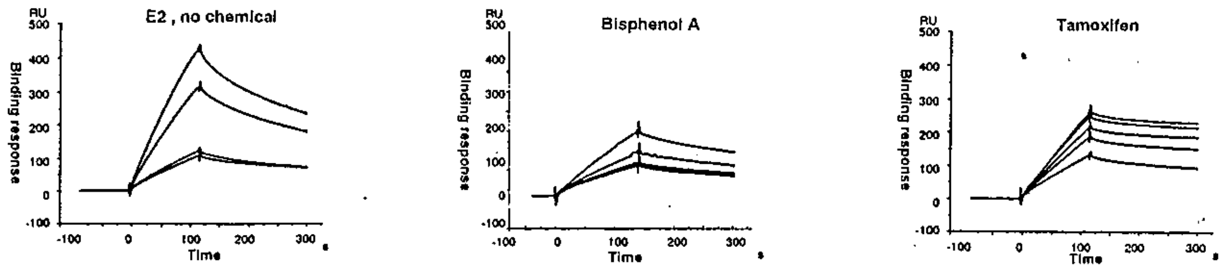

Fig. 11 Different kinetic patterns in the ER-ERE interaction.

Table 1 Results of ER-ERE screening with SPR sensor

No.	CAS No.	Name	1st Screening	2nd Screening
1	000050-28-2	Estradiol	H	H
2	000057-91-0	Estra-1,3,5(10)-triene-3,17-diol (17α)	H	H
3	000053-16-7	Estrone	H	H
4	000057-63-6	19-Nor-17-alpha-pregna-1,3,5(10)-trien-20-yne-3,17-diol	H	H
5	000362-05-0	Estra-1,3,5(10)-triene-2,3,17-beta-triol	H	H
6	000362-07-2	Estra-1,3,5(10)-triene-3,17-diol, 2-methoxy-, (17β)-	L	L
7	000068-22-4	19-Nor-17-alpha-pregn-4-en-20-yn-3-one, 17-hydroxy-	L	L
8	000063-05-8	Androst-4-ene-3,17-dione	N	N
9	000057-83-0	Progesterone	N	N
10	000501-24-6	3-Pentadecylphenol	N	N
11	005153-25-3	Benzoic acid, 4-hydroxy-, 2-ethylhexyl ester	N	L
12	001034-01-1	Gallic acid, octyl ester	N	N
13	006807-17-6	4,4'-(1,3-Dimethylbutylidene)bisphenol	L	L
14	027955-94-8	Phenol, 4,4',4''-ethylidynetri-	N	N
15	000081-92-5	Benzenemethanol, 2-[bis(4-hydroxyphenyl)methyl]-	L	L
16	000081-90-3	o-Toluic acid, .alpha.,.alpha.-bis(p-hydroxyphenyl)-	N	N
17	000978-86-9	4-(Triphenylmethyl)phenol	L	L
18	062625-31-4	Phenol, 4,4'-(3H-1,2-benzoxathiol-3-ylidene)bis 3-methyl-, S,S-dioxide, monosod	N	N
19	005384-21-4	Phenol, 4,4'-methylenebis[2,6-dimethyl-	L	L
20	005613-46-7	2,6-Xylenol, 4,4'-isopropylidenedi-	L	L
21	000084-16-2	Phenol, 4,4'-(1,2-diethylethylene)di-, meso-	H	H
22	000084-17-3	Phenol, 4,4'-(diethylideneethylene)di-	L	H
23	56-53-1	diethylstilbestrol	H	H
24	006893-02-3	Alanine, 3-(4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl)-, L-	N	N
25	000500-38-9	Nordihydroguaiaretic acid	N	N
26	023239-51-2	Benzyl alcohol, p-hydroxy-alpha-(1-((p-hydroxyphenethyl)amino)ethyl)-, hydrochlo	N	N
27	001050-28-8	L-Tyrosine, N-L-tyrosyl-	N	N
28	000145-50-6	1(4H)-Naphthalenone, 4- .alpha.-(4-hydroxy-1-naphthyl)benzylidene -	L	L
29	000446-72-0	Genistein	L	L
30	000080-05-7	Bisphenol A	L	L

H, High responder; L, low responder; N, non-responder.

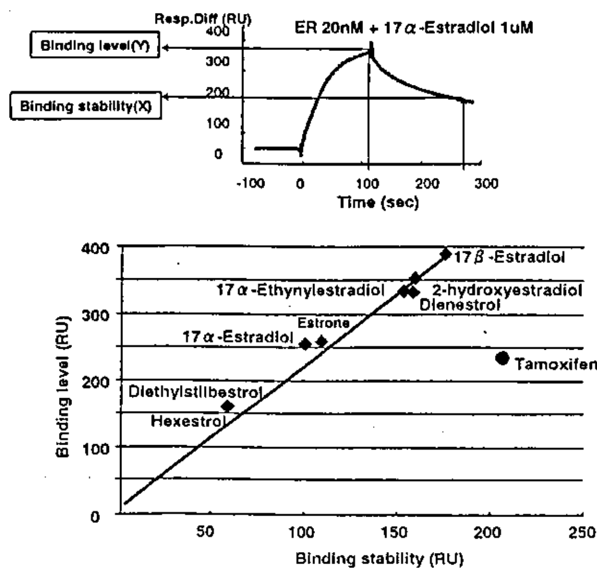

Fig. 12 Binding level vs. binding stability plot.

Discussion

We established a cell free screening method while focusing on the mechanism of the hormone receptor using a surface plasmon resonance sensor. We developed an assay method to detect estrogenic activities of the chemicals with changes in the binding level of ER to ERE by preincubating the chemicals with ER.

It was also suggested that the agonitst and the antagonists had different effects on the interaction of ER and ERE from an analysis of the binding level of ER during the association and dissociation processes. With the conventional end point assay used to monitor only the binding signals, it was impossible to distinguish the agonists from the antagonists. The real time analysis, which is the main feature of the surface plasmon resonance sensor enabled the classification of the agonists and the antagonists. When running the cell based hormone assay, it must be taken into account any unexpected effects of the chemicals to the other components than the receptors of the cells. On the other hand, the cell free assays simply show the

direct effects of chemicals to the receptor-signal transduction systems. Our new assay, based on the hormone receptor mechanism, can rapidly screen a large number of the chemicals for their hormonal activities.

Since other hormone receptors employ similar mechanism as ER for the activation of the gene expression, it is possible to develop same assays for other hormone receptors. A newly developed ER assay is both reliable and efficient as a primary screening method of chemicals for estrogenic activities.

Acknowledgements

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Exacerbation of benzene pneumotoxicity in connexin 32 knockout mice: enhanced proliferation of CYP2E1-immunoreactive alveolar epithelial cells

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Abstract

The pulmonary pathogenesis triggered by benzene exposure was studied. Since the role of the connexin 32 (Cx32) gap junction protein in mouse pulmonary pathogenesis has been suggested, in the present study, we explored a possible role of Cx32 in benzene-induced pulmonary pathogenesis using the wild-type (WT) and Cx32 knockout (KO) mice. The mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for a total of 26 weeks, and then sacrificed to evaluate the pneumotoxicity or allowed to live out their life span to evaluate the reversibility of the lesions and tumor incidence. Our results clearly revealed exacerbated pneumotoxicity in the benzene-exposed Cx32 KO mice, characterized by diffuse granulomatous interstitial pneumonia, markedly increased mucin secretion of bronchial/bronchiolar and alveolar epithelial cells, and hyperplastic alveolar epithelial cells positive for CYP2E1. But the results did not indicate any enhancement of pulmonary tumorigenesis in the Cx32 KO mice though the number of animals was small.
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Keywords: Benzene; Connexin 32; Cx32 knockout mice; CYP2E1; Interstitial pneumonia; Pneumotoxicity

1. Introduction

Benzene has been reported to be a carcinogen capable of producing not only hemopoietic malignancies but also various solid tumors including lung cancers in mice, chronically exposed to it by ingestion or inhalation (Snyder et al., 1988; Huff et al., 1989; Farris et al., 1993).

Benzene toxicity and benzene-induced tumor development in the lung should be taken into consideration for the risk assessment in humans, since the lung is one of the benzene target organs and inhalation is the most common route through which humans are exposed to benzene. Furthermore, a strong relationship

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between benzene exposure and lung cancer development in humans has been assumed for the past decades (Aksoy, 1985, 1989). In addition, benzene metabolites such as benzene oxide, benzene dihydrodiol and diolepoxide have been shown to induce lung tumorigenesis in mouse neonates (Busby et al., 1990). However, little information is available on the pulmonary pathogenesis triggered by benzene exposure.

Intercellular communication through gap junction proteins (GJICs) plays an important role in cellular homeostasis by regulating cell growth, cell differentiation, and apoptosis (Yamasaki, 1996). Based on this concept, alteration in GJICs has been demonstrated to be closely associated with the pathogenesis and carcinogenesis induced by chemicals, particularly by nongenotoxic agents (Yamasaki et al., 1995; Kolaja et al., 2000). Furthermore, down modulation of GJICs is known to induce cytochrome P450s by other chemicals that may be involved in benzene metabolism (Neveu et al., 1994; Snyder and Hedli, 1996; Shoda et al., 2000). We, therefore, hypothesized that GJICs may contribute also to the processes of benzene-induced pneumotoxicity and lung carcinogenesis.

As the presence and the functional role of connexin 32 (C32) gap junction protein in the mouse lung tissue have been suggested in previous in vitro and in vivo studies (Albright et al., 1990; Lee et al., 1997; Ruch et al., 1998; Abraham et al., 1999, 2001), in the present study, we explored a possible role of Cx32 in the lung pathogenesis induced by chronic exposure to benzene, using Cx32 knockout (KO) mice. For this purpose, wild-type (WT) and Cx32 KO mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for 26 weeks. Then the pathological changes were determined based on the results of histopathology, histochemistry for detecting mucin secretion, and immunohistochemistry for detecting CYP2E1 and proliferating cell nuclear antigen (PCNA). The tumor incidence in the pulmonary tissue was also compared between the benzene-exposed WT and Cx32 KO mice.

2. Materials and methods

2.1. Animals

Cx32 KO mice, from the Institut für Genetik, Universität, Bonn, Germany (Moennikes et al., 2000), were maintained as heterozygous KO mice at the animal facility of National Institute of Health Sciences (NIHS), Japan. Because the *Cx32* gene is linked to the X-chromosome, we generated Cx32 WT ($Cx32^{+/Y}$) and KO male ($Cx32^{-/Y}$) mice for this study by cross breeding female $Cx32^{+/-}$ heterozygous mice and male C57BL/6 wild type mice. The Cx32 genotypes of the neonates were identified by the standard PCR assay (Moennikes et al., 2000). The WT and Cx32 KO mice aged 8–9 weeks were used in the study. During the study, the mice were housed within stainless-steel wire cages in inhalation chambers that were maintained on a 12-h light-dark cycle. The basal pellet diet (CRF-1; Funabashi Farm, Tokyo, Japan) was provided ad libitum, except during the 6-h daily inhalation of benzene when the food was withdrawn. Water was automatically supplied throughout the study.

2.2. Benzene exposure

Benzene was purchased from Wako Chemical Company (Osaka, Japan). The mice were randomized and exposed to benzene in 1.3 m^3 inhalation chambers, as described elsewhere (Yoon et al., 2001). Briefly, the benzene vapor was generated by heating liquid benzene to 16 °C and directed into the inhalation chambers (Sibata Scientific Technology Ltd., Tokyo, Japan) with a room temperature of 24 ± 1 °C. The flow rate of benzene was about 650 l/min, and the benzene concentration in the chambers was measured at 30-min intervals during the daily exposures using a gas chromatograph (Shimadzu Co., Kyoto, Japan). The temperature and humidity in the chambers were automatically controlled at 24 ± 1 °C and 55 ± 10%, respectively. As described in the previous Section 2.1, mice were supplied water ad libitum but withdrew the food pellets during the exposure.

The WT and Cx32 KO mice were, respectively, divided into the sham-exposed control group and the benzene-exposed groups; each group was composed of ten to twelve mice. The experimental group was exposed to 300 ppm benzene for 6 h per day, 5 days per week, for 26 weeks and the sham-exposed control group was maintained under the same conditions but without benzene inhalation. Five to six mice from each group were first sacrificed after the 26-week exposure to evaluate pneumotoxicity and the remaining five to seven mice from each group were allowed to

live out their lives to further evaluate their recovery from pulmonary lesions and the incidence of the pulmonary tumor.

2.3. *Measurement of food consumption and body weight*

Food consumption and body weight were measured every Friday throughout the 26-week benzene exposure.

2.4. *Autopsy, organ weight measurement and histopathology*

After the 26-week benzene exposure, five to six mice from each group were sacrificed under ethyl ether anesthesia for autopsy. Gross morphological examination of the mice was performed and the major visceral organs were weighed and analyzed. For the histopathological examination, tissues from both lungs were fixed in 10% neutral buffered formaldehyde for 24 h. Pulmonary tissues were sliced and immediately immersed in the fixative. After routine processing, the paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope.

2.5. *Immunohistochemistry and histochemistry*

The avidin–biotin–peroxidase complex (ABC) method was used for immunohistochemistry to detect the expression of the P450 CYP2E1 enzyme and PCNA. After the lung tissue sections mounted on poly-L-lysine-coated slides were deparaffinized and hydrated, endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min. The lung tissue sections in a Caplin jar containing 1 mM citric acid (pH 6.0) were microwaved for 10 min for retrieval of PCNA. After washing in phosphate-buffered saline (PBS, pH 7.4) for 15 min, the tissue sections were incubated with 10% normal serum at room temperature for 60 min to block nonspecific binding sites. The sections were then incubated with a mouse anti-PCNA monoclonal antibody (1:300, Sigma–Aldrich, Amherst, NY, USA) for 50 min at room temperature and a goat anti-rat CYP2E1 polyclonal antibody (1:1000, Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) overnight at 4 °C. The tissue sections were washed three times in PBS, incubated with the corresponding biotinylated secondary antibodies for 40 min at room temperature, and subsequently incubated with the ABC reagent for 30 min at room temperature. As a chromogen, 0.5% 3,3′-diaminobenzidine tetrahydrochloride was used, and the sections were counterstained with methylene blue. As a positive control for PCNA and CYP2E1, normal testis and kidney sections were used, respectively, and as a negative control, PBS instead of the primary antibodies was applied to the sections.

Periodic acid–Schiff (PAS) reaction was performed to detect mucus secretion. After deparaffinization, the tissue sections were immersed in 0.5% periodic acid solution. After washing with distilled water, the sections were incubated with the Schiff reagent for 15 min, washed with warm tap water for 10 min, and then counterstained with hematoxylin.

2.6. *Statistical analysis*

ANOVA was performed to evaluate the significant differences in food consumption and body weight between the nonexposed sham exposed control and benzene-exposed groups of WT and Cx32 KO mice as well as between WT and Cx32 KO mice of each group.

3. Results

3.1. *Changes in body weight during the 26-week benzene exposure*

No significant difference was observed between WT and Cx32 KO mice of the nonexposed sham-control group throughout the study, even when the mean body weight of Cx32 KO mice was slightly less than that of WT mice at the late stage of this study (Fig. 1). Benzene exposure induced a significant decrease in the body weight of the benzene-exposed group of both WT and Cx32 KO mice compared with the nonexposed sham-control mice. The reduction was much more marked in Cx32 KO mice (Fig. 1), which was observed after seven weeks of exposure ($P < 0.05$). On the other hand, in WT mice, a significant difference in body weight was observed after the fourteenth week of exposure (Fig. 1). Furthermore, after the twelfth week

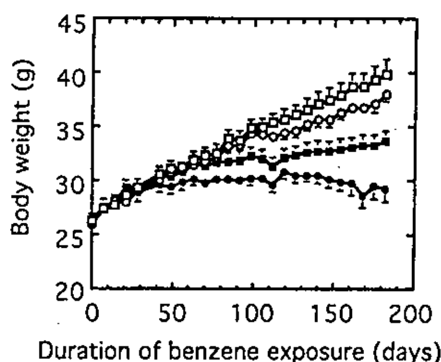

Fig. 1. Changes in body weights of WT and Cx32 KO mice during benzene exposure. Benzene (300 ppm) was inhaled for 6 h per day, 5 days per week for 26 weeks. Eleven to 12 mice per group were used. (□) WT-sham group; (○) Cx32 KO-sham group; (■) WT-benzene-exposed group; (●) Cx32KO-benzene-exposed group. There is significant difference between benzene-exposed group from the corresponding sham-control group after 10 weeks exposure for the Cx32 KO and 14 weeks exposure for WT. Vertical bars mean standard errors.

of exposure, the mean body weight was significantly different between benzene-exposed WT and Cx32 KO mice ($P < 0.05$) (Fig. 1).

During the benzene exposure for 26 weeks, there had been no significant difference in food consumption between the nonexposed group and the benzene-exposed group of both WT and Cx32 KO mice and between WT and Cx32 KO mice of both groups (data not shown).

3.2. Weight of the lung

Significant increases were noted in the absolute lung weight of Cx32 KO mice and in the relative lung weights of both the WT ($P < 0.05$) and Cx32 KO mice ($P < 0.05$) after the twenty-sixth week of exposure to 300 ppm benzene (Fig. 2).

3.3. Histopathology and histochemistry

Severe diffuse interstitial pneumonia was observed in the lungs of the benzene-exposed Cx32 KO mice, which was comparable with that in the lungs of the WT mice showing much milder pulmonary lesions (Table 1, Fig. 3B and E). The alveolar walls were thickened by heavy infiltration of macrophages, the

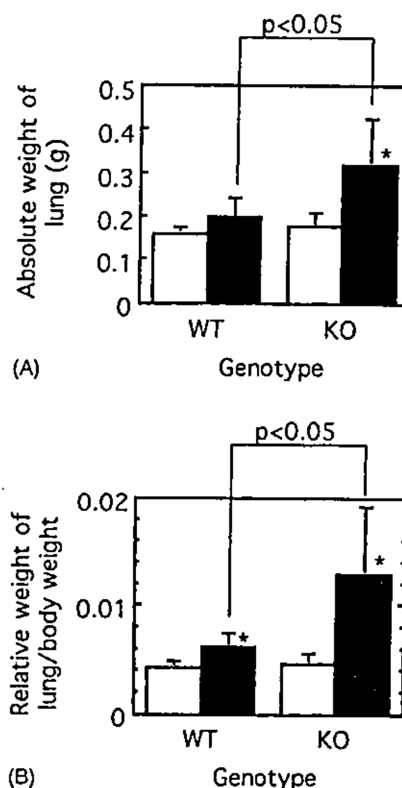

Fig. 2. Changes in weights of the lungs of mice exposed to 300 ppm benzene for 26 weeks. Open column; sham-control group, closed column; benzene-exposed group. Vertical bars mean standard deviations. Symbol (*) indicates significantly different from the corresponding sham-control group at $P < 0.05$.

presence of a small number of lymphocytes and neutrophils, and a considerably increased number of type II alveolar epithelial cells (Fig. 3C). The proliferation of basophilic epithelial cells in the terminal bronchioles and alveolar ducts was frequently noted in the lungs of benzene-exposed Cx32 KO mice (Fig. 3F), while the lungs of benzene-exposed WT mice had mild and a few basophilic proliferating epithelial cell-proliferating foci. The numbers of mucus-secreting epithelial cells increased in the bronchi and bronchioli of both WT and Cx32 KO mice exposed to benzene for 26 weeks (Fig. 4C and D). In particular, in the benzene-exposed Cx32 KO mouse lungs, aggregates composed of mucin-secreting alveolar epithelial cells were occasionally detected (Fig. 4D).

Table 1
Pathological findings in the lungs of the wild-type (WT) and Cx32 knockout (KO) mice exposed to 300 ppm benzene for 26 weeks

Group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
Histopathology/no. of animals examined	6	5	5	5
Interstitial pneumonia granulomatous, diffuse	0 (0.0)	4 (80.0)	0 (0.0)	5 (100.0)
Moderate		4 (80.0)		1 (20.0)
Severe		0 (0.0)		4 (80.0)
Hyperplastic basophilic cell foci	0 (0.0)	1 (20.0)	0 (0.0)	4 (80.0)
Alveolar and bronchiolar epithelial cells		1 (20.0)		4 (80.0)
Mucin-secreting cells	0 (0.0)	5 (100.0)	1 (20.0)	5 (100.0)
Bronchial/bronchiolar epithelial cells		5 (100.0)	1 (20.0)	5 (100.0)
Alveolar epithelial cells		0 (0.0)	0 (0.0)	3 (60.0)

Number in parentheses represents the percentage (%) of the lesions.

3.4. Immunohistochemistry for PCNA and CYP2E1

The labeling indices for PCNA, compared with those of the corresponding control groups, significantly increased in both benzene-exposed WT and Cx32 KO mice; from 79.9 to 162.3‰ ($P < 0.005$) and 92.7 to 533.0‰ ($P < 0.002$), respectively (Fig. 5).

A few bronchial and bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for the CYP2E1 enzyme (Fig. 6A). The numbers of

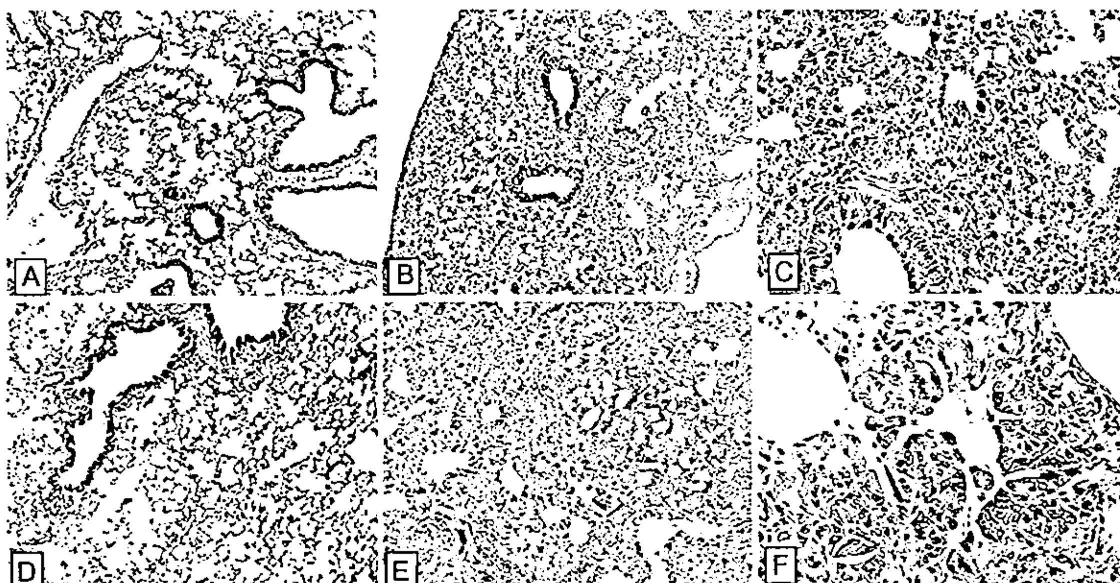

Fig. 3. Histopathological changes of the lungs of benzene-exposed WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) sham-control WT mice, (B and C) benzene-exposed WT mice, (D) sham-control Cx32 KO mice, (E and F) benzene-exposed Cx32 KO mice. Note the granulomatous interstitial pneumonia in the lungs of benzene-exposed WT and Cx32 KO mice, and basophilic epithelial cell-proliferating foci frequently observed in the lungs of benzene-exposed Cx32 KO mice (F). Original magnification: (A) ×100; (B) ×100; (C) ×200; (D) ×100; (E) ×100; (F) ×400. Hematoxylin- and eosin-stained.

Fig. 4. Histochemistry for detection mucin secretion. (A) Sham-control WT mice, (B) sham-control Cx32 KO mice, (C) benzene-exposed WT mice, (D) benzene-exposed Cx32 KO mice. Note the enhanced mucin secretion from bronchiolar epithelial cells of WT mice (C) and Cx32 KO mice (Inset in D), and the aggregated cells releasing mucin occasionally observed in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400.

CYP2E1-positive bronchial and bronchiolar epithelial cells considerably increased following long-term benzene exposure in both WT and Cx32 KO mice (Fig. 6B). The proliferating basophilic alveolar epithelial cells frequently observed in the benzene-exposed Cx32 KO mice were strongly positive for CYP2E1

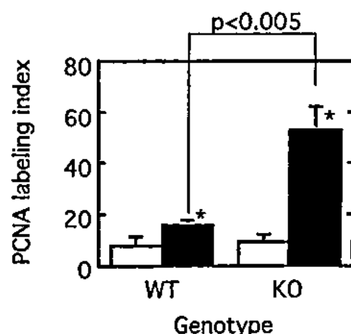

Fig. 5. PCNA labeling indices in the mouse lung tissues exposed to 300 ppm benzene for 26 weeks. Values represent the number of PCNA-positive cell per 1000 cells. More than 3,000 alveolar epithelial cells were counted under a light microscope at a high magnification (×400).

(Fig. 6D), which was significantly comparable with the WT mice in which these findings were rarely observed.

3.5. Survival curves for life time observation

Five to seven mice were randomly selected and allowed to live their life span to evaluate their recovery from pulmonary lesions and the incidence of pulmonary tumor. Survival curves for each group are shown in Fig. 7. In each group the number of mice were limited to 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 control group indicated by open symbols, circles for WT mice and squares for Cx32 KO mice, show a longer life span than the benzene-exposed group indicated by closed symbols, circles for WT mice and squares for Cx32 KO mice. Interestingly, in the exposed group, Cx32 KO mice showed a longer life span than the wild-type mice, although the sham-exposed group does not show much difference between wild-type mice and Cx32 KO mice. During the observation period, all the mice that

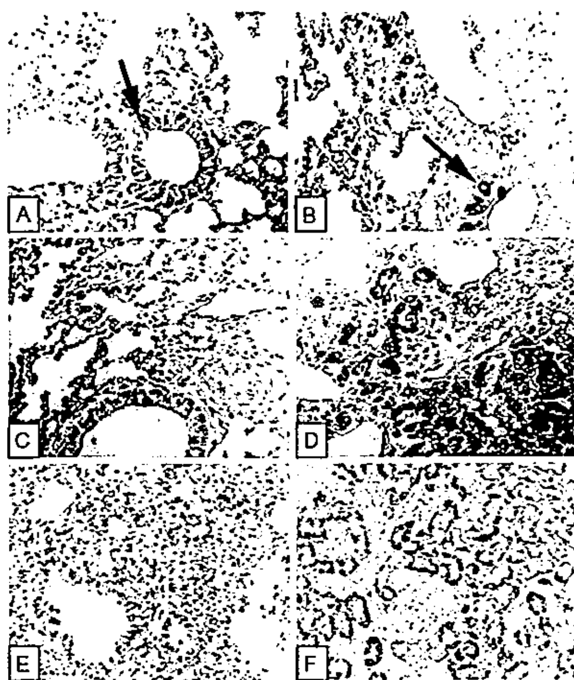

Fig. 6. Immunohistochemistry for P450 CYP2E1 in the lungs of WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) Sham-control WT mice, (B) benzene-exposed WT mice, (C) sham-control Cx32 KO mice, (D) benzene-exposed Cx32 KO mice, and (E and F) negative and positive control, respectively. 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

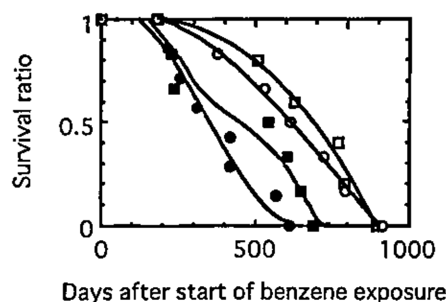

Fig. 7. Survival curves of groups for lifetime observation. The number of mice was limited about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed group, indicated by open symbols, circles for wild-type mice and squares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

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Evaluation of developmental toxicity of 1-butanol given to rats in drinking water throughout pregnancy

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Abstract

The objective of this study was to evaluate the developmental toxicity of 1-butanol in rats. Pregnant rats were given drinking water containing 1-butanol at 0.2%, 1.0% or 5.0% (316, 1454 or 5654 mg/kg/day) on days 0–20 of pregnancy. A significant decrease in maternal body weight gain accompanied by reduced food and water consumption was found at 5.0%. No significant increase in the incidence of pre- and postimplantation embryonic loss was observed in any groups treated with 1-butanol. Fetal weight was significantly lowered at 5.0%. Although a significant increase in the incidence of fetuses with skeletal variations and decreased degree of ossification was found at 5.0%, no increase in the incidence of fetuses with external, skeletal and internal abnormalities was detected in any groups treated with 1-butanol. The data demonstrate that 1-butanol is developmental toxic only at maternal toxic doses. No evidence for teratogenicity of 1-butanol was noted in rats. Based on the significant decreases in maternal body weight gain and fetal weight, it is concluded that the no observed adverse effect levels (NOAELs) of 1-butanol for both dams and fetuses are 1.0% (1454 mg/kg/day) in rats.
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Keywords: 1-Butanol; Developmental toxicity; Teratogenicity; Fetal abnormality; Rat

1. Introduction

1-Butanol (CAS no. 71-36-3, *n*-butanol; *n*-butyl alcohol), a flammable colorless liquid with a rancid sweet odor, is widely used as an organic solvent and intermediate in the manufacture of other organic chemicals (IPCS/WHO, 1987). Exposure of the general population is mainly through its natural occurrence in food and beverages and its use as a flavoring agent (IPCS/WHO, 1987).

Several reports on the developmental toxicity of 1-butanol are available. Nelson et al. (1989a) reported the results of a developmental toxicity study in which SD rats were exposed to 1-butanol by inhalation for 7 hr/day on days 1–19 of pregnancy at 3500, 6000 and 8000 ppm (equivalent to estimated daily absorbed doses of 350, 600 and 800 mg/kg). They observed maternal deaths at 8000 ppm, decreases in maternal food consumption and fetal weight at 6000 and 8000 ppm, and an increased incidence of rudimentary cervical ribs at 8000 ppm, and concluded that 1-butanol was not a selective developmental toxicant in rats. Nelson et al. (1989b) conducted a behavioral teratology study in which female SD rats were given 1-butanol by inhalation at 3000 or 6000 ppm for 7 hr/day throughout pregnancy (the maternal exposure group); male rats were

Abbreviations: NOAEL, no observed adverse effect level

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similarly exposed for 6 weeks and mated to unexposed females (the paternal exposure group), and offspring were behaviorally and neurochemically examined. The data from all tests in their study were within the range of control data in other research conducted by their laboratory. Sitarek et al. (1994) reported a significant increase in the incidence of fetuses with abnormalities after administration of 1-butanol at 0.24–4.0% (300–5000 mg/kg/day) in drinking water during the pre-mating period for 8 weeks and throughout the mating and pregnant period. No maternal toxicity was found at any dose of 1-butanol. The no observed adverse effect level (NOAEL) was not derived from the results of their study, because significant increases in the incidence of fetuses with dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain were found even at the lowest dose (0.24%). They have concluded that 1-butanol is a developmental toxicant and produces anomalies in the skeleton and central nervous system.

The present study was conducted to determine whether or not morphological abnormalities could be produced in fetuses of rats given 1-butanol prenatally and designed to replicate the observations of the study by Sitarek et al. (1994).

2. Materials and methods

This study was performed in compliance with regulatory guidelines (MHW, 1997a) and accordance with the principles for Good Laboratory Practice (MHW, 1997b) and "Guidance for Animal Care and Use" of Ina Research, Inc.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 10 weeks of age and females at 9 weeks of age were purchased from Tsukuba Breeding Center, Charles River Japan, Inc., (Yokohama, Japan). The rats were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum and maintained in an air-conditioned room at 21–25 °C, with a relative humidity of 40–70%, a 12-h light/dark cycle, and ventilation with 16 air charges/hour. Virgin female rats were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 217–273 g and 10–11 weeks of age, were distributed using a computerized randomization procedure (TOXstaff 21 system) into 4 groups of 20 rats each and housed individually.

2.2. Chemicals and dosing

1-Butanol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 1-butanol used in this study was 99.9% pure and a special grade reagent (Lot no. CER5688), and it was kept in a dark place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before and after the study. Rats were given 1-butanol in their drinking water at a concentration of 0 (control), 0.2%, 1.0% or 5.0% on day 0 through day 20 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of 1-butanol in the drinking water on days 0–20 of pregnancy caused decreases in maternal body weight gain and food and water consumption and tended to reduce in fetal weight at 4% and 7% in rats. 1-Butanol was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan). The control rats were given only water. The stability of formulations in a dark and cool place under airtight conditions has been confirmed for up to 3 days. During use, the formulations were maintained under such conditions for no more than 3 days and were 95.7–103.5% of the target concentration.

2.3. Observations

The maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. The pregnant rats were euthanized by exsanguinations under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the numbers of corpora lutea, implantation sites and live and dead fetuses and resorptions were counted. The live fetuses removed from the uterus were sexed, weighed, measured among their crown-rump length, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Dawson, 1926) and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to a free-hand razor-blade sectioning (Wilson, 1973) and the thoracic areas were subjected to microdissecting (Nishimura, 1974) to reveal internal abnormalities. The placental weight was also measured.

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. The initial body

weight, body weight gain and food and water consumption of pregnant rats, numbers of corpora lutea, implantations and live fetuses per litter, fetal weight and crown-rump length and placental weight were analyzed with Bartlett's test (Snedecor and Cochran, 1980) for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test (Dunnett, 1955) to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the 1-butanol-treated groups was compared with that of the control group with the Dunnett type test. The Dunnett type test was used for the incidences of pre- and postimplantation embryonic loss and fetal anomalies and sex ratio of fetuses to compare the mean rank of groups treated with 1-butanol and that of the control group. The incidence of dams with anomalous fetuses was analyzed by Chi-square test or Fisher's exact test. The significance of differences from the control group was estimated at probability levels of 1% and 5%.

3. Results

Table 1 shows the maternal findings in rats given 1-butanol during pregnancy. No death was found in female rats of any group. All females in all groups became pregnant. The body weight gains on days 0–7 of pregnancy were significantly reduced at 5.0%. The body weight gain during the whole period of pregnancy was also significantly decreased at 5.0%. No significant decrease in the body weight gain was noted at 0.2 or 1.0, except for a transient decrease on days 0–2 of pregnancy at 1.0%. The food consumption on days 0–7, days 7–14, days 14–20 and days 0–20 of pregnancy was significantly lower in the 1.0% and 5.0% groups than the control group. The water consumption on days 0–7 at 1.0 and 5.0% and on days 7–14, days 14–20 and days 0–20 at 5.0% was significantly decreased. The mean daily intakes of 1-butanol were 316 mg/kg for the 0.2% group, 1454 mg/kg for the 1.0% group and 5654 mg/kg for the 5.0% group.

Reproductive findings in rats given 1-butanol during pregnancy are presented in Table 2. No litters totally resorbed were found in any group. No effects of the administration of 1-butanol were observed on the numbers of corpora lutea, implantations, pre- or postimplantation loss, resorptions or dead or live fetuses or sex ratio of live fetuses. The body weights of male and female fetuses were significantly lower in the 5.0% group than in the control group. There was no significant difference in the crown-rump length of male and female fetuses or placental weight between the control and groups treated with 1-butanol.

A summary of morphological findings in live fetuses of rats given 1-butanol during pregnancy is shown in Table 3. One fetus with spina bifida in the control group and one fetus with thread-like tail and anal atresia in the 0.2% group were observed. Skeletal examination

Table 1
Maternal findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of rats	20	20	20	20
No. of pregnant rats	20	20	20	20
No. of dead rats	0	0	0	0
Initial body weight	245 ± 14	247 ± 13	245 ± 11	244 ± 12
Body weight gain during pregnancy (g)[a]				
Days 0–7	44 ± 7	45 ± 7	40 ± 6	20 ± 28**
Days 7–14	40 ± 6	41 ± 5	41 ± 7	42 ± 10
Days 14–20	78 ± 14	82 ± 8	84 ± 7	75 ± 11
Days 0–20	162 ± 19	168 ± 16	165 ± 15	146 ± 16**
Food consumption during pregnancy (g)[a]				
Days 0–7	179 ± 12	180 ± 16	164 ± 12*	138 ± 21**
Days 7–14	193 ± 14	194 ± 17	177 ± 14**	160 ± 11**
Days 14–20	176 ± 14	175 ± 15	161 ± 12**	143 ± 11**
Days 0–20	548 ± 38	548 ± 46	503 ± 34**	441 ± 34**
Water consumption during pregnancy (ml)[a]				
Days 0–7	284 ± 28	305 ± 37	258 ± 29*	175 ± 34**
Days 7–14	318 ± 35	337 ± 48	299 ± 40	239 ± 80**
Days 14–20	328 ± 47	342 ± 47	334 ± 46	256 ± 85**
Days 0–20	930 ± 105	983 ± 126	890 ± 106	669 ± 182**
Mean daily intakes of 1-butanol (mg/kg)[a]	0	316 ± 30	1454 ± 186	5654 ± 1402

*,** Significantly different from the control, $^*P < 0.05$ and $^{**}P < 0.01$.
[a] Values are given as the mean ± SD.

Table 2
Reproductive findings in rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
No. of litters	20	20	20	20
No. of litters totally resorbed	0	0	0	0
No. of corpora lutea per litter[a]	16.4 ± 3.6	16.7 ± 3.0[d]	16.1 ± 2.1	16.3 ± 2.6
No. of implantations per litter[a]	14.3 ± 2.8	15.1 ± 1.7	15.2 ± 1.2	14.7 ± 2.5
% Preimplantation loss per litter[b]	9.0	9.0[d]	4.4	9.2
% Postimplantation loss per litter[c]	6.0	5.4	3.7	8.0
No. of live fetuses per litter[a]	13.4 ± 2.6	14.3 ± 1.4	14.7 ± 1.5	13.5 ± 2.5
Sex ratio of live fetuses (male/female)	128/139	145/140	149/144	131/139
Body weight of live fetuses (g)[a]				
Male	4.18 ± 0.27	4.00 ± 0.24	4.04 ± 0.25	3.83 ± 0.18**
Female	3.97 ± 0.25	3.86 ± 0.20	3.83 ± 0.16	3.59 ± 0.17**
Fetal crown-rump length (mm)[a]				
Male	40.5 ± 1.2	40.3 ± 1.4	40.2 ± 1.2	39.7 ± 1.3
Female	39.4 ± 1.2	39.4 ± 1.2	39.3 ± 1.1	38.5 ± 1.4
Placental weight (g)				
Male	0.50 ± 0.05	0.49 ± 0.05	0.48 ± 0.06	0.50 ± 0.06
Female	0.49 ± 0.05	0.48 ± 0.05	0.47 ± 0.05	0.49 ± 0.06

** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± SD.
[b] (No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c] (No. of resorptions and dead fetuses/no. implantations) × 100.
[d] Value was obtained from 19 pregnant rats.

revealed one fetus with supernumerary thoracic vertebral bodies and malpositioned thoracic vertebrae at 1.0%. Although the total number of fetuses with skeletal variations was significantly increased at 5.0%, the number of fetuses with individual skeletal variations was not significantly increased, except for fetuses with short supernumerary ribs at 5.0%. A significantly lower number of forepaw proximal phalanges was observed at 5.0%. Membranous ventricular septum defect occurred in one fetus of the control and 0.2% groups and 3 fetuses in 3 dams of the 5.0% group. One fetus with a double aorta in the control group and one fetus with a left umbilical artery in the control and 2.0% groups were observed. Thymic remnants in the neck were found in 4–11 fetuses of the control and groups treated with 1-butanol. However, there was no significant difference in the incidence of fetuses with internal abnormalities between the control and groups treated with 1-butanol.

4. Discussion

The present study was conducted to determine the developmental toxicity of 1-butanol and designed to replicate the observations of the study by Sitarek et al. (1994). The data showed that prenatal administration of 1-butanol did not produce morphological anomalies in fetuses of rats. Thus, we have been unable to confirm the results of Sitarek's study in which prenatal exposure to 1-butanol produced fetal anomalies.

The doses of 1-butanol used in the present study expected to induce maternal and/or developmental toxicity, such as a decrease in maternal body weight gain and fetal weight, were given to pregnant rats during the whole period of pregnancy to characterize the effects of 1-butanol on embryonic/fetal development. Maternal toxicity, a significant decrease in body weight gain, was found at 5.0%. Maternal food and water consumptions were also reduced in this dose group. Although the only significant decrease in maternal body weight gain was observed on days 0–2 of pregnancy at 1.0%, this decrease was occasional and discontinuous and seems unlikely to be of toxicological significance. In this dose group, decreases in the maternal food consumption during the whole period of pregnancy and water consumption during the early period of pregnancy, which were unaccompanied by the continuous changes in body weight gain, were observed. No significant changes in maternal parameters were noted in the 0.2% group. These findings in maternal rats indicate that 1-butanol exerts maternal toxicity at 5.0% (equivalent to 5654 mg/kg/day) when administered during the entire period of pregnancy in rats.

No significant increase in the incidence of postimplantation loss was found at any dose of 1-butanol, and significantly decreased weights of male and female fetuses were found at 5.0%. No significant adverse effects on reproductive parameters were detected at 0.2% and 1.0%. These findings indicate that 1-butanol is not toxic to embryonic/fetal survival up to 5.0% or fetal growth up to 1.0% when administered during the whole period of pregnancy.

As for morphological examinations in the fetuses of exposed mothers, a few fetuses with external, skeletal

Table 3
Morphological examinations in fetuses of rats given 1-butanol on days 0–20 of pregnancy

Dose (%)	0 (Control)	0.2	1.0	5.0
External examination				
Total no. of fetuses (litters) examined	267 (20)	285 (20)	293 (20)	270 (20)
Total no. of fetuses (litters) with abnormalities	1 (1)	1 (1)	0	0
Spina bifida	1 (1)	0	0	0
Thread-like tail and anal atresia	0	1 (1)	0	0
Skeletal examination				
Total no. of fetuses (litters) examined	139 (20)	147 (20)	152 (20)	140 (20)
Total no. of fetuses (litters) with abnormalities	0	0	1 (1)	0
Supernumerary of thoracic vertebral bodies and malpositioned thoracic vertebrae	0	0	1 (1)	0
Total no. of fetuses (litters) with variations	28 (11)	23 (12)	52 (17)	69 (20)**
Bipartite ossification of thoracic centra	1 (1)	1 (1)	1 (1)	7 (5)
Dumbbell ossification of thoracic centra	0	1 (1)	2 (2)	3 (3)
Bipartite ossification of lumbar centra	0	0	0	2 (2)
Supernumerary lumbar vertebrae	4 (1)	1 (1)	5 (3)	5 (2)
Lumbarization	0	0	1 (1)	1 (1)
Bipartite ossification of sternebrae	1 (1)	1 (1)	1 (1)	1 (1)
Misaligned sternebrae	0	0	0	1 (1)
Cervical ribs	2 (2)	3 (3)	3 (3)	7 (5)
Full supernumerary ribs	5 (2)	1 (1)	10 (5)	9 (5)
Short supernumerary ribs	20 (10)	18 (9)	43 (16)	55 (19)**
Wavy ribs	0	0	0	1 (1)
Degree of ossification[a]				
No. of sacral and caudal vertebrae	8.4 ± 0.5	8.4 ± 0.4	8.3 ± 0.5	8.1 ± 0.3
No. of sternebrae	5.9 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.2
No. of forepaw proximal phalanges	1.6 ± 1.3	1.6 ± 0.9	1.2 ± 1.1	0.3 ± 0.4**
Internal examination				
Total no. of fetuses (litters) examined	128 (20)	138 (20)	141 (20)	130 (20)
Total no. of fetuses (litters) with abnormalities	7 (6)	9 (6)	11 (8)	14 (9)
Membranous ventricular septum defect	1 (1)	1 (1)	0	3 (3)
Double aorta	1(1)	0	0	0
Left umbilical artery	1 (1)	0	1 (1)	0
Thymic remnant in neck	4 (4)	8 (5)	10 (8)	11 (8)

** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± SD.

and/or internal abnormalities were found in all groups. The abnormalities observed in the present study are not thought to be due to the administration of 1-butanol, because they have occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). Several types of skeletal variations were also found in the control and groups treated with 1-butanol. These skeletal variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). In the 5.0% group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with short supernumerary ribs, but not full supernumerary ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e., full supernumerary ribs, is a warning sign of possible teratogenicity, short supernumerary ribs, sternebral variations, and bilobed centra of the vertebral column are normal variations (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphogenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes in fetuses observed in the present study do not indicate a teratogenic response and that 1-butanol possesses no teratogenic potential in rats.

In Sitarek's study (1994), significant increases in the incidences of wavy ribs at 300 mg/kg/day, dilation of the subarachnoid space and dilation of the lateral ventricle and/or third ventricle of the brain at 300 mg/kg/day and higher, dilation of the renal pelvis and external hydrocephaly at 1000 mg/kg/day, internal hydrocephaly at 1000 mg/kg/day and higher, and supernumerary ribs and delayed ossification at 5000 mg/kg/day were found. A significant decrease in fetal crown-rump length was

also observed at 5000 mg/kg/day. Based on these findings, Sitarek et al. (1994) concluded that 1-butanol had adverse effects on the morphological development of fetuses in rats. However, we did not confirm their findings. We have demonstrated here that prenatal 1-butanol has no adverse effect on the morphological development of rat offspring. There are some differences between Sitarek's study and the present study in experimental conditions, such as duration of administration and rat strain used in the experiments. Sitarek et al. (1994) administered 1-butanol to female rats for 8 weeks before mating and throughout the mating and pregnancy period and found fetal anomalies, such as hydrocephaly and dilation of the cerebral ventricles and the renal pelvis. On the other hand, we gave 1-butanol to female rats during the whole period of pregnancy and did not detect fetuses with these anomalies. Administration during the premating and mating period is thought to be excluded from the susceptible period for induction of morphological anomalies such as hydrocephaly/dilation of the cerebral ventricles and dilation of the renal pelvis, because rat fetuses are susceptible to induction of these anomalies during mid and late pregnancy (Wood and Hoar, 1972; Kameyama, 1985). The strain difference of rats used in the experiments may explain the discrepancy in the findings regarding fetal anomalies between the studies. In Sitarek's study (1994), ImP: DAK rats obtained from their own breeding colony were used. No detailed information on this strain of rats was available (Sitarek et al., 1994). In their study, dilation of the lateral ventricle and/or third ventricle of the brain was observed in 2% of fetuses (one of the 12 litters) in the control group. In their another study using Imp: DAK rats, extension of the lateral ventricle and/or third ventricle of the brain was observed in 11.7% of fetuses (8 of the 17 litters) in the control group (Sitarek et al., 1996). However, these anomalies were not found in the control group of their studies using Wistar rats (Baranski et al., 1982), Imp: Lodz rats (Sitarek, 1999, 2001) and Imp: WIST rats (Sitarek and Sapota, 2003). The incidences of dilation of the cerebral ventricles in Imp: DAK rats are thought to be higher than those in the background control data of other strains of rats. The fetal incidence of hydrocephaly/dilation of cerebral ventricles in the control rats of reproductive studies conducted between 1986 and 1993 in 63 research institutes is reported to be 0–0.09% and 0–0.26%, respectively (Nakatsuka et al., 1997). In Crj: CD (SD) IGS rats which were used in the present study, the incidence of dilation of the lateral ventricles of the brain in 19 studies conducted during 1998–2000 is reported to be 0–0.06% in fetuses and 0–0.44% in litters (Barnett et al., 2000). Thus, hydrocephaly/dilation of the cerebral ventricle is not commonly observed in fetuses of common strains of rats.

The difference in terminology used for classification of structural anomalies in fetuses may also explain the discrepancy in the findings regarding fetal anomalies between the studies. Sitarek et al. (1996) stated that minor abnormalities, such as enlarged lateral ventricle and/or third ventricle, are quite frequent in rat fetuses and without having the dose-dependent relationship should not be taken alone as evidence of tested chemical fetotoxicity. However, the Fourth Berlin Workshop on Terminology in Developmental Toxicity noted that changes affecting brain ventricles are more likely to be classified as malformations and classification should be based on the historical control incidences, the nature of the organ affected and the severity (Solecki et al., 2003). In Sitarek's study (1994), dilation of the subarachnoid space was observed in fetuses of rats given 1-butanol at 300 mg/kg/day and higher. This anomaly was also found in fetuses in Imp: DAK rats given *N*-cyclohexyl-2-benzothiazolesulfenamide (Sitarek et al., 1996) and Imp: Lodz rats given *N*-methylmorpholine (Sitarek, 1999). No information on the definition of this anomaly was available in their reports. We are unaware of this anomaly in other literature (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Horimoto et al., 1998; Barnett et al., 2000; Solecki et al., 2003).

In conclusion, the administration of 1-butanol to pregnant rats throughout pregnancy had adverse effects on maternal rats and embryonic/fetal growth but had no adverse effects on fetal morphological development even at a maternally toxic dose. The data indicate that 1-butanol induces developmental toxicity only at maternally toxic doses in rats. Based on the significant decreases in maternal body weight gain and fetal weight at 5.0%, it is concluded that the NOAELs of 1-butanol for both dams and fetuses are 1454 mg/kg/day (1.0% in drinking water) in rats.

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