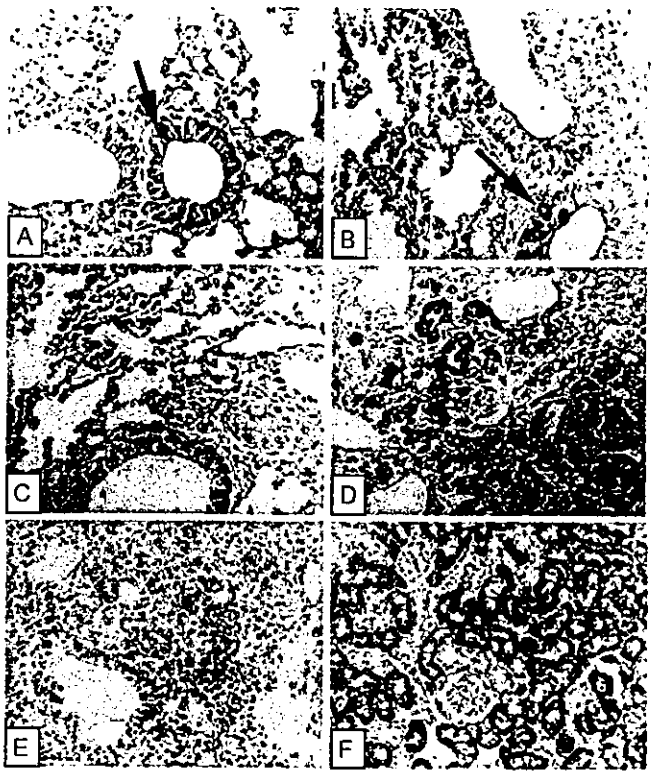

Fig. 6. Immunohistochemistry for P450 CYP2E1 in the lungs of WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) Sham-control WT mice, (B) benzene-exposed WT mice, (C) sham-control Cx32 KO mice, (D) benzene-exposed Cx32 KO mice, and (E and F) negative and positive control, respectively. A few bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for CYP2E1 (arrows). Benzene exposure induced increases in the numbers of the CYP2E1-producing bronchial/bronchiolar and alveolar epithelial cells in WT and Cx32 KO mice. Note the proliferating basophilic alveolar epithelial cells positive for CYP2E1 in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400; (E) ×200; (F) ×200.

died were immediately autopsied, whenever possible, and histopathological examinations were performed.

3.6. Tumor incidence and recovery of pulmonary lesions

Results of histopathological observation are shown in Table 2. No pulmonary tumors were observed in WT and Cx32 KO C57BL/6 mice sacrificed after the 26th week of exposure to 300 ppm benzene. Pulmonary adenoma developed in one Cx32 sham-control mouse. Pulmonary adenoma and adenocarcinoma developed only in two out of the seven benzene-exposed WT

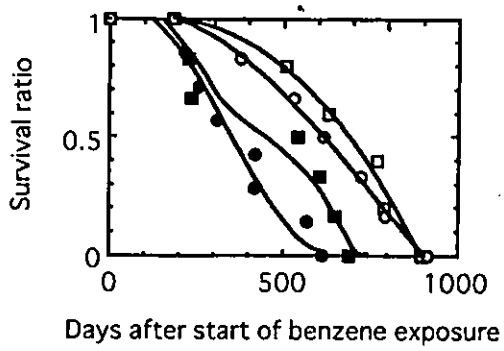

Fig. 7. Survival curves of groups for lifetime observation. The number of mice was limited about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed group, indicated by open symbols, circles for wild-type mice and squares for Cx32 KO mice, show a longer life span than the 300-ppm benzene-exposed group indicated by closed symbols, circles for wild-type mice and squares for Cx32 KO mice.

mice, at 59.7 weeks and 87.3 weeks of the study, respectively (Table 2). The pulmonary lesions observed in the WT and Cx32 KO mice exposed to benzene for 26 weeks were considerably attenuated and regressed with time after cessation of the exposure (data not shown).

Most of the benzene-exposed WT and Cx32 KO mice, which were allowed to live out their lives after termination of benzene exposure, died far earlier than the sham-control mice of each genotype due to malignant lymphomas, squamous cell carcinomas, spindle cell sarcoma and hepatomas or a combination of these tumors (Table 2).

The incidence of hemopoietic neoplasia in C57BL/6 mice was enhanced by 300 ppm benzene exposure as previously reported elsewhere (Snyder et al., 1980; Cronkite et al., 1985; Kawasaki et al., unpublished observation). In the Cx32 KO group, incidences of hemopoietic neoplasia were identical for both the benzene-exposed and sham-exposed control mice, although peak incidences were earlier in the benzene-exposed mice than in the sham-exposed control mice. (see Fig. 7).

4. Discussion

Benzene has been suspected for years as an agent that induces human pulmonary cancer (Aksoy, 1989)

Table 2
Tumor development in the wild-type (WT) and Cx32 knockout (KO) mice that were allowed to live out their life span after termination of 26-week exposure to 300 ppm benzene

Tumor/group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
No. of animals examined	6	7	5	6
Animals bearing tumor(s)	3 (50.0)	6 (85.7)	4 (80.0)	6 (100.0)
Pulmonary adenoma/adenocarcinoma	0 (0.00)	2 (28.6)	0 (0.0)	0 (0.0)
Hemopoietic neoplasia	2 (33.3)	5 (71.4)	4 (80.0)	5 (83.3)
Hepatoma	2 (33.3)[a]	0 (0.0)	1 (20.0)[a]	1 (16.7)
Squamous cell carcinoma	0 (0.0)	2 (28.6)[b]	0 (0.0)	4 (66.7)[c]
Spindle cell sarcoma	0 (0.0)	1 (14.3)	0 (0.0)	1 (16.7)
Animals without tumor(s)[d]	3 (50.0)	1 (14.3)	1 (20.0)	0 (0.0)

Number in parentheses represents the percentage (%) of the lesions.
[a] Concomitant with malignant lymphomas.
[b] Concomitant with pulmonary adenocarcinoma and spindle cell sarcoma.
[c] Concomitant with spindle cell sarcoma for one mouse, granulocytic leukemia for two mice and hepatoma for one mouse.
[d] Mice without tumor in the WT sham-control and the 300-ppm benzene-exposed groups had auricular thrombosis and one mouse without tumor in the Cx32 KO-control group died of ascending nephritis and renal infarction.

and the long-term exposure of mice to benzene had been shown to notably increase the incidence of pulmonary adenoma and adenocarcinoma (Huff et al., 1989; Maltoni et al., 1989; Farris et al., 1993). However, little information is available on the mechanism by which benzene exerts its pneumotoxicity and induces lung cancer.

Experimental conditions of benzene exposure and the incidence of hemopoietic neoplasia occurring in groups for lifetime observation were identical to those previously reported by Snyder et al. (1980, Fig. 4, p. 326 in their article) and also to our separate large-scale study (Kawasaki et al., unpublished observation). In the present study, we specifically focused on a possible role of Cx32 in benzene-induced pneumotoxicity and the pathogenesis of pulmonary tumor using bioengineered Cx32 KO and the WT mice.

Although Cx32-deficient mice have a late-onset peripheral neuropathy, a condition with features similar to those of Charcot–Marie–Tooth disease in humans, their gross morphology had been reported to be normal independent of age (7–28 weeks) and gender, except for a slightly lower body weight than the wild-type mice of the same genetic background (Nelles et al., 1996; Anzini et al., 1997). In agreement with a previous report, the body weight of Cx32 KO mice was lower than that of WT mice at the late stage of this study, although this difference was not significantly different (Fig. 1). The organ weight and histological findings consistently indicated that the decrease in body weight observed during long-term exposure to benzene closely correlated with the development of pulmonary lesions, characterized by diffuse granulomatous interstitial pneumonia, regenerating alveolar epithelial cell proliferation, and increased mucus secretion (Table 1, Figs. 2–5). The pulmonary lesions were far severer in Cx32 KO mice than in WT mice, strongly suggesting that Cx32 prevents the benzene-induced lung pathogenesis.

It has generally been accepted that the metabolism of benzene by the CYP2E1 enzyme to phenolic metabolites is a critical event in its toxic and carcinogenic mechanisms. A noteworthy finding of our study was the active proliferation of bronchiolar–alveolar epithelial cells expressing the CYP2E1 enzyme in the lungs of benzene-exposed Cx32 KO mice (Figs. 4D and 6F). This suggests that benzene exposure stimulates CYP2E1-producing epithelial cells in the lungs through a pathway that is regulated by the Cx32 gap junction protein. The activation of CYP2E1-producing epithelial cells may enhance the metabolism of benzene to metabolites that are potentially pneumotoxic such as benzene oxide, phenol and hydroquinone, resulting in exacerbation of

benzene-induced pneumotoxicity. CYP2E1 has been detected in the lungs of humans and rats (Tindberg and Ingelman-Sundberg, 1989; Carlson and Day, 1992; Wheeler et al., 1992). Moreover, recent studies have shown the important role of the enzyme in benzene metabolism resulting in its pneumotoxicity (Powley and Carlson, 2000, 2001), which is also supported by the finding of benzene metabolism inhibition by a CYP2E1 inhibitor, diethyldithiocarbamate (Chaney and Carlson, 1995). The metabolic level of benzene in the pulmonary tissue has not been evaluated yet. However, a previous study showed that pulmonary microsomes can metabolize benzene at similar rates to those of hepatic microsomes, and that they are likely more efficient in generating hydroquinone (Chaney and Carlson, 1995). Recently, with regards to CYP2E1-mediated 1,1-dichlroethylene-induced lung toxicity, Forkert et al. (2001) reported good correlations among the amount of the enzyme, metabolism of 1,1-dichlroethylene to a toxic metabolite, and lung cytotoxicity.

Activation of alveolar pneumocytes by benzene was considered to be another possible important event for the pneumotoxicity of benzene observed in the present study, as shown in the lungs of benzene-exposed groups of WT and Cx32 KO mice (Fig. 3). Alveolar epithelial cells, containing a large amount of peroxidase, are capable of metabolizing phenolic compounds to genotoxic reactive species that can induce DNA adducts and generate oxygen-free radicals (Brieland et al., 1987; Schlosser et al., 1989; Smith et al., 1989) and of producing nitric oxide by themselves (Laskin et al., 1995). The production of reactive oxygen intermediates has been implicated in cytotoxicity and carcinogenesis, by inhibiting GJICs as well as causing DNA damage (Kuo et al., 1998; Upham et al., 1997, 1998). Several investigators have shown that oxygen radicals from benzene-activated alveolar epithelial cells play an important role in the genotoxic and nongenotoxic mechanisms of benzene-target organs (Subrahmanyam et al., 1991; Kolachana et al., 1993; Laskin et al., 1995). In the lung, Suleiman (1987) showed that benzene induces lipid peroxidation and increases the amount of the lysosomal enzyme released by activating alveolar epithelial cells, contributing to the pathological changes. The formation of oxygen radicals and related reactive oxygen species is highly controlled in a biological system by physiological antioxidant defense mechanisms. In a study by Kojima et al. (1996), a potential role of Cx32 in the regulation of oxygen radical production in cultured hepatocytes has been suggested based on the correlation found between the expression of Cx32 and the effect of oxygen radical scavengers. Therefore, it can also be hypothesized in our present study that the disregulation of reactive oxygen species production by benzene in lung tissues due to a dysfunction of GJICs caused by Cx32 might contribute to the exacerbation of pulmonary lesions in Cx32 KO mice. Further studies will be required to prove this hypothesis.

Despite the finding that the Cx32-mediated disruption of GJICs enhanced the pneumotoxicity of benzene, our present study, though with a small number of animals did not indicate any enhancement of the development of pulmonary tumor in the Cx32 KO mice (Table 2).

Therefore, the pathological lesions exacerbated in Cx32 KO mice may not seem to be critical changes for pulmonary carcinogenesis of benzene. This was supported by their recovery from the pulmonary lesions after removal of benzene and the absence of tumor incidence in benzene-exposed Cx32 KO mice.

In conclusion, our present study indicates that Cx32 attenuates the pneumotoxicity of benzene, particularly in the case of chronic exposure in vivo, most likely by regulating proliferation of CYP2E1-producing lung cells population. However, the role of Cx32 in benzene-induced pulmonary tumorigenesis was not clarified in the present study.

Acknowledgements

The authors thank Ms. C. Aoyagi, Ms. K. Aihara and Ms. E. Tachihara for excellent technical assistance.

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Toxicologic Pathology, 32(Suppl. 2):12–16, 2004

ISSN: 0192-6233 print / 1533-1601 online
DOI: 10.1080/01926230490451725

Mechanism of Benzene-Induced Hematotoxicity and Leukemogenicity: Current Review with Implication of Microarray Analyses

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ABSTRACT

Benzene is a potent human leukemogen but the mechanism underlying benzene-induced leukemia remains an enigma due to a number of questions regarding the requirement of extraordinarily long exposure, a relatively low incidence of leukemia for genotoxicity of metabolites and a narrow dose range for leukemogenicity over marrow aplasia (overdoses tend to result in marrow aplasia). Moreover, there were previous controversies as to whether the cell cycle is upregulated or suppressed by the benzene exposure. Subsequently, it was found that the cell cycle is suppressed, but how leukemia develops under such suppression of hemopoiesis remains to be clarified. These questions were fortunately resolved with much effort. Benzene exposure was found to induce the expression of p21, an interlocking counterdevice for cell cycle: due to *p53* upregulation, thereby inducing the immediate suppression of the kinetics of hemopoietic progenitors followed by the prominent suppression of hemopoiesis. Intermittent benzene exposure (i.e., cessation of exposure during weekends, for example) allowed an immediate recovery from marrow suppression after terminating exposure, which induced continuous oscillatory changes in marrow hemopoiesis. Benzene-induced leukemia was chiefly due to such an oscillatory change in hemopoiesis, which epigenetically developed leukemia more than 1 year later. The mechanisms of benzene-induced leukemogenicity seem to differ between wild-type mice and mice lacking *p53*. For *p53* knockout mice, DNA damage such as weak mutagenicity or chromosomal damage was retained, and such damage induced consequent activation of proto-oncogenes and related genes, which led cells to undergo further neoplastic changes. In contrast, for wild-type mice carrying the *p53* gene, a marked oscillatory change in the cell cycle of the stem cell compartment seems to be important. Compatible and discriminative gene expression profiling between the *p53* knockout mice and wild-type mice was observed after benzene exposure by microarray analyses.

Keywords. Benzene; hematotoxicity; leukemogenicity; gene chip array; BUUV method; *p53*-KO; AhR-KO; hemopoietic progenitor cells.

INTRODUCTION

The mechanism of benzene-induced leukemia had long been an enigma until recently, when the unique cell kinetics of stem/progenitor cells during benzene exposure was elucidated. Leukemia induction by benzene inhalation was first reported in 1897, when Le Noire described multiple cases of leukemia among Parisian cobblers (Le Noir and Claude, 1897). However, the experimental induction of leukemia by benzene exposure was first reported about 20 years ago by Snyder et al. (1980) and our group (Cronkite et al., 1984, 1989). Recently, we demonstrated marked oscillatory changes in peripheral blood and bone marrow (BM)[1] cellularities during and following benzene inhalation, preceding the development of leukemia by about 1 year (Hirabayashi et al., 1998; Kawasaki et al., 2001; Yoon et al., 2001).

Benzene-induced leukemia is unique in that it has been associated only with a weak mutagenicity in the benzene metabolites, phenol and hydroquinone. Another interesting observation is the controversial experimental data concerning the level of actively cycling hematopoietic cells following benzene exposure. While all researchers observed a decrease in peripheral blood and BM cellularities, some observed a suppression of the cell cycle of BM, as measured by tritiated thymidine incorporation (Moeschlin and Speck, 1967), whereas others observed a marked increase in the number of cycling stem/progenitor cells in BM and peripheral blood (Table 1). Careful analysis of these apparently conflicting data revealed an enhancement of the cell cycle occurring at least 2 hours after the termination of benzene exposure. Thus, the higher tritiated thymidine incorporation documented by Cronkite et al. (1982) 18 hours after the termination of benzene exposure probably reflects a recovery phase. Based on these findings, we conducted a series of studies since 1997 to elucidate the leukemogenic effect of benzene in wild-type mice.

The *p53*-knockout (KO) mouse (Tsukada et al., 1993) showed further unique characteristics of benzene-induced leukemia. Using *p53*-KO mouse, we confirmed that benzene has a moderate genotoxic effect, as measured by the micronucleus test performed 4 weeks after the initiation of benzene inhalation (Kawasaki et al., 2001; Li et al., 2003). Moreover, *p53*-deficient mice manifest increased susceptibility to

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[1]Abbreviations: BM, bone marrow; KO, knockout; UV, ultraviolet; BUUV, incorporation of bromodeoxyuridine followed by ultraviolet-light cytocide to evaluate the hemopoietic stem/progenitor cell kinetics in vivo; AhR, aryl hydrocarbon receptor; $AhR^{+/+}$, AhR wild-type; $AhR^{+/-}$, AhR heterozygous-deficient; $AhR^{-/-}$, AhR homozygous-deficient; CFU-GM, granulo-macrophage colony forming unit; CYP, cytochrome P450; FGF, fibroblast growth factor; TGF, tumor growth factor; I.V., intravenous; I.P., intraperitoneal; aft, after; dur, during; expos, exposure.

TABLE 1.—Summary of the results the hemopoietic stem/progenitor cell kinetics during and after benzene exposure by tritiated thymidine (^{3}H-TdR) cytocide assay.

				Evaluation cell and assay methods					
		Cellularity		BM cells			CFU		
Year	Reference	Blood	BM	Kinetics	Labeling[a,1]	Label point	Kinetics	Labeling	Label point
1967	Moeschlin and Speck	↓	↓	↓	In vivo	At pancytopenia	—	—	—
1979	Irons et al.	↓	↓	↑	In vivo[2]	6 days aft. expos-IP	—	—	—
1982	Cronkite et al.	↓	↓	—	—	—	↑	In vitro	18 h aft. expos.
1998	Lee et al.	↓	↓	↓	In vivo[3]	30 min aft. single IP	—	—	—
		↓	↓	↓	In vitro	Dur. and aft. expos.	—	—	—
1997	Farris et al.	↓	↓	→↓	In vivo[4]	Soon aft. expos.	↑	In vitro	2 h aft. expos.

1. ^{3}H-TdR was injected intravenous (IV) at in vivo labeling except indications.
2. ^{3}H-TdR was injected intraperitoneal (IP) 6 days after cessation of benzene.
3. Benzene was treated single IP, and ^{3}H-TdR label was starting 30 minutes after benzene treatment.
4. Instead of ^{3}H-TdR, BrdUrd was used for assay.

benzene-induced leukemogenicity (Kawasaki et al., 2001). Similar findings with regard to increased leukemogenicity following benzene exposure have been documented by French et al. of the National Institute of Environmental Health Sciences (French et al., 2001). Contrary to the result in *p53*-KO mice, benzene-induced leukemia had not been detected in earlier studies in wild-type mice because its manifestations had been masked either by pancytopenia due to severe myelosuppression or by the use of a benzene dose too low to induce pancytopenia or leukemia (Kawasaki et al., 2001). Aryl hydrocarbon receptor (AhR)-KO mice (Mimura et al., 1997) also elucidated the characteristic underlying mechanism of benzene-induced hematotoxicity (Yoon et al., 2002).

In the mechanism underlying benzene toxicity in BM tissue analyzed using a microarray system, various signaling pathways have been suggested to be implicated including cell cycle regulation, DNA-damage/repair-related genes, oxidative-stress-related genes, growth-factor-related genes, oncogenes, and hemopoiesis-related genes in general (Yoon et al., 2003).

Oscillatory Changes in Bone Marrow Cellularity in Wild-Type Mice

BM cellularity decreases markedly during benzene inhalation but recovers rapidly following the termination of benzene exposure (Yoon et al., 2001). The oscillatory nature of the resultant curve is comparable to the response reported by Cronkite et al. (1984, 1989), and suggests that benzene does not only induce BM cell suppression but also activates cell-cycle-regulating genes, resulting in compensatory myelopoiesis.

We used the BUUV (bromodeoxyuridine + UV exposure) method to study stem/progenitor cell kinetics during or after benzene exposure (Hirabayashi et al., 1998; Yoon et al., 2001). Using this method, we were able to measure the labeling rate, cycling fraction of clonogenic progenitor cells, and other cell cycle parameters. Interestingly, the cycling fraction of stem/progenitor cells was found not to turn into active hematopoiesis but to remain low during benzene inhalation. Furthermore, rapid recovery was observed after benzene inhalation was terminated (Figure 1). However, although the exact mechanism of this phenomenon is not yet known, we found the evidence that the cycling fraction depression may be mediated in part by the suppression of stem/progenitor cell cycling per se, owing to the *p53*-dependent upregulation of p21 (Yoon et al., 2001). Thus, the mechanism of benzene-induced leukemia in the wild-type mice may be due to continuous oscillatory changes in hemopoiesis during and after the benzene exposure, which leads to genetic instability followed by the consequent epigenetic leukemogenicity.

p53-Deficient Mice Develop Leukemia by Different Mechanisms

Leukemogenicity induced in *p53*-KO mice, because of the lack of the *p53* gene, results in the noninduction of p21 expression even during the benzene exposure, with subsequent insufficient DNA repair and accumulation of DNA damage. These pathways are shown in Figure 2 for benzene-induced possible toxicological changes in both wild-type and *p53*-KO mice. In *p53*-KO mice, cell cycle suppression, DNA repair, and apoptosis of damaged cells, which, in general, occur in the wild-type mice after benzene exposure, are all suppressed. This is much more likely genotoxic leukemogenesis, in which reactive oxygen species, dysfunction of topoisomerase, and covalent binding of adduct formation to DNA,

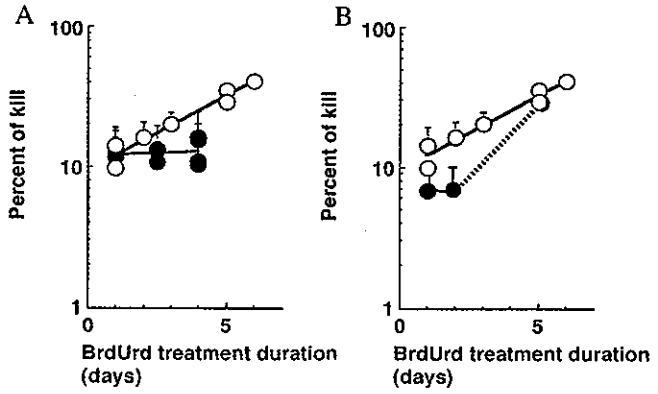

FIGURE 1.—Hemopoietic progenitor cell (CFU-GM) kinetics during (A) and after (B) benzene inhalation. Open circle: sham; Closed circle: during or after inhalation of 300 ppm benzene, 6 h/day, 5 days/week ×2 weeks. (A) For the benzene-treated group, all the mice were sacrificed just after the 5th day of the 2nd week of benzene-inhalation. The osmotic minipump filled with BrdUrd was implanted into donor mice day(s) before sacrificing as indicated on the abscissa. (B) For the benzene-treated group, the BrdUrd-pump was implanted into donor mice after the 5th day of the 2nd week of benzene-inhalation and sacrificed on the day as indicated on the abscissa. Each point represents at least 2 mice as a donor for the CFU-GM assay, and colony assays were performed in triplicate.

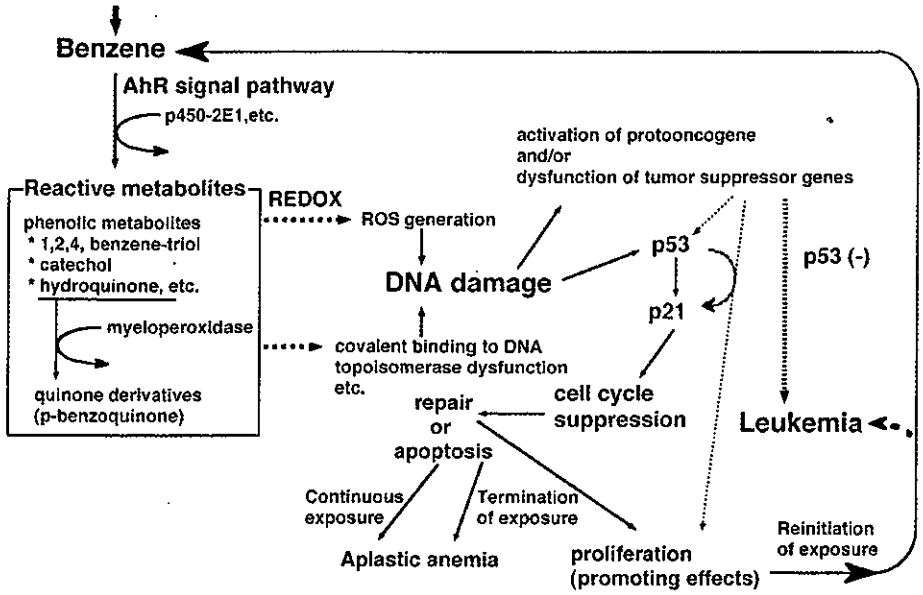

FIGURE 2.—Benzene metabolism and possible mechanism of benzene-induced leukemogenesis.

all synergistically participate in further leukemogenic development without repairing the system (see Figure 2). Thus, leukemogenicity seems to be clearly different between the mice carrying wild-type *p53* and the mice lacking *p53* (Yoon et al., 2001; Hirabayashi et al., 2002).

ARYLHYDROCARBON-RECEPTOR-MEDIATED BENZENE METABOLISM

We investigated the involvement of the aryl hydrocarbon receptor (AhR), a ligand-activated basic helix-loop-helix transcription factor, in hematotoxicity using AhR wild-type ($AhR^{+/+}$), heterozygous-KO ($AhR^{+/-}$) and homozygous-KO ($AhR^{-/-}$) male mice (Mimura et al., 1997; Yoon et al., 2002). Following a 2-week inhalation of benzene at 300 ppm, we evaluated the changes in cellularity of the peripheral blood and BM, and the levels of granulocyte-macrophage colony-forming units (CFU-GM) in the BM (Figure 3). The expression of the cyclin-dependent kinase inhibitor, p21, in BM cells and cytochrome P450 (CYP) 2E1 in hepatic tissues were evaluated by Western blot analysis after benzene exposure. Our results clearly showed that $AhR^{-/-}$ mice are much more resistant to the benzene-induced hematotoxicity than $AhR^{+/+}$ wild-type mice. No changes in p21 expression level by BM cells were detected in $AhR^{-/-}$ mice, whereas a marked up-regulation of p21 expression by BM cells was observed in $AhR^{+/+}$ mice. This finding is a further proof of the resistance of $AhR^{-/-}$ mice to benzene-induced hematotoxicity. The benzene resistance of $AhR^{-/-}$ mice was abrogated by exposure to a combination of 2 major metabolites, phenol and hydroquinone, strongly supporting the notion that AhR participates in benzene metabolism. CYP species involved in such metabolism are under investigation. The results obtained imply that pollutants that react with AhR confer marked susceptibility to benzene-induced hematotoxicity.

IMPLICATION OF MICROARRAY ANALYSIS

In the mechanism underlying benzene toxicity in BM tissue, various signaling pathways have been suggested to be implicated including metabolism, genotoxicity, cell cycle regulation, and apoptosis (Table 2). Our microarray analysis

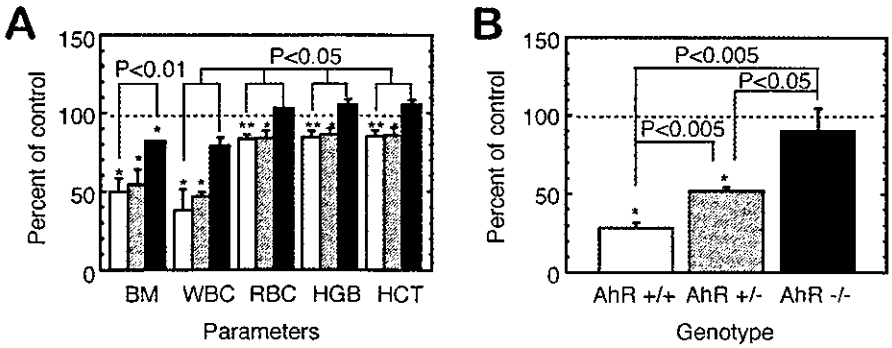

FIGURE 3.—Changes in peripheral blood parameters and BM cellularity (A) and CFU-GM per 2 femurs (B) in the AhR wild-type ($AhR^{+/+}$:open bar), heterozygous-KO ($AhR^{+/-}$:shaded bar) and homozygous-KO mice ($AhR^{-/-}$:closed bar) exposed to 300 ppm benzene for 2 weeks. The mean BM cellularities for the $AhR^{+/+}$, $AhR^{+/-}$, and $AhR^{-/-}$ mice were 4.8, 5.6, and 4.8 $\times$ 10^7/2 femurs, respectively, and the mean numbers of CFU-GM's per 8 $\times$ 10^4 BM cells was 79, 78, and 72, respectively. *, **: Significantly different from each corresponding control group at $p < 0.05$ and $p < 0.01$, respectively.

TABLE 2.—Reported genes whose expression changed during and/or after benzene inhalation.

Category	Gene name	Reference
Metabolic enzyme	CYP 2E1	Zhang et al., 2002
	Myeloperoxidase	Schattenberg et al., 1994
Cell cycle	*p53*	Boley et al., 2002
	p21 (waf 1)	
	Cyclin G	
	Gadd 45	
Apoptosis	Bax-alpha	Boley et al., 2002
Oncogene	c-fos	Ho and Witz, 1997

elucidated the up- or downregulation of genes functioning after 2-week exposure to 300 ppm benzene (Table 3): First, among cell-cycle-related genes, in addition to *p53* and p21 which are known to be upregulated to various extents depending upon the time course and the detection methods, Rb-related genes, such as the Rb-related protein p130 and the Rb-binding protein p48 are significantly upregulated; furthermore, elongation factor 1-delta shows a high expression level associated with the G2/M cell cycle checkpoint; vice versa, a significant downregulation of cyclin D1 and BimB is also recognized. Less significant changes in expression of cyclin G and Gadd45 are noted as previously reported. Second, among DNA-damage/repair-related genes, those encoding ADP-ribosylation factor-like protein 1 and Rad51 are significantly upregulated. The altered expression of other genes in the same category such as Metaxin, ERCC-3, and the DTR111 precursor are also noted, although *p*-values are not statistically significant. Third, among oxidative-stress-related genes, mitogen-activated protein kinase 2, which responds primarily to stress and inflammatory stimuli, is significantly upregulated, and the known typical ROS absorber genes, such as those encoding GST-1 and UDP glucuronosyltransferase show mild but significant increases. C3h-dioxin-inducible cytosolic aldehyde dehydrogenase-3, Cytochrome c oxidase Vb, and lactate dehydrogenase are also upregulated. Fourth, among growth-factor-related genes, those encoding the hepatocyte-growth factor-like protein shows significant upregulation, associated with a slight increase/decrease in expression level, with less significant *p*-values, for the following genes: fibroblast growth factor (FGF)-15, FGF-b, G protein-coupled receptor, growth factor-induced delayed-early-response protein, insulin-like growth factor I receptor precursor, insulin-induced growth response protein cl-6, tumor growth factor (TGF)-beta 1, TGF-beta 1 masking protein, and tumor necrosis factor alpha. Fifth, among the gene expression profiling of oncogenes, RhoB, which is possibly related to the genotoxic effect of benzene metabolites, shows a high expression level. Finally, hemopoiesis-related genes also show particular changes in their expression level, but the profiling of such genes led to the elucidation that benzene generally induces suppression of cell proliferation without an increase in cytokine gene expression levels.

TABLE 3.—*p53*-related genes whose expression level decreased or increased by benzene exposure, but unchanged in the wild-type mice.

WT: unchanged *p53*-KO: decreased	CalDAG-GEFI, Cbfa2, Dctn1, Fr1, Grl-1, Ig/EBP, Klra3, Mek5, MEP, Mlp1, B-myb, Nog, PBX2, Prkm3, PTPalpha, Rad50, Rad51, Zfp94
WT: unchanged *p53*-KO: increased	24p3, 4E-BP2, Abcg2, ACRP, Activine, Ahd3, Alp, Anx3, AOE372, Apaf1, BAG-1, BAP, bcl-2, Calcyclin, Canexin, Caspase 9, Caspase 9S, CCR1, CD3 theta, CD71, CD143, Cox5b, Cox7a1, COX8H, Ctla-2a, Cu/Zn-SOD, Cyclin B1, DCIR, Dnmt2, Dpagt2, E4BP4, EPO, FACS, Fes, elk1, G6PD, G6PD-2, Galbp, Gapdh, Gcdh, Gdi2, Growth hormone, Gnb-1, Gng3lg, H-2T18, HES-1, IGF-1, IL1bc, IL-4, IL-9, JSR1, LDH-1, LDH-2, mLigI, Lipo 1, Lrf, Ly-3, Ly-40, Jam, JNK2, Kcc1, KSR1, M-CSF, Mac-1 alpha, Mch6, Mg11, MHR23A, MmCEN3, Mrad17, MRP14, Mtx2, NFATp, NL, Nmo1, OERK, PAFR, Pde8, PERK, PGRP, Pla2g2c, PLGF, Pop2, Prkm9, Prtn3, RBP-L, Rga, S100A13, Siva, Smad 6, SPRR2J, Stat4, Stat 5B, TCF4, TOM1, Trypsin 2, Tst

See reference, Yoon et al. (2003).

It is of interest to determine gene expression in *p53*-KO mice with or without exposure by benzene inhalation (Yoon et al., 2003). In Table 3, the annotated genes were all downregulated (top) or upregulated (bottom) after benzene exposure, although their expressions were not altered in the wild-type mice, implying that the expressions of these genes are masked by the homeostasis governed by *p53* gene regulation. Thus, this study on *p53*-KO mice led to the elucidation of hidden gene alterations in wild-type mice, which we do not generally observe in toxicological examination.

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Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice

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Received 13 May 2002; received in revised form 10 September 2003; accepted 16 October 2003

Abstract

In rodents, steroid hormones are thought to be transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses may have higher serum levels of estradiol, and lower serum levels of testosterone, relative to siblings of the same sex that develop between two male fetuses. The consequence in the variation of postnatal growth, development, and function in the intrauterine position, using various parameters such as anogenital distance, preputial separation and vaginal opening, estrous cycle, locomotor activity, and growth of reproductive organs, were examined in Sprague–Dawley rats. ICR mice were treated with 17β-estradiol before copulation and during pregnancy to address the interaction with endogenous estradiol during pregnancy. In rats, no evidence of effects of prior intrauterine position was observed for any of the parameters examined. Mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. The results of this study suggested that the intrauterine position of the embryos/fetuses did not affect the postnatal growth of the reproductive organs, sexual maturation, or behavior in rats and mice.

Keywords: Intrauterine position; Postnatal growth; Sexual maturation; Behavior; Anogenital distance; Rats; Mice

1. Introduction

The development of sexually differentiated phenotypes depends upon the hormonal environment during a critical period of growth [1]. Testosterone secretion by the fetal testis causes a longer anogenital distance (AGD), seen in neonatal males, relative to females. The AGD of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position of the animals [1–4]. A longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus. Females with a male fetus on only one side are immediate [4].

In all litter-bearing species that have been examined to date, the intrauterine position that a fetus occupies relative to fetuses of the same or opposite sex has profound effects on its reproductive, behavioral, and morphological traits measured during adult life [4–7]. Gerbil males and females that developed in utero between two female fetuses or two male fetuses, respectively, did not differ in relative hippocampal size [8].

The effects of intrauterine position are apparently not the result of the position itself, but rather of the movement of steroid hormones between the fetuses, and variations in the hormonal environment relative to the proximity of an individual fetus to other fetuses of the same or opposite sex [9]. Male rats located between two females had elevated serum estradiol and larger prostates than males located between two males, which had elevated serum testosterone and larger seminal vesicles [10]. The effect of intrauterine position in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity [11,12].

Recently, intrauterine position has been the focus of discussions in the toxicology community because of its potential to alter the susceptibility of fetuses to endogenous hormones and endocrine disrupting chemicals [13,14]. In this regard, failure to account for intrauterine position in endocrine disrupting chemical toxicology studies could lead to false negative results, especially when adverse alterations

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0890-6238/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2003.10.009

are produced by low doses in fetuses from only one intrauterine position [14,15]. This possibility has been raised because of investigations into estrogenic compounds in mice. In rats, consistent effects due to intrauterine position on testosterone concentrations, and therefore potential interactions with endocrine disrupting chemicals, have not been found. Howdeshell and vom Saal [16] demonstrated that the greatest response to the estrogenic chemical, bisphenol A, occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A within the range of human exposure, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development. It has been demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin interacted with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses [17].

The objectives of this study were to determine the effects of intrauterine position, under normal physiological conditions, on the development of rat offspring, as well as sexual maturation, estrous cycle, behavior, and reproductive organ development. Another objective of this study was to determine whether the intrauterine position of mouse fetuses, which is related to background levels of estradiol and testosterone, would influence the response of the postnatal growth of gonads, including sexual maturation, to low dose 17β-estradiol.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Crj:CD, IGS), and ICR mice (Crj:CD-1) were purchased from Charles River, Laboratories, Inc. (Atsugi, Japan). Twenty-seven male rats (9 weeks of age), 84 female rats (8 weeks of age), 130 male mice (9 weeks of age), and 130 female mice (8 weeks of age), were used. The rats and mice arrived with mean weights of 301.1 ± 7.9 g for males and 216.2 ± 8.1 g for females, and 37.2 ± 1.2 g for males and 29.1 ± 0.9 g for females (mean ± S.D.), respectively. The animals were acclimated to the laboratory for 7–14 days prior to the start of the experiments to evaluate weight gain and any gross signs of disease or injury. The animals were housed individually in stainless steel, wire-mesh cages in a room with controlled temperature (22–25 °C) and humidity (50–65%), with lights on from 07:00 to 19:00 h daily. The animals were given access to food (NIH-07-PLD: phytoestrogen low diet, Oriental Yeast Co., Japan) and tap water through metal pipes (distilled water, Wako Pure Chem., Japan) ad libitum. In a few instances, the temperature and humidity were outside the standard ranges, but the magnitude and duration of these incidents were minimal and judged to be of no consequence. The contents of genistein and daidzein in the diet and wood bedding (ALPHA-dri, Shepherd Specialty Paper, USA) used in the present study were determined. Neither genistein nor daidzein were not detected in the diet or wood bedding (detection limit: 0.5 mg/100 g in each individual phytoestrogen, by HPLC).

, Animal care and use conformed to published guidelines [18].

2.2. Experiment I (examination of intrauterine position effect on postnatal growth in rats)

2.2.1. Cesarean delivery and fostering

Estrous female rats at 10–11 weeks of age were cohabited overnight with a single male to obtain 66 pregnant females within 4 days. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Thirty-three pregnant females were killed by CO_2 asphyxiation and cervical dislocation, and subjected to cesarean sectioning on day 21 of gestation. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed. Anogenital distance (AGD) was measured with a digital micrometer (reproductive precision of 0.01 mm, Digimatic caliper CD-15C, Mitutoyo Co., Kanagawa, Japan) under an Olympus dissecting microscope for each fetus, and the average was taken. The subject was held steady and in the same position during measurement. Measurements were made without knowledge of intrauterine position by one person. The AGD was measured from the center of the phallus to the center of the anus. The fetuses obtained by cesarean delivery were fostered to 33 dams that had just given birth naturally (one litter to each female). The original littermates remained together when cross-fostered. The litter sizes were similar for each cross-fostered dam. The day of cesarean section was considered as postnatal day (PND) 0. Pup body weights were recorded on PND 21 (day of weaning). Following weaning, and until 10 weeks of age, offspring were weighed once a week.

Neonates from 33 pregnant females were categorized as occupying six different intrauterine positions: 2M (male fetus located between two male fetuses; number of pups and litters on PND 0 = 36 and 19); 1M (male fetus that located between a male fetus and a female fetus; n = 73 and 27); 0M (male fetus located between two female fetuses; n = 45 and 24); 2F (female fetus located between two female fetuses; n = 38 and 18); 1F (female fetus located between a female fetus and a male fetus; n = 83 and 29); 0F (female fetus located between two male fetuses; n = 41 and 27). Fetuses adjacent to dead embryos (resorptions or macerated fetuses), and fetuses that were closest to each ovary or the cervix, were discarded from further analyses.

2.2.2. Observations of postnatal growth

2.2.2.1. Measurement of AGD and reproductive organ weights, and evaluation of sexual maturation. On PND

4, the AGD was measured for pups in each group using calipers with a reproductive precision of 0.01 mm. On PND 21, all pups were weaned and half of the pups in each group (2M = 13, 1M = 37, 0M = 11, 2F = 14, 1F = 43, 0F = 12) were subjected to necropsy, and the testes, epididymides, and prostates with seminal vesicles (fluid was not removed and all lobes were included) in males, and uteri and ovaries in females, were weighed. For the remaining male and female pups in each litter (2M = 21, 1M = 32, 0M = 30, 2F = 23, 1F = 36, 0F = 26), as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 28), and preputial separation for males (beginning on PND 35), were assessed, and each rat was weighed when these criteria were achieved.

2.2.2.2. Postweaning tests of behavior, evaluation of estrous cycle, and histological observation of reproductive organs. One male and one female were randomly selected from each litter in each group (number of rats examined: 2M = 18; 1M = 27; 0M = 25; 2F = 17; 1F = 27; 0F = 25), and were subjected to an open field test and wheel cage activity test to assess the emotionality and regulatory running activity, respectively. At 4 weeks of age, the rats were placed into a circular area (140 cm in diameter) surrounded by a wall (40 cm in height). The light and noise levels averaged 500 lx and 50 dB, respectively, at the center of the circular area. Rearing, grooming, defecation, and urination were counted, and ambulation was recorded automatically on a computer (Unicom, Inc., Japan), during a 3-min trial between 13:00 and 16:00 h on one day. At 7 weeks of age, the rats were placed into a wheel cage (Nippon Cage, Inc., Japan), 32 cm in diameter and 10 cm in width, as a measure of spontaneous activity. Each rat was kept within the wheel for 24 h with free access to food (NIH-07-PLD) and distilled water in the same animal room. The number of revolutions was automatically recorded with a 20-channel digital counter (Seiko Denki, Inc., Japan).

Each morning (9:00–10:00 h), from 6 to 10 weeks of age, all females in each group were subjected to vaginal lavage. The lavage fluid was applied to a glass slide, air-dried, and stained with Wright–Giemsa stain. Cytology was evaluated and the stage of the estrous cycle was determined using the method of Everett [19].

At 10 weeks of age, 3–5 males in each group were weighed, and anesthetized. Transcardial perfusions were carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the prostate gland was sampled, rinsed three times in phosphate buffer, postfixed for 2 h at 4 °C in 2% osmium tetroxide, and dehydrated in alcohol; the prostate gland was embedded in epoxy resin. Ultrathin sections of the prostates were stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7100, Hitachi, Japan). The remaining males in each group (2M = 18, 1M = 27, 0M = 25) were weighed and subjected to necropsy, and the testes, epididymides, ventral prostate, and dorsal prostates with seminal vesicles, were weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. All females (2F = 17, 1F = 27, 0F = 25) were weighed and subjected to necropsy when the stage of the estrous cycle was diestrus. The ovaries and uteri were then weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. These reproductive organs were embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.3. Experiment II (examination of low-dose in utero effects of 17β-estradiol in mice)

The objective of this experiment was to determine whether the intrauterine position of male fetuses, which is related to background levels of estradiol (elevated in males located between two female fetuses) and testosterone (elevated in males located between two male fetuses), would influence the response of the developing prostate to low dose 17β-estradiol. In addition, we examined whether the intrauterine position of male and female fetuses would affect the postnatal growth of other reproductive organs and sexual maturation.

2.3.1. Administration, cesarean delivery and fostering

Thirty female mice at 9 weeks of age were administered 17β-estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μg/kg per day for 7 days before mating, during a mating period of 7 days at the longest, and on day 0 through 17 of gestation. In a preliminary study, the offspring of the ICR pregnant females exposed to 17β-estradiol at 0.05 μg/kg per day on day 0 through 17 of gestation showed no changes in weight and histological morphology of reproductive organs in adulthood. However, the offspring of dams exposed to 17β-estradiol at 0.1 μg/kg per day on these gestational days showed changes in the parameters in adulthood (data not shown). In the present study, 30 control females were administered corn oil (Nacalai Tesque, Co., Tokyo). After the administration for 7 days before mating, female mice were caged with untreated males overnight and examined for a vaginal plug the next morning. The day on which a plug was found was termed day 0 of gestation. In this study, 30 female mice in the 17β-estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO_2 asphyxiation, and subjected to cesarean sectioning. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed, and then the AGD was measured. The fetuses obtained by cesarean delivery were fostered to 60 dams that had just given birth naturally (one litter to each female). The day of cesarean section was considered as PND 0. Pup body weights were recorded on PND 21 (day of weaning), and at 5, 7, and 10 weeks of age.

Neonates from 30 pregnant females exposed to corn oil and 30 pregnant females exposed to 17β-estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17β-estradiol exposed group and the control group: 38 and 41) and 0M (33 and 32), and 2F (41 and 32) and 0F (28 and 37). Fetuses adjacent to dead embryos, and fetuses that were closest to each ovary or the cervix, were discarded. In this experiment, fetuses of two intrauterine positions, 1M and 1F, were also discarded.

2.3.2. Observations of postnatal growth

2.3.2.1. Evaluation of sexual maturation. On PND 21, all male and female pups (2M, 0M, 2F, 0F) in each litter were weaned. For all male and female mice in each litter, as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 25), and preputial separation for males (beginning on PND 30), were assessed, and each pup was weighed when these criteria were achieved.

At 10 weeks of age, five males in each group were weighed and processed to the transcardial perfusion to observe the histological alteration of the prostate by electron microscope. The remaining males in each group were weighed and subjected to necropsy, and the testes, epididymides and seminal vesicles, were weighed. All females were weighed and subjected to necropsy. The ovaries were then weighed. These reproductive organs including prostates and uteri were fixed in 0.1 M phosphate-buffered 10% formalin solution and embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.4. Data analyses

Statistical analysis of the data for the offspring (AGD, body weight and organ weight, organ/body weight ratios, timing of vaginal opening and preputial separation) was performed using the litter as the unit [20,21]. The AGD, body weight and organ weight, organ/body weight ratios (relative organ weight), timing of vaginal opening and preputial separation, were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect the significances among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, or there was a group whose variance was zero, Kruskal–Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. Comparisons between groups were made using $P \leq 0.05$ as the level of significance.

3. Results

3.1. Experiment 1

3.1.1. AGD and body weights of fetuses at cesarean section and pups at PND 4

Table 1 shows the AGD, body weight, AGD/body weight (AGDI: anogenital distance index), and AGD/$\sqrt[3]{\text{body weight}}$ of fetuses at various intrauterine positions and pups at PND 4. It is reasonable to anticipate that the AGD might vary with body weight of fetus or pup. It has been proposed that the relationship between AGD and body weight should be more properly evaluated using the cube root of the body weight [22–25]. If it is desirable to normalize AGD to body weight, the AGD/$\sqrt[3]{\text{body weight}}$ seems to provide a more appropriate adjustment.

There were no statistically significant differences in any parameter evaluated at cesarean section (PND 0) or PND 4

Table 1
Effects of prior intrauterine position on anogenital distance in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
AGD of fetuses at cesarean section						
No. of litters	19	27	24	18	29	27
No. of pups	36	73	43	38	83	41
Body weight (g)	5.6 ± 0.4[a]	5.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4
AGD	2.43 ± 0.22	2.42 ± 0.22	2.42 ± 0.28	1.21 ± 0.20	1.23 ± 0.19	1.22 ± 0.24
AGD/body weight	0.43 ± 0.04	0.42 ± 0.04	0.42 ± 0.05	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
AGD/$\sqrt[3]{\text{body weight}}$	1.36 ± 0.12	1.36 ± 0.14	1.35 ± 0.18	0.69 ± 0.08	0.70 ± 0.07	0.70 ± 0.08
AGD of pups on PND 4						
No. of litters	19	27	24	18	29	27
No. of pups	34	69	41	37	79	38
Body weight (g)	10.9 ± 1.5	11.2 ± 1.5	10.8 ± 1.1	10.4 ± 1.4	10.3 ± 1.1	10.4 ± 1.3
AGD	4.57 ± 0.54	4.41 ± 0.48	4.43 ± 0.51	2.00 ± 0.22	1.99 ± 0.19	2.00 ± 0.21
AGD/body weight	0.42 ± 0.06	0.40 ± 0.05	0.41 ± 0.03	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.04
AGD/$\sqrt[3]{\text{body weight}}$	2.06 ± 0.22	1.99 ± 0.19	1.99 ± 0.19	0.92 ± 0.12	0.91 ± 0.11	0.92 ± 0.13

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus: 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses. No significant differences were observed between groups.

[a] Mean ± S.D.

Table 2
Effects of prior intrauterine position on reproductive organs before maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight on PND 21						
No. of litters	18	27	24	17	27	25
No. of offspring	13	37	11	14	43	12
Body weight (g)	40.9 ± 6.3[a]	40.5 ± 6.5	40.2 ± 9.6	38.8 ± 6.3	38.2 ± 7.0	40.7 ± 7.0
Testes (mg)[b]	169.3 ± 27.5	172.2 ± 22.2	164.9 ± 26.1			
Testes[c]	416.3 ± 48.4	429.2 ± 40.3	418.2 ± 46.7			
Epididymides (mg)[b]	23.3 ± 3.1	23.5 ± 4.8	21.9 ± 4.4			
Epididymides[c]	60.5 ± 10.8	58.2 ± 7.8	55.2 ± 7.0			
Prostate + SV (mg)[b,d]	47.2 ± 9.9	46.7 ± 10.3	45.9 ± 7.9			
Prostate + SV[c,d]	115.6 ± 18.0	115.7 ± 19.0	117.2 ± 19.5			
Ovaries (mg)[b]				24.3 ± 4.0	22.9 ± 3.9	24.8 ± 3.6
Ovaries[c]				63.5 ± 10.1	60.8 ± 9.5	61.5 ± 7.3
Uterus (mg)[b]				10.2 ± 2.0	11.2 ± 3.7	11.8 ± 2.9
Uterus[c]				26.4 ± 4.5	28.8 ± 6.8	29.1 ± 6.0

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (g or mg per 100 g body weight).
[d] Seminal vesicle.

between groups 2M, 1M and 0M in males, or groups 2F, 1F and 0F in females.

No significant differences in viability of fetuses at cesarean section (PND 0), or that from PND 0 to PND 4 (the number of pups died; 2M = 2, 1M = 4, 0M = 2, 2F = 1, 1F = 4, 0F = 3), were detected between the groups. In addition, there were no statistically significant differences in body weight at PND 0 and 4.

3.1.2. Body weight and reproductive organ weight of offspring at PND 21

The absolute and relative weights of testes, epididymides, and prostates with seminal vesicles in males, and ovaries and uteri in females, as well as body weight of offspring at PND 21 are shown in Table 2. Irrespective of the intrauterine position, no significant differences were detected between the groups in absolute or relative reproductive organ weights, or body weights of male and female weanlings, suggesting that the intrauterine position did not affect postnatal growth before weaning in rats.

3.1.3. Sexual maturation and estrous cycle of offspring

Table 3 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups. The estrous cycle of female offspring from 6 to 10 weeks of age is shown in Table 4. No significant differences were detected between the groups in mean estrous cycle length, or the frequency of females showing each stage of estrous cycle.

Table 3
Effects of prior intrauterine position on sexual maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
No. of litters	18	27	24	17	27	25
No. of offspring	21	32	30	23	36	26
Day of preputial separation	43.3 ± 1.3[a]	43.4 ± 1.2	44.0 ± 1.8			
Body weight (g)[b]	211.8 ± 5.5	212.1 ± 4.3	212.9 ± 5.2			
Day of vaginal opening				33.8 ± 2.2	33.8 ± 1.8	34.1 ± 1.7
Body weight (g)[b]				125.6 ± 4.1	124.6 ± 4.4	126.1 ± 3.9

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Body weight when the criterion was achieved.

Table 4
Effects of prior intrauterine position on estrous cycle in Sprague–Dawley rats

	Group		
	2F	1F	0F
No. of litters	17	27	25
No. of female offspring	23	36	26
Mean estrous cycle length (day)	4.16 ± 0.29[a]	4.08 ± 0.30	4.20 ± 0.42
No. of females showing Regular cycle (%)	18 (78.3)	28 (77.8)	21 (80.8)
No. of females showing Irregular cycle (%)	5 (21.7)	8 (22.2)	5 (19.2)

2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses. No significant differences were observed between groups.
[a] Mean ± S.D.

3.1.4. Behavior and locomotor activity of offspring

Table 5 shows the results of an open field test at 4 weeks of age, and spontaneous activity within the wheel for 24 h at 7 weeks of age, for male and female offspring. There were no significant differences between groups 2M, 1M and 0M in latency, ambulation, rearing, grooming, defecation and urination, or number of revolutions for 24 h in a wheel cage. In the females, urination in group 0F was significantly increased as compared with that in group 2F, whereas other behavioral parameters, including the number of revolutions in a wheel cage were comparable between groups 2F, 1F and 0F.

3.1.5. Weights and histology of reproductive organs of offspring in adulthood

Table 6 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed in any of the reproductive organs, including the prostates, of the offspring.

3.2. Experiment II

3.2.1. AGD and body weights of fetuses at cesarean section

Table 7 shows the body weight, AGD, AGD/body weight, and AGD/$\sqrt[3]{\text{body weight}}$, of embryonic day 18 (PND 0) fetuses exposed to corn oil or 17β-estradiol. There were no significant differences in any of the parameters between the groups. No significant differences in viability of fetuses at cesarean section, or that from PND 0 to PND 21 were detected between the groups (the number of pups died from PND 0 to PND 21: see Tables 7 and 8).

3.2.2. Sexual maturation of offspring

Table 8 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups.

Table 5
Effects of prior intrauterine position on postnatal behavior in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Open field						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Latency (s)	20.4 ± 40.8[a]	17.9 ± 16.9	15.3 ± 16.2	12.0 ± 9.4	13.8 ± 12.3	16.9 ± 36.1
Ambulation (cm)	676.3 ± 411.3	627.1 ± 417.2	659.0 ± 501.9	940.6 ± 538.1	1039.8 ± 436.3	970.7 ± 449.8
No. of rearing	2.3 ± 3.1	3.0 ± 3.2	1.5 ± 1.4	3.5 ± 2.1	4.5 ± 3.4	3.8 ± 2.3
No. of grooming	0.6 ± 0.9	0.7 ± 0.7	1.1 ± 1.2	0.8 ± 0.9	0.4 ± 0.5	0.8 ± 0.8
No. of defecation	2.8 ± 1.9	2.1 ± 1.5	3.3 ± 2.2	1.9 ± 1.9	1.7 ± 1.8	1.8 ± 1.9
No. of urination	0.4 ± 0.6	0.4 ± 0.6	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.7 ± 0.6**
Spontaneous activity						
Count/24 h	1547 ± 467	1789 ± 697	1559 ± 638	4107 ± 1140	4429 ± 1501	4746 ± 1831

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
**Significantly different from group 2F, $P < 0.01$ (by multiple comparison and Student t-test).
[a] Mean ± S.D.

Table 6
Effects of prior intrauterine position on reproductive organs after maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight at 10 weeks old						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Body weight (g)	417.2 ± 31.6[a]	416.1 ± 34.4	413.6 ± 36.9	270.0 ± 23.2	271.8 ± 28.9	273.6 ± 29.1
Testes (mg)[b]	3.00 ± 0.20	2.98 ± 0.15	3.00 ± 0.17			–
Testes[c]	0.72 ± 0.05	0.72 ± 0.06	0.73 ± 0.07			–
Epididymides (mg)[b]	0.77 ± 0.05	0.78 ± 0.07	0.76 ± 0.06			–
Epididymides[c]	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.02			–
Ventral prostate (g)[b]	0.46 ± 0.08	0.44 ± 0.08	0.43 ± 0.10			–
Ventral prostate[c]	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.03			–
Dorsal prostate (g) + SV[b,d]	1.53 ± 0.28	1.56 ± 0.24	1.52 ± 0.27			–
Dorsal prostate + SV[c,d]	0.37 ± 0.07	0.38 ± 0.05	0.37 ± 0.07			–
Ovaries (mg)[b]				92.6 ± 13.3	91.8 ± 13.7	95.4 ± 16.9
Ovaries[c]				34.3 ± 3.6	33.8 ± 3.4	35.0 ± 5.8
Uterus (g)[b]				0.36 ± 0.06	0.38 ± 0.06	0.38 ± 0.05
Uterus[c]				0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.02

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (g or mg per 100 g body weight).
[d] Seminal vesicle.

Table 7
Effects of prior intrauterine position on anogenital distance in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	41	32	32	37	38	33	41	28
Body weight (g)	1.41 ± 0.08[a]	1.42 ± 0.05	1.32 ± 0.05	1.33 ± 0.04	1.42 ± 0.09	1.41 ± 0.10	1.32 ± 0.07	1.30 ± 0.11
AGD	1.92 ± 0.07	1.90 ± 0.06	0.95 ± 0.02	0.95 ± 0.03	1.92 ± 0.08	1.93 ± 0.06	0.93 ± 0.09	0.95 ± 0.05
AGD/body weight	1.36 ± 0.09	1.35 ± 0.10	0.75 ± 0.03	0.73 ± 0.05	1.40 ± 0.09	1.38 ± 0.10	0.75 ± 0.05	0.74 ± 0.07
AGD/$\sqrt[3]{\text{body weight}}$	1.71 ± 0.07	1.70 ± 0.10	0.88 ± 0.03	0.89 ± 0.05	1.70 ± 0.09	1.72 ± 0.11	0.89 ± 0.07	0.88 ± 0.08

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 8
Effects of prior intrauterine position on sexual maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	39	30	31	35	37	31	39	28
Day of preputial separation	27.2 ± 1.5[a]	27.3 ± 1.3			27.0 ± 1.8	26.9 ± 2.0		
Body weight (g)	30.3 ± 1.9	31.1 ± 1.5			30.0 ± 2.1	31.3 ± 1.8		
Day of vaginal opening			24.5 ± 1.6	25.1 ± 1.5			24.4 ± 1.7	24.9 ± 1.6
Body weight (g)			21.5 ± 0.9	21.6 ± 1.2			21.6 ± 1.1	22.0 ± 1.5

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 9
Effects of prior intrauterine position on reproductive organs after maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	34	25	26	30	32	26	34	23
Terminal body weight (g)	51.5 ± 4.2[a]	53.6 ± 4.4	40.3 ± 2.7	41.2 ± 3.4	55.1 ± 5.1	53.1 ± 6.2	41.8 ± 2.1	42.1 ± 3.1
Testes (g)	257.9 ± 28.4[b]	266.5 ± 23.5			259.3 ± 25.5	260.3 ± 19.9		
	486.3 ± 76.3[c]	484.2 ± 71.3			488.3 ± 62.3	479.1 ± 60.9		
Epididymides (mg)	89.5 ± 8.7	92.2 ± 9.3			94.6 ± 7.1	93.1 ± 7.1		
	175.2 ± 21.5	161.9 ± 16.9			161.2 ± 13.6	173.6 ± 4.9		
Seminal vesicle (mg)	413.9 ± 30.6	452.2 ± 13.9			431.3 ± 18.2	454.8 ± 21.0		
	812.8 ± 56.9	802.6 ± 44.2			811.5 ± 42.3	809.3 ± 33.5		
Ovary (mg)			15.3 ± 3.3	14.6 ± 4.2			15.5 ± 3.9	15.3 ± 4.5
			35.6 ± 7.5	33.2 ± 4.6			34.1 ± 6.9	33.6 ± 5.1

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
Five males in each group were processed to the transcardial perfusion. Male pups shown here were subjected to necropsy.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (mg per 100 g body weight).

3.2.3. *Weights and histology of reproductive organs of offspring in adulthood*

Table 9 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed.

4. Discussion

4.1. *Anogenital distance*

The AGD of newborn rats and mice is longer in males than in females, and it has been demonstrated that the AGD varies as a function of the intrauterine position of the animals [1–4]. The AGD is commonly regarded as a hormonally sensitive developmental measure in rodents [26], and it has been reported that a longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus [27,28]. Evidence supports the hypothesis that exposure to testosterone and estrogen in utero are critical components of the intrauterine position effect [29]. Female mouse fetuses located between two males have significantly higher serum testosterone levels and lower estradiol levels than their sisters that were located between two females. Male mice located between two females have significantly higher levels of estradiol and lower levels of testosterone than males located between two males [4,12]. The mechanism for these intrauterine position effects can be traced to amniotic fluid transport between adjacent fetuses in uterus [30,31]. However, our data were not consistent with previous reports showing a significant effect of intrauterine position on AGD in rats and mice [2,32–34].

A failure to replicate the effects of intrauterine position on AGD may have potentially arisen for a number of methodological reasons. A set of potential problems revolves around possible errors in the measurement of the AGD. One possibility was that our calipers were not accurate enough to detect small mean differences between females located in various positions in the uterus, found by other investigators [2,32–34]. However, as the calipers could be read to an accuracy of 0.01 mm, they were clearly accurate enough to detect differences of this magnitude. Another possibility is that of human error. Given the short distances being measured, it was absolutely essential that all fetuses or pups be oriented in exactly the same fashion, as even a slight arching of the animal's back could significantly distort the AGD measurements. Two attempts were made to minimize these sorts of errors: (i) efforts were made to orient all fetuses or pups in exactly the same fashion when measuring, and (ii) two independent measurements were taken for each fetus or pup and averaged to obtain the value used. In most cases, the different measurements were highly similar for the same animal.

Simon and Cologer-Clifford [35] reported an absence of an intrauterine position effect on AGD in CF-1 mice. Their finding is only the second study to examine AGD in CF-1 mice, and the original report was more than 10 years old [2]. Therefore, it is possible that either genetic drift, or differences in the source of the CF-1 breeding stock, may

underlie the discrepant findings. In this context, Jubilan and Nyby [6] also found no effect of intrauterine position on the AGD/body weight (AGDI) in CF-1 offspring, using stock from the same supplier employed by the Simon and Cologer-Clifford [35] report.

4.2. Sexual maturation and estrous cycle

Since prenatal exposure of females to testosterone delays vaginal opening [36,37], it was predicted that females situated proximate to males in utero would display vaginal opening later than females not proximate to males during gestation. However, in the present study in rats and mice, there were no significant differences in days of vaginal opening or preputial separation between the groups (see Tables 3 and 8), suggesting that intrauterine position did not influence the sexual maturation in males and females. vom Saal [4] reported that 2F and 0F mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than 0F (see categorization of the different intrauterine positions shown in Section 2).

Female mouse fetuses occupying an intrauterine position between male fetuses exhibit longer estrous cycles in adulthood than females formerly residing in utero next to other female fetuses [11,27]. Prior intrauterine position is therefore a source of individual variation in the production of, and sensitivity to, cues that modulate the timing of puberty and the length of subsequent estrous cycles in female mice, suggesting that prenatally androgenized females occupying an intrauterine position between male fetuses may have a reproductive advantage over other females at high population densities [4]. In the present study of rats, however, 0F and 2F did not differ significantly in the estrous cycle length, although the estrous cycle length of 2F (4.16 ± 0.29) tended to be shorter than that of 0F (4.20 ± 0.42). Prior studies have shown that, in the absence of males, vaginal estrus does not correlate with ovulation in peripubertal CF-1 female mice [38,39]. Further studies in which ovulation is confirmed by the presence of corpora lutea and tubal ova is thus required.

4.3. Behavior

Kinsley et al. [40] demonstrated that female mice located in utero between two female fetuses exhibited higher levels of regulatory running activity (locomotor activity) in adulthood than females located between two male fetuses. Male mice, which were less active than females, were also influenced by intrauterine contiguity, indicating that intrauterine position influences the behaviors involved in the maintenance of metabolic homeostasis. Previous work has shown that female rats and mice display higher levels of regulatory running activity than males, and that perinatal testosterone is responsible for this sex difference [41–43]. The present study also showed female rats displayed higher levels of running activity than males.

In the present study of rats, however, there were no significant differences in spontaneous activity in the wheel cage, or in ambulation in the circular area, as well as the frequency of rearing, grooming and defecation between the groups in both sexes, suggesting no intrauterine position effects on locomotor activity in rats. Interestingly, the frequency of urination for females that developed in utero between male fetuses was significantly increased, more than in females that developed in utero between female fetuses (see Table 5). Females that were located between female fetuses in utero were found to urine mark at higher rates than females that were located between male fetuses, in adulthood in CF-1 mice [2]. The frequency of urination in the circular area, observed in the present study, would relate to the emotionality of the animals when placed in a novel environment, and differ from urine marking. Female urine marking may play an important role in communication between female mice, as well as in inter-sexual communication. It has been suggested that in natural populations of mice, females urine mark to advertise their dominant breeding status to other females; urine-marking appears to be dependent on female social/reproductive status [44]. Taken together, these observations suggest that the intrauterine position did not affect behavior as evaluated by the open field test and the wheel cage.

4.4. Prostate development

Growth and differentiation of the prostate is primarily under the control of androgen. Expression of the androgen metabolizing enzyme, 5α-reductase, within prostatic mesenchyme cells is also necessary for normal development of the prostate [45]. The possibility that estrogen might be involved in modulating the effects of androgen on prostatic development during early life has been the subject of speculation for over 60 years [46–49]. Timms et al. [50] demonstrated that development of the urogenital system in male and female rat fetuses is influenced by their intrauterine proximity to fetuses of the same or opposite sex, and suggested that exposure to supplemental estradiol (due to being positioned between two female fetuses) induces prostatic bud development in females, and enhances the growth of prostatic buds in both males and females. An enlarged prostate in males located between two female fetuses was hypothesized to be mediated by an elevated level of serum estradiol, relative to males located between two males, due to the transport of estradiol from adjacent female fetuses [4,30]. This hypothesis was confirmed in a study in which estradiol was experimentally elevated by 50% in male mouse fetuses (via maternal administration), and the estrogen-treated males showed both a significant increase in prostatic glandular buds and significantly larger buds during fetal life, as well as enlarged prostates in adulthood [46].

In the present study, however, the weights of the prostates (with seminal vesicles) of the rats at PND 21, and the ventral and dorsal prostates (with seminal vesicles) of the rats at 10

weeks of age, were not significantly different between the groups. In addition, morphological observation of prostates in the rats and mice, in weanlings or adulthood, by light and electron microscope revealed no alteration in males located in any uterine position.

4.5. Developmental exposure to 17β-estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17β-estradiol administration to pregnant mice on the early development of the prostate in male mouse fetuses, with attention being paid to the intrauterine position of the males. Timms et al. [17] reported that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly reduced serum estradiol in males located between two females, but not males located between two males, and also significantly interfered with initial budding and subsequent growth of the prostate in males located between two females or two males. In sharp contrast, the seminal vesicles were larger in the control males located between two males than in control males located between two females, similar to prior findings in mice [29], and TCDD only decreased the size of the seminal vesicles in males located between two males. Taken together, the findings of Timms et al. [17] demonstrate that in utero exposure to TCDD disrupts the development of the prostate, but this disruption depends on an interaction with background levels of estradiol. Howdeshell and vom Saal [16] reported that fetal mouse exposure via the mother to an estrogen-mimicking chemical, bisphenol A, increased the rate of postnatal growth in males and females, and also advanced the timing of puberty in females. They also demonstrated that the greatest response to bisphenol A occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A treatment, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development.

In the present study, however, mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. Therefore, we concluded that exposure to low-dose estrogenic endocrine disrupting chemicals during fetal life does not contribute to the intrauterine position.

5. General discussion

We are at a loss to explain why we were unable to replicate the effects of intrauterine position on AGD, or to find intrauterine position effects upon sexual maturation, and the estrous cycle. However, we know the difficulty in demonstrating intrauterine position effects upon morphology and behavior [35]. In addition, in contrast to earlier work [11] which examined blood androgen titers in mouse fetuses, Baum et al. [51] reported that whole-body androgen levels in female rat fetuses did not vary as a function of intrauterine position, and suggested that intrauterine position effects upon rodent morphology and behavior may not have the robust generality that is generally assumed.

Howdeshell and vom Saal [16] demonstrated that one source of variability in the response of both male and female mouse fetuses to an estrogen-mimicking chemical, bisphenol A, is their background levels of endogenous sex hormones. They suggested that a very small increase in the level of endogenous estradiol may substantially increase the susceptibility of fetuses to endocrine disrupting chemicals consumed or absorbed through the skin or lungs by pregnant animals and humans.

Contiguous [1,52], caudal [53,54], and no effect [51,55–57], due to intrauterine position, have been reported. Hotchkiss et al. [55] in a study with Sprague–Dawley rats examined the effect of intrauterine position on concentrations of testosterone in several different tissues. No effect of either contiguous or caudal intrauterine position on testosterone concentration was detected in fetal carcasses, reproductive tracts, or amniotic fluid. Furthermore, no correlation was found between masculinization due to intrauterine position and increasing anogenital distance. It is unclear at this time why there is such a discrepancy between the previous findings and the present results in rats and mice. However, varied strains of rats and mice, multiple uncontrolled variables, and different criteria for defining the effects of intrauterine positioning, may all contribute to this uncertainty. In addition, the discrepancies in the data may be attributed to such factors as the dietary influences (such as background levels of phytoestrogens and caloric intake), caging (steel versus polycarbonate), bedding, housing (group versus individual), and seasonal variation, as well as differences among the studies in control body and prostate weights [58,59].

The results of the present study clearly showed that intrauterine position of embryos/fetuses did not influence postnatal development, including sexual maturation and behavior.

Acknowledgments

The technical support provided by the reproductive and developmental biology group was essential to this study and was greatly appreciated. This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan.

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