Table 1. Distribution of silver grains on electron microscopic autoradiographs after single oral administration of DEHP.

Group	Organ Cell	Organelle	After administration 6 hr	 24 hr
[3,4,5,6-^{3}H]-Phthalic acid, di-(2-ethylhexyl)ester : DEHP-^{3}H	Testis			
	Sertoli cell	mitochondria	••••••	••
		s-ER	••••••••••••	••••••••
		r-ER		••••
		extracellular space	•••	••••••••••••••
		Golgi apparatus	•	
		lysosome	•	
	spermatogonia			••••••••
	spermatocyte		•••	••••••••
	spermatid			•••
	Liver			
	hepatocyte	mitochondria	••	••••••••••••
		r-ER	••••	•••••
		peroxisome	••••	••
	sinusoid			
	Kidney			
	proximal tubule	brush border	••••••••••	•
	epithelial cell	mitochondria	••••	•••••
		s-ER	••••	
		lysosome		••
		peroxisome	•••	
		Golgi apparatus	•	
Phthalic acid, di-(2-ethyl[1^{3}H]hexyl)ester : ^{3}H-DEHP			ND	ND

ND: not detected, •: count of silver grains.

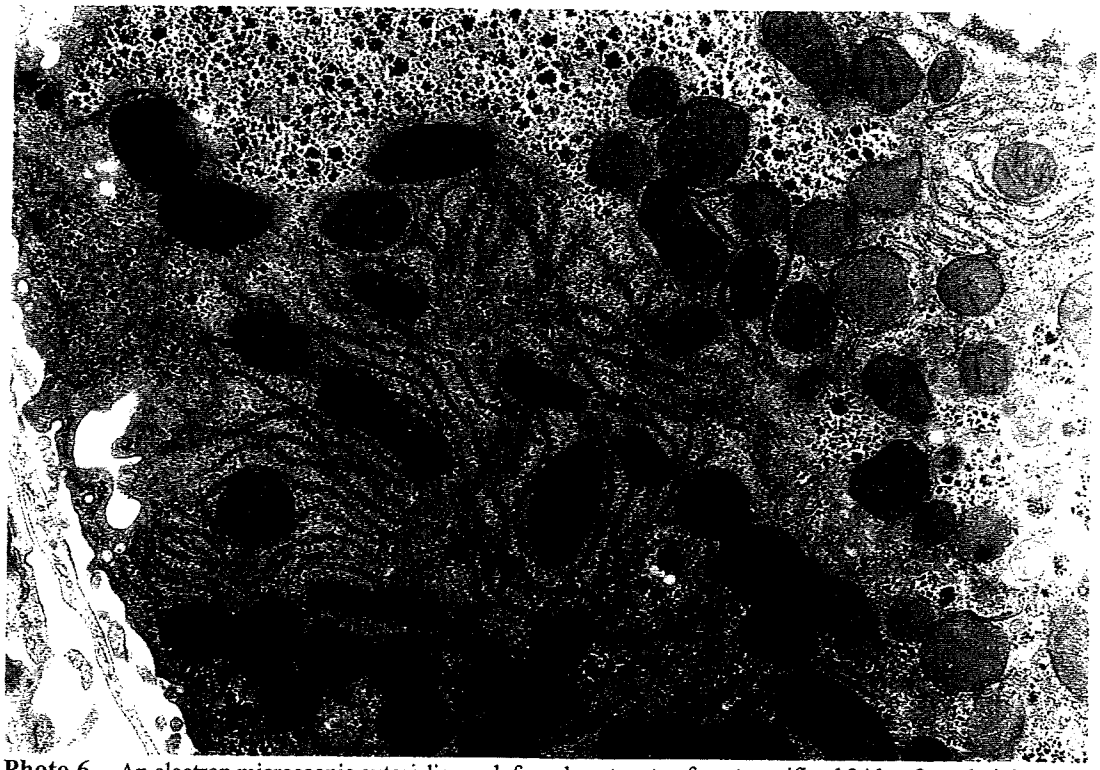


Photo 6. An electron microscopic autoradiograph from hepatocyte of a rat sacrificed 24 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few silver grains are noted on the mitochondria, rough-surfaced endoplasmic reticulum and peroxisome., × 14000.

alcohol was administered to 8-week-old rats of the Sprague-Dawley strain (92.5 MBq/rat). The rats were sacrificed and autopsied at 6 or 24 hr after administration, autoradiographs prepared for the testes, liver, and kidneys, and observed under light microscope or electron microscope.

On the autoradiographs of animals administered with DEHP-^{3}H, labeled at the phthalic acid, considerable distribution of radioactivity was observed in the testes at the basal area of seminiferous tubules at the stages from IX to I of spermatogenic cycle, which corresponded well to the location of DEHP toxicity observed in our previous study (Saitoh *et al.*, 1997). By electron microscopic observation, subcellular localization of radio-sensitized grains was observed at smooth-surfaced endoplasmic reticulum and mitochondria of Sertoli cells. Fewer grains were also noted at the Golgi apparatus and lysosome of Sertoli cells, and at the junctions between the neighboring Sertoli cells or between a Sertoli cell and spermatocytes. On the other hand, on the autoradiographs by administration of ^{3}H-DEHP, labeled at the alcohol moiety, only a few grains were observed by light microscopy in the testes at 6 hr after administration.

There are already many studies on the distribution of DEHP in the body. Schulz and Rubin (1973) studied the metabolism of ^{14}C-DEHP by oral administration in rats and reported that more than 80% of radioactivity was recovered within 24 hr, about 35% in urine and 55% in feces. Williams and Blanchfield (1974) administered ^{14}C-DEHP orally to rats, and observed that most of the radioactivity was excreted in urine and feces by 24 hr after administration and that the distribution of radioactivity among tissues was most highly to the spleen, testes, and adipose tissue, followed by kidneys and liver. Radioactivity levels in tissues reached the peak 4 hr after administration, and no radioactivity was observed 24 hr after administration. On the other hand, the study on oral administration of ^{14}C-DEHP by Daniel (1978) suggested that enterohepatic circulation of DEHP or its metabolites might have occurred, since 14% of radioactivity was excreted in bile for 4 days. Gaunt and Butterworth (1982) have conducted a study of whole-body autorad-

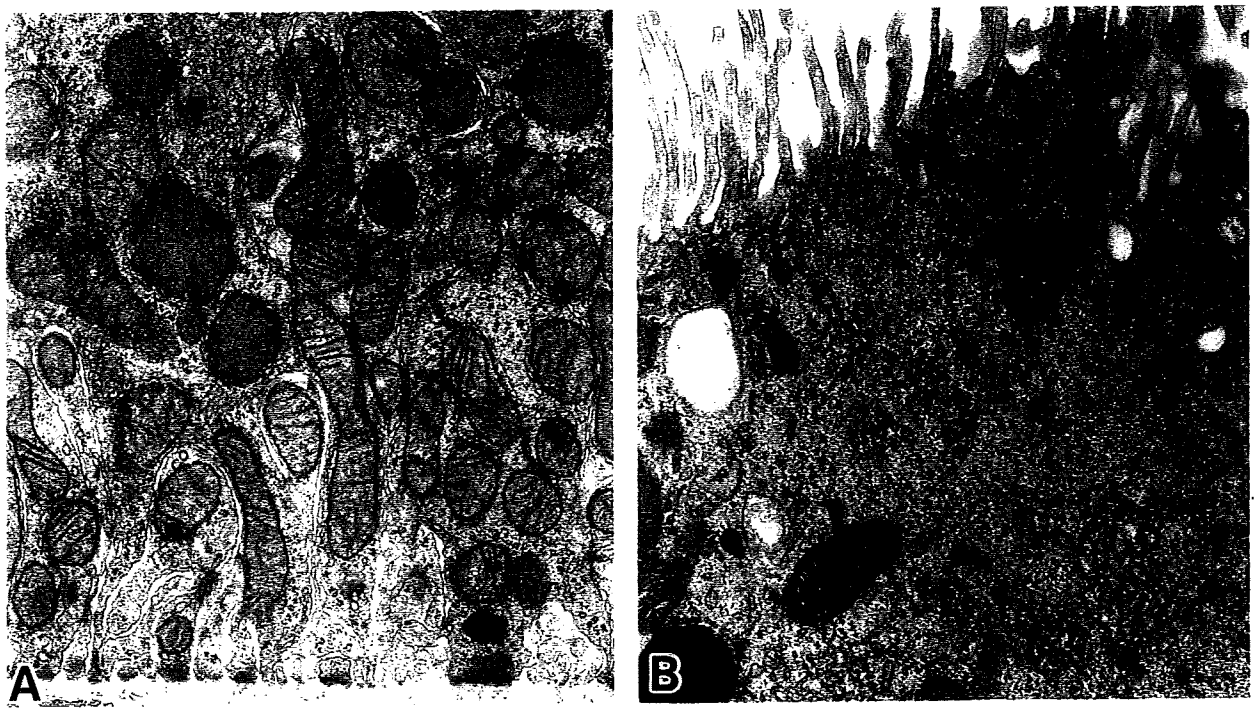


Photo 7. Electron microscopic autoradiographs from renal tubular epithelial cell of a rat sacrificed 6 hr after administration of [3,4,5,6-^{3}H]-phthalic acid di(2-ethylhexyl)ester. A few silver grains are noted on the brush border, smooth surfaced endoplasmic reticulum and mitochondria of the cell.
A: Basal area, × 10640.
B: Adluminal area, × 12160.

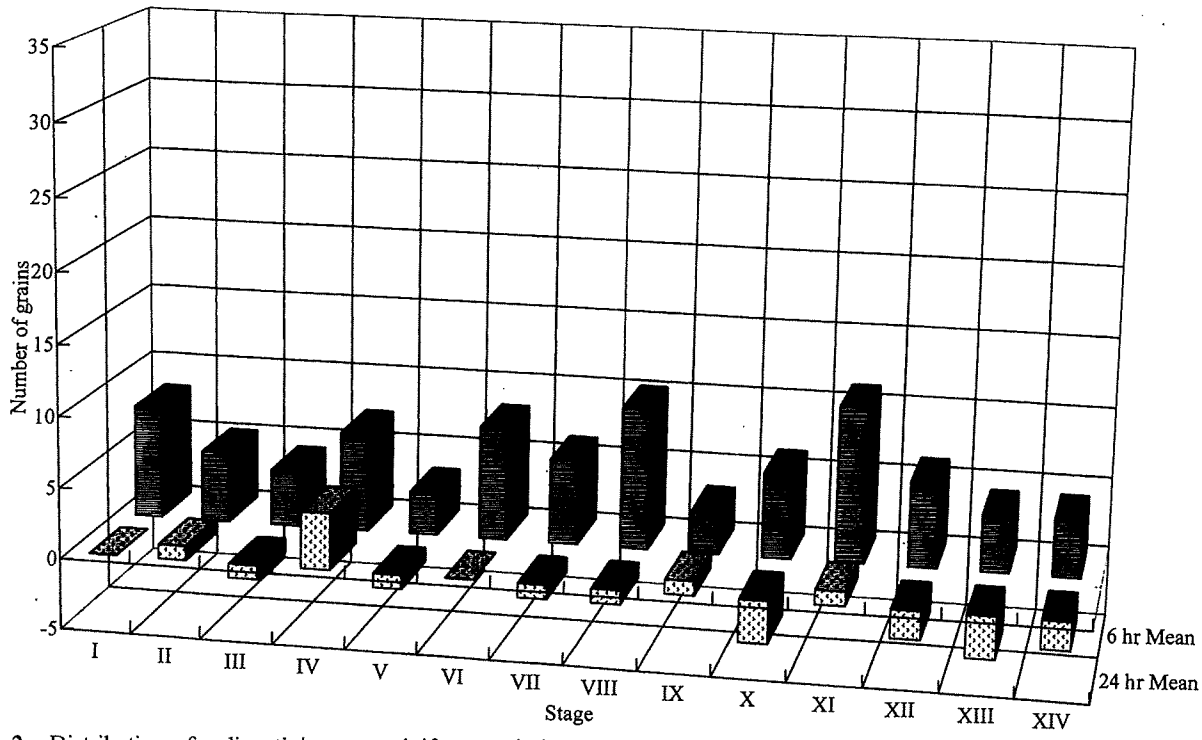
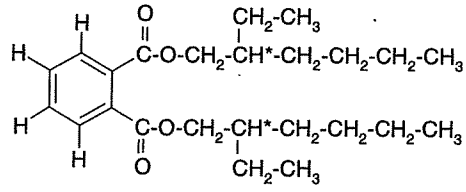
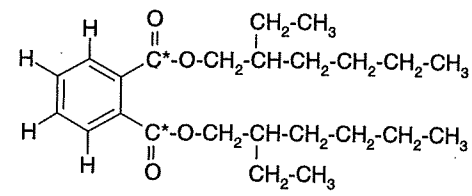


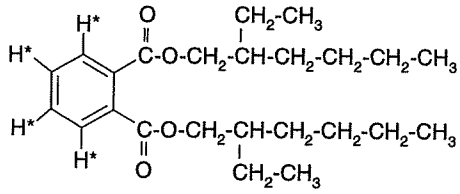
Fig. 2. Distribution of radioactivity to seminiferous tubules of the testis of rats 6 and 24 hr after administration of di(2-ethyl[1-^{3}H]hexyl)ester. Mean counts of radiosensitized grains per seminiferous tubule on the autoradiographs are shown by the stage of spermatogenesis.



Phthalic acid, di (2-ethyl [1-^{3}H] hexyl)ester
: [1-^{3}H] DEHP



[7-^{14}C]-Phthalic acid di (2-ethylhexyl)ester
: [7-^{14}C] DEHP



[3,4,5,6-^{3}H] Phthalic acid di (2-ethylhexyl)ester
: [3,4,5,6-^{3}H] DEHP

Fig. 3. Chemical structures of the labeled compounds used in the present study and used in the study of Gray and Gangolli (1986). The positions of radio-labeling are shown with *.

iography in mice by oral administration of ^{14}C-DEHP and observed that radioactivity levels of the intestinal wall, renal pelvis, bladder, urine, and liver reached their peaks at 4 hr after administration, and that they decreased afterwards except in the renal pelvis. In the present study, distribution of radioactivity observed as grains in testes and kidneys at 6 hr after administration of DEHP-^{3}H, significantly decreased at 24 hr after administration. Thus, DEHP or its metabolites distributed to testes and kidneys after single administration disappeared from the tissue rather fast. On the other hand, the grain count of the liver at 24 hr increased more than at 6 hr after administration, which suggests that DEHP or its metabolites were accumulated in the liver, possibly by enterohepatic circulation.

On autoradiography with oral administration of ^{3}H-DEHP, only a few radiosensitized grains were observed under light microscopic observation of thick sections of any tissues of the testes, liver or kidneys, and no grains were observed on electron microscopic autoradiography. This result implies that DEHP is rapidly splitted in the body into phthalic acid and alcohol, and only the phthalic acid moiety is transported into the tissues. It has been reported that orally administered phthalate diesters such as DEHP are rapidly changed in the gut to monoesters such as MEHP and absorbed, and that MEHP was similarly as potent as DEHP in causing testicular changes (Gray and Gangolli, 1986; Thomas and Thomas, 1984). On the other hand it has been reported that oral administration of phthalic acid did not cause testicular atrophy in rats (Cater *et al.*, 1977). From this and other experimental evidence, MEHP has been assumed to be the proximate toxicant (Albro *et al.*, 1989). In the present study, the radioactivity of DEHP labeled at the phthalic acid moiety penetrated the blood-testis barrier into Sertoli cells, while almost no tissue distribution of radioactivity were observed by autoradiography with DEHP labeled at the alcohol moiety (Fig. 1 and 2). Since MEHP metabolized from ^{3}H-DEHP was still radioactive, it is considered that orally administered DEHP was metabolized to MEHP before intestinal absorption and then further metabolized to the form of phthalic acid and taken into the testicular tissue. Thus, the phthalic acid moiety seems to be responsible for toxicological changes in testes. However, contradictory to the present results, Gray and Gangolli (1986) reported that ^{14}C-MEHP penetrated the blood-testis barrier only to a very limited extent. They used DEHP labeled with ^{14}C supposedly at the 7th position of phthalic acid or carbonyl carbon (Fig. 3). (They have not described the labeled position of the compound, but preceding papers had given the relevant information (Albro *et al.*, 1973; Schultz and Rubin, 1973).) It is chemically implausible that the carbonyl radical is detached so easily from the phthalic acid in the body. The conclusion of Gray and Gangolli (1986) that MEHP did not pass the blood-testis barrier was drawn from their observation that very little ^{14}C-MEHP appeared in rete testis fluid 25 min after intravenous administration. They remarked that MEHP affected Sertoli cells from the outside of the cells. The present study has demonstrated with autoradiography that the radioactivity of DEHP labeled at the phthalic acid moiety did pass the blood-testis barrier into Sertoli cells. Differences of test methods, amount of radioactivity used and timing of measurement may explain the discrepancy.

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Validation Activities in Japan: A Report

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Summary — Japanese activities in the validation of alternative test methods are reviewed. The validation of the *Limulus* endotoxin test as an alternative method to the rabbit pyrogen test was initiated by the Japanese Pharmacopoeia Society, and was successfully conducted under the direction of the National Institute of Health Sciences (NIHS), with the participation of many pharmaceutical company laboratories. The validation of *in vitro* alternatives to the Draize eye test for cosmetics were carried out by the NIHS and cosmetic company laboratories. Research on alternatives in Japan has been promoted by the Japanese Society of Alternatives to Animal Experiments (JSAAE) since 1989. Validation studies on alternatives were also attempted by the JSAAE. Interlaboratory reproducibility studies were carried out by the JSAAE on five different cytotoxicity tests. The JSAAE is planning the validation of *in vitro* alternatives to a local irritation test for intramuscular injection. Cell transformation assays have been carried out in Japan as adjuncts to the *in vivo* carcinogenicity test.

Key words: *cell transformation, endotoxin test,* in vitro *tests, validation.*

Introduction

A previous report on the validation activities of alternative test methods in Japan was made at the First World Congress on Alternatives, held in Baltimore in 1993 (1). In the years since then, several international collaborative validation studies on a large scale have been organised, and some Japanese laboratories have participated. These studies have resulted in the acceptance of the alternative methods in regulatory procedures in a few cases. Established alternatives are limited to those for an acute oral toxicity test, a phototoxicity test and a skin corrosivity test. However, the development of alternatives to other kinds of animal tests for toxicology has not reached full-scale interlaboratory validation. Criteria for validation and regulatory acceptance were established, and intensive efforts for validation of alternatives have been taken by specialised organisations such as the European Centre for the Validation of Alternative Methods (ECVAM) and the (US) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Though we have no such comparable body for validation under governmental auspices in Japan, some alternative research activities have been carried out in Japan.

Research on alternatives to animal experiments in Japan started much later than in Western countries. The Japanese Society of Alternatives to Animal Experiments (JSAAE) was established in 1989. It has now enrolled some 300 scientist members, who are interested in alternative experimental methods to animal experiments. An annual meeting is held for scientific communication, and its official journal, *Alternatives to Animal Tests and Experiments*, is published.

Needs for Alternatives and Validation

As in many other countries, the Japanese government requires many toxicological tests for the safety management of various kinds of chemicals. Different sets of toxicological data are required for different categories of chemicals, such as pharmaceuticals, pesticides, cosmetics, novel chemicals, and materials for medical devices. Most of these toxicological tests are animal tests. Only a limited number of tests have alternatives, such as the acute oral toxicity test, skin and eye irritation tests, and the pyrogenicity test. Many others are waiting for the development of the alternative testing methods. Replacing long-term toxicity tests or reproductive/developmental toxicology studies with non-animal procedures will be very difficult; however, researchers are persevering in their efforts to investigate the possibilities.

Japanese Participation in Validation Studies

The participation of Japanese laboratories in various validation activities is depicted in Table 1. The first four studies listed in the table were international collaborative studies. Several Japanese laboratories have participated individually in these international collaborations; initially, in the validation study for the fixed dose proce-

Table 1: Participation of Japanese laboratories in international and national validation studies

Alternative to	Title of study	Study term	Sponsor/Steering body
Acute oral toxicity (LD50 test)	Fixed Dose Procedure (BTS Method)	1988–1989	EC/UK Home Office
Acute oral toxicity (LD50 test)	Toxic Class Method (BGA Method)	1991–1994	BGA (FRG)/BGA
Acute oral toxicity (LD50 test)	*In vitro* Cytotoxicity (MEIC Project)	1989–1995	SSCT/MEIC
Eye irritation test (Draize test)	*In vitro* Draize Alternatives	1992–1994	EC/UK Home Office
Eye irritation test (Draize test)	JSAAE Cytotoxicity Study	1992–1996	JSAAE/JSAAE Working Group
Eye irritation test (Draize test)	Safety Evaluation of Cosmetic Ingredients	1993–1996	MHW (JPN)/NIHS-JCIA
Pyrogenicity test (rabbit)	Endotoxin Test (*Limulus* Amebocyte Lysate)	1991–1992	Soc. Jpn Pharmacopoeia/ Osaka and Tokyo PMA
Intramuscular irritation test	Safety Evaluation Test for Intramuscular Injections	Planning in progress	JSAAE/JSAAE-JPMA
Carcinogenicity test (Cell transformation)	3T3 Cell Transformation Validation Study	1995–1997 1998–1999	JEMS/JEMS Working Group
In vivo micronucleus test	*In vitro* micronucleus test	1998–2001	JEMS/SFTG

dure of acute oral toxicity test (British Toxicology Society [BTS] method) promoted by the European Commission (EC) and the UK Home Office (2), and then in an international collaborative study for the acute toxic class method, organised by German Bundesgesundheitamt (BGA) (3). The Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) project, conducted by the Scandinavian Society for Cell Toxicology (SSCT), made a unique approach to *in vitro* alternatives to acute toxicity testing, and seven Japanese laboratories have participated in this project (4). A few Japanese laboratories participated in the EC/UK Home Office validation project of *in vitro* alternatives to the Draize eye irritation test.

High Hurdles of International Validation Studies

Through these experiences, the difficulties of international collaborative studies became evident, i.e. different national regulations defining different goals in developing alternative tests; different regulations for chemical transportation, which formed a hurdle against studies proceeding by using common chemicals in a blind manner; different standards for laboratory conditions, such as room temperature and humidity, feed for animals; geographical distances that hinder frequent meetings and sufficient communication; different Good Laboratory Practice (GLP) regulations; language barriers; and, finally, but most importantly, financial matters.

Nevertheless, international and interlaboratory validation studies have been of considerable importance in establishing the robustness of the test procedures across international and interlaboratory barriers.

Validation Studies Within the Country

Thereafter, validation activities involving Japanese laboratories were mostly conducted within the country. In the early 1990s, two major interlaboratory studies were performed in Japan. Both studies were intended to confirm the usefulness of *in vitro* tests as alternatives to Draize eye and skin irritation tests.

The project on the interlaboratory reproducibility of cytotoxicity assays was initiated by the JSAAE, gathering 47 laboratories from among the society's members to participate in the project (5, 6). Comparisons were made between five different cytotoxicity tests, each using two different cell preparations. Data for six surfactants with each test method and each cell line were collected and analysed for reproducibility among the laboratories. Extensive data analysis revealed the least interlaboratory variation with the crystal violet staining method, and the results also showed no superiority of any cells over other kinds of cells in testing for cytotoxicity of surfactant chemicals.

The EC directive to prohibit animal testing for cosmetic ingredients had a great impact internationally, and the Japanese Cosmetic Industry Association (JCIA) entered into a collaboration to evaluate *in vitro* test methods as possible alternatives to the Draize rabbit eye test. The project was supported with a grant from the Ministry of Health and Welfare (MHW) and was conducted by the National Institute of Health Sciences (NIHS) (7). In this study, nine different *in vitro* test methods, i.e. tests using the chorio-allantoic membrane of the hen's egg, sheep erythrocytes, artificial skin models (SKIN^{2}™ and MATREX™), cytotoxicity tests using several different cells, and EYTEX™, were evaluated for reproducibility among the different

laboratories. In total, 17 laboratories participated in this 3-year project, producing data for 45 test chemicals. The results were subsequently published (8), and a scheme for the safety evaluation of cosmetic ingredients has been proposed for regulatory acceptance.

Prior to these two projects concerned with the rabbit eye test, a validation study for an endotoxin test using the *Limulus* amebocyte lysate as the replacement alternative to the pyrogenicity test using rabbits was carried out. The rabbit pyrogen test had been frequently performed, as the test was described in monographs of the *Japanese Pharmacopoeia* (*JP*) for drugs for injection, antibiotics and biological preparations. The project was planned on the initiative of the Society of Japanese Pharmacopoeia and was conducted by the NIHS, in collaboration with 27 laboratories of the Osaka and Tokyo Associations of Pharmaceutical Manufacturers (PMA). They tested all the 67 preparations for the pyrogen test, required in the monograph in *JP XI* (1986). The result was satisfactory, and for most of these preparations, the pyrogen test was replaced by an endotoxin test in the next version of the *JP, JP XII* (1991).

In this validation study, the *Limulus* test actually validated was the "gel-formation method", and *JP XII* described only this method for the endotoxin test. Later, when more-sophisticated photometric methods based on the same principle were developed, these improved procedures were described in the next revision, *JPXIII* (1996), without any large-scale collaborative validation study.

The Intramuscular Irritation Test

The validation of an *in vitro* cytotoxicity test as an alternative to the intramuscular local irritation test is planned by the JSAAE. A local irritation test using intramuscular injections in rabbits is required in Japan for every drug used by intramuscular injection. This requirement was made mandatory after outbreaks of thigh muscle contracture and gait disturbance in children who had received intramuscular injections for the treatment of febrile diseases. The drug preparations were injections of antipyretic pyrazolone and antibiotics. It was demonstrated that the potential tissue-damaging property could be detected by an intramuscular injection test of these drugs using rabbits. This test has since been required for every drug to be used by the intramuscular route. The use of cytotoxicity tests as an alternative to this irritating property test is promising, and the JSAAE is preparing the validation in collaboration with the Japanese Pharmaceutical Manufacturers Association (JPMA). This project has been postponed for some years, because there was a reluctance among pharmaceutical industries intending to drop rather than sustain the intramuscular injection method. Thus, this project has not been launched, although the need for alternatives still exists. A survey by the JSAAE in 2000 revealed that 67 preparations for intramuscular injection have been developed during the past five years.

Alternatives to *In Vivo* Carcinogenicity Studies

Cell transformation assays hold promise for serving as predictive indicators of the carcinogenic potential of chemicals (9), and the transformation assay with Syrian hamster embryonic (SHE) cells has now been proposed for an OECD test guideline. On the other hand, the transformation assay using an established cell line, Balb/c 3T3, has also been developed and recognised as useful. A multi-laboratory validation study for an improved cell transformation assay using Balb/c 3T3 cells was conducted by the Japan Environmental Mutagen Society (JEMS) in 1995–1997 (the first phase), and in 1998–1999 the second phase study took place. The results of the study were published in *ATLA* (10), and are being presented at this Congress by Makoto Umeda.

Instead of a cytogenetic test on whole animals, the usefulness of an *in vitro* micronucleus test using CHL cells and L5178Y cells has been studied by international collaboration with JEMS and the Société Française de Toxicologie Génétique (SFTG), including 38 laboratories, in 1998–2001.

Discussion

The validation of a test method is not only a process for showing its scientific usefulness, but also the way forward to public and regulatory acceptance. The most remarkable progress of the last decade in the area of alternative research has been in developing and agreeing on the criteria for validation and regulatory acceptance.

Japanese scientists have carried out several validation studies within the nation or have participated in international collaborative studies. Through these studies, we have recognised considerable difficulties in the huge workload of validation, as well as its cost and time-consuming nature. In addition to inadequate financial support, we have difficulties in finding sufficient human resources that are capable of conducting a complicated collaboration, dealing with a large volume of data and completing the independent review of the results.

A new trend in genomic sciences is emerging in the world of toxicology. Research in toxicogenomics might produce great changes in toxicology and also in animal use in safety assessment. However, we have not yet focused much upon the validation of this new approach to predicting toxicity. We must make a fresh assessment of validation procedures

for these new methods in toxicology. It is not too early to consider this, since the toxicogenomics investigation is a large-scale enterprise with a very promising future.

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Low-dose bisphenol A does not affect reproductive organs in estrogen-sensitive C57BL/6N mice exposed at the sexually mature, juvenile, or embryonic stage

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Abstract

Bisphenol A (BPA) is used on a large scale in the manufacture of polycarbonate plastics. BPA has been shown to bind weakly to both estrogen receptor (ER) α and ER β. The objective of this study was to evaluate the effects of low-dose BPA on male sexual development after exposure at various stages of development. Mice of the estrogen-sensitive strain C57BL/6N were exposed to BPA orally at doses of 2, 20, or 200 μg/kg at various stages, i.e. adulthood, the immature stage just after weaning, or the embryonic/fetal stage, to evaluate the effects of low-dose BPA on male reproductive organs. Body weight changes, weights of reproductive organs (testes, epididymides, seminal vesicles), cauda epididymal sperm density, and histology of reproductive organs including the ventral prostate were not affected by exposure to BPA at any dose examined. The results of this study indicate that exposure of estrogen-sensitive C57BL/6N mice to low-dose BPA did not reduce sperm density or disrupt development of the male reproductive organs.

Keywords: Bisphenol A; Low-dose exposure; C57BL/6N mice; Reproduction; Testicular toxicity

1. Introduction

Bisphenol A (BPA) is an industrial compound that has generated a great deal of concern on the part of regulatory agencies and scientists due to its high level of production and widespread use. BPA is the monomer used in the manufacture of the resin used to line food and drink cans and from which polycarbonate plastic is made. BPA is also used to make dental sealants, which are often used to protect children's teeth [1]. BPA has been reported to be weakly estrogenic both in vitro and in vivo. Krishnan et al. [2] reported that BPA leached from polycarbonate flasks competed with [^{3}H]-estradiol for binding to estrogen receptors (ER) from rat uterus, induced progesterone receptor expression, and promoted cell proliferation in cultured human mammary cancer cells (MCF-7). BPA binds to both ER α and ER β with low affinity and transactivates reporter genes in vitro [3,4].

Recently, experiments by Nagel et al. [5] and vom Saal et al. [6] indicated that administration of low oral doses of BPA to pregnant mice on days 11 through 17 of gestation produced statistically significant increases in the weights of the prostate and preputial glands, a decrease in epididymis weight, and reduced efficiency of sperm production in male offspring. However, the low-dose effects of BPA have been controversial. Other researchers reported no treatment-related effects of BPA at the same and additional low-dose levels given at the same time of pregnancy to mice [7–12].

Large (more than 16-fold) differences in sensitivity to disruption of juvenile male reproductive development by 17β-estradiol (E_2) were found between strains of mice. Spermatid maturation was eliminated by low doses of E_2 in strains such as C57BL/6J and C17/Jls. In contrast, mice of the widely used CD-1 line showed little or no inhibition of spermatid maturation even in response to 16-fold higher doses of E_2 [13].

In the present study, we examined the effects on development of male reproductive organs in C57BL/6N mice, which were confirmed to be sensitive to estrogens similarly to C57BL/6J, as a result of embryonic or fetal exposure to environmentally relevant doses of BPA. In addition, the effects on reproductive organs in males exposed to low

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doses of BPA at the sexually mature or immature stages after weaning were also investigated in this strain.

2. Materials and methods

2.1. Animals and treatment

C57BL/6N and ICR mice were purchased from Charles River, Atsugi, Japan, at 3 or 8 weeks of age. The animals were acclimated to the laboratory for 3 days to 2 weeks prior to the start of the experiments. Animals were housed individually in polycarbonate cages in a room with controlled temperature (24 ± 1°C) and humidity (50 ± 5%), with lights on from 07:00 to 19:00 daily. Mice were given access to food (PLD, phytoestrogen-low diet, Oriental Japan) and tap water (distilled water) ad libitum. The contents of phytoestrogens in the diet, tap water, and wood bedding were determined, and genistein and daidzein levels were below 0.5 mg/100 g.

2.2. Experiment I (exposure of C57BL/6N and ICR juvenile male mice to E_2)

To compare the susceptibility to 17β-estradiol (E_2, Sigma Chemical Co., St. Louis, MO) between C57BL/6N and ICR males, 6 to 8 males from each strain were treated subcutaneously (s.c.) with E_2 at 10 μg/kg from postnatal day 27 to 48. The administration period was determined according to the protocol of the study by Spearow et al. [13]. Administration was performed at a defined time (12:00). Ten males of each strain were given corn oil (2 ml/kg) as controls. On postnatal day 43, male mice were weighed and subjected to necropsy. Subsequently, the testes, epididymides and seminal vesicles with coagulating glands were weighed. These reproductive organs were fixed in Bouin's solution for histologic observation.

2.3. Experiment II (exposure of C57BL/6N adult males to BPA)

Groups of twenty C57BL/6N male mice at 10 weeks of age were exposed to bisphenol A (BPA, Tokyo Kasei, purity, GC min. 99.0%) at 2, 20, or 200 μg/kg by oral gavage for 6 consecutive days. The dosages were determined on the basis of body weight on the day of the treatment. Administration was performed at a defined time (12:00). Twenty males were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. Six weeks after the final administration, male mice were weighed and 15 were subjected to necropsy. The administration period and the day of necropsy after the last administration were determined based on the results of the previous study by Ohsako et al. [14]. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed. The ventral prostate was not weighed in the present study since it was difficult to sample only the prostate in mice, and to determine the precise weight of this organ. The left cauda epididymis was homogenized in 1 ml distilled water. The homogenates were stained with an IDENT staining kit (Hamilton Thorne prepackaged DNA-specific dye based on Hoechst 33342). The stained samples were placed onto Cell-Vu slides (Fertility Technologie, MA, USA), and the numbers of sperm were counted using an HTM-IVOS analyzer (Hamilton Thorne Research, MA, USA) and the IDENT software supplied with the HTM-IVOS. Other reproductive organs were fixed in Bouin's solution for histologic evaluation. The remaining mice (5 mice per group) were anesthetized. Transcardiac perfusion was carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the testes, epididymides, seminal vesicles, and prostates of these mice were rinsed three times in phosphate buffer, postfixed for 2 h at 4°C in 2% osmium tetroxide, and dehydrated in alcohol; these organs were embedded in epoxy resin. Tissue sections (1 μm thick) were stained with toluidine blue for light microscopy. Ultrathin sections stained with uranyl acetate and lead citrate were observed with an electron microscope (H-7100, Hitachi, Japan).

2.4. Experiment III (exposure of C57BL/6N juvenile males to BPA)

To obtain pregnant animals, 10-week-old virgin C57BL/6N females were cohabited overnight on a 1:1 basis with males of the same strain at 11 weeks of age or older. The next morning, females with vaginal plugs were regarded as pregnant, and the day of gestation was designated as day 0. All pregnant mice were allowed to give birth. On postnatal day 0, all female pups were discarded, and the number of males per litter was adjusted to 3. Male pups (30 males from 10 litters/group) were weaned on postnatal day 21, and exposed to BPA at 2, 20, or 200 μg/kg by oral gavage from postnatal day 21 to 43. The dosages were determined on the basis of body weight on the day of treatment. Administration was performed at a defined time (12:00). Thirty males from 10 litters were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. At 6 weeks old, all males were weighed. Five mice per group were anesthetized and transcardiac perfusion was carried out. The remaining males were subjected to necropsy. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed, and the left cauda epididymis of each male was homogenized to determine the sperm density. Finally, weighed organs were fixed in Bouin's solution for histologic evaluation.

2.5. Experiment IV (exposure of C57BL/6N embryos/fetuses to BPA)

Pregnant mice were obtained as described in Experiment III. Groups of 10 C57BL/6N mice were exposed to BPA at

Table 1
Reproductive organ weights in male ICR and C57BL/6N mice exposed to E_2

Dose (μg/kg/day)	No. males treated	Start of treatment (Administration period)	Age at termination	Final body weight (g)	Testes (g)	Epididymides (g)	Seminal vesicles (g)
ICR							
0	10	27 days old (21 days)	7 weeks old	38.52 ± 0.77[a]	0.221 ± 0.011[b] 0.577 ± 0.037[c]	0.074 ± 0.002 0.192 ± 0.007	0.288 ± 0.023 0.743 ± 0.051
10	10	27 days old (21 days)	7 weeks old	35.69 ± 0.78	0.203 ± 0.012 (92%)[d] 0.563 ± 0.034 (98%)	0.069 ± 0.003 (93%) 0.194 ± 0.007 (101%)	0.263 ± 0.015 (91%) 0.739 ± 0.042 (99%)
C57BL/6N							
0	10	27 days old (21 days)	7 weeks old	19.15 ± 0.45	0.130 ± 0.005 0.673 ± 0.024	0.042 ± 0.002 0.216 ± 0.008	0.100 ± 0.008 0.520 ± 0.030
10	10	27 days old (21 days)	7 weeks old	19.61 ± 0.34	0.108 ± 0.017* (83%) 0.548 ± 0.083* (81%)	0.028 ± 0.004** (67%) 0.141 ± 0.018** (65%)	0.057 ± 0.011** (57%) 0.284 ± 0.050** (55%)

[a]mean ± S.E.
[b]absolute weight.
[c]relative weight (organ weight/terminal body weight) × 100.
[d]percentage of control.
*Significantly different from the control, $P < 0.05$.
**Significantly different from the control, $P < 0.01$.

2, 20, or 200 μg/kg by oral gavage from gestational day 11 through 17. The administration period was determined according to the protocol of the study by Cagen et al. [8]. The dosages were determined on the basis of body weight on the day of treatment. Administration was performed at a defined time (12:00). Ten pregnant mice were given 0.5% carboxymethyl cellulose (5 ml/kg) as controls. Forty untreated female mice were allowed to give birth. All the BPA-treated and control dams were subjected to cesarean section on day 18 of gestation. The day of cesarean section was considered as postnatal day 0. The neonates obtained by cesarean section were fostered to untreated C57BL/6N females, one litter per female. On postnatal day 4, all female pups were discarded, and the number of males per litter was adjusted to 3 for each foster female. The males were weaned on postnatal day 21 and then individually housed in polycarbonate cages, where they were allowed to grow and sexually mature. At 12 weeks of age, all males were weighed. Five males per group were anesthetized and transcardiac perfusion was carried out. The remaining males (25 males per group) were subjected to necropsy. Subsequently, the testes, epididymides, and seminal vesicles with coagulating glands were weighed, and the left cauda epididymis of each male was homogenized to determine the sperm density. Finally, weighed organs were fixed in Bouin's solution for histologic evaluation.

Animal care and use conformed to published guidelines [15].

2.6. Analysis of data

Data were analyzed, where appropriate, to determine the statistical significance of differences between the control and E_2- or BPA-treated groups; $P < 0.05$ and $P < 0.01$ were taken to indicate statistical significance. Individual data or mean values of each litter were treated as single samples, and homogeneity of variance of these samples among groups was analyzed initially using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect significance of differences among groups. If a significant difference was detected among groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous or there was any group in which the variance was zero, Kruskal-Wallis analysis of ranks was applied. If significance was detected among groups, Dunnett's test was applied for multiple comparisons.

3. Results

3.1. Susceptibility to E_2 between C57BL/6N and ICR mice

Table 1 shows the reproductive organ weights of C57BL/6N and ICR males treated s.c. with E_2 from postnatal days 27 to 48. There were no significantly different in the absolute or relative weights (testes, epididymides, and seminal vesicles) between the E_2-treated males and the controls in ICR strain. In marked contrast, significant decreases in the absolute and relative weights of reproductive organs were detected in the E_2-treated males of the C57BL/6N strain as compared with those of the controls. In particular, relative weight of seminal vesicles in the E_2-treated males of this strain was 55% that of the controls. At necropsy, three and five C57BL/6N males treated with E_2 showed marked atrophy of the testes and seminal vesicles, respectively, while no macroscopic changes of the reproductive organs were observed in the ICR males treated with

Table 2
Histopathologic findings in ICR and C57BL/6N male mice exposed to E_2

Strain	ICR															C57BL/6N												
Dose (μg/kg/day)	0							10								0							10					
Animal No.	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	1	2	3	4	5	6
Testis																												
Multinucleated giant cell, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–
Decrease in elongate spermatid, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	–	+	–
Decrease in elongate spermatid, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++
Atrophy of Leydig cell, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	±	+	++
Epididymis																												
Cell debris in lumen, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	±	–
Cell debris in lumen, bilateral																			+	+	–							+
Decrease in sperm, unilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–
Decrease in sperm, bilateral	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	+	+++
Seminal vesicle																												
Atrophy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	++	+++

–, Negative; ±, Very slight; +, Slight; ++, Moderate; +++, Severe.

E_2. Histopathologic findings of the reproductive organs in ICR and C57BL/6N mice treated with E_2 are shown in Table 2, and the representative changes observed in C57BL/6N mice are shown in Fig. 1. Only a few elongate spermatids were observed in seminiferous tubules of C57BL/6N mice treated with E_2. The number or size of Leydig cells of C57BL/6N mice treated with E_2 was decreased as compared with the controls. In addition, no sperm were observed in the lumen of the epididymal ducts, and the lumen of the seminal vesicles contained no secretions. Three other C57BL/6N males treated with E_2 showed similar but slight changes in the testis, epididymis, and seminal vesicles. C57BL/6N males in the control group showed no abnormalities except for cell debris and a slight decrease in sperm in the epididymal duct. In ICR males treated with E_2, no histopathologic changes in the reproductive organs were observed.

3.2. Low-dose effects of BPA in C57BL/6N male mice

No adult or immature C57BL/6N male mice exposed to BPA for 6 days or 3 weeks, respectively, died during the study period. In addition, there were no significant differences in body weight gain from the day of commencement of administration to the day of necropsy between the BPA-treated groups and the controls. No significant differences were found in embryo mortality or viability after birth between the BPA-treated groups and the control, nor in the body weight gain until necropsy (data not shown).

Table 3 shows the percentages of control values for the reproductive organ weights in C57BL/6N males exposed to BPA at various stages. In males exposed to BPA at the mature or immature stage after weaning there were no significant differences between the BPA-treated groups and the controls in terminal body weight or reproductive organ weights (testes, epididymides, seminal vesicles), or their relative weights.

In C57BL/6N males exposed to BPA as embryos at the organogenic stage, a significant decrease in the absolute weight of seminal vesicles in the 2 μg/kg group was found as compared with the controls, but the effect was not dose-dependent, suggesting that the decrease in the weight of the seminal vesicles was not related to BPA administration.

Fig. 2 shows the numbers of sperm per gram cauda epididymis of C57BL/6N males exposed to BPA at various stages. There were no significant differences in the density of sperm between the BPA-treated groups and the controls.

With regard to the histopathologic observations of the testes, epididymides, seminal vesicles, and prostate by light and electron microscopy, slight atrophy of the seminiferous tubules and multinucleated giant cells in immature seminiferous tubules were observed in males exposed to BPA after weaning for 3 weeks in all of the BPA-treated groups and the controls. In one of 30 males exposed to BPA at 2 μg/kg after weaning for 3 weeks, diffuse atrophy of the seminiferous tubules was found. In C57BL/6N males exposed to BPA at the mature stage or embryonic stage, no histopathologic changes that may have resulted in a decreased number of germ cells including mature sperm were observed in any parts of the reproductive tract.

4. Discussion

Significant decreases in weights of reproductive organs were detected in C57BL/6N male mice treated with E_2 from postnatal day 27 to 48 as compared with those of the controls. In particular, relative weight of seminal vesicles in the E_2-treated males of the C57BL/6N was 55% of that of the controls. Male C57BL/6N mice treated with E_2 showed

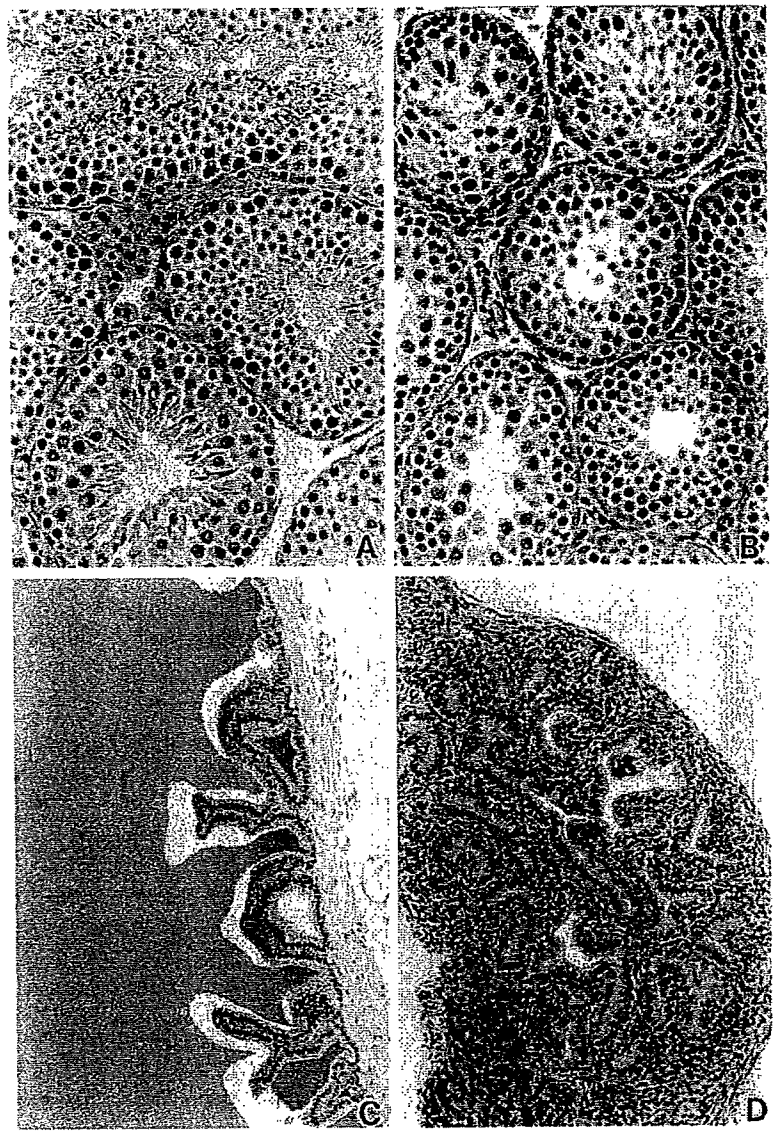

Fig. 1. Histopathologic changes in C57BL/6N male mice exposed s.c. to E_2 from postnatal day 27 to 48. (A) Control male testis. No abnormalities were observed. H&E, ×350. (B) Testis from BPA-treated male. Only a few elongate spermatids were observed in the seminiferous tubules.The number and size of Leydig cells was decreased. H&E, ×350. (C) Control seminal vesicle. No abnormalities were observed. H&E, ×175. (D) Seminal vesicle from BPA-treated male. The lumen contained no secretions. H&E, ×175.

marked atrophy of the testes and seminal vesicle as well as histopathologic changes of the reproductive organs (see Table 2). In contrast, no macroscopic or microscopic changes of the reproductive organs were detected in males of ICR mice treated with E_2. Taken together, these observations suggested that male C57BL/6N mice are extremely sensitive to the effects of E_2 similar to the male C57BL/6J mice reported previously [13], while male ICR mice are extremely resistant to E_2.

In the present study, reproductive organ weights (testes, epididymides, seminal vesicles) in C57BL/6N males exposed to BPA at the mature or immature stage did not differ from those of controls. Ohsako et al. [14] reported that testes weight and daily sperm production of Sprague-Dawley rats 36 days after the first treatment with BPA at 20 μg/kg/day for 6 days were significantly decreased, and concluded that even at very low doses BPA affected spermatogenesis of mature rats. These results were incompatible with those of the present study. There were no significant differences in the density of sperm between the groups exposed to BPA at various stages and the controls. In addition, the numbers of sperm in the BPA-treated and control groups were within the range of the other control data based on information gathered in reproductive and developmental toxicity studies using C57BL/6N mice in our laboratory over a period of 3 years, suggesting that exposure of mice to low-dose BPA at various stages did not affect sperm production.

Table 3
Reproductive organ weights in male C57BL/6N mice exposed to BPA at various stages

Dose (μg/kg/day)	No. mice treated	Start of treatment (Administration period)	Age at termination	No. males/ No. litters	Final body weight (g)	Testes (g)	Epididymides (g)	Seminal vesicles (g)
0	20 males	12 weeks old (6 days)	17 weeks old	15	29.77 ± 0.59[a]	0.150 ± 0.008[b] 0.502 ± 0.042[c]	0.071 ± 0.003 0.237 ± 0.015	0.419 ± 0.016 1.395 ± 0.033
2	20 males	12 weeks old (6 days)	17 weeks old	15	29.00 ± 0.47	0.157 ± 0.001 0.536 ± 0.013	0.072 ± 0.001 0.246 ± 0.006	0.417 ± 0.030 1.431 ± 0.128
20	20 males	12 weeks old (6 days)	17 weeks old	15	29.81 ± 0.77	0.159 ± 0.005 0.539 ± 0.026	0.078 ± 0.003 0.262 ± 0.013	0.407 ± 0.008 1.366 ± 0.050
200	20 males	12 weeks old (6 days)	17 weeks old	15	29.75 ± 0.75	0.159 ± 0.004 0.545 ± 0.017	0.072 ± 0.001 0.246 ± 0.011	0.411 ± 0.020 1.382 ± 0.083
0	30 males	21 days old (21 days)	6 weeks old	25/10	17.15 ± 0.29	0.119 ± 0.002 0.713 ± 0.014	0.031 ± 0.001 0.181 ± 0.006	0.045 ± 0.003 0.259 ± 0.015
2	30 males	21 days old (21 days)	6 weeks old	25/10	18.22 ± 0.67	0.117 ± 0.017 0.663 ± 0.084	0.033 ± 0.003 0.187 ± 0.013	0.044 ± 0.006 0.247 ± 0.028
20	30 males	21 days old (21 days)	6 weeks old	25/10	16.08 ± 0.83	0.116 ± 0.012 0.701 ± 0.056	0.030 ± 0.003 0.184 ± 0.011	0.043 ± 0.008 0.253 ± 0.041
200	30 males	21 days old (21 days)	6 weeks old	25/10	16.83 ± 0.34	0.115 ± 0.006 0.677 ± 0.038	0.030 ± 0.001 0.173 ± 0.007	0.045 ± 0.003 0.263 ± 0.014
0	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	24.70 ± 0.28	0.149 ± 0.003 0.617 ± 0.014	0.060 ± 0.001 0.249 ± 0.004	0.248 ± 0.006 1.028 ± 0.027
2	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	23.99 ± 0.50	0.146 ± 0.003 0.612 ± 0.012	0.058 ± 0.002 0.242 ± 0.006	0.234 ± 0.003* 0.968 ± 0.038
20	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	23.85 ± 0.25	0.142 ± 0.002 0.594 ± 0.009	0.057 ± 0.002 0.239 ± 0.007	0.243 ± 0.011 1.009 ± 0.047
200	10 dams	Gestational day 11 (7 days)	12 weeks old	25/10	24.75 ± 0.52	0.149 ± 0.003 0.608 ± 0.012	0.060 ± 0.001 0.240 ± 0.005	0.250 ± 0.008 1.008 ± 0.022

[a]mean ± S.E.
[b]absolute weight.
[c]relative weight: (organ weight/terminal body weight) × 100.
*Significantly different from the control, $P < 0.05$.
Five males in each group were anesthetized and transcardiac perfusion was performed for histologic observance testes, epididymides, seminal vesicles, and prostates with an electron microscope.

Developmental and reproductive toxicity of high doses of BPA have been demonstrated in rats and mice. Persistent estrus was observed in ovariectomized rats injected twice/day for 3 consecutive days with 100 mg BPA [16]. Intraperitoneal (i.p.) injection of BPA at 125 mg/kg on gestational day 1 through 15 also interfered with the maintenance of pregnancy and reduced the number of live fetuses per litter in Sprague-Dawley rats [17]. However, no significant developmental toxicity of BPA was observed in CD rats or CD-1 mice exposed to BPA (rats, 640 mg/kg; mice 1000 mg/kg) by gavage from gestational day 6 through 15 [18]. In addition, neonatal exposure to BPA at 300 mg/kg did not produce detectable effects on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA), nor in development of male reproductive organs or reproductive function after puberty in Sprague-Dawley rats [19]. Oral administration of BPA at much lower doses has also been reported to affect male reproductive organ parameters such as the prostate gland (increase in fresh tissue weight at 2 or 20 μg/kg/day), preputial glands (increase in tissue weight at 2 μg/kg/day), and epididymis (decrease in tissue weight at 2 μg/kg/day), and the efficiency of sperm production (decrease in daily sperm production per g testis at 20 μg/kg/day) in CF-1 mice exposed to BPA during prenatal development from GD 11 to GD 17 [5,6]. However, the low-dose effects of BPA have been controversial. Other groups reported no treatment-related effects of BPA at the same and additional low-dose levels given at the same time during pregnancy to CF-1 mice [7,8]. In the experiments in Sprague-Dawley rats, Welsch et al. [12] demonstrated the lack of effects of perinatal exposure to low doses of BPA on ventral prostate weight of male offspring. Elswick et al. [9] also reported that rats exposed to low doses of BPA during the perinatal period did not display significant differences in hormone levels, sperm counts, or immunohistochemical ventral prostate androgen receptor (AR) levels. Recently, two- or three-generation reproductive toxicity studies of BPA administered by gastric intubation or in the diet were performed. The results of these multigeneration studies indicated that low doses of BPA between 0.2 and 200 μg/kg or between 0.015 and 75 ppm over 2 or 3 generations did not cause significant compound-related changes in reproductive or developmental parameters in rats [10,20]. At present, we cannot explain the differences between the results of the present study and those of vom Saal [6] and others [5] and the rat study of Ohsako et al. [14]. These

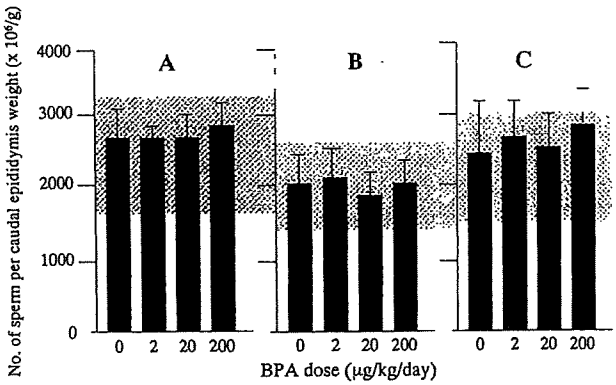

Fig. 2. Numbers of sperm per gram cauda epididymis of male C57BL/6N mice exposed to low-dose BPA at various developmental stages; (A) sexually mature, (B) juvenile, or (C) embryonic/fetal stage. The shadowed regions represent the range of sperm number per gram cauda epididymis in the historical controls in C57BL/6N male mice (left, 17–18 weeks old; middle; 6 weeks old; right, 12–13 weeks old).

discrepancies make the assessment of xenoestrogenic compounds extremely controversial, while concomitantly highlighting the need to resolve these potential important public health concerns [21]. Ashby suggested that some of the failures to repeat observations are probably associated with the many subtle differences that exist between formally identical studies conducted in different laboratories (radio playing quietly in the animal room, soy content of the animal diet, average body weight of the control CF-1 mice, genetic drift of CF-1 mice) [7]. Some chemicals produce small changes in sex-related biologic endpoints that may be difficult to reproduce among laboratories.

Diffuse atrophy of the seminiferous tubules was observed in one C57BL/6N male mouse exposed to BPA at 2 μg/kg after weaning for 3 weeks in the present study. As this was the only case in which an abnormality was found in the testes from males exposed to BPA at various stages, it was not clear whether this change had arisen spontaneously or as a consequence of BPA exposure. In C57BL/6N males exposed to BPA at the mature stage or embryonic stage, no microscopic changes that may have resulted in the decreased number of germ cells including mature sperm were observed in any parts of the reproductive tract. These histopathologic findings were consistent with the lack of effects on sperm density following BPA administration.

When assessing the biologic activity of putative estrogenic compounds in animal model systems, it is important to consider species and strain differences. Strain differences in response to estrogenic stimuli have been demonstrated previously. Gorski and coworkers [22–24] found that the potent estrogens diethylstilbestrol and E_2 induce an overgrowth of lactotropes in the pituitary glands of F344 rats but not in those of outbred strains of rats. Spearow et al. [13] found marked strain-related differences in the susceptibility of mice to estradiol-induced disruption of testicular development. Estradiol treatment during juvenile development resulted in the suppression of testis weight in mouse strains CD-1, C57BL/6J, C17/Jls, S15/Jls, E/Jls and CN-/Jls [13]. Among these, C57BL/6J mice were extremely sensitive with the lowest E_2 dose used (2.5 μg) producing 60% suppression of testis weight in this strain. Unlike C57BL/6J mice in which low to moderate doses of E_2 completely blocked spermatogenesis, CD-1 mice showed very little inhibition of spermatogenesis in response to increasing doses of E_2. Other studies have shown differences in the efficacy of E_2 in stimulation of uterine DNA synthesis between strains of mice [25,26]. In our preliminary study, female ICR and C57BL/6N mice were exposed to various estrogens and endocrine disrupting chemicals during pregnancy, and C57BL/6N embryos were more sensitive (10- to 100-fold) to the lethal effects and disruptive effects of all of these chemicals on development of the reproductive organs than ICR embryos (Nagao, unpublished data).

5. Concluding remarks

In the present study, C57BL/6N mice that are highly susceptible to endocrine disruption by estrogen were used to evaluate the effects of low-dose BPA following exposure at various stages. Based on the results of the present study and the considerable body of literature on the effects of BPA at similar and much higher doses, low-dose BPA should not be considered as a testicular toxicant.

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Premature ovarian failure in androgen receptor-deficient mice

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Premature ovarian failure (POF) syndrome, an early decline of ovarian function in women, is frequently associated with X chromosome abnormalities ranging from various Xq deletions to complete loss of one of the X chromosomes. However, the genetic locus responsible for the POF remains unknown, and no candidate gene has been identified. Using the Cre/LoxP system, we have disrupted the mouse X chromosome androgen receptor (*Ar*) gene. Female $AR^{-/-}$ mice appeared normal but developed the POF phenotype with aberrant ovarian gene expression. Eight-week-old female $AR^{-/-}$ mice are fertile, but they have lower follicle numbers and impaired mammary development, and they produce only half of the normal number of pups per litter. Forty-week-old $AR^{-/-}$ mice are infertile because of complete loss of follicles. Genome-wide microarray analysis of mRNA from $AR^{-/-}$ ovaries revealed that a number of major regulators of folliculogenesis were under transcriptional control by AR. Our findings suggest that AR function is required for normal female reproduction, particularly folliculogenesis, and that AR is a potential therapeutic target in POF syndrome.

male hormone | nuclear receptor | female physiology | folliculogenesis | kit ligand

Premature ovarian failure (POF) is defined as an early decline of ovarian function after seemingly normal folliculogenesis (1). Genetic causes of POF have been frequently associated with X chromosome abnormalities (1, 2). Complete loss of one of the X chromosomes, as in Turner syndrome, and various Xq deletions are commonly identified as a cause of POF. However, responsible X-linked genes and their downstream targets have not been identified so far.

The androgen receptor (*Ar*) gene, which is the only sex hormone receptor gene on the X chromosome, is well known to be essential not only for the male reproductive system, but also for male physiology. In contrast, androgens are considered as male hormones; therefore, little is known about androgens' actions in female physiology, although AR expression in growing follicles has been described (3). However, because excessive androgen production in polycystic ovary syndrome causes infertility with abnormal menstrual cycles (4, 5), it is possible that AR-mediated androgen signaling also plays an important physiological role in the female reproductive system. Recently, using Cre/LoxP system, we generated an AR-null mutant mouse line (6) and demonstrated that inactivation of AR resulted in arrest of testicular development and spermatogenesis, impaired brain masculinization, high-turnover osteopenia, and late onset of obesity in males (7–9). At the same time, no overt physical or growth abnormalities were observed in female $AR^{-/-}$ mice. Therefore, to further examine potential role of AR in female physiology, we characterized female reproductive system in $AR^{-/-}$ females. Herein we show that female $AR^{-/-}$ mice develop the POF phenotype. At 3 weeks of age, $AR^{-/-}$ females had apparently normal ovaries with numbers of follicles similar to those in the wild-type females. However, thereafter the number of healthy follicles in the $AR^{-/-}$ ovary gradually declined, with a marked increase of atretic follicles, and by 40 weeks $AR^{-/-}$ mice became infertile, with no follicle detectable in the ovary. Reflecting this age-dependent progression in ovarian abnormality, several genes known to be involved in the oocyte–granulosa cell regulatory loop were identified by microarray analysis as AR downstream target genes. These findings clearly demonstrate that AR-mediated androgen signaling is indispensable for the maintenance of folliculogenesis and implicate impaired androgen signaling as a potential cause of the POF syndrome.

Materials and Methods

Generation of AR Knockout Mice. *AR* genomic clones were isolated from a TT2 embryonic stem cell genomic library by using human *AR* A/B domain cDNA as a probe (6). The targeting vector consisted of a 7.6-kb 5′ region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and a neo cassette with two loxP sites (10). Targeted clones (FB-18 and FC-61) were aggregated with single eight-cell embryos from CD-1 mice (11, 12). Floxed *AR* mice (C57BL/6) were then crossed with CMV-Cre transgenic mice (6). The two lines exhibited the same phenotypic abnormalities. The chromosomal sex of each pup was determined by genomic PCR amplification of the Y chromosome *Sry* gene (13).

Western Blot Analysis. To detect AR protein expression, ovarian cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes (14). Membranes were probed with polyclonal AR antibodies (N-20; Santa Cruz Biotechnology), and blots were visualized by using peroxidase-conjugated second antibody and an ECL detection kit (Amersham Pharmacia Biosciences).

Morphologic Classification of Growing Follicles. Sections were taken at intervals of 30 μm, and 6-μm paraffin-embedded sections were mounted on slides. Routine hematoxylin and eosin staining was performed for histologic examination by light microscopy. Follicle numbers in 12 sections per ovary were evaluated as primary follicles (oocyte surrounded by a single layer of cuboidal granulosa cells), preantral follicles (oocyte surrounded by two or

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Abbreviations: AR, androgen receptor; DHT, 5α-dihydrotestosterone; POF, premature ovarian failure.

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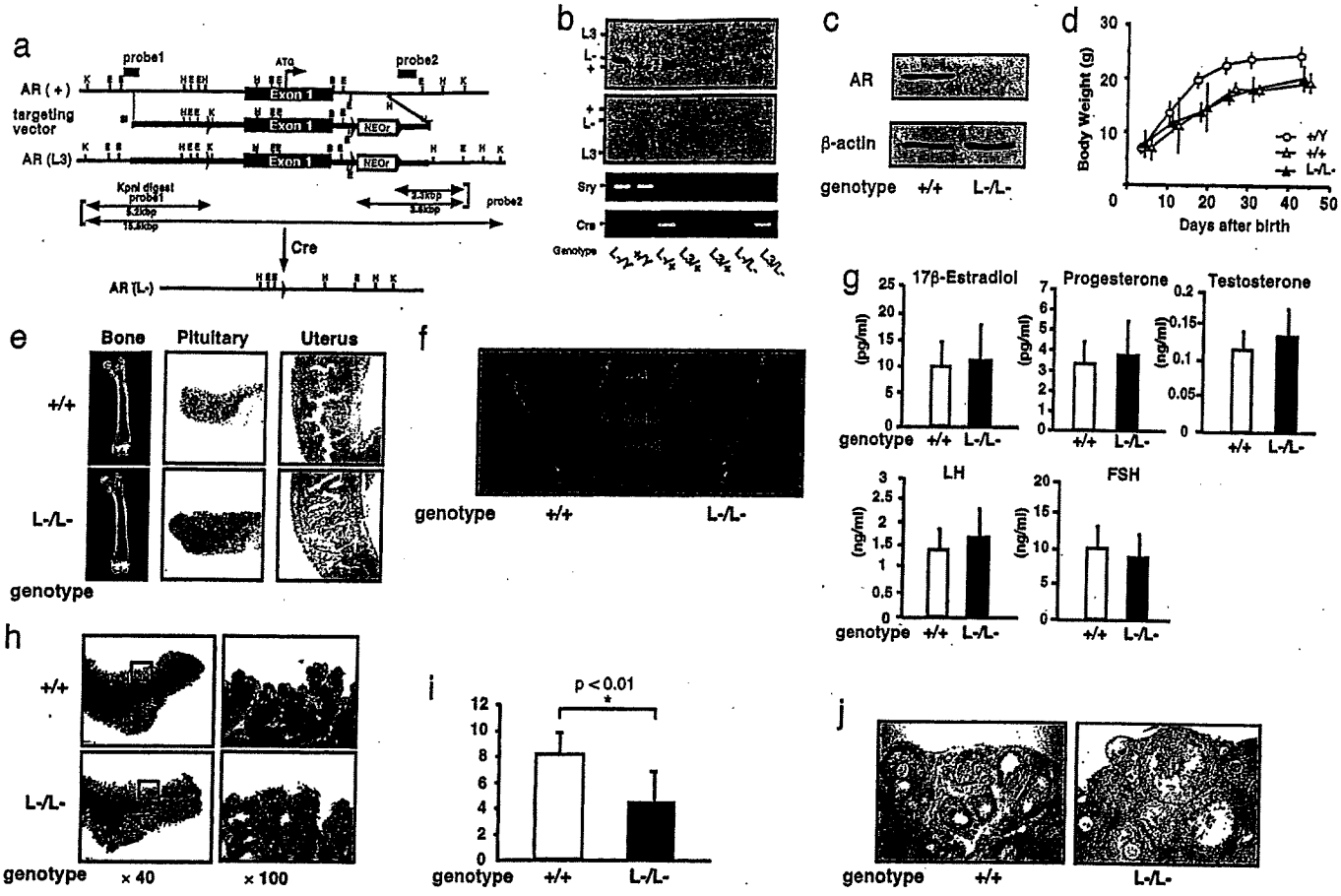

Fig. 1. Phenotypic characterization of AR knockout female mice. (*a*) Diagram of the wild-type *Ar* genomic locus (+), floxed *AR* L3 allele (L3), and *AR* allele (L-) obtained after Cre-mediated excision of exon 1. K, KpnI; E, EcoRI; H, HindIII; B, BamHI. LoxP sites are indicated by arrowheads. The targeting vector consisted of a 7.6-kb 5′ homologous region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and the neo cassette with two loxP sites. (*b*) Detection of the Y chromosome-specific *Sry* gene in $AR^{-/Y}$ mice by PCR. (*c*) Absence of AR protein in $AR^{-/-}$ mice ovaries by Western blot analysis using a specific C-terminal antibody. (*d*) Normal weight gain in $AR^{-/-}$ females. (*e*) Histology of pituitary, uterus, and bone tissues in $AR^{+/+}$ and $AR^{-/-}$ females at 8 weeks of age. (*f*) Female reproductive organs were macroscopically normal in $AR^{-/-}$ mice. (*g*) Serum hormone levels at the proestrus stage in $AR^{-/-}$ mice were not significantly altered. Serum 17β-estradiol, progesterone, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels in $AR^{+/+}$ ($n = 13$) and $AR^{-/-}$ ($n = 10$) females at 8–10 weeks of age are shown. (*h*) Lobuloalveolar development is impaired in $AR^{-/-}$ mammary glands. Whole mount of inguinal mammary glands (*Left*) and its higher magnification (*Right*) were prepared on day 3 of lactation. (*i*) Average number of pups per litter is markedly reduced in $AR^{-/-}$ mice at 8 weeks of age. Data are shown as mean ± SEM and analyzed by using Student's *t* test. (*j*) AR immunocytochemistry in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Sections were counterstained with eosin.

more layers of granulosa cells with no antrum), or antral follicles (antrum within the granulosa cell layers enclosing the oocyte). Follicles were determined to be atretic if they displayed two or more of the following criteria within a single cross section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane, or uneven granulosa cell layers (15).

Immunohistochemistry. Sections were subjected to a microwave antigen retrieval technique by boiling in 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min (16). The cooled sections were incubated in 1% H_2O_2 for 30 min to quench endogenous peroxidase and then incubated with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in normal goat serum for 1 h at 4°C. Sections were then incubated with anti-AR (1:100) or anti-cleaved caspase-3 (1:100) in 3% BSA overnight at 4°C. Negative controls were incubated in 3% BSA without primary antibody. The ABC method was used to visualize signals according to the manufacturer's instructions. Sections were incubated in biotinylated goat anti-rabbit IgG (1:200 dilution) for 2 h at room temperature, washed with PBS, and incubated in avidin–biotin–horseradish peroxidase for 1 h. After thorough washing in PBS, sections were developed with 3,3′-diaminobendizine tetrahydrochloride substrate, slightly counterstained with eosin, dehydrated through an ethanol series and xylene, and mounted.

Estrus Cycles and Fertility Test. To determine the stage of the estrus cycle (proestrus, estrus, and diestrus), vaginal smears were taken every morning and stained with Giemsa solution. For evaluation of female fertility for 15 weeks, an 8- or 24-week-old wild-type or $AR^{-/-}$ female was mated with a wild-type fertile male, replaced every 2 weeks with the other fertile male. Cages were monitored daily and for an additional 23 days, and the presence of seminal plugs and number of litters were recorded.

RNA Extraction and Quantitative Competitive RT-PCR. Total ovarian RNA was extracted by using TRIzol (Invitrogen) (16). Oligo-dT-primed cDNA was synthesized from 1 μg of ovarian RNA by using SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20-μl reaction volume, 1 μl of which was then diluted serially (2- to 128-fold) and used to PCR-amplify an internal control gene, *cycA*, to allow concentration estimation.

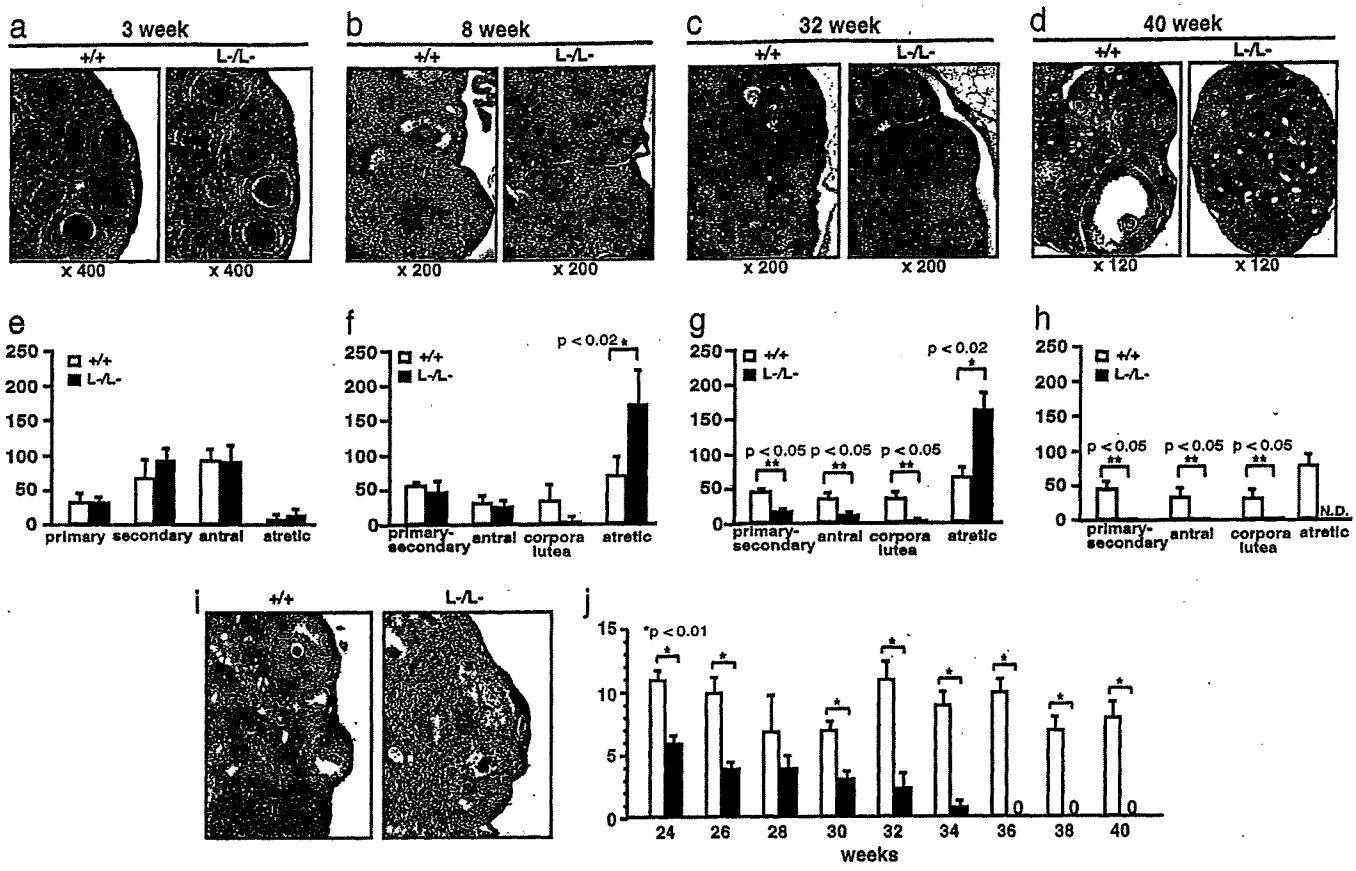

Fig. 2. POF in $AR^{-/-}$ female mice. (*a–d*) Histology of $AR^{+/+}$ and $AR^{-/-}$ ovaries at 3 weeks, 8 weeks, 32 weeks, and 40 weeks of age. All sections were stained with hematoxylin and eosin. An asterisk marks the atretic follicle. CL, corpus luteum. (*e–h*) Relative follicle counts at 3 weeks (*e*), 8 weeks (*f*), 32 weeks (*g*), and 40 weeks (*h*) of age. Numbers represent total counts of every fifth section from serially sectioned ovaries ($n = 4$ animals per genotype). (*i*) Immunohistochemical study for activated, cleaved caspase-3 revealed increased positive cells (apoptotic cells) in $AR^{-/-}$ ovaries. Sections were counterstained with hematoxylin. An asterisk marks the caspase-3-positive cell. CL, corpus luteum. (*j*) Age-dependent reduction in the number of pups per litter in $AR^{-/-}$ female mice. A continuous breeding assay was started at 24 weeks of age ($n = 6–10$ animals per genotype). For all panels, data are shown as mean ± SEM and were analyzed by using Student's *t* test.

Primers were designed from cDNA sequences of *Kitl* (M57647; nucleotides 1099–1751), Gdf9 (NM008110; nucleotides 720-1532), *Bmp15* (NM009757; nucleotides 146–973), *Ers2* (NM010157; nucleotides 1139–1921), *Pgr* (NM008829; nucleotides 1587–2425), *Cyp11a1* (NM019779; nucleotides 761-1697), *Cyp17a1* (M64863; nucleotides 522–932), *Cyp19* (D00659; nucleotides 699-1049), *Fshr* (AF095642; nucleotides 625-1427), *Lhr* (M81310; nucleotides 592-1331), *Ptgs2* (AF338730; nucleotides 3–605), and *Ccnd2* (NM009829; nucleotides 150-1065) and chosen from different exons to avoid amplification from genomic DNA.

GeneChip Analysis. Ovaries were isolated and stabilized in RNA*later* RNA Stabilization Reagent (Ambion, Austin, TX) before RNA purification (17). Total RNA was purified by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of RNA by using 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCAC-TATAGGGAGGCGG-$(dT)_{24}$-3′], 1× first-strand buffer, and 0.5 mM dNTPs at 42°C for 1 h. Second-strand synthesis was performed by incubating first-strand cDNA with 10 units of *Escherichia coli* ligase (Invitrogen), 40 units of DNA polymerase I (Invitrogen), 2 units of RNase H (Invitrogen), 1× reaction buffer, and 0.2 mM dNTPs at 16°C for 2 h, followed by 10 units of T4 DNA polymerase (Invitrogen) and incubation for another 5 min at 16°C. Double-stranded cDNA was purified by using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions and labeled by *in vitro* transcription by using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, dsDNA was mixed with 1× HY reaction buffer, 1× biotin-labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1× DTT, 1× RNase inhibitor mix, and 1× T7 RNA polymerase and incubated at 37°C for 4 h. Labeled cRNA was then purified by using GeneChip Sample Cleanup Module and fragmented in 1× fragmentation buffer at 94°C for 35 min. For hybridization to the GeneChip Mouse Expression Array 430A or 430B or Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, and 1× hybridization buffer in a 45°C rotisserie oven for 16 h. Washing and staining were performed by using a GeneChip Fluidic Station (Affymetrix) according to the manufacturer's protocol. Phycoerythrin-stained arrays were scanned as digital image files and analyzed with GENECHIP OPERATING SOFTWARE (Affymetrix) (17).

Luciferase Assay. The *Kitl* promoter region (−2866 to −1 bp) was inserted into the pGL3-basic vector (Promega) for assay using the Luciferase Assay System (Promega) (14, 16). Cells at 40–50% confluence were transfected with a reference pRL-CMV