2.5. Statistical analyses

The means and standard errors (S.E.) were calculated for the labeling index obtained by ELISA for each treatment group. The stimulation index (SI) relative to the concurrent AOO-treated vehicle control value was then calculated. The BrdU labeling index was analyzed simultaneously using the Bartlett test for homogeneity of variance. If the variances were homogeneous at a level of 5% significance, a one-way analysis of variance (one way-ANOVA) was performed. If the one way ANOVA produced a significant difference, the difference between the control group and each of the experimental groups was analyzed using the Dunnett test. If the variances were not homogeneous, the Kruskal–Wallis test was employed. If this test produced a significant difference, the difference between the control group and each of the experimental groups was analyzed using the non-parametric Dunnett test (Bruning and Kintz, 1997). The estimated concentration of a chemical required to induce a SI of three relative to vehicle-treated controls (EC3 value) was derived by linear interpolation as described previously (Basketter et al., 2000). The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and one immediately below, the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the following equation:

$$EC3 = c + [(3 - d)/(b - d)](a - c)$$

3. Results

HCA has been reported to induce stable responses in the standard LLNA (Dearman et al., 2001). To evaluate the utility of various statistical methods for enhancing the sensitivity of the non-RI LLNA two independent experiments were performed. HCA was tested at application concentrations ranging from 0 to 50% in each of the two experiments. In both cases clear dose responses were observed (Fig. 1). In the context of BrdU incorporation it is apparent that concentrations of HCA in excess of 25% were required to provoke a SI of 3 or greater; the criterion for a positive response in the standard LLNA (Kimber and Basketter, 1992; Dearman et al., 1999). However, if the statistical significance of allergen-induced changes in LNC turnover relative to vehicle control values was measured instead then a different picture emerged. A significant treatment related increase in BrdU incorporation was recorded with 25% HCA in Experiment 1, and with 25 and 50% HCA in Experiment 2 (Table 1).

4. Discussion

The standard LLNA is used for the identification of chemicals that have the potential to cause skin sensitization (Kimber et al., 1994; Loveless et al., 1996). The standard LLNA using radioisotopes is a robust and reliable method for hazard identification and has been evaluated fully and validated formally for this purpose. However, it requires specific facility and handling procedures to accommodate a RI endpoint. For this reason there has been some interest in exploring whether other, non-RI endpoints could be used as a read-

Table 1
Results of non-RI LLNA with HCA in two separate experiments

Group	*N*	Mean	S.E.	SI	Probability (%)
Experiment 1					
0(AOO)	4	0.068	0.002		
3.125%	4	0.079	0.009	1.16	91.20
6.25%	4	0.108	0.024	1.59	40.81
12.5%	4	0.127	0.011	1.87	6.64
25%	4	0.165*	0.034	2.42	2.14
Experiment 2					
0(AOO)	4	0.120	0.012		
12.5%	4	0.190	0.036	1.58	37.14
25%	4	0.289*	0.030	2.40	1.17
50%	4	0.436*	0.049	3.63	0.01

N, number of animals; S.E., standard error; SI, stimulation index. Significantly different from the concurrent vehicle control (parametric or non-parametric Dunnett test, $P < 0.05$).

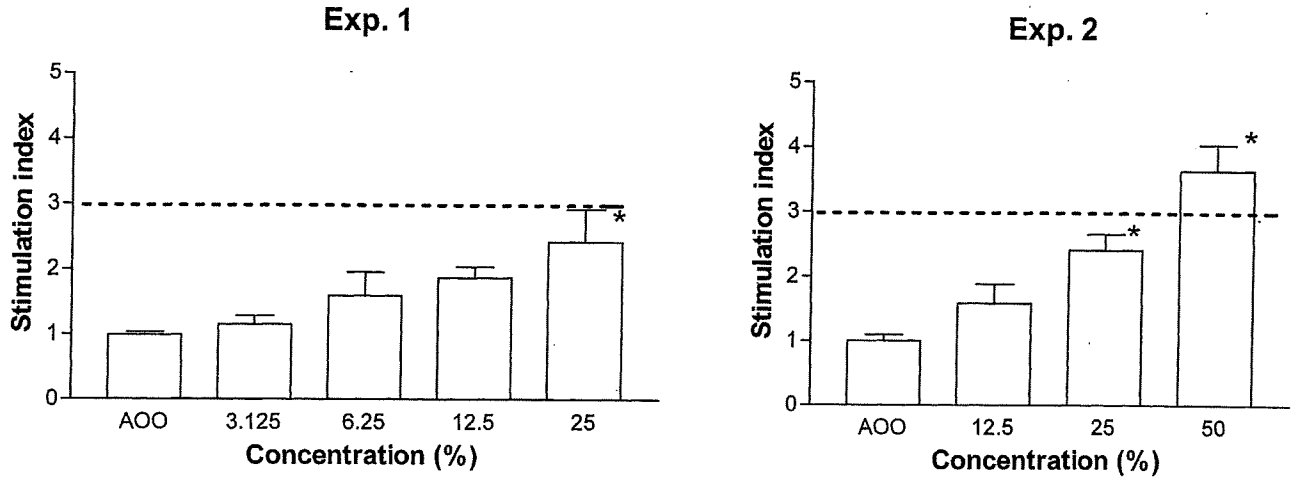

Fig. 1. Dose responses in non-RI LLNA with HCA. * Significantly different from the concurrent vehicle control (parametric or non-parametric Dunnett test, $P < 0.05$).

out for the LLNA. To this end, we have explored the utility of BrdU (Takeyoshi et al., 2001). Several other approaches to find non-RI endpoints for determining a proliferative response in LN have been reported such as the histochemical detection of BrdU (Boussiquet-Leroux et al., 1995), the measurement of interleukin 2 production by LNC (Hatao et al., 1995), flow cytometric analysis of B cell marker on LNC (Gerberick et al., 2002) and flow cytometric analysis of cell division as a function of carboxyfluoresein succinimidyl ether (CFSE) incorporation in LNC (Humphreys et al., 2003). Among them, B cell marker based analysis was found to elicit 1.93 of SI with 50% HCA using CBA/J or CBA/Ca mice, while EC3 of HCA with CFSE based cell division assay in BALB/c mice was estimated as around 5% which close to the EC3 value obtainable in the standard LLNA. In this study, EC3 of HCA was estimated as 37.2% using CBA/JN mice. SIs of 50% HCA for BALB/c and CBA mice were reported to be 10.9 and 9.4, respectively, (Woolhiser et al., 2000). These suggest that non-RI LLNA based on BrdU incorporation was apparently less sensitive than standard LLNA based on radioactive thymidine incorporation. We have no data with regard to the strain-related difference in sensitivity to BrdU. To establish the high sensitive non-RI endpoint, it is necessary to investigate the difference in the strain-related responsiveness to BrdU.

In these investigations, the alternative non-RI method required HCA concentrations of greater than 25% to elicit a positive response based on the criterion for classification as a skin sensitizer in the standard LLNA (the ability at one or more test concentrations to provoke a 3-fold or greater increase in LNC proliferation compared with concurrent vehicle controls; Kimber and Basketter, 1992). Nevertheless, dose responses to HCA in the alternative method were consistent in both experiments and we examined whether the use of an endpoint based upon the statistical significance of induced changes in LNC turnover, rather than an SI of 3 or greater, might provide for additional sensitivity. The results in a series of experiments with HCA clearly support the previous recommendation that an equivocal result would suggest to conduct a repeat test if possible using higher application concentrations (Kimber and Basketter, 1992), and a statistical analysis would also provide effective information to make decision whether the test chemical is a sensitizer or non-sensitizer. The results reported here demonstrate that with HCA at least significant responses were, in each of the two experiments, recorded following exposure of mice to 25% of the chemical. These data suggest that this approach may be more satisfactory—at least when BrdU incorporation is measured. However, even when such a statistical approach is employed, it is clear that this modification of the LLNA is rather less sensitive than the standard

method. It has been reported previously that when employing the standard version of the LLNA positive responses to HCA are observed at application concentrations lower than those required here. Thus, it has been calculated when using the standard assay that between 7 and 12.2% of HCA is necessary to provoke a 3-fold increase in LNC proliferative activity compared with vehicle controls (Dearman et al., 2001). Taken together the data reported here suggest that a modified LLNA in which BrdU is used in place of radioisotope incorporation shows some promise, but that in its present form, even with the use of a statistical endpoint, lacks some of the sensitivity of the standard method. The challenge is to develop strategies for further refinement of this approach.

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MOLECULAR TOXICOLOGY

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Development of a high-performance reporter plasmid for detection of chemicals with androgenic activity

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Abstract A number of chemicals are present in the environment, and some synthetic chemicals may disrupt endocrine function of wild animals and humans. An effective procedure to screen chemicals for endocrine modulating activity has been needed to ensure the safety of chemicals, and the reporter gene assay technique may provide a powerful tool for screening endocrine-disrupting chemicals. We have developed a high-performance reporter plasmid that can trigger high androgen-dependent induction with high selectivity by using mouse mammary tumor virus (MMTV) androgen-responsive elements and a partial fragment of the rat α_{2u}-globulin promoter region. This new type plasmid can induce higher transcriptional activation than a commercial PGV-P-based construct bearing the SV40 promoter fragment, and the basal induction level of this plasmid is much lower than that of the PGV-P-based construct. Moreover, only androgen derivatives could selectively induce a high response in the reporter gene assay with the new reporter plasmid. This new type of reporter plasmid, ARE-AUG-*Luc*+, should be of value in endocrine research and in screening to identify endocrine-modulating chemicals.

Keywords Androgen · α_{2u}-Globulin · Promoter · Endocrine · Reporter gene

Introduction

A number of chemicals are present in the environment, and some synthetic chemicals may disrupt the endocrine function of wild animals and humans. To ensure the safety of chemicals, an effective procedure to screen chemicals for endocrine-modulating activity has been needed by regulatory agencies in several countries, such as the United States Environment Protection Agency (US EPA) and Japan (EDSTAC 1998; OECD 2001). The US EPA developed a chemical screening and testing program consisting of a tiered system to evaluate the endocrine-disrupting effects of chemicals (Earl-Gray 1998). In this program, the hormone receptor-mediated reporter gene assay system is proposed for pre-screening and for the Tier 1 screening battery. The reporter gene assay technique has been used as a tool to investigate gene function, especially to test for enhancer or promoter activity of the regulatory sequences of various genes (Boffelli et al. 1999; Zhang and Teng 2000). Most natural hormones and endocrine-modulating chemicals exert their effects through the transcriptional activation of hormone-responsive genes. Thus, the reporter gene assay technique may be suitable for detecting hormonal activity of chemicals because it has been used to detect enhancers and promoter activity of genes. The reporter gene assay system may also provide a powerful tool to screen for endocrine-disrupting chemicals (Takeyoshi et al. 2002; Yamasaki et al. 2002).

We report here the establishment of a high-performance reporter plasmid that can trigger high androgen-dependent induction with high selectivity by using mouse mammary tumor virus (MMTV) androgen-responsive elements and a partial fragment of the rat α_{2u}-globulin promoter.

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Materials and methods

Test chemicals

The chemicals used in this study are listed in Table 1. All chemicals were dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) at concentrations of 10 mM, and the solutions were serially diluted in the same solvent at a common ratio of 1:10 with an automated pipetting device (Biomek 2000; Beckman Coulter Company, Tokyo, Japan) to prepare stock solutions with concentrations of 1 mM, 100 μM, 10 μM, 1 μM, 100 nM and 10 nM.

Table 1 Test chemicals and results of reporter gene assay using a new reporter plasmid, ARE-AUG200-*Luc*+

Category	Test chemical			Transcriptional activity	
	Name	CAS No.	Manufacturer	PC10(M)	PC50(M)
Natural and synthetic estrogen derivatives	17β-Estradiol	50-28-2	Wako	–	–
	17α-Estradiol	57-91-0	Wako	–	–
	Estrone	53-16-7	Wako	–	–
	Estriol	50-27-1	Wako	–	–
	Ethynyl estradiol	57-63-6	Aldrich	3.55E−06	–
3- or 5- Derivatives of estradiol	Estradiol valerate	979-32-8	Sigma	–	–
	β-Estradiol 3-benzoate	50-50-0	Wako	–	–
	β-Estradiol 3-benzoate 17-*n*-butyrate	63042-18-2	Sigma	–	–
	β-Estradiol 17-acetate	1743-60-8	Sigma	–	–
	Estriol 3-methyl ether	1474-53-9	Sigma	–	–
	Estrone 3-methyl ether	1624-62-0	Sigma	–	–
	Estradiol dipropionate	113-38-2	Sigma	–	–
	Mestranol	72-33-3	Wako	–	–
	β-Estradiol 17-cypionate	313-06-4	Sigma	–	–
	β-Estradiol 3-carboxymethyl ether	41164-36-7	Sigma	–	–
	β-Estradiol 17-hemisuccinate	7698-93-3	Sigma	–	–
	β-Estradiol 17-enanthate	4956-37-0	Sigma	–	–
	17α-Ethynylestradiol 3-cyclopentyl ether	152-43-2	Sigma	–	–
	Estrone acetate	901-93-9	Sigma	–	–
	Estradiol 3-carboxymethyl ether	69260-14-6	Sigma	–	–
	β-Estradiol 17-propionate	3758-34-7	Sigma	–	–
	Estriol 3-benzyl ether	18650-87-8	Sigma	–	–
16 or 17- Stereoisomers/derivatives of estradiol	17-Epiestriol	1228-72-4	Sigma	–	–
	Estradiol 16,17-diacetate	805-26-5	Sigma	–	–
	Estriol triacetate	2284-32-4	Sigma	–	–
	16α-Hydroxyestrone	566-76-7	Sigma	–	–
	16-Ketoestradiol	566-75-6	Sigma	–	–
1- or 3- Derivatives of estradiol	2-Hydroxyestradiol	362-05-0	Sigma	–	–
	4-Hydroxyestradiol	5976-61-4	Sigma	–	–
	2-methoxy-β-estradiol	362-07-2	Aldrich	–	–
	2-Hydroxyestriol	1232-80-0	Sigma	–	–
	3-Deoxyestrone	53-45-2	Sigma	3.29E−07	–
	4-Hydroxyestrone	3131-23-5	Sigma	–	–
	2-Methoxyestrone	362-08-3	Sigma	–	–
Other estradiol derivatives	6β-Hydroxyestradiol-17β	547-81-9	Sigma	–	–
	6α-Hydroxyestradiol	1229-24-9	Sigma	–	–
	6-Ketoestradiol	571-92-6	Sigma	–	–
	6-Dehydroestrone	2208-12-0	Sigma	–	–
	6-Ketoestradiol 6-(*o*-carboxymetyl)oxime	35048-47-6	Sigma	–	–
	6-Ketoestrone	1476-34-2	Sigma	–	–
	Equilin	474-86-2	Sigma	–	–
Androgen derivatives	Testosterone	58-22-0	Wako	1.40E−09	1.24E−08
	Methyltestosterone	58-18-4	Wako	2.00E−11	1.71E−10
	4,5α-Dihydrotestosterone	521-18-6	Wako	2.98E−10	2.93E−09
	Norethynodrel	68-23-5	Sigma	2.52E−09	2.13E0−7
	Norethindrone	68-22-4	Sigma	7.57E−10	3.57E−09
	4-Androstene-3,17-dion	63-05-8	Sigma	3.97E−09	2.86E−08
	Levonorogestrel	797-63-7	Sigma	3.19E−11	4.15E−10
	Testosterone enanthate	315-37-7	Wako	2.22E−08	5.55E−07
Other steroids	Androsterone	53-41-8	Wako	1.18E−07	–
	5α-Androstane	438-22-2	Sigma	–	–
	5α-Androstan-3β-ol	1224-92-6	Sigma	–	–
	Progesterone	57-83-0	Sigma	7.05E−09	–
	Corticosterone	50-22-6	Sigma	–	–
	5α-Androstane-3β,17β-diol	571-20-0	Sigma	6.70E−06	–
	Dehydroisoandrosterone	53-43-0	Wako	–	–
Non-steroids	*p-sec*-Butylphenol	99-71-8	TCI	–	–
	p-t-Butylphenol	98-54-4	TCI	–	–
	Nonylphenol	84852-15-3	TCI	–	–
	Bisphenol A	80-05-7	TCI	–	–
	Diethylstilbestrol	56-53-1	Wako	–	–

Cells

A *Cercopithecus aethiops* kidney cell line (CV-1, ATCC No. CCL-70) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and supplemented with 10% dextran-coated-charcoal (DCC)-treated fetal bovine serum (FCS; Gibco BRL, Rockville, MD, USA) at 37°C in a humid atmosphere containing 5% CO_2 (Miller et al. 2000). The cells were passaged every 3–5 days at 60–80% confluence.

Plasmid construction and transient transfection assay

The full open reading frame of human androgen receptor (AR) coding cDNA (Genbank Accession No. M20132) was cloned into the pcDNA3.1 mammalian expression vector (Invitrogen Corp., Groningen, Netherlands) to make AR/pcDNA3.1. Rat α_{2u}-globulin promoter fragment (Genbank Accession No. U28152) was cloned by using the TaKaRa LA PCR in vitro cloning Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions with gene-specific and adapter-specific primers from rat genomic DNA. An oligonucleotide containing three copies of the mouse mammary tumor virus (MMTV) androgen-responsive element (ARE), 5′-TGAGGTACCA AGCTAGAACA GCATGTTCTG ATCAAGCTAG AACAGCATGT TCTGATCAAG CTAGAACAGC ATGTTCTGAT CGAGCTCTTG-3′, was synthesized and concatenated in the upstream region of the partial rat α_{2u}-globulin promoter fragments. This concatenated fragment was cloned into the multiple cloning site of PGV-B vector (PicaGene basic vector; Toyo Ink MFG Co. Ltd., Tokyo, Japan) to create reporter vector ARE-AUG-*Luc*+. PGV-P vector (PicaGene promoter vector; Toyo Ink MFG Co. Ltd.) bearing SV40 promoter, which was also employed to evaluate promoter performance.

CV-1 cells grown in 90-mm dishes to 60–80% confluence were washed twice with phosphate-buffered saline (PBS). The cells were then transiently transfected with both 2 μg AR/pcDNA3.1, designed to express human AR, and 4 μg ARE-AUG-*Luc*+, designed to detect ARE-mediated transcriptional activation, with 12 μl LipofectAMINE (Gibco BRL) and 60 μl PLUS reagent (Gibco BRL) according to the manufacturer's protocol. After overnight incubation, the cells were trypsinized, resuspended in EMEM without phenol red containing 10% DCC-treated fetal bovine serum, and plated on a flat-bottomed microplate (Corning Coster Corp., Cambridge, MA, USA) at a density of 10^4 cells/well. Each test chemical, diluted in DMSO, was added to the wells at final concentrations of 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-11}–10^{-5} M), in quadruplicate. Positive control wells ($n=6$) treated with a natural ligand (10 nM dihydrotestosterone, DHT), and negative control wells ($n=6$) treated with DMSO alone were also prepared on every assay plate. After adding the chemicals to induce the reporter gene product, the assay plates were incubated for 24 h. After washing three times with PBS, the cells were lysed with cell culture lysis reagent (CCLR; Promega Corp., Madison, WI, USA). Luciferase activity was measured with the commercial luciferase assay reagent (Promega Corp.) and a luminometer (LUMIstar; BMG, Durham, NC, USA) as the integrated value over 5 s. The luminescence signal data were processed, and the average and standard deviation for the negative control wells were calculated. The integrated value for each test well was divided by the average integrated value of the negative control wells to obtain individual relative transcriptional activity. The average transcriptional activity was then calculated for each concentration of the test chemical. The PC50 and PC10 values were calculated to evaluate the AR agonist potency of each chemical. These PC values were defined as the concentration of chemical estimated to cause 50% or 10%, respectively, of activity of the positive control response (Takeyoshi et al. 2002; Yamasaki et al. 2002). Descriptions of these parameters are provided in Fig. 1.

Results

We prepared three types of plasmids, ARE-AUG150-*Luc*+, ARE-AUG200-*Luc*+, and ARE-AUG250-*Luc*+, concatenated with different lengths of rat α_{2u}-globulin promoter fragment (Fig. 2). PC50 values calculated for each plasmid were 2.85×10^{-9} M (ARE/PGV), 2.34×10^{-9} M (ARE-AUG100-*Luc*+), 2.93×10^{-9} M (ARE-AUG150-*Luc*+) and 3.21×10^{-9} M (ARE-AUG200-*Luc*+). The values (PC50) were identical for each of the plasmids used in the assay system. However, since ARE-AUG200-*Luc*+ showed the highest induction level for dihydrotestosterone (Fig. 3), suggesting that ARE-AUG200-*Luc*+ had a suitable promoter length for detection of androgen receptor-mediated transcriptional activation, we used ARE-AUG200-*Luc*+ as the standard plasmid to test chemicals for androgenic activity. We confirmed antagonist detectability with these plasmids when cyproterone acetate was used as antagonist against 0.5 nM of DHT (data are not shown).

In the experiment with the positive control substance, 10 nM DHT, the androgen-inducible transcriptional activation level of the plasmid was higher than that of the commercial PGV-P-based construct bearing the SV40 promoter fragment, and the basal induction level of the plasmid was much lower than that of the PGV-P-based construct (Fig. 4).

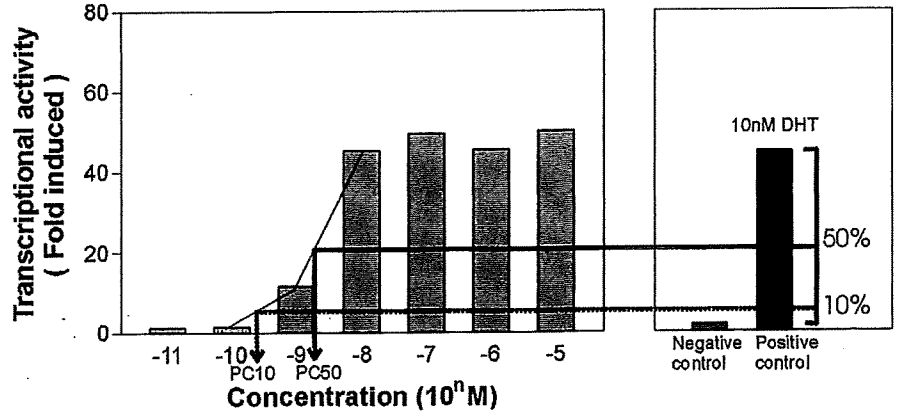

Fig. 1 Schema for the estimation of PC50 and PC10 values for evaluation of the hormonal activity of chemicals (*DHT* dihydrotestosterone)

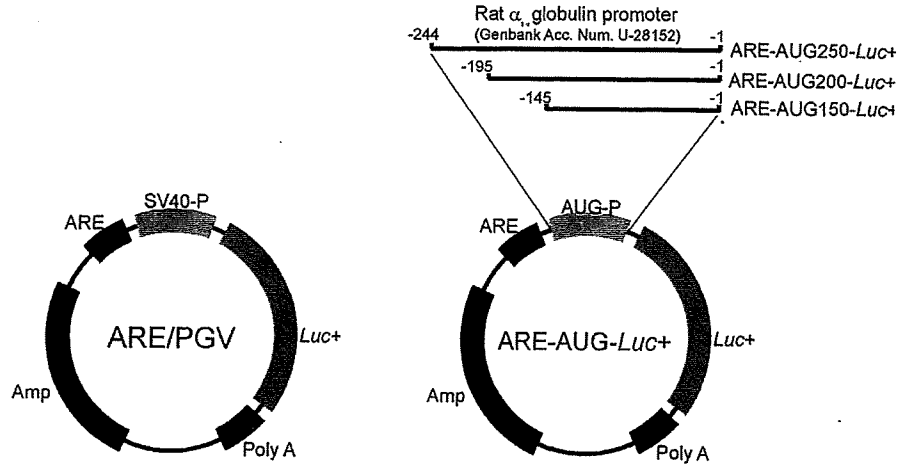

Fig. 2 Structure of hormone-responsive reporter plasmid (ARE-AUG-*Luc*+) used in this study, together with that of a commercially available plasmid (ARE/PGV) *ARE* androgen-responsive elements, *AUG-P* rat α_{2u}-globulin promoter fragment

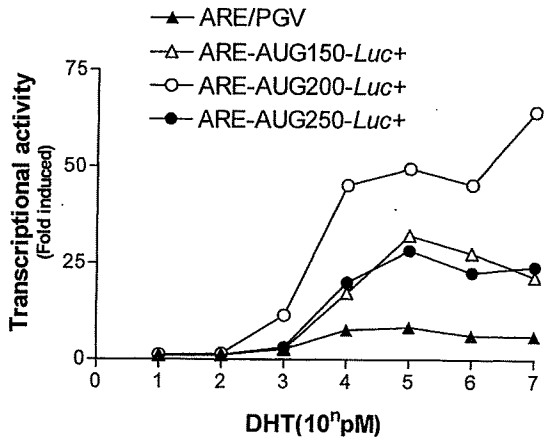

Fig. 3 Transcriptional activity of the androgen-responsive elements (ARE)-mediated reporter gene assay with different types of reporter plasmids. Values are presented as the mean transcriptional activity (of dihydrotestosterone) from quadruplicate assays

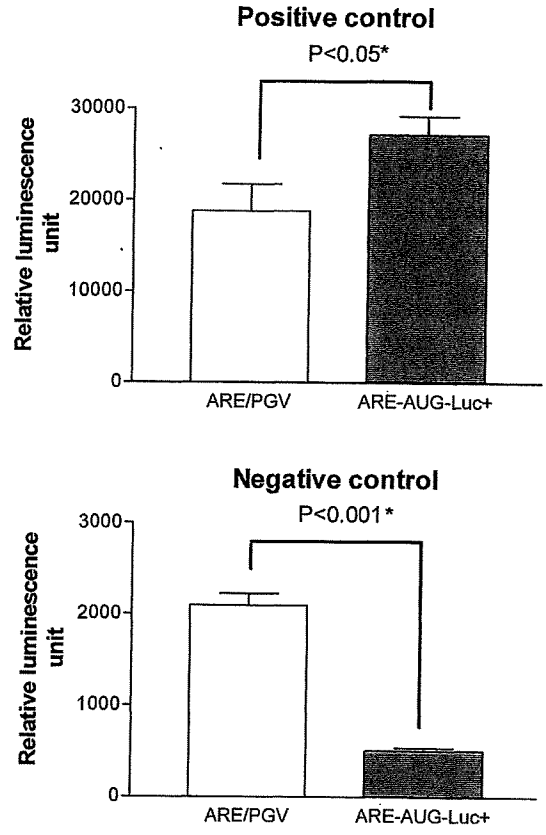

Fig. 4 Comparison of positive and negative control responses in reporter gene assay using ARE-AUG200-*Luc*+ or commercially available plasmid (PGV/ARE). *Data were analysed by Student's *t*-test

The results of a pilot study of 56 steroid derivatives and five non-steroid chemicals are shown in Table 1. PC50 values were calculated only for androgen derivatives, including testosterone, methyltestosterone, 5α-dihydrotestosterone, norethynodrel, norethindrone, 4-androstene-3,17-dione, levonorogestrel and testosterone enanthate (Fig. 5). PC10 values were calculated for ethinyl estradiol, 3-deoxyestrone, androsterone, progesterone, and 5α-androstene-3β,17β-diol, in addition to the compounds for which the PC50 was calculated.

Discussion

Tens of thousands of chemicals are currently used around us and many of them escape into the environment as pollutants. However, since some of the synthetic chemicals that are widely distributed in our environment may mimic estrogens or otherwise disrupt the endocrine system, an effective procedure to screen chemicals for endocrine-modulating activity has been needed by regulatory agencies in several countries, including the USA and Japan, to ensure the safety of chemicals (EDSTAC 1998; OECD 2001). The US EPA developed a chemical screening and testing program consists of a tiered system to evaluate the endocrine-disrupting effects of chemicals (Earl-Gray 1998), and the hormone receptor-mediated reporter gene assay system is proposed for pre-screening and in the Tier 1 screening battery. Since no effective high-throughput screening procedure is available to detect androgen-active compounds, in this study we have developed a new plasmid construct that can be used for the reporter gene assay system to screen chemicals

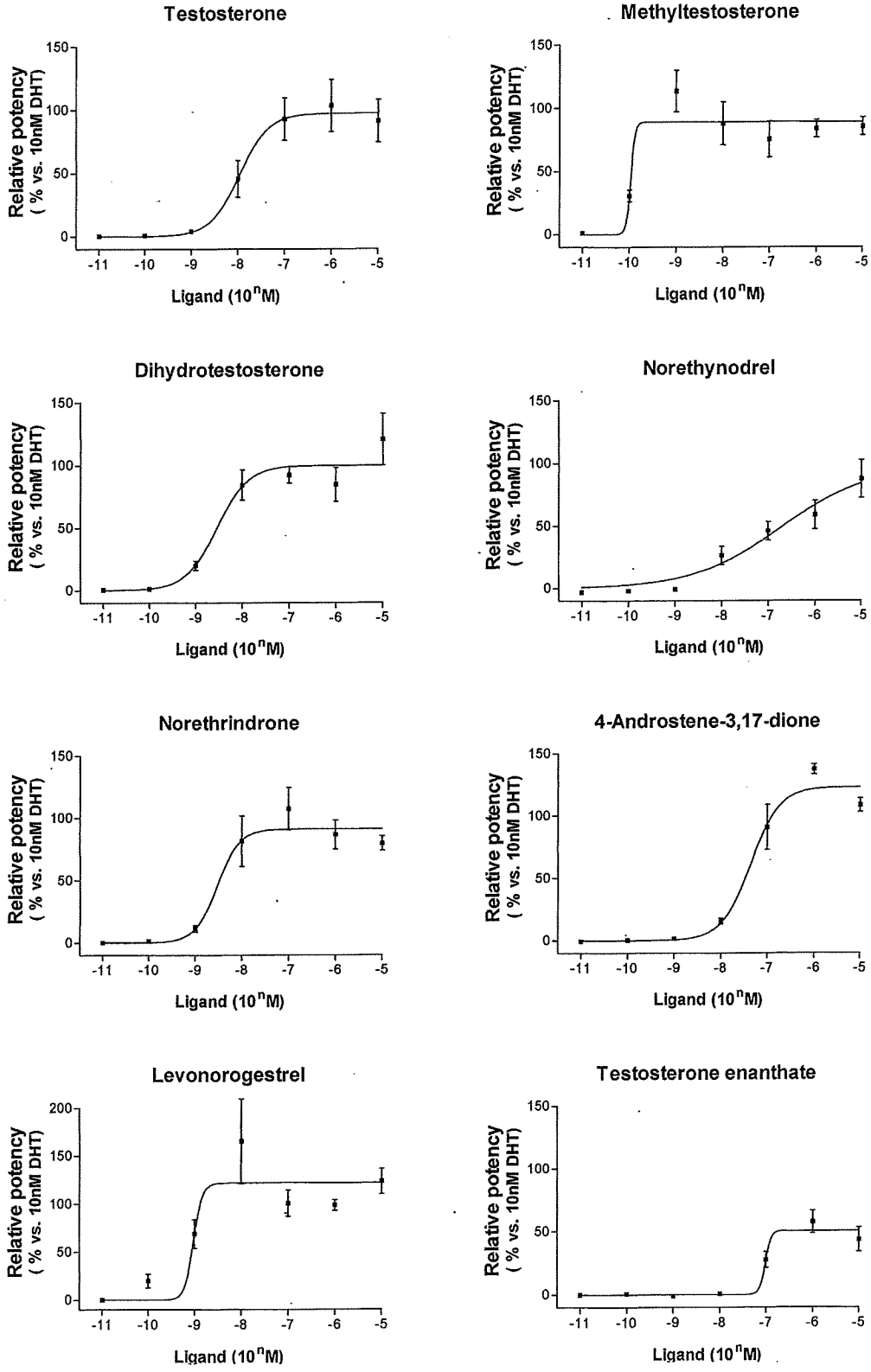

Fig. 5 Induction of luciferase activity by androgen derivatives in the HeLa cell transiently transfected with AR/pcDNA3.1 and ARE-AUG200-*Luc*+. Luciferase activities were expressed as the mean relative potency ±SE, relative to the positive control response induced by 10 nM dihydrotestosterone

for androgenic potential. A partial fragment of the rat α_{2u}-globulin promoter sequence was used as the basal promoter unit. The rat α_{2u}-globulin is known as a hormone-inducible protein, and its biosynthesis is under multi-hormonal regulation (Kulkarni et al. 1985; Kurtz et al. 1976; Roy and Neuhaus 1967; Roy et al. 1987).

The rat α_{2u}-globulin promoter fragment used in this plasmid has no hormone-responsive motifs, but the fragment seems to have properties suitable for constructing reporter plasmids to detect hormonal activity of chemicals. In this study, a new plasmid bearing rat α_{2u}-globulin promoter showed high hormonal induction with remarkably low background induction. A higher assay background induction for negative control with a lower maximum induction for positive control result in less sensitivity of the assay system because it would lead to a narrow dynamic range in the assay system. The new reporter plasmid ARE-AUG-*Luc*+ has excellent properties with regard to the assay background and positive induction levels when compared with a commercial plasmid. Accordingly, a sensitive reporter gene assay system would be established with this new reporter plasmid. Moreover, among 56 test chemicals consisting of several types of steroids, only the androgen derivatives selectively triggered a high response in the reporter gene assay using the new reporter plasmid, ARE-AUG-*Luc*+. Recently several reporter gene systems for detecting endocrine-active chemicals have been described (Gagne et al. 1994; Vinggaad et al. 1999; Balaguer et al. 1999, 2001; Miller et al. 2000) but most of them were intended for detecting estrogenic activity of chemicals, whereas our new plasmid construct is suitable for detecting the androgenic activity of chemicals. Adoption of high-throughput screening for the androgenic and anti-androgenic activity of chemicals will surely lead to safer chemicals, and the reporter gene assay system should provide a powerful tool to screen for endocrine-disrupting chemicals (Takeyoshi et al. 2002; Yamasaki et al. 2002). This new type of reporter plasmid, ARE-AUG-*Luc*+, will be of value in endocrine research and in the screening for endocrine-modulating chemicals.

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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.

Table 4. Histopathological findings of testes of fetuses exposed to di-(2-ethylhexyl) phthalate (DEHP) or ethinyl estradiol (EE) during gestational days 7-18 (Experiment 1).

Group	DEHP 0 mg/kg					DEHP 500 mg/kg					DEHP 1000 mg/kg					EE 0.25 mg/kg					EE 0.5 mg/kg				
Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Gestational day 12	(12)					(10)					(10)										(10)				
Degeneration of fetal tissue	12	0	0	0	0	10	0	0	0	0	8	0	0	2	0						2	0	0	8	0
Gestational day 14	(9)					(9)					(9)					(9)					(9)				
Multinucleated germ cells	8	1	0	0	0	9	0	0	0	0	8	1	0	0	0	9	0	0	0	0	8	1	0	0	0
Gestational day 16	(10)					(10)					(12)										(4)				
Degeneration of germ cells	10	0	0	0	0	10	0	0	0	0	11	0	0	1	0						4	0	0	0	0
Multinucleated germ cells	10	0	0	0	0	9	1	0	0	0	10	2	0	0	0						4	0	0	0	0
Gestational day 18	(20)					(20)					(10)					(11)					(16)				
Multinucleated germ cells	20	0	0	0	0	18	2	0	0	0	10	0	0	0	0	11	0	0	0	0	14	2	0	0	0
Increased germ cells in a cord	20	0	0	0	0	20	0	0	0	0	8	2	0	0	0	11	0	0	0	0	16	0	0	0	0
Hyperplasia of interstitial cells	20	0	0	0	0	8	12	0	0	0	0	3	7	0	0	11	0	0	0	0	16	0	0	0	0
Decrease in testicular size	20	0	0	0	0	20	0	0	0	0	4	0	6	0	0	11	0	0	0	0	9	7	0	0	0
Gestational day 20	(17)					(17)					(18)										(8)				
Multinucleated germ cells	16	1	0	0	0	0	0	10	7	0	0	3	13	2	0						4	4	0	0	0
Increased germ cells in a cord	17	0	0	0	0	14	3	0	0	0	1	12	5	0	0						5	3	0	0	0
Hyperplasia of interstitial cells	17	0	0	0	0	0	0	14	0	0	0	1	17	0	0						8	0	0	0	0
Decrease in testicular size	17	0	0	0	0	17	0	0	0	0	5	8	5	0	0						5	3	0	0	0

− : negative, ± : very slight, + : slight, ++ : moderate, +++ : severe.
Figures in parentheses show number of dams examined.

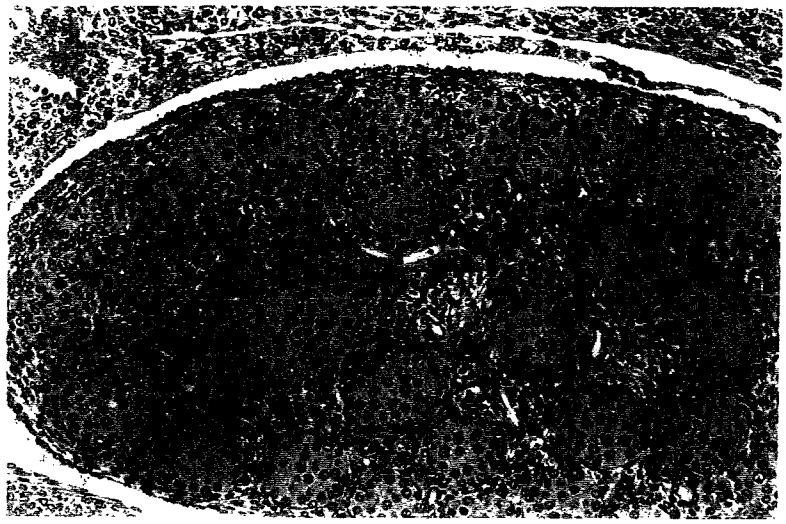

Photo 1-a. Transverse section of a fetus from the control group on G16 showing the genital ridge. HE stain, ×160.

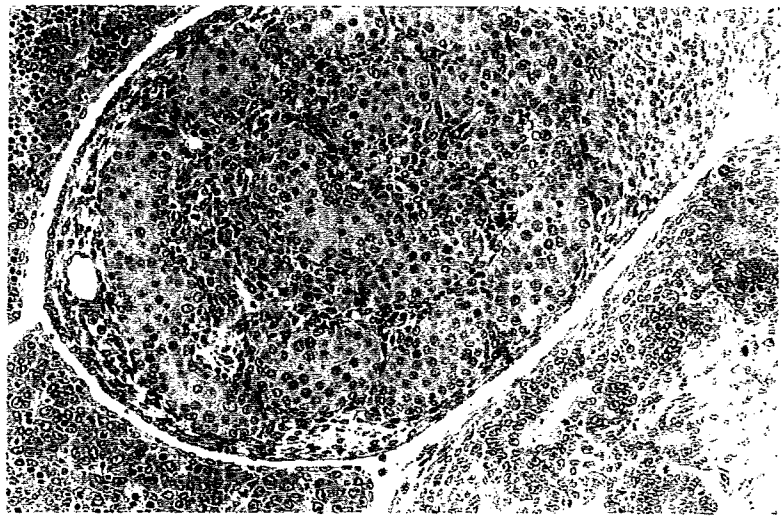

Photo 1-b. Transverse section of a fetus from the 1000 mg/kg DEHP group on G16 showing no abnormality in the genital ridge. HE stain, ×160.

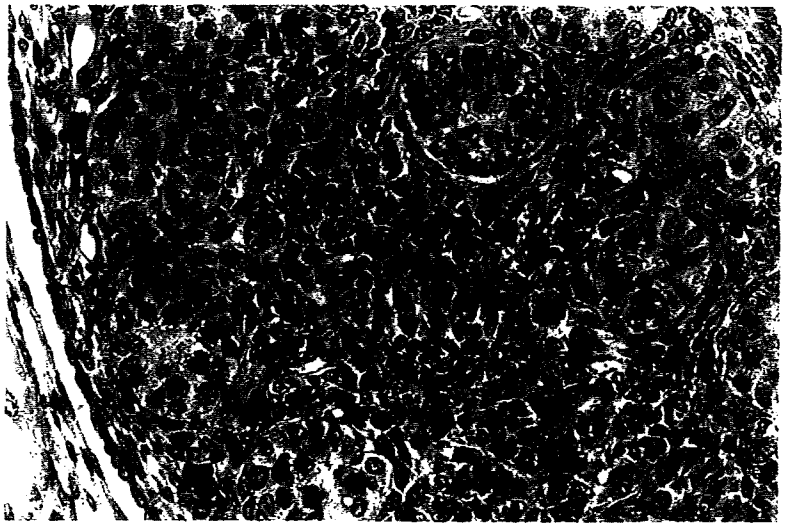

Photo 1-c. Transverse section of a fetus from the 1000 mg/kg DEHP group on G16 showing the genital ridge. Many germ cells are degenerated and densely stained. HE stain, ×310.

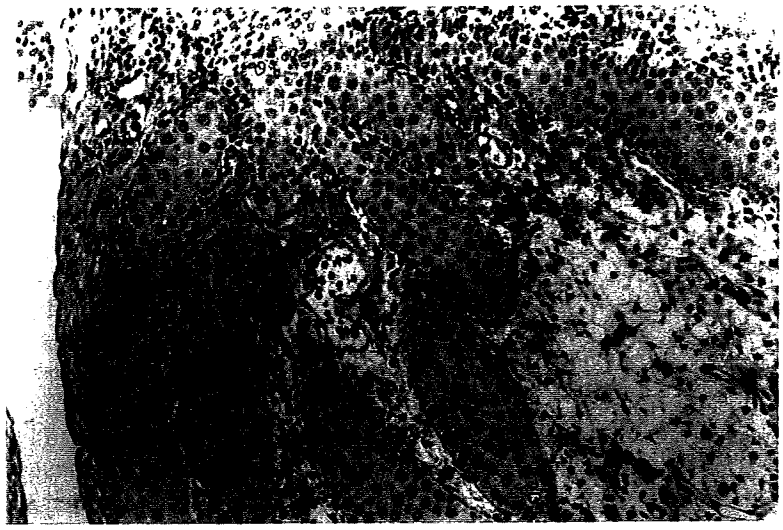

Photo 2-a. Testis of a G18 rat fetus from the control group. HE stain, ×160.

Photo 2-b. Testis of a G18 fetus from a rat treated with 500 mg/kg of DEHP showing hyperplasia of interstitial cells. HE stain, ×160.

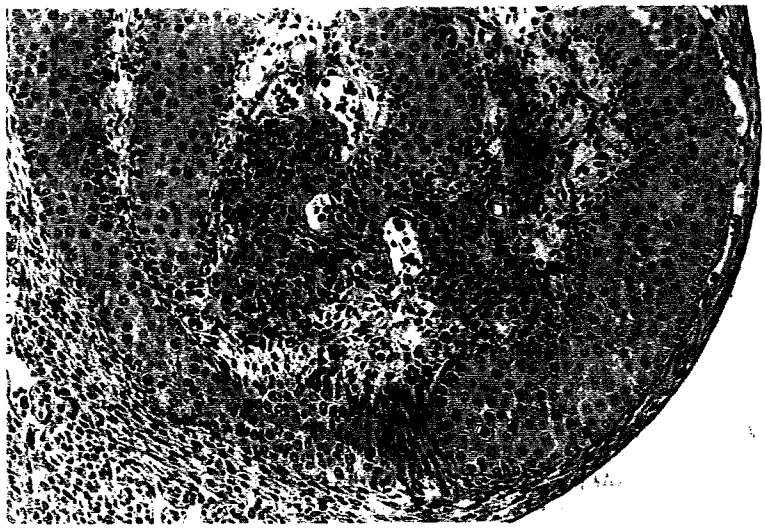

Photo 2-c. Testis of a G18 fetus from a rat treated with 1000 mg/kg DEHP showing hyperplasia of interstitial cells. HE stain, ×160.

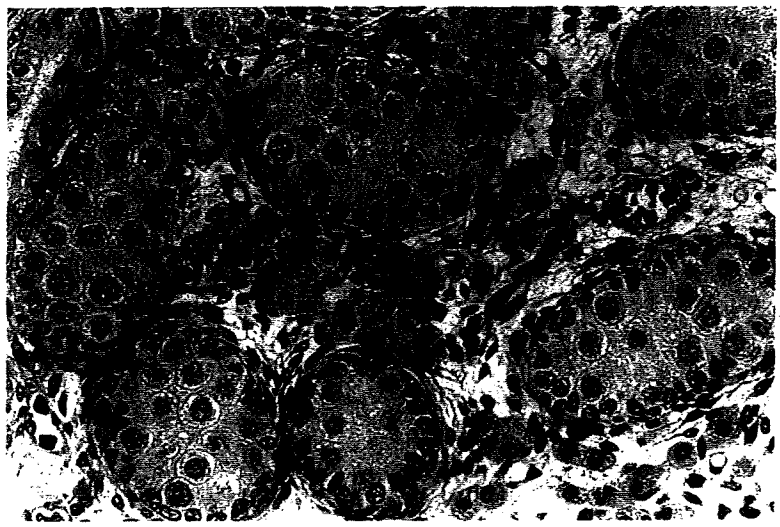

Photo 3-a. Testis of a G20 fetus from the control group showing the seminiferous cords and interstitial cells. HE stain, ×310.

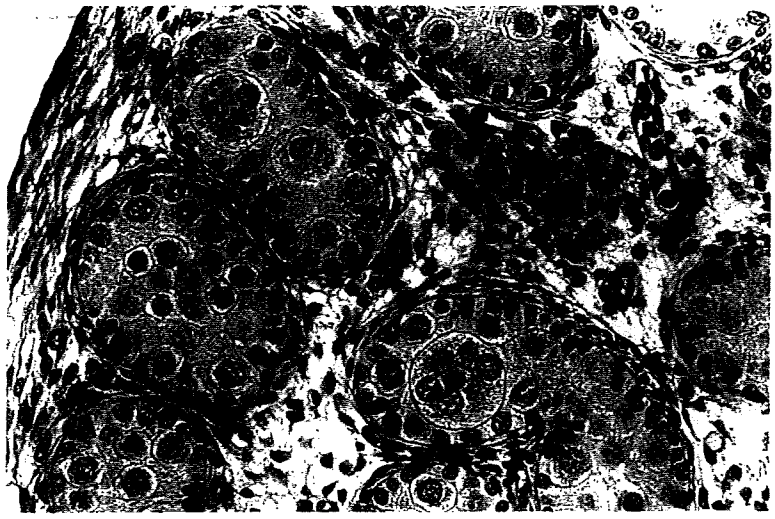

Photo 3-b. Testis of a G20 fetus from the group treated with 500 mg/kg of DEHP showing multinucleated germ cells in seminiferous cords. HE stain, ×310.

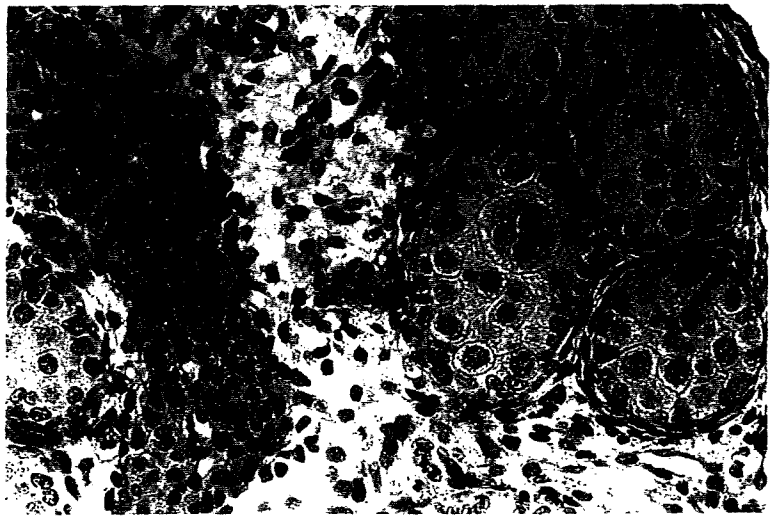

Photo 3-c. Testis of a G20 fetus from the group treated with 1000 mg/kg of DEHP showing multinucleated germ cells in seminiferous cords, and hyperplastic smaller-sized interstitial cells. HE stain, ×310.

5a, 5b). In their epididymides, atrophy was found in all of the animals and cell debris in the epididymal lumen was also found (Photos 6a, 6b).

Effects of the lower doses of DEHP on testicular development were examined in Experiment 2. Table 6 summarizes histopathological findings of fetal testes on G20 and testes of offspring at 5 and 10 weeks of age in Experiment 2. Multinucleated germ cells were found in the fetal testes of all the groups exposed to DEHP, although its incidence was very low in the 125 mg/kg group. In the groups exposed to 250 mg/kg and 500 mg/kg of DEHP, partly thickened germinal cords due to aggregation of increased number of germ cells and hyperplasia of the interstitial cells were observed. Degenerated germ cells and apoptosis were observed in a few animals in the group exposed to 500 mg/kg of DEHP. These findings are comparable to those in DEHP-exposed testes at the same dose in Experiment 1.

In contrast to the findings of the fetal testes, no abnormalities were found in testes of the offspring at 5 and 10 weeks of age in any group in histopathological examination. Furthermore, the seminiferous cycles in the testis determined at 5 weeks of age were comparable between control and DEHP-exposed groups (Table 7).

Electron microscopic findings of fetuses

Electron microscopic examination of fetal testes was performed in Experiment 1. In the fetal testis of the groups exposed to DEHP at 500 and 1000 mg/kg, degenerated germ cells were found in the testicular cord on G16 (Photo 7), and smaller-sized interstitial cells containing fewer lipid droplets were noted on G18 (Photo 8a). These changes of the interstitial cells became more obvious on G20 (Photo 8b).

In the fetal testis from the group exposed to EE at 0.5 mg/kg, degeneration of germ cells was found only on G14. No abnormalities such as those observed with DEHP treatment were found on G16, 18 and 20. Slightly swollen mitochondria and hyperplastic smooth endoplasmic reticulum were noted in interstitial cells on G18 and 20. Furthermore, degeneration of interstitial cells surrounded by neutrophils infiltration were observed on G20.

In examination of offspring at 5 and 10 weeks after birth in Experiment 2, ultrastructural changes were not observed in the testis and epididymides of any

Table 5. Histopathological findings in the testis and epididymis of offspring exposed to DEHP during gestational days 7-18 (Experiment 1) 7 weeks after birth.

Group	DEHP 500 mg/kg (6)					DEHP 1000 mg/kg (12)				
Grade	−	±	+	++	+++	−	±	+	++	+++
Testis										
Atrophy of seminiferous tubules	6	0	0	0	0	2	2	4	3	1
Multinucleated giant cells	5	1	0	0	0	0	3	5	4	0
Dilatation of seminiferous tubules	6	0	0	0	0	2	0	4	6	0
Dilatation of rete testis	6	0	0	0	0	8	2	1	1	0
Hyperplasia of interstitial cells	6	0	0	0	0	10	1	1	0	0
Necrosis	6	0	0	0	0	11	0	0	0	1
Mineralization	6	0	0	0	0	10	0	1	1	0
Foreign body giant cells	6	0	0	0	0	10	0	1	1	0
Focal loss of seminiferous tubules	6	0	0	0	0	11	0	1	0	0
Malformation of seminiferous tubules	6	0	0	0	0	11	0	1	0	0
Epididymis										
Atrophy	6	0	0	0	0	0	1	2	2	7
Cell debris in lumens	0	0	6	0	0	3	3	6	0	0
Dilatation of lumens	6	0	0	0	0	8	0	3	1	0
Infiltration of lymphocytes	6	0	0	0	0	8	2	2	0	0
Granuloma	6	0	0	0	0	11	0	0	1	0

Figures in parentheses indicate number of offspring examined.
− : not observed, ± : very slight, + : slight, ++ : moderate, +++ : severe.