treatment with ENNG succeeded in increasing the incidence of endometrial adenocarcinomas, but concurrently induced serious bleeding into the lumina of the uteri or abdominal cavity from hematomas or angiosarcomas (Table 5). In addition, associated exfoliation of endometrial epithelium resulted in difficulty of detailed histopathological analysis.

Table 4. Sequential changes in the uterine endometrium of Donryu rats after ENNG treatment [Modified ref. 14].

	3	6	9	12	15(months of age)
Control group					
Number of rats examined	4	4	4	8	30
Hyperplasia					
Slight	0	0	0	1	7
Moderate	0	0	0	0	6
Severe	0	0	0	1	0
Adenocarcinoma	0	0	0	0	0
ENNG-treated group					
Number of rats examined	6	6	6	8	49
Hyperplasia					
Slight	0	1	2	1	7
Moderate	0	0	1	1	7
Severe	0	0	1	2	6
Adenocarcinoma	0	0	1	4	24

Table 5. Comparison of data for uterine adenocarcinomas and hematomas, and cause of death, with repeated ENNG treatment.

	Repeated ENNG treatment
Number of rats examined	29
Incidence of uterine lesions	
Uterine hematoma	9
Endometrial adenocarcinoma	16
Angiosarcoma	5
Cause of death	
Uterine hematoma	8
Endometrial adenocarcinoma	10
Uterine stromal sarcoma	3
Uterine angiosarcoma	4
Uterine choriocarcinoma	1
Mammary tumor	1
Leiomyosarcoma in small intestine	1
Pneumonia	1

Relatively high E2 status driven uterine carcinogenesis

The simplest pathway for uterine carcinogenesis in the Donryu rat model is relatively high estrogen status (elevated E2:P ratio)-mediated (Figure 3). The early occurrence of ovarian hormonal imbalance leading to elevation of serum E2 levels relative to P as compared to other rat strains, a characteristic of Donryu rats, was described above. This imbalance is morphologically reflected in atrophic ovaries with small polycystic atretic follicles and lack of corpora lutea showing vaginal cornification or a PE status of vaginal cytology. In contrast, other rat strains such as Sprague-Dawley or Fisher 344 rats demonstrate corpus luteum predominant ovaries with increasing age, with vaginal mucification and only low incidences of endometrial adenocarcinomas [5]. This ovarian hormonal imbalance and the associated changes in ovarian morphology with cystic follicles and lack of corpus lutea are crucial for rat uterine carcinogenesis.

One example of promoting effects is provided by concurrent oral administration of ethylenethiourea (ETU) and sodium nitrate, which was found to cause an early occurrence of PE (Table 6) and enhance endometrial adenocarcinoma development at the termination (Table 7) [16]. In this study, the rats were initiated by *N-ethyl-N-nitrosourea* (ENU) and its profile of uterine endometrial adenocarcinoma development was very similar to that initiated with ENNG [16]. As an example of inhibitory effects, long-term dietary treatment with hydroxymatairesinol (HMR), a lignan derived from spruce trees, delayed the occurrence of PE (Table 8) and also exerted an inhibitory effect on uterine carcinogenesis in this rat model (Table 9) [15].

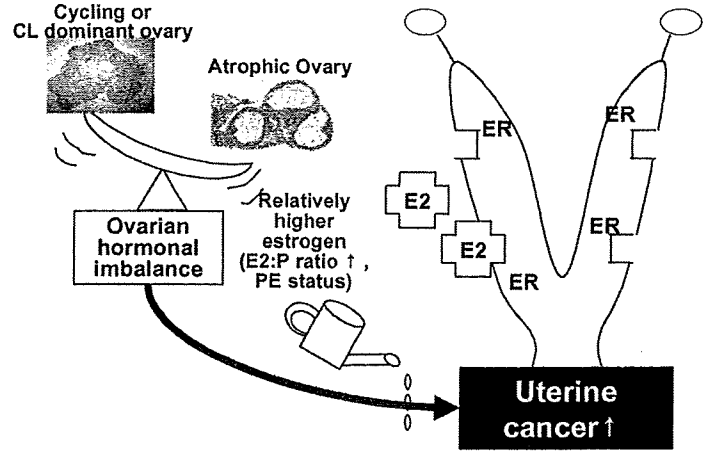

Figure 3. Relatively high E2 driven pathway of uterine carcinogenesis using the rat model. CL, Corpus luteum; PE, persistent estrus; E2, 17β-estradiol; P, progesterone; ER, estrogen receptor.

Table 6. Sequential changes in the incidence of persistent estrus [Modified ref.16].

	Incidence (%) of persistent estrus				
Group	3	4	5	6	7 (months of age)
Control	0	0	50.0	87.5	87.5
ETU+sodium nitrate	0	44.4	66.6	77.8.	87.5
ETU+sodium nitrate+ENU	0	80.0	80.0	80.0	80.0

Control, Oral administration of distilled water once a week by stomach tube without ETU+sodium nitrate, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube.
ETU+sodium nitrate+ENU, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube after ENU initiation (15mg/kg) into the uteri instead of ENNG treatment.
Estrous cycles were checked monthly in 8-10 rats in each group by vaginal smear.

Table 7. Uterine proliferative lesions at the termination [Modified ref.16].

Group	Control	ENU	ETU+ Sodium nitrate	ENU+ETU Sodium nitrate
No. of rats examined	21	21	31	37
Endometrial hyperplasia				
Slight	4	1	12	2
Moderate	1	10*	6	9
Severe	1	1	7	5
Endometrial adenocarcinoma	0	6*	4	21**

Control, Oral administration of distilled water once a week by stomach tube.
ENU, intrauterine treatment with ENU (15mg/kg) instead of ENNG
ETU+sodium nitrate, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube.
ETU+sodium nitrate+ENU, Concurrent oral administration of ETU(80mg/kg) and sodium nitrate (56mg/kg) in distilled water once a week by stomach tube after ENU initiation (15mg/kg) into the uteri instead of ENNG treatment.
*;**, Significantly different from the control group ($p<0.05$ and $p<0.01$, respectively)

Table 8. Delay of persistent estrus by hydroxymatairesinol(HMR) dosing [15].

Group	Mean week of age of persistent estrus start
Control-conventional diet (CRF-1)	30.3±1.2
Control-basal diet (1324 diet alone)	32.1±1.3
200 ppm HMR in basal diet	35.4±1.6*
600 ppm HMR in basal diet	35.3±1.6*

Values are mean±SEM, for n=25, 27, 27, and 26 rats in the groups, respectively. Means of 200 and 600 ppm HMR in basal diet groups are significantly different (*$p<0.05$) from both control values.

Table 9. Numbers and incidences of uterine endometrial proliferative lesions at 15 months of age in the four experimental groups [Modified ref. 15].

	No. of rats examined	Atypical hyperplasia None	Slight	Moderate	Severe	Adenocarcinoma
Control-conventional diet (CRF-1)	25	0	2	8	6	9
Control-basal diet (1324 diet alone)	27	2	2	8	7	8
200 ppm HMR in basal diet	27	3	5	9	7	3*
600 ppm HMR in basal diet	26	1	4	12	5	4*

Significantly different from both control values (*p<0.05).

None, animal bearing no atypical hyperplasia and/or adenocarcinoma

Although precise mechanisms of the promotive or inhibitory effects of these compounds on the ovarian function have yet to be fully determined, these results clearly demonstrate that treatment-related ovarian changes play essential roles. In human beings, anovulatory women with the polycystic ovary syndrome are defined as a high risk group for endometrial cancer [18]. Any factors that might disturb the ovarian hormone balance would be expected to modulate risk of uterine tumorigenesis in women as well as experimental animals.

Long term exporsure to estrogens or estrogenic compound driven uterine carcinogenesis

A second pathway of uterine carcinogenesis is with long-term with estrogen or estrogenic compound treatment (Figure 4). Enhancement of uterine carcinogenesis by estrogenic compounds involves binding to ERα and consequent shift of E2 dependent tissues into a proliferation mode. Long term treatment with effective doses of E2 is well known to promote endometrial uterine carcinogenesis in rats [4, 7], whereas extremely high dose E2-exposure does not induce uterine cancers, but rather development of squamous metaplasia in the luminal and/or glandular epithelium in the uteri or serious pyometra in rats, suggesting over dose treatment with estrognes can not induce any uterine cancer in rats.

Katsuda et al. [2] reported that long-term subcutaneous injection of high dose *p-tert* octylphenol (100mg/kg/day), a weak environmental xenoestrogen, significantly promotes endometrial adenocarcinoma development in our rat model (ovary intact females) whereas no adenocarcinomas developed in ovarictomized (OVX) rats exposed to *p-tert* octylphenol as the same dose and manner (Table 10). The dose level examined is known to be effective for estrogenic activity in rats [19, 20]. In addition, the *p-tert* octhylphenol treatment disturbed the vaginal cytology with large amounts of epithelial cells

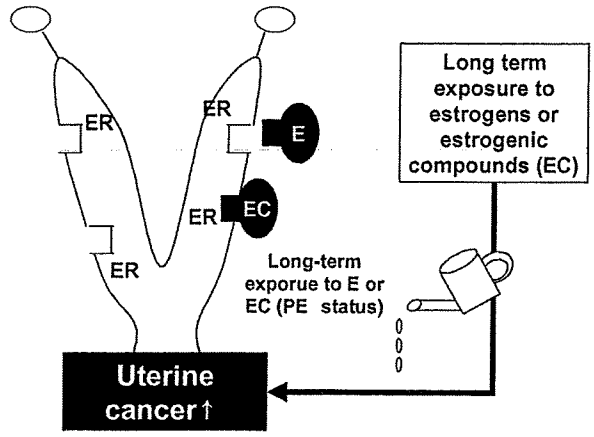

Figure 4. Long term exposure to estrogens or estrogenic compound driven pathway of uterine carcinogenesis using the rat model. E, excess estrogens; EC, estrogenic compounds; PE, persistent estrus; ER, estrogen receptor.

Table 10. Incidence of uterine proliferative lesions [Modified ref. 2].

Age (months)	Group	n		Hyperplasia			Adenocarcinoma
			None	Slight	Moderate	Severe	
9	Control	6	1	5	0	0	0
	OP-treated	6	2	4	0	0	0
	Control(OVX)	3	3	0	0	0	0
	OP-treated(OVX)	6	6	0	0	0	0
12	Control	6	1	2	3	0	0
	OP-treated	8	0	4	1	0	3
	Control(OVX)	3	3	0	0	0	0
	OP-treated(OVX)	7	6	1	0	0	0
15	Control	23	2	2	8	7	4
	OP-treated	26	0	1	8	5	12*
	Control(OVX)	15	15	0	0	0	0
	OP-treated(OVX)	29	25	3	1	0	0

*, Significantly different from the control value. ($p<0.05$)
OP, *p-tert* octylphenol
None, Animal bearing no atypical hyperplasia and/or adenocarcinoma
OVX, ovariectomized

at metestrus and/or diestrus stages in ovary-intact rats and uterotropic effects in OVX rats. On the other hand, dietary treatment with *p-tert* octylphenol at concentrations of 10, 100 and 1000ppm, which are neither uterotrophic nor steroid hormone modulating, did not exhibit any promotive effects on uterine carcinogenesis in the rat model. Therefore, estrogens and/or estrogenic compounds that impact directly upon the uterus by binding to ER can promote uterine carcinogenesis.

Estrogen metabolite or catechol estrogen driven uterine carcinogenesis

Recently, a hypothesis has been presented that high tissue levels of E2 could promote carcinogenesis via two mechanisms; stimulating proliferation as described above and by producing DNA damage [1]. 4-Hydroxyestradiol (4HE), a hydroxylation metabolite of E2, has been reported to be a stronger carcinogen then the parent E2 due to production of DNA damage [1, 21-23]. Of the cytochrome P450 enzymes related to estrogen metabolism, CYP1B1 which is widely distributed in many human and mammalian tissue has been nominated as important for transformation of E2 into 4-hydroxyestradiol (4HE) [24, 25]. Concerning E2 metabolites, 2-hydroxyl estradiol (2HE), which is a major metabolite by 2-hydroxylation route in the liver, results in little estrogenic activity, and therefore limited carcinogenicity effects in estrogen dependent target organs [26]. On the other hand, 4HE, which is a minor product of estrogen metabolism in the liver, has proven to enhance tumorigenesis via production of DNA adducts, despite this catechol estrogen having weak estrogenic activity compared to parent E2. There is also experimental evidence that 4HE promotes tumor development in the hamster kidney and mouse uteri [27, 28]. Furthermore, in the Donryu rat model long term subcutaneous treatment with 4HE promote uterine carcinogenesis in terms of both incidence and multiplicity (Table 11)[29].

Recently, a number of 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 [30, 31]. Since these products exert no direct estrogenic activity on target organs, their induction of CYPs and consequent modulation of estrogen metabolism indirectly impacts on estrogen dependent organ carcinogenesis. Indole-3-carbinol (I3C), a cruciferous vegetable, is reported to induce the CYP1 family in the liver [31, 33] and has been shown to suppress or promote carcinogenesis depending on the animal model [33-35]. Regarding its preventive effects, I3C acts as an anti-estrogen and can induce apoptosis [36-38], but precise mechanisms remain to be determined. Nevertheless, I3C does not exert any estrogenic or anti-estrogenic activity to the rat uteri using uterotropic assays in OVX rats or any disturbance of the ovarian hormone balance [29]. When I3C was administered to Donryu rats at dietary concentrations of 500 or 2000ppm for 12 months, incidences of uterine adenocarcinomas and/or multiplicities of uterine neoplastic lesions were increased (Table 11). In addition, I3C treatment caused consistent elevation of estradiol 2- and more especially 4-hydroxylase activities in the liver, but no effects on estradiol 16α-hydroxylase activity (Figure 5). Expression of mRNAs for CYP1A1, 1A2 and 1B1 was increased in the liver by the treatment (Figure 6), with

translation confirmed immunohistochemically. These studies provide new evidence that modulation of estrogen metabolism to increase 4HE through induction of CYP 1family members is a new pathway to promote uterine carcinogenesis in rats (Figure 7).

Table 11. Incidences of uterine proliferative lesions and their multiplicities at 15 months of age [Modified ref. 29].

	None	Atypical hyperplasia			Adenocarcinoma	Multiplicity(a)
		Slight	Moderate	Severe		
Control (n=24)	4	2	5	7	6	1.04±0.62
I3C 500ppm (n=30)	1	2	3	7	17*	1.50± 0.63*
Control (n=18)	2	2	7	3	4	1.17±0.62
I3C 2000ppm (n=18)	1	2	5	2	8	1.78±0.73**
E2 1μg/kg (n=16)	0	3	2	3	8	1.50±0.52
4HE 5μg/kg (n=16)	0	0	5	1	10*	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

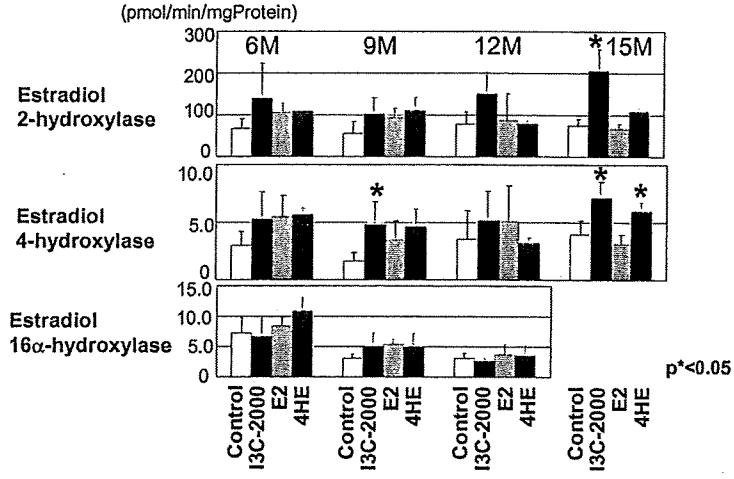

Figure 5. Sequential changes of enzyme activity related to estrogen metabolism in the liver. M, months of age; Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000ppm in basal diet; E2, subcutaneous injection of 17β-estradiol at 1μg/kg; 4HE, subcutaneous injection of 4-hydroxyestradiol at 5μg/kg.

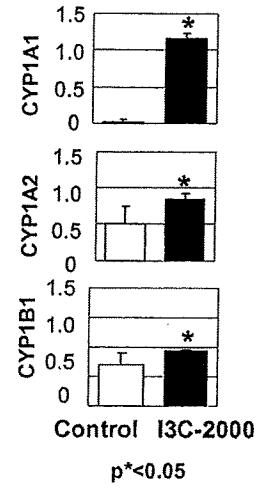

Figure 6. Levels of expression of cytochrome P450s 1A1, 1A2 and 1B1 mRNA relative to GAPDH mRNA in the liver (calculated as %). Control, control group given basal diet only; I3C-2000, indole-3-carbinol at 2000ppm in basal diet. [Modified ref. 29]

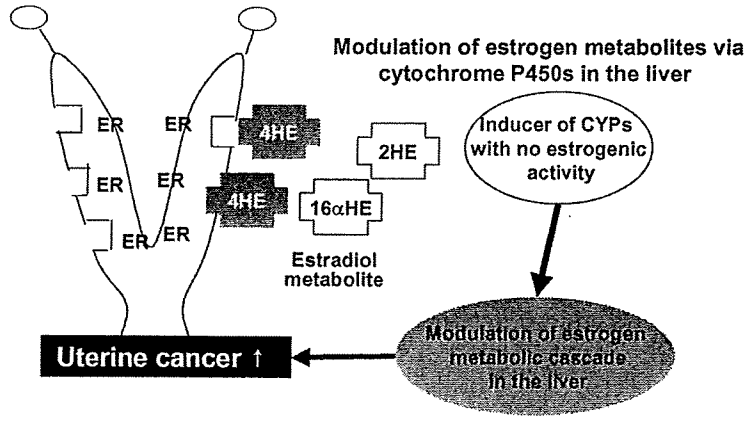

Figure 7. Modulation of estrogen metabolite or catechol estrogen driven pathway of uterine carcinogenesis using rat model. CYP, cytochrome P450s; ER, estrogen receptor; 2HE, 2 hydroxyestradiol; 4HE, 4 hydroxyestradiol; 16α-E, 16α-hydroxyestradiol. [Modified ref. 29]

Molecular evidence for rat uterine carcinogenesis

In women, most molecular studies of endometrial carcinoma have been performed on small series of cases from single institutions [1]. In rodents, there have only been a few molecular based attempts to elucidate endometrial adenocarcinoma development. Nonetheless, the studies have produced several findings common with human cases as described below.

K-ras mutation

Mutations in the *ras* oncogene have been identified in approximately 20% of endometrioid carcinomas but not in serous tumors in women [39-43]. In rodents, *K-ras* point mutations have also been detected in endometrial hyperplasia and adenocarcinomas but not in normal endometrium in Donryu rats [43], suggesting that alteration in the *K-ras* gene may be an important initiating event in this strain of rats, comparable to the endometrioid carcinoma in the human case.

P53 mutation

Mutations in the p53 tumor suppressor gene and accumulation of p53 protein have been detected in approximately 90% of serous carcinomas [45, 46], whereas they are comparatively rare in endometrioid carcinomas and AH [47]. In Donryu rats, such changes are only features of poorly-differentiated adenocarcinomas [11], although mutations have been detected in some well-differentiated endometrial adenocarcinomas in F344 rats [48]. These results suggest that p53 mutation might be late stage event of uterine carcinogenesis or dedifferentiation in Donryu rats, but not in F344 rats, suggesting the profile in former rat strain to be similar to the endometrioid type and the latter to the serous one.

PTEN (phophatase and tensin homolog) mutation

PTEN is a tumor suppressor gene and its mutation as well as loss of PTEN protein are suggested to be early events in uterine carcinogenesis in women [49]. However, in Donryu rats, immunohistochemical results and/or mRNA expression profiling of uterine proliferative lesions which were laser-microdissected from ethanol-fixed, paraffin-embedded tissues did not show any decrease or loss of PTEN [11], suggesting no relation with endometrial adenocarcinoma development in this rat.

β-Catenin mutation

Nuclear accumulation of β-catenin in atypical hyperplasias and carcinomas is more frequent than in simple/complex hyperplasias of the endometrium in women [49] but in rats no nuclear accumulation of β-catenin could be detected in any uterine proliferative lesions immunohistochemically [11].

To conclude, findings for expression and mutation of various genes during endometrial adenocarcinoma development are summarized in Figure 8. Unfortunately, useful early endpoint markers based on molecular biology have yet to be established for uterine cancer, and this needs to be a focus of future research.

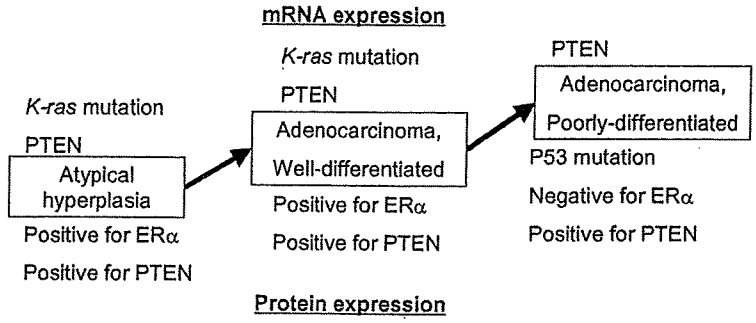

Figure 8. Gene and protein expression profiles for different stages of uterine carcinogenesis in the Donryu rat. ERα, estrogen receptor α.

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雑　誌

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INFLUENCE OF DI-(2-ETHYLHEXYL)PHTHALATE ON FETAL TESTICULAR DEVELOPMENT BY ORAL ADMINISTRATION TO PREGNANT RATS

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ABSTRACT — Influence of di-(2-ethylhexyl)phthalate (DEHP) on testicular development was studied by oral administration of DEHP at doses of 500 and 1000 mg/kg/day to pregnant rats on gestational days (G) 7 to 18. Ethinyl estradiol (EE) at dose levels of 0.25 and 0.5 mg/kg/day was used as a reference substance. Each 5-6 pregnant rats were sacrificed and their fetuses were examined on G12, 14, 16, 18 and 20. Fetal deaths averaging 20-36% were observed at every examination in the group receiving 1000 mg/kg of DEHP. Increases of fetal deaths over 50% were also observed in the reference group that received 0.5 mg/kg of EE. Microscopic examination of the fetal testis in groups treated with DEHP revealed degeneration of germ cells in G16 fetuses and localized proliferation or hyperplasia of interstitial cells in G18 and 20 fetuses. Germ cells having more than two nuclei were observed in a few cases including the control testes of G14 fetuses. These multinucleated cells were observed frequently in G20 fetuses treated with DEHP. Examination of testes of naturally delivered offspring of dams treated with 1000 mg/kg of DEHP at 7 weeks of age revealed scattered atrophy or dilatation of seminiferous tubules.

Another experiment was carried out to confirm the dose of DEHP affecting testicular development and spermatogenesis. DEHP was given to pregnant rats at doses of 125, 250 and 500 mg/kg/day during G7-18. Similar histopathological changes were observed in fetal testes of the group exposed to 500 and 250 mg/kg of DEHP, but not in those exposed to 125 mg/kg. In postnatal examinations, however, no abnormality was found in the testes at 5 and 10 weeks after birth in any of the treated groups. Furthermore, no abnormal findings were observed in the function of sperm, sperm counts and sperm morphology in the offspring of the group treated with DEHP during the fetal period at 10 weeks of age. Thus, 125 mg/kg/day is considered the no-observed-effect-level of DEHP on testicular development of rats by exposure *in utero* during the period of organogenesis.

KEY WORDS: Phthalic acid ester, Developmental toxicity, Testicular toxicity, Sertoli cells, Sperm function, Rats

INTRODUCTION

It has been shown that high doses of phthalic acid esters exert testicular toxicity in animals (Calley *et al.*, 1966; Gangolli, 1982). Toxic effects on the testis were similarly observed with a variety of phthalate esters such as di-(2-ethylhexyl) phthalate (DEHP) (Gray *et al.*, 1977), dibutylphthalate, (Cater *et al.*, 1977) and di-n-pentylphthalate (Creasy *et al.*, 1983, 1987). Among a variety of phthalate esters, DEHP has been investigated most frequently as a representative substance of phthalic acid esters. The mechanism of the testicular toxicity of phthalates is not yet wholly clear, although the damaging effect on Sertoli cells and blood-testis

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barrier has been considered (Gray and Butterworth, 1980). We have conducted a series of experiments on testicular toxicity of DEHP in rats, and have clarified that ultrastructural changes were induced in seminiferous tubules at stages from IX to I of the spermatogenic cycle in 8 week-old Sprague-Dawley rats, 3 to 18 hr after single-dose administration of 2,800 mg/kg of DEHP (Saitoh *et al.*, 1997). Noteworthy changes were degeneration of spermatocytes, dilatation of rough-surfaced endoplasmic reticulum, especially those in the vicinity of the tight junction of ectoplasmic specialization of Sertoli cells, and disintegration of the intercellular junction between Sertoli cells. In a study utilizing electron microscopic autoradiography, we have demonstrated the distribution of phthalic acid into the testis, especially to Sertoli cells (Ono *et al.*, 2004). We have also observed that clear structural changes of testes were induced with single oral dose of 1400 mg/kg, and that the non-toxic dose level of DEHP on testes was 700 mg/kg in mature rats. Furthermore, we have employed a lanthanum trace method to examine the effects of DEHP on Sertoli cell function, especially on the condition of blood-testis barrier in rats (Saitoh *et al.*, 1997). In this study, lanthanum particles were observed 6 hr after administration at the tight junction between Sertoli cells, which showed that the function of Sertoli cells to maintain the blood-testis-barrier was affected with DEHP as early as 6 hr after oral administration, but had recovered by 24 hr. The fetal stage is known to be vulnerable to chemical exposure, and the effects on gonadal and endocrine systems are of special concern. In this context, de Kretser and Kerr (1994) described that the blood-testis barrier in rats was established during 16~19 days of postnatal life. In the present study, influence of *in utero* exposure to DEHP on development of testes in rats was examined. Ethinyl estradiol was used as a reference substance for estrogenic activity of DEHP, if any.

MATERIALS AND METHODS

Materials

Di-(2-ethylhexyl)phthalate (DEHP) was purchased from Wako Pure Chemical Industries Ltd. and was diluted with corn oil (Nacalai Tesque Inc.) to a concentration appropriate for administration at the constant volume of 5 mL/kg. Ethinyl estradiol (EE, Wako Pure Chemical) was suspended in corn oil on the same principle and used as the reference compound.

Animals

Adult rats of Sprague-Dawley strain (Crj: CD IGS) were purchased from Charles River Japan Inc., and were kept for a week to acclimatize them to the laboratory condition. The animals were reared individually in a metallic cage sized 220×270×190 mm, in a room with conditioned temperature at 24~26°C and relative humidity within 50~65%. Lighting was alternated at 12 hr intervals (lights on 7:00~19:00). Appropriate bedding material such as White flake (Charles River) was provided for pregnant and nursing rats. The animals were fed with pellet food (CE-2, CLEA Japan Inc.) *ad libitum* and were supplied with tap water.

A female rat was mated with a male and a vaginal smear specimen was examined every morning. The day when a vaginal plug or sperm in the specimen was confirmed was defined as gestational day (G) 0. The pregnant animals were allocated to groups in a random fashion stratified by body weight on the day of administration (G7).

Dosage and administration

Preliminary dose-finding study showed that administration of 2000 mg/kg/day DEHP to pregnant rats from G7 to G18 caused high incidence of absorption of embryos and fetal deaths. Similar administration of 1000 mg/kg/day of DEHP caused a few fetal deaths and some pathological findings in the testis. Thus the doses of DEHP were decided on 1000 mg/kg for the highest and 500 mg/kg for the lowest in the first experiment. The doses of DEHP in Experiment 2 were selected to be 500, 250 and 125 mg/kg, considering the results of the first experiment. The doses of EE were set at 0.5 and 0.25 mg/kg referring to the study by Yasuda *et al.* (1985) in mice. Oral administration by gavage was started on G7 and continued till G18.

Experimental design

The study was designed in two phases; observation of the histopathological changes of testicular development by intra-uterine exposure to DEHP was made in Experiment 1, including the dose finding, and in Experiment 2 a search for the no-effect level was attempted, together with confirmation of the findings in Experiment 1.

In Experiment 1, 28-30 dams per group were given oral administration of DEHP, EE or the vehicle from G7 to G18. Each 6 of these dams were killed by ether inhalation on G12, 14, 16, 18 and 20 to examine their fetuses. In addition, each 5 dams of groups given 500 and 1000 mg/kg of DEHP were allowed to deliver

spontaneously to examine postnatal changes in the testis and epididymis of their offspring. The male offspring were reared and kept until examination at 7 weeks of age.

In Experiment 2, each 11-12 pregnant females were given oral administration of DEHP or vehicle. Each 3 dams of the groups were submitted to Caesarean section on G20 to examine their fetuses. Other dams were allowed to deliver spontaneously and male offspring chosen for examination at 5 and 10 weeks of age. The day of delivery was defined as Day 0 of lactation.

Observations of dams

Dams were examined daily for general conditions in all experiments and body weight was measured occasionally. Delivery and nursing conditions were observed and the numbers of fetuses delivered and live offspring were determined. From these data and the number of implantations counted at the necropsy, viability of the offspring, namely, delivery index (fetuses delivered/implantation sites, %), birth index (live offspring at birth/implantation sites, %), viability index (live offspring on day 4 of lactation/live offspring at birth, %) and weaning index (live offspring on day 21 of lactation/live offspring on day 4 of lactation, %), were determined.

Examination of the fetuses and offspring

In Experiment 1, fetuses on G12 were collected only for histopathological examination. Live fetuses collected on G14, 16, 18 and 20 were weighed and the external appearances examined. Whole bodies and testes from these live fetuses were submitted for histopathological or electron microscopic examination. The testes and epididymides of male offspring of the DEHP-treated groups were collected at 7 weeks of age for histopathological examination.

For histopathological examination, the specimens were fixated in Bouin's solution and then immersed in a buffered neutral formalin solution. The fixed tissues were embedded in paraffin and cut in 4 μm slices. These sections were stained with hematoxylin and eosin (HE) and were examined under light microscopy.

For electron microscopic examination, the tissues were immersed in an ice-cold mixture of 2% paraformaldehyde buffered with 0.1 M s-collidine and 1.25% glutaraldehyde for 3 hr. The fixed tissues were cut into small pieces and post-fixed with 2% osmium tetraoxide buffered with 0.1 M s-collidine. The post-fixed tissues were dehydrated in ethanol and embedded in epoxy resin (Quetol-651, Nissin EM, Tokyo). Semithin sections (1 μm) were stained with toluidine blue and observed under a light microscope. Representative areas were selected from the testis preparations and ultra-thin sections were prepared and stained with uranyl acetate and lead citrate, and then examined with an electron microscope (H-7100, Hitachi, Tokyo).

In Experiment 2, all of the live fetuses examined on G20 were weighed by sex and examined for their external appearance, and then testes were dissected from live male fetuses for histopathological examination as described in Experiment 1, and for staining of androgen receptors. The offspring were weighed and reared until examination. Each 4 male offspring from each group were killed at 5 and 10 weeks of age, and testes with epididymides were dissected and HE-stained thin sections prepared as described above. For electron microscopic examination, each 2 male offspring were used and fixation was performed by a systemic perfusion of a mixture solution of 2% paraformaldehyde buffered with 0.1 M s-collidine and 2.5% glutaraldehyde from the aorta to the body with a perfusion pump under sodium pentobarbital anesthesia. The testes were submitted to electron microscopic observation. The other 4 offspring of each group were killed by ether inhalation at 10 weeks of age to obtain their testes and epididymides for sperm examination.

Immunohistochemistry of androgen receptors

In addition, in order to confirm the development of hormone receptors, expression of androgen receptors in the testis was observed by an immunohistochemical method (Dalgaard *et al.*, 2001), using a rabbit polyclonal antibody for N-terminal of the androgen receptor (AR-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Examination of spermatogenesis

In Experiment 2 the seminiferous epithelium cycle was examined on testis sections stained with HE obtained at 5 weeks of age, and the spermatogenic stage was determined according to the simplified method described by Matsui *et al.* (1996). Briefly, seminiferous tubules on a specimen were classified into four groups by spermatogenic stages I-VI, VII-VIII, IX-XI and XII-XIV (Dym and Clermont, 1970). One corresponding section of the testis was stained with periodic acid Schiff (PAS) to confirm acrosomes of spermatogonia. Each 5 seminiferous tubules belonging to 4 groups were chosen and the numbers of germ

cells and Sertoli cells in a tubule determined to calculate a ratio of germ cells to Sertoli cells in each group.

Analysis of morphology and function of sperm

Sperm were collected through micropuncture of the cauda epididymis of rats at 10 weeks of age, and were examined as previously described (Sato *et al.*, 2000). Sperm motility was measured using a computer-assisted sperm motion analysis system (HTM-IVOS ver 10.6, Hamilton-Thorne Research, Beverly, MA, USA) and for morphological analysis of spermatozoa as described previously (Sato *et al.*, 2002a). After the collection of sperm for motility analysis, the cauda epididymis was dissected at the transition point to the vas deferens and at the middle of the cauda and body of epididymis, weighed and then stored at –20°C. The frozen cauda epididymis was thawed to room temperature and homogenized in distilled water. The sperm heads in the homogenate were counted with HTM-IVOS as previously described (Sato *et al.*, 2002b).

Statistical analysis

When uniformity of variance was confirmed among the groups by the method of Bartlett, data obtained were analyzed by ANOVA (Yoshimura, 1986). When the uniformity was not confirmed, Kruskal-Wallis's rank-sum test was applied instead (Yoshimura, 1986). When significant differences between groups were observed in either of the analyses, Dunnett test was applied for a comparison between the control and treated groups of either DEHP or EE (Yoshimura, 1986). A p value less than 0.05 was considered statistically significant.

RESULTS

Effects of DEHP treatment on dams

Daily oral administration of DEHP at a level of 1000 mg/kg and EE at levels of 0.25 and 0.5 mg/kg slightly suppressed body weight gain of pregnant rats during the treatment period. Administration of the lower dose levels of DEHP did not affect maternal body weight (Tables 1 and 2).

Effects of maternal treatment with DEHP on fetuses and offspring

Reproductive performance data, including fetal weights on G14, 16, 18 and 20 in Experiment 1, are summarized in Table 1. Oral DEHP treatment at 1000 mg/kg reduced fetal body weights at G14 and 18 significantly ($p<0.01$) as compared with those of the control group. Furthermore, 1000 mg/kg of DEHP treatment increased intrauterine mortality to 20-36%. DEHP treatment at 500 mg/kg did not cause increase in fetal deaths and changes in fetal body weight significantly. Treatment with 0.5 mg/kg of EE also increased intrauterine mortality of fetuses, even to more than 50% on G16 and 20.

External observation of fetuses on G20 revealed various malformations in treated groups. Two fetuses with branchyury from a single dam given 500 mg/kg DEHP were observed and each one fetus with general edema, club foot or anal atresia and 3 fetuses with kinked tail from a single dam given 1000 mg/kg of DEHP were observed. In the group treated with 0.5 mg/kg of EE, one fetus with kinked tail was observed. Two out of 5 dams given 500 mg/kg DEHP did not deliver any offspring because of early embryonic loss. However, 1000 mg/kg of DEHP did not cause any abnormality in delivery.

In Experiment 2, DEHP-treatment up to 500 mg/kg did not show any marked effect on fetuses (Table 2). External malformations observed in the 500 mg/kg group in Experiment 1 were not reproduced in Experiment 2. Birth weights of the offspring were significantly higher in the groups exposed to DEHP at 250 and 500 mg/kg than control. Viability and growth rate of the offspring are summarized in Table 3. Differences of body weight among the groups were insignificant on the 4th day of lactation.

Histopathological findings of fetuses and offspring

Histopathological findings of fetal testes in Experiment 1 are summarized in Table 4. Representative photographs are shown in Photos 1~3. The testis was not distinguishable in the fetuses on G12, when a few round germ cells with clear cytoplasm were scattered in mesenchyma around mesonephros. The testis was distinguished morphologically on G14, when the germinal ridge was formed and a few germ cells, some showing mitosis, were seen in the gonadal cord. On G16, the testicular cord became prominent, containing many round nucleated germ cells and Sertoli cells on its margin (Photo 1a). On G18, the interstitium was widened in the center of the gonad containing rich interstitial cells (Photo 2a), when the density of germ cells in the reproductive tract was increased. On G20, the testicular cord developed further, although the tubular structure was not yet formed (Photo 3a).

No abnormalities were observed in any group on G14. On G16, degeneration of germ cells was noted in

Table 1. Viability and development of fetuses exposed to di-(2-ethylhexyl)phthalate (DEHP) or ethinyl estradiol (EE) during gestational days 7-18 (Experiment 1).

	DEHP (mg/kg)			EE (mg/kg)	
	0 [a]	500	1000	0.25	0.5
Gestational day 14	(6)	(6)	(6)	(5)	(5)
Maternal body weight (g)	336.9 ± 17.4	326.5 ± 31.0	320.2 ± 14.9**	295.7 ± 14.7**	278.4 ± 22.7**
Implantations	17.0 ± 1.4	15.0 ± 1.7	16.2 ± 1.2	15.6 ± 1.1	13.6 ± 4.7
Intrauterine mortality (%)	7.1 ± 5.9	3.0 ± 5.0	20.3 ± 18.4	3.8 ± 5.6	6.9 ± 9.4
Live fetuses	15.8 ± 2.1	14.5 ± 1.2	12.8 ± 2.9	15.0 ± 1.4	12.6 ± 4.5
Mean fetal weight (g)	0.16 ± 0.02	0.15 ± 0.01	0.12 ± 0.02**	0.15 ± 0.01	0.15 ± 0.02
Gestational day 16	(5)	(6)	(6)	(0)	(5)
Maternal body weight (g)	344.9 ± 4.9	344.3 ± 21.3	311.4 ± 20.0**		285.7 ± 30.4**
Implantations	15.4 ± 1.3	16.0 ± 1.3	15.3 ± 1.6		13.6 ± 6.2
Intrauterine mortality (%)	1.3 ± 2.8	12.4 ± 7.6	33.1 ± 31.3		72.0 ± 36.9
Live fetuses	15.2 ± 1.3	14.0 ± 1.4	10.2 ± 4.8		4.2 ± 5.4
Mean fetal weight (g)	0.44 ± 0.02	0.43 ± 0.02	0.37 ± 0.04		0.42 ± 0.02 [b]
Gestational day 18	(6)	(6)	(6)	(4)	(5)
Maternal body weight (g)	370.7 ± 36.5	360.0 ± 36.6	335.5 ± 20.2*	327.6 ± 42.4*	321.7 ± 16.3**
Implantations	14.5 ± 1.4	15.2 ± 2.6	14.8 ± 1.6	15.3 ± 2.2	14.4 ± 2.4
Intrauterine mortality (%)	3.6 ± 6.3	1.0 ± 2.4	35.6 ± 36.5	5.7 ± 7.9	1.3 ± 3.0
Live fetuses	14.0 ± 1.8	15.0 ± 2.5	9.5 ± 5.6	14.3 ± 1.0	14.2 ± 2.4
Mean fetal weight (g)	1.35 ± 0.07	1.32 ± 0.06	1.03 ± 0.13**	1.33 ± 0.05	1.25 ± 0.10
Gestational day 20	(6)	(6)	(6)	(0)	(2)
Maternal body weight (g)	404.2 ± 6.5	410.8 ± 30.2	365.0 ± 25.4**		322.8
Implantations	14.7 ± 1.6	14.8 ± 2.6	14.5 ± 1.5		15.5
Intrauterine mortality (%)	0.0 ± 0.0	2.7 ± 4.4	36.4 ± 26.5		50.8
Live fetuses	14.7 ± 1.6	14.5 ± 2.9	9.0 ± 3.5		7.5
Mean fetal weight (g)	3.68 ± 0.20	3.52 ± 0.14	2.82 ± 0.11		2.90
External malformations	0.0 ± 0.0	2.22 ± 5.44	6.25 ± 15.31		7.14

[a] Vehicle control (corn oil, 5 mL/kg). [b] Data from 3 dams having live fetuses.
* Significantly different from control ($p<0.05$). ** Significantly different from control ($p<0.01$).

Table 2. Reproductive parameters on gestational day 20 in rats treated with di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Gestational day 20				
Dams examined	3	3	3	3
Maternal body weight (g)	408.3 ± 32.6	428.8 ± 42.5	399.3 ± 43.4	427.9 ± 50.8
Implantations	14.7 ± 0.6	15.0 ± 2.6	14.0 ± 1.7	16.0 ± 1.7
Intrauterine mortality (%)	2.2 ± 3.9	0	2.8 ± 4.8	4.1 ± 3.6
Live fetuses	14.3 ± 0.6	15.0 ± 2.6	13.7 ± 2.3	15.3 ± 1.5
Males	5.3 ± 1.2	7.0 ± 3.5	6.3 ± 2.1	9.3 ± 2.1
Females	9.0 ± 1.0	8.0 ± 1.0	7.3 ± 0.6	6.0 ± 1.0
Sex ratio (%)	37.2 ± 7.6	44.7 ± 17.2	45.5 ± 8.5	60.5 ± 9.1
Fetal body weight (g)	14.0 ± 1.8	15.0 ± 2.5	9.5 ± 5.6	14.2 ± 2.4
Males	3.77 ± 0.13	3.86 ± 0.40	4.02 ± 0.13	3.57 ± 0.14
Females	3.51 ± 0.14	3.67 ± 0.34	3.77 ± 0.16	3.40 ± 0.03
External malformations	0	0	0	0

Values represent mean ± S.D.
[a] Vehicle control (corn oil, 5 mL/kg).

one of 12 examined fetuses of the 1000 mg/kg DEHP group (Photos 1b, 1c). No such findings were noted in other fetuses of the group exposed to DEHP at 1000 mg/kg and also at 500 mg/kg. On G18, interstitial cells were increased in number and aggregated topically in the 500 mg/kg DEHP group (Photo 2b), and the hyperplasia of interstitial cells was intensified in the 1000 mg/kg DEHP group (Photo 2c), while such findings were not noted in any testes of fetuses exposed to EE. Testicular size was smaller in the groups of 1000 mg/kg DEHP and 0.5 mg/kg EE on G18 and G20. On G20, germ cells having more than two nuclei were noted and thickened seminiferous cords containing rich germ cells were seldom observed in the 500 mg/kg DEHP group. In fetal testes of the 1000 mg/kg DEHP group hyperplasia of interstitial cells, multinucleated germ cells were also seen (Photos 3b, 3c). Topically thickened seminiferous cords due to aggregation of germ cells were observed frequently in this group.

Table 5 summarizes histopathological findings in the testis of the offspring in Experiment 1. Representative pictures are shown in Photos 4~6. In the offspring at 7 weeks after birth prenatally exposed to DEHP at a level of 500 mg/kg, no obvious abnormalities were found except for multinucleated giant cells in the seminiferous tubules and cell debris in the epididymal lumens (Photos 4a, 4b). In the 1000 mg/kg-exposed group, however, most of the animals had developed abnormalities, such as branched seminiferous tubules with atrophy and/or dilatation, multinucleated giant cells and dilatation of rete testis (Photos 4c, 5a, 5b). In addition to these findings, testes from several animals in this group showed hyperplasia of the interstitial cells (Photo 4c), necrosis and/or mineralization of testes, foreign body giant cells, focal loss of seminiferous tubules and malformed seminiferous tubules (Photos

Table 3. Reproductive data and development of the offspring treated with di-(2-ethylhexyl) phthalate (DEHP) during gestational days 7-18 (Experiment 2).

	DEHP (mg/kg)			
	0 [a]	125	250	500
Dams examined	8	9	8	8
Gestation length (days)	21.8 ± 0.5	22.0 ± 0.0	22.0 ± 0.0	22.0 ± 0.0
Implantation sites	15.4 ± 1.2	15.6 ± 2.4	15.4 ± 1.1	14.9 ± 1.2
At birth (Day 0 of lactation)				
Live offspring	14.0 ± 2.1	14.6 ± 2.6	14.4 ± 1.7	14.1 ± 1.2
Birth index (%) [b]	90.8 ± 9.0	93.2 ± 6.3	93.5 ± 8.8	95.1 ± 5.7
Sex ratio (%)	42.0 ± 10.4	45.7 ± 8.9	42.9 ± 12.3	50.5 ± 12.3
Body weight, males (g)	6.5 ± 0.3	6.7 ± 0.5	7.0 ± 0.5	7.1 ± 0.3*
Body weight, females (g)	6.1 ± 0.3	6.3 ± 0.5	6.7 ± 0.6*	6.7 ± 0.3*
Day 4 of lactation				
Live offspring	13.9 ± 2.2	14.3 ± 2.5	14.4 ± 1.7	14.0 ± 1.3
Viability (%)	99.0 ± 2.7	98.6 ± 2.8	100.0 ± 0.0	99.1 ± 2.5
Sex ratio (%)	42.3 ± 10.1	46.3 ± 8.5	42.9 ± 12.3	50.9 ± 11.4
Body weight, males (g)	10.3 ± 1.1	10.4 ± 1.0	10.7 ± 0.7	10.5 ± 1.3
Body weight, females (g)	9.8 ± 1.2	9.7 ± 1.0	10.3 ± 0.8	10.0 ± 1.3
Body weight, preserved males (g) [c]	10.4 ± 0.9	10.7 ± 0.7	11.0 ± 0.5	10.7 ± 0.1
Day 7 of lactation				
Body weight, preserved males (g)	17.1 ± 2.3	16.9 ± 1.0	18.2 ± 0.7	17.2 ± 0.2
Day 14 of lactation				
Body weight, preserved males (g)	36.1 ± 2.8	34.5 ± 1.5	37.5 ± 0.8	37.6 ± 1.3
At weaning (Day 21 of lactation)				
Body weight, preserved males (g)	58.7 ± 4.7	57.1 ± 4.1	62.2 ± 1.5	60.5 ± 3.3
Weaning index (%)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Values represent mean ± S.D. * Significantly different from control ($p<0.05$).

[a] Vehicle control (corn oil, 5 mL/kg). [b] Live offspring/implantation sites.

[c] Each 2~3 male offspring from dams were preserved.