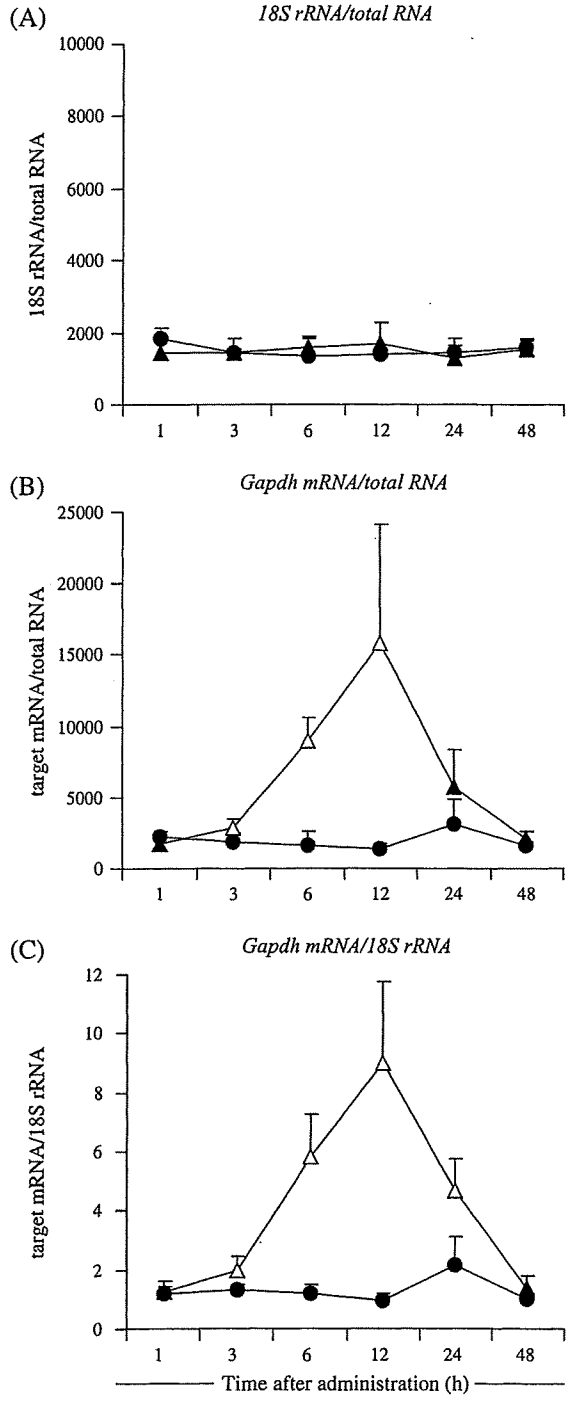

FIG. 2. Effects of EE on the expression of reference genes (A, 18S rRNA; B and C: *Gapdh*) in the uterus of immature female rats. Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

(Fig. 4A). An increase of the complement C3 mRNA in the 3-μg/kg EE group reached a peak at 24 h (43.90-fold) after administration (Table 3). Since complement C3 mRNA was markedly induced after EE administration, this gene was considered as a useful biomarker for evaluating the effects of estrogenic compounds in the uterus.

The expression of *Igf1* mRNA significantly increased between 3 and 12 h (the peak: 9.73-fold at 3 h) after treatment with 3 μg/kg EE (Fig. 4B, Table 3). However, thereafter, the expression of *Igf1* mRNA kept decreasing and significantly decreased more than that of the time-matched vehicle group at 48 h (0.52-fold) (Table 3).

Time courses of the expression of these genes (particularly the maximal response time) were greatly different individually. Consequently, it is considered important to closely investigate the time course of gene expressions for evaluating the estrogen-like effects of chemicals based on changes in mRNA expression level.

Effects of EE on the Expression of Wnt Genes in the Uterus of Immature Female Rats

Wnt4, *Wnt5a*, and *Wnt7a* mRNAs were selected to evaluate the effect of EE on the expression of *Wnt* genes (Carta and Sassoon, 2004; Hou *et al.*, 2004; Mericskay *et al.*, 2004; Miller *et al.*, 1998b).

The expression of *Wnt4* mRNA increased between 3 and 12 h after treatment with 3 μg/kg EE, as compared with those in the time-matched vehicle groups, and significant differences were noted at 6 h (1.71-fold) and 12 h (1.95-fold) after administration (Fig. 5A, Table 3). However, thereafter, the expression of *Wnt4* mRNA kept decreasing, and significant differences were noted at 24 h (0.51-fold) and 48 h (0.43-fold) after administration.

The expression of *Wnt5a* mRNA significantly increased at 3 h (2.12-fold) after treatment with 3 μg/kg EE as compared with that in the time-matched vehicle group (Fig. 5B, Table 3). However, thereafter, the expression of *Wnt5a* mRNA kept decreasing, and significant differences were noted at 24 h (0.45-fold) and 48 h (0.39-fold) after administration.

The expression of *Wnt7a* mRNA significantly decreased from 1 h after treatment with 3 μg/kg EE (0.70-fold) as compared with those in the time-matched vehicle groups (Fig. 5C, Table 3). No statistically significant difference was noted in the expression of *Wnt7a* mRNA at 3 h after treatment with 3 μg/kg EE as compared with that in the time-matched vehicle group. However, the mean *Wnt7a* mRNA level was similar to that observed at 1 h (0.70-fold) after administration. Thereafter, the expression of *Wnt7a* mRNA significantly decreased between 6 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups. The decrease of *Wnt7a* mRNA in the 3-μg/kg EE group fell to the lowest at 24 h (0.15-fold) after administration.

Effects of EE on the Expression of β-Catenin/TCF Target Genes in the Uterus of Immature Female Rats

Anti-Mullerian hormone type 2 receptor (*Amhr2*, also known as Mullerian inhibiting substance type II receptor: Hossain and Saunders, 2003), bone morphogenetic protein 4 (*Bmp4*: Kim

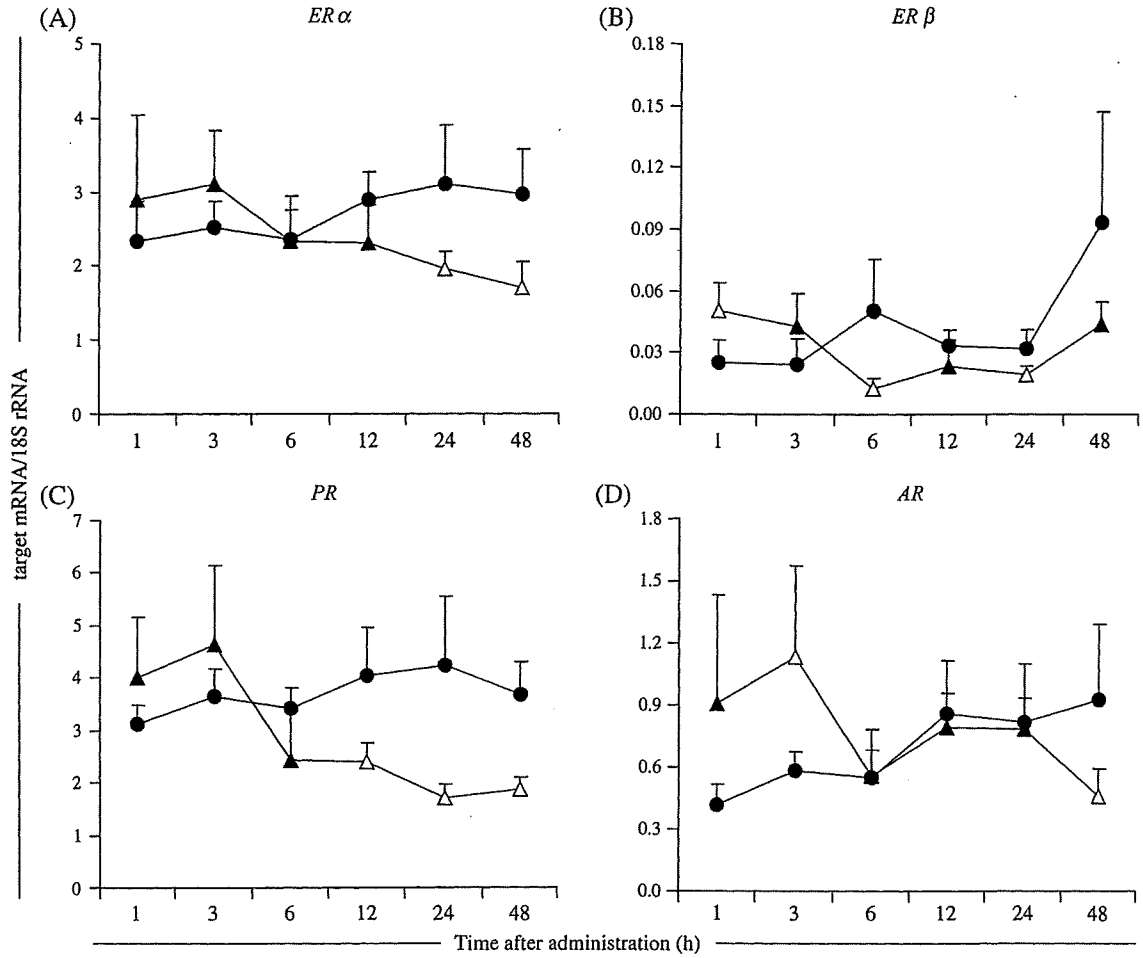

FIG. 3. Effects of EE on the expression of sex steroid hormone receptor genes (A, *ERα*; B, *ERβ*; C, *PR*; D, *AR*) in the uterus of immature female rats. Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

et al., 2002; Schwartz *et al.*, 2003), fibroblast growth factor 9 (*Fgf9*: Imai *et al.*, 2002; Schwartz *et al.*, 2003), cyclin D1 (Schwartz *et al.*, 2003; Shtutman *et al.*, 1999), follistatin (Willert *et al.*, 2002), fibronectin (Gradl *et al.*, 1999), and matrix metalloproteinase 7 (*Mmp7*: Brabletz *et al.*, 1999; Schwartz *et al.*, 2003) mRNAs were selected to evaluate the effect of EE on the expression of β-catenin/TCF target genes.

The expression of *Amhr2* mRNA significantly decreased from 6 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups, and they kept decreasing until 48 h after administration (Fig. 6A, Table 3). Significant differences were noted between 6 and 24 h (the lowest: 0.14-fold at 6 h) after administration.

The expression of *Bmp4* mRNA significantly decreased between 6 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 6B, Table 3). A decrease of the *Bmp4* mRNA in the 3-μg/kg EE group fell to the lowest at 12 h (0.13-fold) after administration.

The expression of *Fgf9* mRNA significantly decreased at 24 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 6C, Table 3). A decrease of the *Fgf9* mRNA in the 3-μg/kg EE group fell to the lowest at 24 h (0.40-fold) after administration.

The expression of cyclin D1 mRNA significantly increased between 3 and 12 h (the peak: 1.89-fold at 3 h) after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 7A, Table 3). However, thereafter, the expression of cyclin D1 mRNA kept decreasing and significantly decreased more than that of the time-matched vehicle group at 24 h (0.45-fold) after administration.

The expression of follistatin mRNA significantly increased between 1 and 6 h (the peak: 4.18-fold at 6 h) after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 7B, Table 3). However, thereafter, the expression of follistatin mRNA significantly decreased more than those of the time-matched vehicle groups between 12 and 48 h (the lowest: 0.32-fold at 24 h).

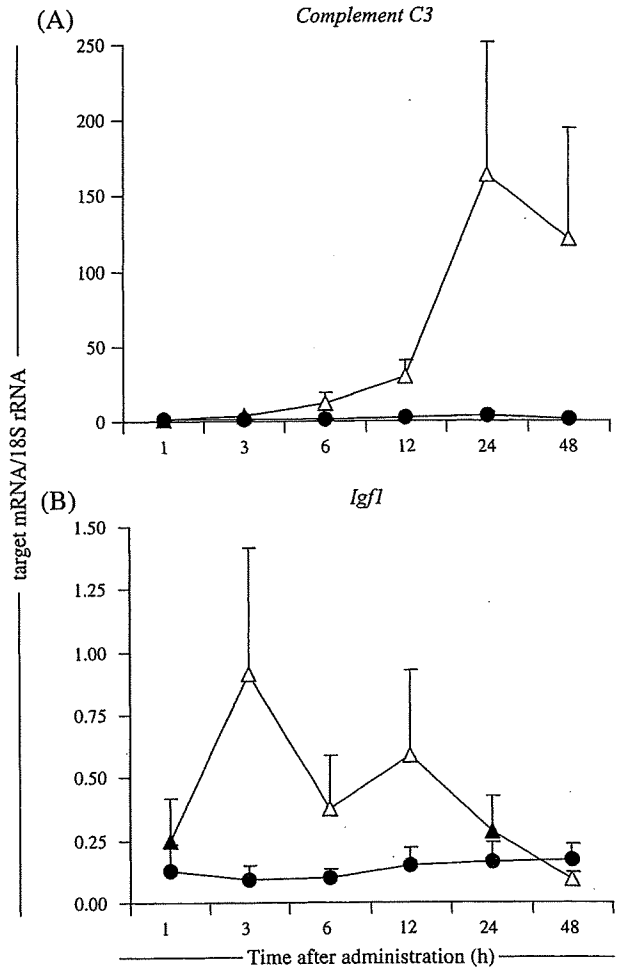

FIG. 4. Effects of EE on the expression of well-known estrogen target genes (A, complement C3; B, *Igf1*) in the uterus of immature female rats. Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

The expression of fibronectin mRNA significantly increased at 1 and 3 h (the peak: 3.06-fold at 3 h) after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 7C, Table 3). However, thereafter, the expression of fibronectin mRNA decreased remarkably, and no statistically significant difference was noted from 6 to 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups.

The expression of *Mmp7* mRNA increased remarkably from 3 to 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 7D, Table 3). A statistically significant difference was noted in the expression of *Mmp7* mRNA at 6, 12, and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups, respectively. An increase of the *Mmp7* mRNA in the 3-μg/kg EE group reached a peak at 6 h (41.93-fold) after administration.

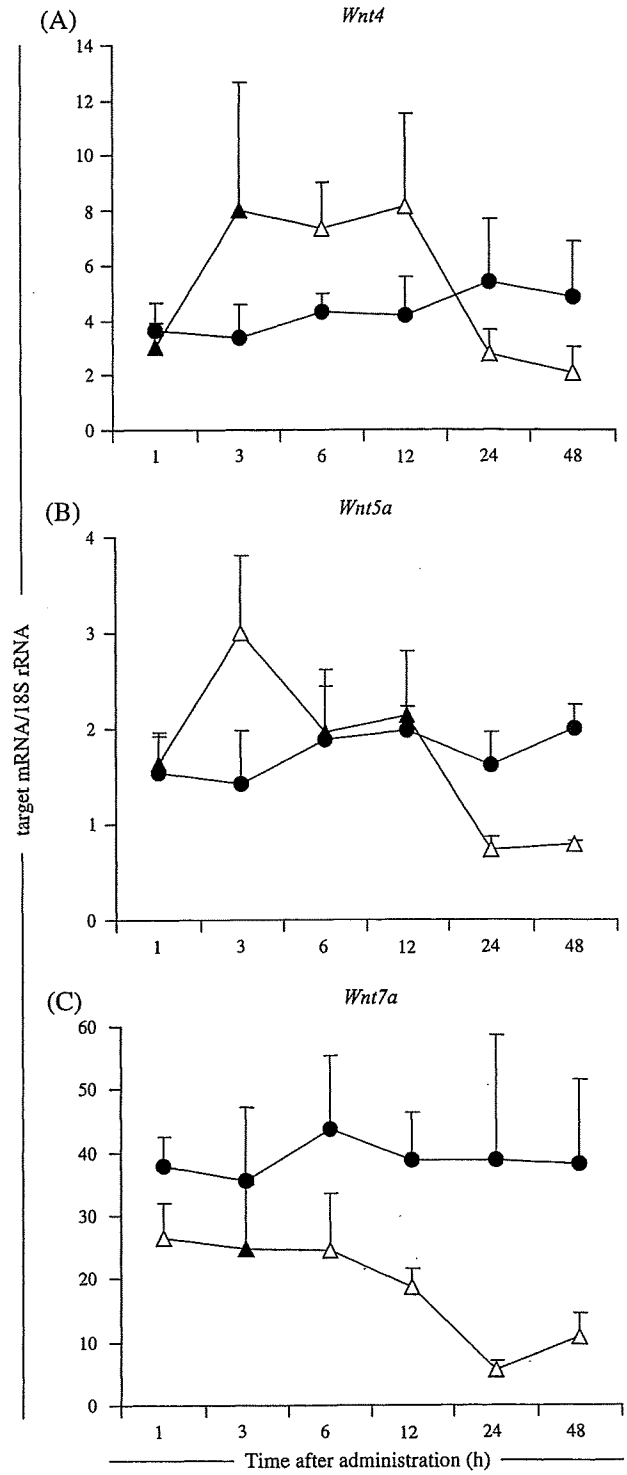

FIG. 5. Effects of EE on the expression of *Wnt* genes (A, *Wnt4*; B, *Wnt5a*; C, *Wnt7a*) in the uterus of immature female rats. Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

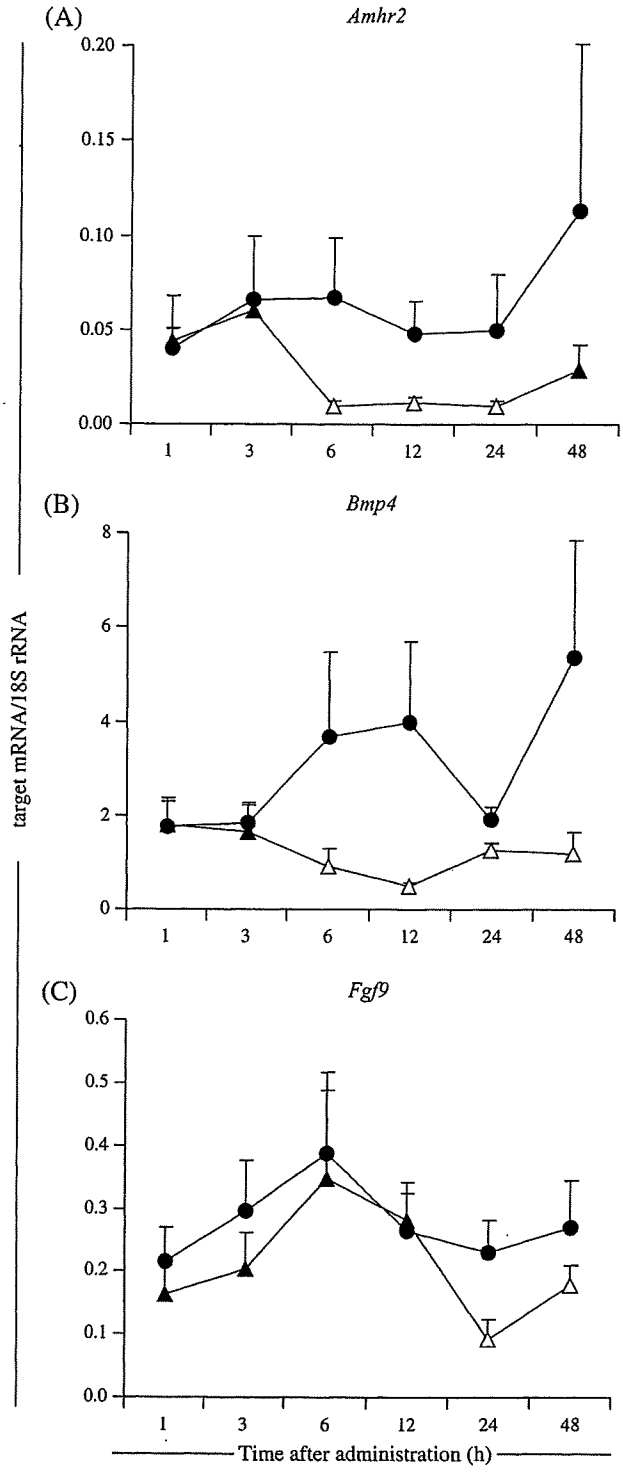

FIG. 6. Effects of EE on the expression of β-catenin/TCF target genes (A, *Amhr2*; B, *Bmp4*; C, *Fgf9*) in the uterus of immature female rats. Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

DISCUSSION

Exposure to estrogenic compounds during critical *in utero* and early postnatal stages of development is the particular concern because many feedback mechanisms functioning in the adult are absent and adverse effects may be noted at doses lower than those observed in the adult (Colborn *et al.*, 1993; Crisp *et al.*, 1998). Furthermore, it may lead to permanent alterations in the development of reproductive organs and other tissues with ER (Bigsby *et al.*, 1999). Therefore, it is important to understand whether estrogenic compounds interfere with the expression of morphogenesis-related gene in the immature uterus.

We demonstrated that EE altered the mRNA expression of *Wnt* genes and β-catenin/TCF target genes by various time-course patterns in the uterus of immature female rats. *Wnt7a* is the key target gene assumed to be responsible for the effects of diethylstilbestrol (Mericskay *et al.*, 2005; Miller *et al.*, 1998a; Sassoon, 1999). In this study, *Wnt7a* mRNA was drastically downregulated after EE administration, decreasing to 15% of the time-matched control level at 24 h. Although the time courses of *Wnt4*, *Wnt5a*, and *Wnt7a* mRNA varied until 12 h after EE administration, all of them were downregulated at 24 and 48 h. Since these genes are intimately involved with development and differentiation of organs (Heikkila *et al.*, 2001; Wodarz and Nusse, 1998), simultaneous downregulation of *Wnt4*, *Wnt5a*, and *Wnt7a* mRNA by EE may cause altered development of the female reproductive tract, indicating that estrogenic compounds affect the Wnt signaling pathway regulated by Wnt4, Wnt5a, and Wnt7a.

The Wnt/β-catenin pathway is the most understood Wnt signaling pathway. In the absence of Wnt signals, free β-catenin is phosphorylated by glycogen synthase kinase-3β in the cytosol. Adenomatous polyposis coli and Axin are part of the large multiprotein complex that facilitates this phosphorylation process (Rubinfeld *et al.*, 1993). Phosphorylated β-catenin is ubiquitinated and ultimately degraded by the proteasome (Orford *et al.*, 1997). In the presence of a Wnt signal, this phosphorylation of β-catenin is blocked, and the free cytosolic β-catenin is translocated to the nucleus and heterodimerized with one of the TCF/LEF family members and can regulate the transcription of β-catenin/TCF target genes (Giles *et al.*, 2003).

If ER agonist interfered with the Wnt/β-catenin pathway in the immature uterus, the mRNA expression of β-catenin/TCF target genes should also alter in the uterus of immature rats. Expression patterns of β-catenin/TCF target gene after EE administration were roughly classified into four patterns. Group 1, consisting of *Amhr2*, *Bmp4*, and *Fgf9* mRNA, revealed downregulation in expression levels after EE administration. *Amhr2* and *Bmp4* mRNA were downregulated after EE administration, demonstrating time courses linked closely to *Wnt7a* mRNA. *Fgf9* mRNA was downregulated during the period when *Wnt7a* mRNA was downregulated most remarkably. Consequently, it was suggested that mRNA expressions of

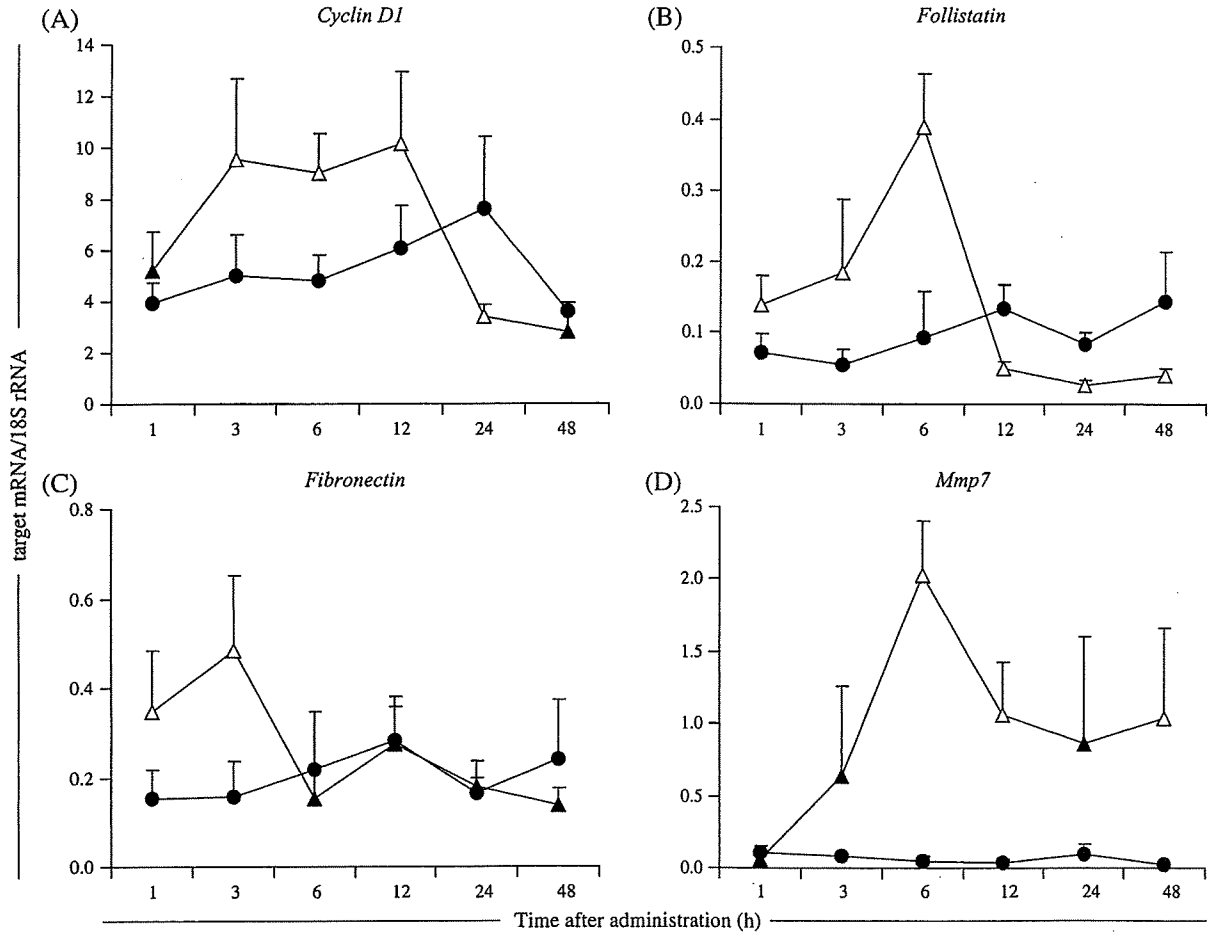

FIG. 7. Effects of EE on the expression of β-catenin/TCF target genes (A, cyclin D1; B, follistatin; C, fibronectin; D, *Mmp7*) in the uterus of immature female rats. Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

Amhr2, *Bmp4*, and *Fgf9* after EE administration might be affected by the downregulation of *Wnt7a* but not *Wnt4* and *Wnt5a*. Group 2, consisting of cyclin D1 and follistatin mRNA, revealed upregulation at the early phase after EE administration followed by downregulation, as compared with those in the time-matched vehicle groups. Time-course expression patterns of these genes were similar to that of *Wnt4* mRNA. Hou *et al.* (2004) reported that estrogen in an ER-independent manner rapidly upregulates the mRNA expressions of *Wnt4* and *Wnt5a* in the uterus of ovariectomized wild-type or *ERα* (−/−) mice. Terada *et al.* (2003) showed that the overexpression of Wnt4 and β-catenin promoted the cell cycle and increased the promoter activity and protein expression of cyclin D1 in LLC-PK1 cells. Yao *et al.* (2004) reported that follistatin is a downstream component of Wnt4 signaling and proposed that Wnt4 acts through follistatin to regulate vascular boundaries and maintain germ cell survival in the ovary. Therefore, it was suggested that the upregulation of cyclin D1 and follistatin mRNA at the early phase after EE administration might be affected by the upregulation of *Wnt4*. Group 3, consisting of fibronectin mRNA, revealed upregulation at the early phase after EE administration, followed by changes similar to the level of the time-matched vehicle group. Group 4, consisting of *Mmp7* mRNA, revealed continuous upregulation after EE administration. *Mmp7* is well known as the β-catenin/TCF target gene described above; however, it is also well known as a gene that increases in response to estrogen (Naciff *et al.*, 2003). In this study, *Mmp7* mRNA was remarkably upregulated after EE administration regardless of the alteration of expression levels of *Wnt4*, *Wnt5a*, and *Wnt7a*. Therefore, it was suggested that the *Mmp7* mRNA was not downregulated as the β-catenin/TCF target gene but was remarkably upregulated as the estrogen target gene in the uterus of immature rats. According to these results, mRNA expression level of the β-catenin/TCF target genes in the uterus after EE administration was considered dependent on the balance between estrogen and Wnt signaling pathway under the presence of multiple stimuli. In the ongoing experiment, to determine whether the gene expression changes caused by EE were ER-mediated reactions, immature female rats were treated once by oral gavage with vehicle, EE, or EE

plus ICI 182,780 (ER antagonist). At the present moment, we confirmed that the mRNA expression of *Wnt5a*, *Wnt7a*, *Amhr2*, and *Fgf9* was regulated by ER (data not shown).

Wnt5a is known as a ligand of the Wnt/Ca^{2+} pathway, and it plays a role in inhibiting the Wnt/β-catenin pathway (Kuhl *et al.*, 2000; Veeman *et al.*, 2003). However, the signaling pathway of Wnt4 and Wnt7a is complex, and there are still many uncertain aspects of their molecular mechanism of action. For example, it has been reported that the signal of Wnt7a might be transmitted via both the β-catenin–dependent pathway (Lyu and Joo, 2005; Shimizu *et al.*, 1997) and the β-catenin–independent pathway (Kengaku *et al.*, 1998; Lyu and Joo, 2005). Lyu and Joo (2005) demonstrated that Wnt7a induced the accumulation of cytosolic β-catenin and the activation of small GTPase Rac and β-catenin in the SV40-immortalized human corneal epithelial cells. The regulation of β-catenin–independent pathway by Wnt7a depended on the activation of Rac and c-Jun, suggesting that Wnt7a could activate the Wnt/JNK pathway. In this study, the precise mechanisms of action of ER agonist on the expression of *Wnt* genes and β-catenin/TCF target genes remain to be elucidated. Therefore, further studies are needed to examine whether the expression of *Wnt4*, *Wnt5a*, and *Wnt7a*, which are altered by ER agonist, influences the expression of the β-catenin/TCF target genes through β-catenin-dependent pathway and/or β-catenin–independent pathway.

It is rare that the cell is exposed only to the single extracellular or intracellular signal in the process of development and differentiation of organs. Actually, it is thought that the multiple signal transduction pathways are activated and/or inhibited at the same time in one cell, and they influence each other. In this study, we demonstrated that ER agonist influenced not only the expression of sex steroid hormone receptor genes and well-known estrogen target genes but also *Wnt* genes and β-catenin/TCF target genes in the uterus of immature rats, indicating that their molecules are the potential players affected by estrogenic stimuli. Our findings in this study will provide important clues for evaluating the effects of estrogenic compounds in the immature uterus.

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REGULATORY TOXICOLOGY

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Subacute oral toxicity study of diethylphthalate based on the draft protocol for "Enhanced OECD Test Guideline no. 407"

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Abstract We performed a 28-day repeated-dose toxicity study of diethylphthalate based on the draft protocol of the "Enhanced OECD Test Guideline 407" to investigate whether it has endocrine-mediated properties according to this assay. Diethylphthalate was orally administered to SD rats at doses of 0, 40, 200, and 1,000 mg/kg/day for at least 28 days, but no endocrine-mediated effects were detected based on any of the parameters examined, suggesting that diethylphthalate does not possess endocrine properties according to this assay.

Keywords Diethylphthalate · Enhanced Test Guideline 407 · Rat · Endocrine effects

Introduction

Since a considerable number of chemicals have been reported to have possible endocrine-disrupting activities in humans and animals (McLachlan 1993; McLachlan and Korach 1995), the Organisation for Economic Co-operation and Development (OECD) has proposed the use of a uterotrophic assay, Hershberger assay, and Enhanced OECD Test Guideline no. 407 (enhanced TG 407) as in vivo screening tests to detect endocrine properties (OECD 1999). With the enhanced TG 407, the chemical compounds are orally administered for at least 28 days, and the OECD has prepared the establishment of guidelines from 2001 (OECD 2001). An international validation study of the enhanced TG 407 for various chemicals coordinated by the OECD has been conducted, but the results have not yet been published. The enhanced TG 407 has recently been performed on various chemicals in laboratories, and its usefulness as an in vivo screening test to detect endocrine-mediated effects has been confirmed (Toyoda et al. 2000; Andrews et al. 2001; Okazaki et al. 2001, 2002a, b; Yamasaki et al. 2002a, c; Kennel et al. 2003; Mellert et al. 2003; Wason et al. 2003; Kunimatsu et al. 2004).

Diethylphthalate is used as a plasticizer in a wide variety of products. However, a number of phthalates and their metabolites are suspected of having endocrine-disrupting effects. Diethylphthalate has been reported to have binding affinity for hepatic estrogen receptors (Lutz and Kloas 1999), and phthalic acid di-*n*-amyl ester has been reported to have an androgenic antagonistic effect in the Hershberger assay (Yamasaki et al. 2004b). We, therefore, performed enhanced TG 407 assay of diethylphthalate according to the OECD draft protocol (OECD 1999, 2000) to investigate whether it has endocrine-mediated activity.

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Materials and methods

The present study was performed under Good Laboratory Practice guidelines.

Chemicals

The materials were obtained from the following manufacturers: diethylphthalate (DEP, CAS No. 84-66-2, Lot No. LDQ5211, 99.8% pure), Wako Pure Chemical Industries (Tokyo, Japan); corn oil (Lot No. WAL7450), Wako Pure Chemical Industries.

Animals

Crj:CD (SD) rats were purchased from Charles River Japan, Inc (Shiga, Japan). Animals were weighed, weight-ranked, and randomly assigned to each of the treatment groups and control group before administration, and then housed individually in stainless steel,

wire-mesh cages throughout the study. Rats were provided with water automatically and with a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan). The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and was artificially illuminated with fluorescent light on a 12-h light/dark cycle (600–1,800 h). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by *the Japanese Association for Laboratory Animal Science.*

Study design

Rats were orally gavaged with 0, 40, 200, and 1,000 mg/kg/day DEP for at least 28 days beginning at 8 weeks of age. In the preliminary test, rats were orally gavaged with 0, 40, 200, and 1,000 mg/kg/day for 14 days and no abnormalities were detected in each parameter, so we selected these dose levels in this study. A vehicle control group was gavaged with corn oil alone. The volume of the corn oil solution containing DEP for gavage was 10 ml/kg. The concentration and stability of the DEP were confirmed. Each group consisted of 10 males and 10 females. Animals were killed by exsanguination under ether anesthesia, and blood samples were obtained from the abdominal aorta and examined for hematological, clinical biochemistry, and hormonal parameters.

General observations

Clinical signs were recorded daily. Detailed clinical observations of all animals were made outside the home cage, once before the first dose and once a week thereafter. The signs for which the animals were examined included changes in skin, fur, eyes, and mucous membranes, frequency of urine and feces, and autonomic activity (e.g., lacrimation, piloerection, pupil size, respiratory pattern). Changes in gait, posture, response to handling, the occurrence of clonic or tonic movements, stereotypes (e.g., excessive grooming, circling), or bizarre behavior (e.g., self-mutilation, walking backwards), were also recorded. In the fourth week, a functional observation battery (FOB) that tested sensory reactivity to stimuli of different types (e.g., auditory, visual, and proprioceptive) assessed grip strength, and assessed motor activity.

Body weight and food consumption

Individual body weight was recorded twice weekly and immediately before necropsy. Food consumption was measured weekly.

Hematology

The following were examined in the hematology examinations: red blood cell count, white blood cell count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, reticulocyte count, prothrombin time, activated partial thromboplastin time, and differential leukocyte count.

Clinical biochemistry

Serum levels of the following were measured in the clinical biochemistry examination: glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, cholinesterase, γ-glutamyl transpeptidase, total cholesterol, triglyceride, glucose, total protein, albumin, blood urea nitrogen, creatinine, total bilirubin, calcium, inorganic phosphorus, sodium, potassium and chlorine, and the albumin–globulin ratio were calculated.

Hormone analysis

The serum concentrations of the following hormones were measured at the end of the test period: thyroid-stimulating hormone (TSH), thyroxin (T4), triiodothyronine (T3), testosterone, follicle-stimulating hormone (FSH), luteinising hormone (LH), and estradiol. T3 and T4 were determined with an automatic immunoassay system (IMX, Abott laboratories), and TSH, testosterone, FSH, and LH were measured with a microplate reader (UV max, Molecular Devices). Estradiol was analyzed by the Panafarm Laboratory (Udo, Japan) using a radioimmunoassay system (Auto-Gamma 5530 Gamma Counting System, Packard). The analyses of testosterone, FSH, LH, and estradiol were performed in addition to those in the current enhanced TG 407.

Spermatology

Sperm morphology (200 formalin-fixed, Giemza-stained spermatozoa) and (heated to 70°C) the sperm count were determined by examining specimens obtained from the right epididymis. The number of homogenization-resistant (0.9% NaCl solution plus Triton-X100) sperm was determined on specimens from the right testis.

Estrous cycling

The estrous cycle of all females was assessed daily from day 22 until the day of sacrifice by examining vaginal smears stained with Giemsa stain.

Necropsy

Males were necropsied on day 29. Females were necropsied after having been dosed for at least 29 days and sacrificed on days 30–34 to allow them to be sacrificed in the diestrous stage.

Organ weight

The following organs were weighed after necropsy: testes, epididymes, ventral prostate, dorsolateral prostate, seminal vesicles, ovaries, uterus, adrenals, liver, spleen, kidneys, heart, brain, and thymus, as fresh organs, and the thyroid and pituitary glands, after organ fixation.

Histopathology

The following organs were fixed in 10% neutral buffered formalin and examined: prostate, including ventral prostate and dorsolateral prostate, seminal vesicles, ovaries, uterus, vagina, mammary gland, brain, thyroid, adrenals, liver, spleen, kidneys, stomach, intestine, pancreas, thymus, parathyroids, and pituitary gland. The epididymes and testes were fixed in Bouin's solution before examining them.

Statistical analysis

Body weight, food consumption, hematological data, clinical biochemical data, organ weight, spermatological data (sperm counts), and FOB data were analyzed by the Bartlett's test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. If a significant difference was found, the difference between the control group and each of the dosage groups was analyzed by Dunnett's test. If the variance was not homogeneous, the Kruskal-Wallis test was used. If a significant difference was found, the difference between the control group and each of the dosage groups was analyzed by the nonparametric Dunnett's test. FOB numerical data and spermatological data (sperm morphological data) were analyzed by the Kruskal-Wallis test. If a significant difference was found, the difference between the control group and each of the dosage groups was analyzed by the nonparametric Dunnett's test.

Results

Body weight and food consumption

Although there was no significant difference in the body weights and food consumption between each treated

Table 1 Body and relative organ weights (mean ± SD) in rats given diethylphthalate

	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)
Male				
Body weights (g)				
Initial	307.1 ± 5.5	308.6 ± 7.5	307.3 ± 8.9	305.0 ± 8.0
Terminal	442.8 ± 22.6	434.0 ± 24.6	430.0 ± 26.0	417.5 ± 19.4
Organ weights				
Testes (g/100 g)	0.70 ± 0.05	0.74 ± 0.08	0.74 ± 0.07	0.78 ± 0.07
Epididymes (g/100 g)	0.23 ± 0.02	0.25 ± 0.01	0.25 ± 0.02	0.25 ± 0.02
Prostate (g/100 g)[a]	0.22 ± 0.03	0.24 ± 0.05	0.24 ± 0.03	0.25 ± 0.03
Ventral prostate (g/100 g)	0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.15 ± 0.02
Dorsolateral prostate (g/100 g)	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02
Seminal vesicle (g/100 g)	0.33 ± 0.04	0.32 ± 0.05	0.31 ± 0.05	0.35 ± 0.03
Pituitary gland (mg/100 g)	2.7 ± 0.3	2.6 ± 0.3	2.7 ± 0.3	2.8 ± 0.4
Liver (g/100 g)	3.66 ± 0.16	3.57 ± 0.19	3.56 ± 0.17	3.60 ± 0.11
Kidney (g/100 g)	0.61 ± 0.05	0.62 ± 0.07	0.60 ± 0.02	0.65 ± 0.04
Heart (g/100 g)	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.03	0.29 ± 0.02
Spleen (g/100 g)	0.15 ± 0.02	0.14 ± 0.01	0.15 ± 0.02	0.15 ± 0.03
Adrenals (mg/100 g)	11.8 ± 2.1	11.0 ± 1.0	11.0 ± 1.2	12.1 ± 1.5
Thymus (mg/100 g)	87.8 ± 23.2	90.9 ± 19.3	81.9 ± 16.5	87.6 ± 20.6
Thyroid (mg/100 g)	4.2 ± 0.7	4.9 ± 1.1	4.9 ± 0.9	4.7 ± 0.7
Brain (g/100 g)	0.46 ± 0.02	0.47 ± 0.03	0.47 ± 0.04	0.48 ± 0.02
Female				
Body weights (g)				
Initial	198.4 ± 8.4	194.7 ± 7.2	196.0 ± 5.5	194.9 ± 8.7
Terminal	263.2 ± 15.2	253.2 ± 9.0	264.9 ± 9.2	261.2 ± 13.8
Organ weights				
Ovary (mg/100 g)	28.0 ± 4.6	28.1 ± 2.2	28.7 ± 4.8	30.1 ± 2.9
Uterus (g/100 g)	0.18 ± 0.03	0.17 ± 0.03	0.17 ± 0.02	0.19 ± 0.02
Pituitary gland (mg/100 g)	4.9 ± 0.7	5.3 ± 0.5	5.5 ± 0.8	5.2 ± 0.5
Liver (g/100 g)	3.50 ± 0.17	3.35 ± 0.27	3.47 ± 0.16	3.60 ± 0.19
Kidney (g/100 g)	0.60 ± 0.04	0.65 ± 0.03*	0.62 ± 0.04	0.64 ± 0.03*
Heart (g/100 g)	0.32 ± 0.03	0.32 ± 0.03	0.34 ± 0.01	0.34 ± 0.01
Spleen (g/100 g)	0.19 ± 0.02	0.20 ± 0.03	0.18 ± 0.02	0.21 ± 0.02
Adrenals (mg/100 g)	21.8 ± 2.4	21.8 ± 2.8	22.2 ± 2.0	24.8 ± 2.3*
Thymus (mg/100 g)	142.8 ± 34.9	135.0 ± 24.5	135.0 ± 30.5	140.2 ± 24.5
Thyroid (mg/100 g)	6.7 ± 1.2	7.2 ± 1.3	6.5 ± 1.2	6.9 ± 1.1
Brain (g/100 g)	0.71 ± 0.05	0.74 ± 0.04	0.71 ± 0.04	0.72 ± 0.04

[a]Prostate: ventral prostate and dorsolateral prostate
*Significantly different from control at $P < 0.05$

group and control group, the terminal body weights in the males in the 1,000 mg/kg group were depressed in comparison with the control group (Table 1).

General observations

Frequency of urination was significantly increased in the males in the 1,000 mg/kg/day group after 4 weeks of administration (Table 2). No abnormalities were detected in other clinical signs and defects by the FOB.

Hematological findings

Activated partial thromboplastin time was prolonged in the male rats in the 40 and 1,000 mg/kg groups, but not in the 200 mg/kg group (Table 3). No abnormalities were detected in other parameters.

Clinical biochemical findings

A decrease in glutamic-oxaloacetic transaminase and an increase in γ-glutamyl transpeptidase were observed in the male rats in the 40 and 1,000 mg/kg groups, and an increase in albumin–globulin ratio and decrease in creatinine were observed in the male rats in the 1,000 mg/kg group (Table 3). No abnormalities were detected in other parameters.

Hormone analysis

The estradiol value was decreased in the male rats in the 1,000 mg/kg group (Table 4). No abnormalities were detected in other hormonal values.

Spermatological analysis

There were no abnormal spermatological findings in the rats given DEP.

Estrous cycles

There were no abnormalities in the estrous cycles of the rats given DEP.

Gross findings

No dose-related changes in gross findings were detected in either the male or female rats.

Organ weights

The results for the relative organ weight changes are shown in Table 2. In the female rats, increased kidney

Table 2 Frequency of urination (count/min, mean ± SD) in rats given diethylphthalate

Weeks	Male				Female			
	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)
Pre-dosing	0.10 ± 0.32	0.00 ± 0.00	0.20 ± 0.63	0.00 ± 0.00	0.90 ± 2.23	0.00 ± 0.00	0.10 ± 0.32	0.10 ± 0.32
1	0.10 ± 0.32	0.20 ± 0.42	0.20 ± 0.63	0.80 ± 1.14	0.10 ± 0.32	0.20 ± 0.63	0.00 ± 0.00	0.10 ± 0.32
2	0.00 ± 0.00	0.10 ± 0.32	0.50 ± 1.58	0.60 ± 1.07	0.20 ± 0.63	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.32
3	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.32	0.40 ± 0.70	0.10 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.70 ± 1.34*	0.10 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

*Significantly different from control at $P<0.05$

Table 3 Activated partial thromboplastin time (APTT), glutamic-oxaloacetic transaminase (GOT), γ-glutamyl transpeptidase (γ-GTP), albumin–globulin ratio (A/G ratio), and creatinine (mean ± SD) in rats given diethylphthalate

Items	Male				Female			
	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)
APTT (sec)	17.8 ± 2.1	22.0 ± 2.2*	19.5 ± 2.8	21.6 ± 2.7*	20.5 ± 2.6	20.6 ± 1.6	20.4 ± 2.3	20.1 ± 2.4
GOT (IU/l)	73 ± 14	56 ± 6*	70 ± 10	62 ± 7*	91 ± 18	74 ± 17	88 ± 21	75 ± 13
γ-GTP (IU/l)	0.3 ± 0.2	0.5 ± 0.2*	0.3 ± 0.2	0.6 ± 0.2*	0.6 ± 0.2	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.3
A/G ratio	0.89 ± 0.05	0.90 ± 0.05	0.89 ± 0.10	0.98 ± 0.06*	0.98 ± 0.08	0.96 ± 0.05	0.97 ± 0.08	0.96 ± 0.04
Creatinine (mg/dl)	0.26 ± 0.04	0.23 ± 0.03	0.24 ± 0.02	0.22 ± 0.01*	0.29 ± 0.03	0.27 ± 0.03	0.29 ± 0.02	0.28 ± 0.03

*Significantly different from control at $P < 0.05$

Table 4 Hormonal values (mean ± SD) in rats given diethylphthalate

Items	Male				Female			
	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)	Control ($n=10$)	40 mg/kg/day ($n=10$)	200 mg/kg/day ($n=10$)	1000 mg/kg/day ($n=10$)
T3 (ng/dl)	43 ± 9	43 ± 6	40 ± 9	42 ± 9	57 ± 8	64 ± 10	61 ± 8	60 ± 9
T4 (μg/dl)	4.05 ± 0.86	4.04 ± 0.53	4.09 ± 0.84	3.70 ± 0.60	4.46 ± 0.50	4.83 ± 0.83	4.54 ± 0.53	4.58 ± 0.52
TSH (ng/ml)	8.2 ± 1.4	9.6 ± 2.9	8.2 ± 1.2	8.3 ± 0.8	8.2 ± 1.5	10.3 ± 4.0	9.5 ± 2.6	8.3 ± 2.5
LH (ng/ml)	6.0 ± 1.7	6.0 ± 1.2	7.1 ± 2.4	6.6 ± 2.3	4.8 ± 1.6	4.7 ± 1.8	4.8 ± 1.3	4.6 ± 1.5
FSH (ng/ml)	94.5 ± 19.2	90.0 ± 21.6	110.7 ± 53.6	96.7 ± 30.2	71.7 ± 18.1	90.0 ± 19.3	89.2 ± 24.9	83.0 ± 24.5
Testosterone(ng/ml)	3.07 ± 1.79	2.89 ± 2.76	3.08 ± 2.86	3.77 ± 2.16	0.17 ± 0.04	0.16 ± 0.03	0.14 ± 0.04	0.15 ± 0.03
Estradiol (pg/ml)	7.9 ± 4.4	6.8 ± 3.3	6.2 ± 3.8	3.6 ± 1.6*	14.1 ± 7.4	16.8 ± 8.6	17.3 ± 8.6	18.9 ± 6.6

T3 Triiodothyronine, *T4* thyroxin, *TSH* thyroid-stimulating hormone, *LH* luteinising hormone, *FSH* follicle-stimulating hormone*Significantly different from control at $P < 0.05$

weight was observed in the 40 and 1,000 mg/kg groups, and increased adrenal weight was detected in the 1,000 mg/kg group.

Histopathological findings

No dose-related histological changes were detected in either the male or female rats.

Discussion

In the previous study, we performed the Hershberger assays on three phthalate esters having no receptor-binding affinity, i.e., phthalic acid di-*n*-hexyl ester, phthalic acid di-*n*-amyl ester, and phthalic acid di-*n*-propyl ester, and the results showed weak androgen antagonistic affinity in the assay of phthalic acid di-*n*-amyl ester (Yamasaki et al. 2004b). We also performed the uterotrophic assay on these three chemicals, and the results showed no uterotrophic property in any of the three chemicals (Yamasaki et al. 2004a). Nor was any estrogenic agonistic property detected in dibutylphthalate in the uterotrophic assay (Yamasaki et al. 2002b). On the other hand, it has been reported that neither di-(2-ethylhexyl)phthalate nor its metabolites interact with human or rodent androgen receptors in transcriptional activation assays (Foster et al. 2001) and in utero and lactational di-(2-ethylhexyl)phthalate exposure inhibits sexually dimorphic central nervous system development (Moore et al. 2001). Since these findings demonstrated that some phthalates possess endocrine-mediated properties while others do not, we performed the present study to investigate whether the endocrine-mediated properties of DEP would be detected by the enhanced TG 407. It has been reported that estrogenic receptor-binding affinity has been confirmed for DEP and that its affinity is higher than that of bisphenol A, 4-nonyphenol, and 4-octhylphenol (Lutz and Kloas 1999). Other members of the phthalate family, such as di-(2-ethylhexyl) phthalate, on the other hand, have androgen antagonistic properties (Moore et al. 2001). The estradiol levels in the present study were decreased only in the males in the 1,000 mg/kg group, but it is difficult to determine whether the decrease was related to the endocrine-mediated effect of DEP. An increase in estradiol levels by the androgenic antagonist flutamide has been detected in the enhanced TG 407, but the increase was accompanied by other endocrine-mediated changes, i.e., a decrease in accessory sex gland weight and an increase in testosterone level (Toyoda et al. 2000). The estradiol changes observed in the males in the 1,000 mg/kg group were unaccompanied by any other endocrine-mediated changes in hormonal, organ weight, or histopathological parameters, and the estradiol values in the 1,000 mg/kg group were within our laboratory control data (1,000 mg/kg group: 3.6 ± 1.6 pg/ml; control data: 5.4 ± 3.9 pg/ml). On the other hand, estradiol levels in the enhanced TG 407 were found to be unchanged by the androgen agonistic chemical 17β-methyltestosterone, and estrogen agonistic and antagonistic chemicals genistein, tamoxifen, bisphenol A, methoxychlor, and ethynyl estradiol (Okazaki et al. 2001, 2002a; Yamasaki et al. 2002c; Kennel et al. 2003; Wason et al. 2003). We therefore concluded that the estradiol level changes observed in the males in the 1,000 mg/kg group were unrelated to endocrine-mediated effects.

Di-(2-ethylhexyl)phthalate and other phthalates have been reported to cause testicular toxicity (Gray and Butterworth 1980; Gangolli 1982; Cammack et al. 2003; Akingbemi et al. 2004). However, no testicular histopathological or organ weight changes and no abnormal findings in the spermatological analysis or serum hormone analysis were detected in this study, suggesting that DEP does not cause testicular disorders.

The decrease in body weight in the males in the 1,000 mg/kg/kg group was considered to be a toxicological effect of DEP, suggesting that the no-observed-effect level for DEP in this assay is 200 mg/kg/day. In addition, activated partial thromboplastin time was prolonged, glutamic-oxaloacetic transaminase and creatinine were decreased, and γ-glutamyl transpeptidase and albumin–globulin ratio were increased in the males in the 1,000 mg/kg group. Liver injury, manifested by an increase in serum glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, and other parameters, by DEP has been detected in rats given 50 ppm DEP in their diet for 120 days (Sonde et al. 2000). In the present study, the decrease in glutamic-oxaloacetic transaminase and increase in γ-glutamyl transpeptidase were unaccompanied by liver weight changes or histopathological changes in the liver, and no dose-relationship was apparent. We therefore concluded that there was no clear evidence of liver injury in this study. The changes in activated partial thromboplastin time, albumin–globulin ratio, and creatinine levels were mild, and they were also concluded not to be abnormal based on our reference laboratory data. We could not explain why the changes of activated partial thromboplastin time, glutamic-oxaloacetic transaminase, and γ-glutamyl transpeptidase were not detected in the 200 mg/kg group and the changes of activated partial thromboplastin time, glutamic-oxaloacetic transaminase, γ-glutamyl transpeptidase, albumin–globulin ratio, and creatinine were only observed in male rats.

Histopathological examination is considered to be a good method of detecting endocrine-mediated effects and other toxicity effects (Toyoda et al. 2000; Yamasaki et al. 2002a, c; Kennel et al. 2003; Wason et al. 2003). Although a slight increase in kidney weight in the 40 and 1,000 mg/kg groups and an increase in adrenal weight in the 1,000 mg/kg group were observed, no histopathological changes were detected in these organs. Therefore, these organ weight changes related to DEP treatment were judged to be of little significance. On the other hand, we were unable to identify the mechanism of increase in urination frequency in the males in the 1,000 mg/kg group after

4 weeks of administration. This change may be related to osmotic diuresis.

In conclusion, we performed the enhanced TG 407 on one of the phthalates, DEP, and no endocrine-mediated properties were detected by the assay.

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TOXICOKINETICS AND METABOLISM

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Subacute oral toxicity study of di(2-ethylhexyl)adipate based on the draft protocol for the "Enhanced OECD Test Guideline no. 407"

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Abstract We performed a 28-day repeated-dose toxicity study of di(2-ethylhexyl)adipate (DEHA) based on the draft protocol of the "Enhanced OECD Test Guideline 407" to investigate whether it has endocrine-mediated properties according to this assay. DEHA was orally administered to SD rats at doses of 0, 40, 200 and 1,000 mg/kg/day for at least 28 days, and disturbance of the estrous cycle and increased ovarian follicle atresia were detected in the 1,000 mg/kg group.

Keywords Di(2-ethylhexyl)adipate · Enhanced · TG 407 · Rat · Endocrine effects

Introduction

Because a considerable number of chemicals have been reported to possibly have endocrine-disrupting activities in humans and animals (McLachlan 1993; McLachlan and Korach 1995), the Organisation for Economic Co-operation and Development (OECD) has proposed the use of uterotrophic assay, the Hershberger assay, and enhanced OECD test guideline no. 407 (enhanced TG 407) as in vivo screening tests to detect endocrine properties (OECD 1999). An international validation study of the enhanced TG 407 coordinated by the OECD has been conducted for various chemicals, but the final results have not yet been published. The enhanced TG 407 for various chemicals has recently been performed in several laboratories, and its usefulness as an in vivo screening test to detect endocrine-mediated effects has been confirmed (Toyoda et al. 2000; Andrews et al. 2001; Okazaki et al. 2001; Okazaki et al., 2002a, b; Yamasaki et al. 2002a, b; Kennel et al. 2003; Mellert et al. 2003; Wason et al. 2003; Kunimatsu et al. 2004).

Di(2-ethylhexyl)adipate (DEHA) is widely used as a plasticizer in an extensive array of products, and it also has been detected in ready-to-eat baby food (Petersen et al. 1998, 2000). A prolonged gestation period in the dams and an increase in postnatal deaths among their offspring have been reported when rats were given DEHA from pregnancy day 7 to weaning day 17 (Dalgaard et al. 2003). Urethal deformity and skeletal abnormalities have also been reported in the offspring of rats given DEHA on pregnancy days 1–22 (Hodge 1991). We therefore subjected DEHA to the enhanced TG 407 according to the OECD draft protocol for the enhanced TG 407 (OECD 1999, 2000) to investigate whether it has endocrine-mediated activities.

Materials and Methods

The present study was performed using Good Laboratory Practice guideline.

Chemicals

The di(2-ethylhexyl)adipate (DEHA, CAS No. 103-23-1, Lot No. LDJ4348, 99.8% pure) and corn oil (Lot No. WAL7450) used in this study were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Animals

Crj:CD (SD) rats were purchased from Charles River Japan, Inc (Shiga, Japan). Animals were weighed, weight-ranked, and randomly assigned to each of the treatment groups and control group before administration, and then housed individually in stainless steel, wire-mesh cages throughout the study. Rats were provided with water automatically and with a commercial

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diet (MF, Oriental Yeast Co., Tokyo, Japan). The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and was artificially illuminated with fluorescent light on a 12-h light/dark cycle (0700–1900 h). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by *the Japanese Association for Laboratory Animal Science.*

Study Design

Rats were orally gavaged with 0, 40, 200, and 1,000 mg/kg/day DEHA for at least 28 days from 8 weeks of age. The doses were selected on the basis of our preliminary test. A vehicle control group was gavaged with corn oil alone. The volume of the corn oil solution containing DEHA for gavage was 10 ml/kg. The concentration and stability of the DEHA were confirmed. Each group consisted of ten males and ten females. Animals were killed by exsanguination under ether anesthesia, and blood samples were obtained from the abdominal aorta and examined for hematological, clinical biochemistry and hormonal parameters.

General observations

Clinical signs were recorded daily. Once before the first dose and once a week thereafter, detailed clinical observations of all animals were made outside the home cage. The signs for which the animals were examined included changes in skin, fur, eyes, and mucous membranes, the frequency of urine and feces, and autonomic activity (e.g., lacrimation, piloerection, pupil size, respiratory pattern). Changes in gait, posture, response to handling, the occurrence of clonic or tonic movements, stereotypes (e.g., excessive grooming, circling), or bizarre behavior (e.g., self-mutilation, walking backwards), were also recorded. In the 4th week, a functional observation battery (FOB) that tested sensory reactivity to stimuli of different types (e.g., auditory, visual, and proprioceptive), assessed of grip strength, and assessed motor activity, was also conducted.

Body weight and food consumption

Individual body weight was recorded twice weekly and immediately before necropsy. Food consumption was measured weekly.

Hematology

The following were examined in the hematology examinations: red blood cell count, white blood cell count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count, reticulocyte count, prothrombin time, activated partial thromboplastin time, and differential leukocyte count.

Clinical biochemistry

Serum levels of the following were measured in the clinical biochemistry examination: glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, cholinesterase, γ-glutamyl transpeptidase, total cholesterol, triglyceride, glucose, total protein, albumin, blood urea nitrogen, creatinine, total bilirubin, calcium, inorganic phosphorus, sodium, potassium and chlorine, and the albumin–globulin ratio was calculated.

Hormone analysis

The serum concentrations of the following hormones were measured at the end of the test period: thyroid-stimulating hormone (TSH), thyroxin (T4), triiodothyronine (T3), testosterone, follicle-stimulating hormone (FSH), luteinising hormone (LH), and estradiol. T3 and T4 were determined with an automatic immunoassay system (IMX, Abott laboratories), and TSH, testosterone, FSH, and LH were measured with a microplate reader (UV max, Molecular Devices). Estradiol was analyzed by the Panafarm Laboratory (Udo, Japan) using a radioimmunoassay system (Auto-Gamma®5530 Gamma Counting System, Packard). The analyses of testosterone, FSH, LH, and estradiol were performed in addition to those in the current enhanced TG 407.

Spermatology

Sperm morphology (200 formalin-fixed, Giemza stained spermatozoa) and (heated to 70°C) the sperm count were determined by examining by using specimens obtained from the right epididymis. The number of homogenization-resistant (0.9% NaCl solution plus Triton-X100) sperm was determined on specimens from the right testis.

Estrous cycling

The estrous cycle of all females were assessed daily from day 22 to the day of sacrifice by examining vaginal smears stained with Giemza stain.

Necropsy

Males were necropsied on day 29. Females were necropsied after having been dosed for at least 29 days and sacrificed on days 30–34 in the diestrous stage.

Organ weights

The following organs were weighed after necropsy: testes, epididymides, ventral prostate, dorsolateral prostate, seminal vesicles, ovaries, uterus, adrenals, liver, spleen, kidneys, heart, brain, and thymus, as fresh organs, and the thyroid and pituitary gland after organ fixation.

Histopathology

The following organs were fixed in 10% neutral buffered formalin and examined: prostate, including ventral prostate and dorsolateral prostate, seminal vesicles, ovaries, uterus, vagina, mammary gland, brain, thyroid, adrenals, liver, spleen, kidneys, stomach, intestine, pancreas, thymus, parathyroids, and pituitary glands. The epididymes and testes were fixed in Bouin's solution before examining them.

Statistical analysis

Body weight, food consumption, hematological data, clinical biochemical data, organ weight, spermatological data (sperm counts) and FOB data were analyzed by the Bartlett test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. Following a significant difference in this analysis, the difference between the control group and each of the treatment groups was analyzed by the Dunnett's test. When the variances were not homogeneous, the Kruskal-Wallis test was used. Following a significant difference in this test, the difference between the control group and each of the treatment groups was analyzed by the nonparametric Dunnett's test. FOB countable data and spermatological data (sperm morphological data) were analyzed using the Kruskal-Wallis test. When there was a significant difference in this analysis, the difference between the control group and each of the treatment groups was analyzed by the nonparametric Dunnett's test.

Results

Body weight and food consumption

There were no abnormal findings in regard to body weight or food consumption (Table 1).

General observations

Although a female rat in the 200 mg/kg group died as a result of technical error of dosing 14 days after administration, there was no evidence of toxicity in any of the groups.

Hematological findings

No abnormalities were detected in either the male or the female rats.

Clinical biochemistry findings

No abnormal findings were detected in either the male or the female rats.

Hormonal analysis

No abnormal findings were detected in either the male or the female rats.

Spermatological analysis

No abnormalities were detected in the male rats.

Estrous cycles

In two rats in the 1,000 mg/kg group, the estrous stage persisted until the day of sacrifice 4 and 10 days, respectively, later (Table 2).

Gross findings

A clear spotty pattern was detected in the kidneys of two male rats in the 1,000 mg/kg group.

Organ weights

The results for the relative organ weight changes are shown in Table 1. In the male rats, increased kidney weight was observed in the 200 and 1,000 mg/kg groups, and increased liver weight in the 1,000 mg/kg group. In the female rats, increased liver, kidney, and adrenal weights were observed in the 1,000 mg/kg group.

Histopathological findings

The abnormal histopathological findings are summarized in Table 3. In the male rats, increased eosinophilic bodies and hyaline droplets were observed in the kidneys of the 1,000 mg/kg group. In the female rats, increased ovarian follicle atresia was detected in four rats in the 1,000 mg/kg group, and prolongation of the estrous stage was detected in two of these four rats.

Discussion

Although DEHA is widely used as a plasticizer in an extensive array of products, few data from toxicity

Table 1 Body and relative organ weights (mean ± SD) in rats given di(2-ethylhexyl)adipate

	Control	40 mg/kg/day	200 mg/kg/day	1,000 mg/kg/day
Male				
Body weights (g)				
Initial	310.7 ± 13.4	311.6 ± 14.3	308.3 ± 13.3	311.0 ± 13.5
Terminal	428.6 ± 31.3	427.7 ± 38.2	420.7 ± 37.8	427.4 ± 34.0
Organ weights				
Testes (g/100 g)	0.78 ± 0.05	0.75 ± 0.07	0.78 ± 0.06	0.76 ± 0.06
Epididymides (g/100 g)	0.26 ± 0.02	0.26 ± 0.03	0.26 ± 0.01	0.25 ± 0.03
Prostate (g/100 g)	0.23 ± 0.03	0.24 ± 0.05	0.22 ± 0.05	0.23 ± 0.03
Ventral prostate (g/100 g)	0.13 ± 0.02	0.15 ± 0.04	0.14 ± 0.03	0.14 ± 0.02
Dorsolateral prostate (g/100 g)	0.10 ± 0.02	0.10 ± 0.03	0.09 ± 0.02	0.10 ± 0.01
Seminal vesicle (g/100 g)	0.33 ± 0.05	0.36 ± 0.06	0.33 ± 0.06	0.33 ± 0.04
Pituitary gland (mg/100 g)	2.7 ± 0.4	2.6 ± 0.2	2.8 ± 0.2	2.6 ± 0.1
Liver (g/100 g)	3.55 ± 0.27	3.50 ± 0.18	3.78 ± 0.38	4.29 ± 0.24**
Kidneys (g/100 g)	0.59 ± 0.04	0.59 ± 0.04	0.66 ± 0.04**	0.68 ± 0.05**
Heart (g/100 g)	0.30 ± 0.02	0.30 ± 0.01	0.31 ± 0.02	0.29 ± 0.02
Spleen (g/100 g)	0.15 ± 0.01	0.14 ± 0.01	0.16 ± 0.03	0.15 ± 0.02
Adrenals (mg/100 g)	11.6 ± 1.5	11.9 ± 1.6	12.0 ± 1.6	11.8 ± 1.7
Thymus (mg/100 g)	92.2 ± 16.2	82.7 ± 18.6	88.1 ± 25.0	83.5 ± 15.5
Thyroid (mg/100 g)	4.9 ± 1.4	4.6 ± 0.8	5.4 ± 1.4	4.4 ± 0.3
Brain (g/100 g)	0.47 ± 0.03	0.47 ± 0.04	0.48 ± 0.04	0.46 ± 0.03
Female				
Body weights (g)				
Initial	197.8 ± 8.1	197.7 ± 9.8	198.7 ± 9.7	196.9 ± 8.2
Terminal	257.4 ± 16.1	259.9 ± 22.8	254.9 ± 14.6	249.2 ± 16.2
Organ weights				
Ovaries (mg/100 g)	28.2 ± 4.5	29.4 ± 4.0	27.3 ± 2.0	24.1 ± 5.6
Uterus (g/100 g)	0.18 ± 0.03	0.17 ± 0.03	0.16 ± 0.03	0.19 ± 0.07
Pituitary gland (mg/100 g)	5.6 ± 0.6	5.2 ± 0.8	5.7 ± 0.6	5.1 ± 0.8
Liver (g/100 g)	3.60 ± 0.23	3.46 ± 0.30	3.51 ± 0.20	4.12 ± 0.12**
Kidneys (g/100 g)	0.61 ± 0.04	0.63 ± 0.05	0.63 ± 0.04	0.67 ± 0.04*
Heart (g/100 g)	0.33 ± 0.03	0.33 ± 0.02	0.33 ± 0.03	0.33 ± 0.02
Spleen (g/100 g)	0.22 ± 0.04	0.20 ± 0.04	0.18 ± 0.03	0.18 ± 0.02
Adrenals (mg/100 g)	23.3 ± 2.9	23.6 ± 2.2	23.2 ± 3.1	24.1 ± 3.2*
Thymus (mg/100 g)	148.3 ± 23.9	154.6 ± 39.0	141.9 ± 16.8	123.2 ± 34.2
Thyroid (mg/100 g)	6.9 ± 1.2	6.6 ± 1.3	6.7 ± 1.5	7.1 ± 1.6
Brain (g/100 g)	0.73 ± 0.05	0.72 ± 0.06	0.74 ± 0.07	0.74 ± 0.06

*Significantly different from control at $P < 0.05$; **Significantly different from control at $P < 0.01$

Table 2 Duration of estrous stage in female rats given di(2-ethylhexyl)adipate

Period of estrous stage	Number of rats showing abnormal estrous cycle			
	Control (n = 10)	40 mg/kg/day (n = 10)	200 mg/kg/day (n = 9)	1,000 mg/kg/day (n = 10)
1 day	9	10	9	8
2 days	1	0	0	0
3 days	0	0	0	0
4 days	0	0	0	1
10 days	0	0	0	1

Estrous cycle analysis was performed from the 22nd day to the day of sacrifice

studies to detect the endocrine-mediated effects have been published. Since the OECD proposed the enhanced TG 407 assay as a screening assay to detect endocrine-mediated effects (OECD 1999, 2000), we performed enhanced TG 407 of DEHA according to the OECD draft protocol for the enhanced TG 407 to investigate whether it has endocrine-mediated activities.

In the present study, ovarian follicle atresia was increased in four rats in the 1,000 mg/kg group, and two of these four rats had a prolonged estrous cycle. Since a change in the estrous cycle has been reported to be one of the important endpoints for detecting of endocrine-mediated effects in the enhanced TG 407 assay and other assays (Yamasaki et al. 2002b; Sawaki et al. 2003), the abnormal estrous cycles accompanied by histopathological changes appeared to be related to the endocrine-mediated effects of DEHA. No binding activity of DEHA to the estrogen receptors was detected in the

Table 3 Histopathological findings in rats given di(2-ethylhexyl)adipate

	Control	40 mg/kg/day	200 mg/kg/day	1,000 mg/kg/day
Male				
Kidney				
Increased eosinophilic bodies	1/10[a]	0/10	0/10	7/10
Increased hyaline droplets	1/10	0/10	0/10	8/10
Female				
Ovary				
Increase of follicle atresia	0/10	0/10	0/9	4/10

[a]affected number/examined number

yeast two-hybrid assay (Nishihara et al. 2000), and thus the findings in this study may be attributable to the disturbance of ovarian function according to the hypothalamic-pituitary-gonad axis following DEHA exposure.

A prolonged gestation period in dams and a dose-related increase in postnatal death among their offspring were observed in an in utero and lactational exposure assay of DEHA (Dalgaard et al. 2003), and the authors had stated that DEHA did not exhibit any antiandrogenic effects similar to those exerted by di(2-ethylhrxyl)phthalate, even though their chemical structure was similar and they both had a common metabolite, 2-ethtylhexanol. No abnormal findings were detected in the male rats in the present study. On the other hand, it should be noted that no clear endocrine-mediated effects of DEHA were detected in an in utero and lactational exposure assay, but that the effects of DEHA were detected in growing rats by the enhanced TG 407, suggesting that the enhanced TG 407 is a suitable screening assay for detection of endocrine-mediated activities.

Increased kidney weight and histopathological changes were observed in the male in the 200 and 1,000 mg/kg groups, and these changes were toxic effects of DEHA. Increased liver weight was also detected in the male and female rats in the 1,000 mg/kg group, and this change was appeared to be a toxic effect of DEHA. These findings indicated that the no-observed-effect level for DEHA in this assay is 40 mg/kg/day. We do not know the cause of the increased kidney weight and adrenal weight in the female rats in the 1,000 mg/kg group, but the increases were not accompanied by histopathological changes.

In conclusion, we performed the enhanced TG 407 of DEHA and detected a prolonged estrous stage associated with histopathological changes in the ovary by this assay.

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