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Original Article

A chronic toxicity study of diphenylarsinic acid in the drinking water of C57BL/6J mice for 52 weeks

Takashi Yamaguchi[1], Min Gi[1], Masaki Fujioka[1], Yoshiyuki Tago[1], Anna Kakehashi[1], and Hideki Wanibuchi[1]*

[1] Department of Molecular Pathology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan

Abstract: Diphenylarsinic acid (DPAA), a neurotoxic organic arsenical, is present in the groundwater and soil in some regions of Japan due to illegal dumping. The purpose of the present study was to evaluate the potential toxicity of DPAA when administered to mice in their drinking water for 52 weeks. DPAA was administered to mice at concentrations of 0, 6.25, 12.5, and 25 ppm in their drinking water for 52 weeks. There were no significant differences in final body weights between the control groups and the DPAA treatment groups in male or female mice. Relative liver weights were significantly increased in males treated with 25 ppm DPAA, and absolute liver weights were significantly decreased in female mice treated with 25 ppm DPAA. In female mice, cholangitis and simple bile duct hyperplasia were observed in the 12.5 and 25 ppm DPAA groups, and focal necrosis of hepatocytes was observed in the 25 ppm DPAA group. Proteomic analysis and Ingenuity Pathway Analysis identified 18 proteins related to hepatotoxicity that were overexpressed in the female 25 ppm group. The phase I metabolic enzyme CYP2E1 was one of these overexpressed proteins. Immunostaining confirmed high expression of CYP2E1 in the livers of females in the 25 ppm group. These results suggest that DPAA is toxic to the intrahepatic bile duct epithelium and hepatocytes in female mice and that CYP2E1 might be involved in DPAA-associated toxicity. The no-observed-adverse-effect levels of DPAA were 12.5 ppm (1.6 mg/kg bw/day) for males and 6.25 ppm (1.1 mg/kg bw/day) for females under the conditions of this study. (DOI: 10.1293/tox.2018-0067; J Toxicol Pathol 2019; 32: 127–134)

Key words: diphenylarsinic acid, chronic toxicity, bile duct toxicity, C57BL/6J mice

Introduction

Diphenylarsine chloride and diphenylarsine cyanide were synthesized as chemical weapons during World War II[1–3]. Diphenylarsinic acid (DPAA), an organic arsenical, is a synthetic intermediate and environmental degradation product of these chemical warfare agents. In 2003, inhabitants chronically exposed to DPAA through drinking of contaminated well water due to illegal dumping of chemical warfare agents after World War II in Kamisu City, Ibaraki, Japan, were found to suffer from neurological symptoms such as vertigo, visual disorder, myoclonus, and tremors[4].

Short-term toxicological studies have shown that DPAA induces oxidative and nitrosative stress in Purkinje cells[5] and increases exploratory behavior, impairs learning behavior, and decreases the cerebellar glutathione (GSH) concentration in rats[6]. DPAA also produced behavioral effects in subchronic and chronic toxicity studies in mice[7]. In humans, inorganic arsenic is known to cause skin cancer, urinary bladder cancer, and lung cancer and to possibly cause liver and kidney cancers in populations chronically exposed through contaminated drinking water[8]. Organic metabolites of inorganic arsenicals in humans, such dimethylarsenic acid (DMA), monomethylarsonic acid, and trimethylarsine oxide, have been shown to induce urinary bladder cancer and promote liver and kidney carcinogenesis in rats and to enhance skin and lung carcinogenesis in mice[9–11]. We recently demonstrated that DPAA promotes diethylnitrosamine (DEN)-induced liver carcinogenesis in a medium-term rat liver bioassay[12]. DPAA is also toxic to the intrahepatic and extrahepatic bile duct epithelium in male and female rats as evidenced by intrahepatic bile duct hyperplasia and dilation of the common bile duct accompanied by stenosis of the papilla of Vater in a 52-week rat chronic study[13], although DPAA was not carcinogenic in a 104-week rat carcinogenicity study[14]. However, little is known about chronic toxicity of DPAA in mice. The purpose of the present study was to evaluate the chronic toxicity of DPAA when administered to mice in their drinking water for 52 weeks.

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*Corresponding author: H Wanibuchi
(e-mail: wani@med.osaka-cu.ac.jp)

Materials and Methods

Chemicals

DPAA with a purity of more than 99.9% was provided by Tri Chemical Laboratories (Yamanashi, Japan). The purity of the DPAA and its stability in tap water for 28 days at room temperature were verified using ion chromatography (IC; IC7000, Yokogawa Analytical System Inc., Tokyo, Japan) coupled with inductively coupled plasma mass spectrometry (IC-ICP-MS; HP 4500, Agilent Technologies, Santa Clara, CA, USA) at Osaka City University Graduate School of Medicine.

Animals

Male and female C57BL/6J mice at 5 weeks of age were obtained from Charles River Laboratories Japan, Inc. (Tsukuba, Ibaragi, Japan). The animals were housed in polycarbonate cages (5 mice/cage) in experimental animal rooms with a targeted temperature of 24 ± 2°C, relative humidity of 50 ± 10%, and a 12-h light/dark cycle. All animals were acclimated for 1 week before the beginning of the experiment. Diet and drinking water were available *ad libitum* throughout the study. Body weights, food consumption, and water intake were measured weekly until week 13 and every 4 weeks thereafter.

Experimental design

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine. A total of 40 male and 40 female C57BL/6J mice were divided into groups of 10 mice each by sex (8 groups in total; Table 1). DPAA was dissolved in the tap water and administered to the mice for 52 weeks at 0, 6.25, 12.5, or 25 ppm in their drinking water. Fresh drinking water containing DPAA was supplied to the animals twice weekly. The highest dose of 25 ppm was determined based on the results of a 4-week preliminary study in which a 50 ppm dose significantly decreased body weight and induced focal necrosis in the livers of male (7/10) and female (7/10) mice and a dose of 25 ppm DPAA induced inflammatory cell infiltration around the bile duct (4/10) and single cell necrosis (3/10) in the livers of female mice but not male mice. At the end of 52 weeks, mice were fasted overnight and euthanized by inhalation of an overdose of isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a Small Animal Anesthetizer (MK-A110D, Muromachi Kikai Co., Ltd., Tokyo, Japan) coupled with an Anesthetic Gas Scavenging System (MK-T 100E, Muromachi Kikai Co., Ltd., Tokyo, Japan). The final body weights and the weights of the heart, liver, spleen, kidneys, adrenals, thymus, testes, and brain of all mice surviving to the end of the study were measured.

Serum biochemistry

Whole blood samples were collected via the inferior vena cava under deep anesthesia at necropsy. Serum biochemical parameters were measured in all mice surviving to the end of the study by LSI Medience Corporation, Tokyo, Japan. Serum biochemical parameters included total protein (TP), albumin (ALB), total bilirubin (T-BIL), triglycerides (TG), total cholesterol (T-Cho), blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (γ-GPT).

Histopathology

The testes from males were fixed in Bouin's solution. The remaining tissues from all animals were fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin and processed for histopathological examination: the lymph nodes (cervical and mesenteric), intrathoracic aorta, submaxillary gland, sublingual gland, thymus, trachea, lung, heart, thyroids, parathyroids, tongue, esophagus, forestomach, glandular stomach, duodenum, small intestine (jejunum and ileum), large intestine (cecum, colon, and rectum), liver, pancreas, spleen, kidneys, adrenals, urinary bladder, seminal vesicles, prostate, testes, epididymides, ovaries, oviduct, uterus, vagina, brain, pituitary, sciatic nerve, skeletal muscle, spinal cord (cervical and lumbar), eye, Harderian gland, sternum, femur, skull bone, nasal cavity, and sites of macroscopic abnormality were examined.

Table 1. Survival, Food Consumption, Water and Diphenylarsinic Acid (DPAA) Intake, and Final Body Weight of C57BL/6J Mice Treated with DPAA in Their Drinking Water for 52 Weeks

DPAA (ppm)	Initial no. of mice	No. of surviving mice (%)	Final body weight (g)	Average food consumption (g/day/mouse)	Average water intake (g/day/mouse)	Average DPAA intake (mg/kg bw/day/mouse)	Total DPAA intake (mg/kg bw/mouse)
Male							
0	10	10 (100)	35.3 ± 1.7	3.1	3.9	0	0
6.25	10	10 (100)	35.4 ± 2.0	3.2	3.9	0.8	273.7
12.5	10	10 (100)	33.9 ± 2.9	3.1	3.8	1.6	573.0
25	10	10 (100)	34.1 ± 2.1	3.5	4.0	3.2	1,153.2
Female							
0	10	10 (100)	27.1 ± 1.9	3.4	4.7	0	0
6.25	10	9 (90)	27.0 ± 1.5	3.3	4.2	1.1	383.4
12.5	10	9 (90)	26.3 ± 1.4	3.5	4.8	2.7	997.8
25	10	8 (80)	25.3 ± 1.1	3.4	4.4	4.8	1,745.1

Protein extraction and QSTAR Elite LC/MS/MS (liquid chromatography-tandem mass spectrometry) analysis

The livers of 3 female mice in the 25 ppm group and 3 female mice in the control group were processed for proteomic analysis. Protein extraction was performed using T-PER™ Tissue Protein Extraction Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentrations were determined using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Twenty micrograms of protein from each mouse was used for proteome analysis as described previously[15]. Briefly, protein reduction, alkylation, digestion, and subsequent peptide labeling were performed using an AB Sciex iTRAQ Reagents Multiplex Kit (AB Sciex, Framingham, MA, USA) according to the manufacturer's instructions. Peptides were fractionated by six concentrations of KCl solutions using ICAT cation exchange cartridges (AB Sciex). After desalting and concentration of the fractions, peptides of each fraction were quantified using a DiNa-AI nano LC System (KYA Technologies, Tokyo, Japan) coupled to a QSTAR Elite MS/MS through a NanoSpray ion source (AB Sciex, Concord, ON, Canada). The Protein Pilot 2.0 software (AB Sciex) with the Paragon Algorithm was used for identification and relative quantification of proteins. Protein quantitative ratio statistics were calculated as the median of all peptide ratios. Proteins showing a fold-change of at least 1.2 at a *P*-value <0.05 were considered differentially expressed. Functional annotation and pathway analysis of differentially expressed proteins was performed using Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA). Pathways with z-scores ≥2.0 were defined as activated, and pathways with z-scores ≤−2.0 were defined as inactivated.

Immunohistochemistry of CYP2E1

Serial sections (4-μm thickness) cut from paraffin-embedded livers of males and females in the control and 25 ppm groups were examined for expression of CYP2E1 by immunohistochemical staining using the avidin-biotin-peroxidase complex (ABC) method. Antigen retrieval was performed by microwaving at 98°C for 20 min in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in distilled water for 5 min, and the slides were then incubated with serum at 37°C for 30 min to block nonspecific binding. Sections were incubated with rabbit polyclonal anti-CYP2E1 antibody (ab28146, Abcam, Cambridge, MA, USA) at a dilution of 1:100 overnight at 4°C. Immunoreactivity was detected using a VECTASTAIN Elite ABC Kit (PK-6101, Vector Laboratories, Burlingame, CA, USA) and 3,30-diaminobenzidine hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA). Omission of the primary antibody served as the negative control and was included with each staining procedure. Animals that died before the scheduled sacrifice at week 52 were not included in immunohistochemistry analyses, as postmortem changes hindered accurate evaluation.

Statistical analysis

All mean values are reported as the mean ± SD. Statistical analyses were performed using the Statlight program (Yukms Co., Ltd., Tokyo, Japan). Homogeneity of variance was tested by Bartlett's test. The significance of differences between the controls and treated groups in body weight, food consumption, water intake, organ weights, and biochemical parameters was assessed by 2-tailed Dunnett's test when the variances were homogeneous and by 2-tailed Steel's test when the variances were heterogeneous. Histopathological examination results were compared using the 2-tailed Fisher's exact test. *P* values less than 0.05 were considered significant.

Results

Survival, body weight, food consumption, water and DPAA intake

The number of mice surviving to the end of the study, final body weights, average food consumption, and water and DPAA intake are summarized in Table 1, and body weight curves are shown in Fig. 1. One, one, and two female mice in the 6.25, 12.5, and 25 ppm groups died at weeks 45, 50, 40, and 45, respectively. All the remaining animals survived to the end of study in good condition. No clinical signs or symptoms of neurotoxicity were observed in any of

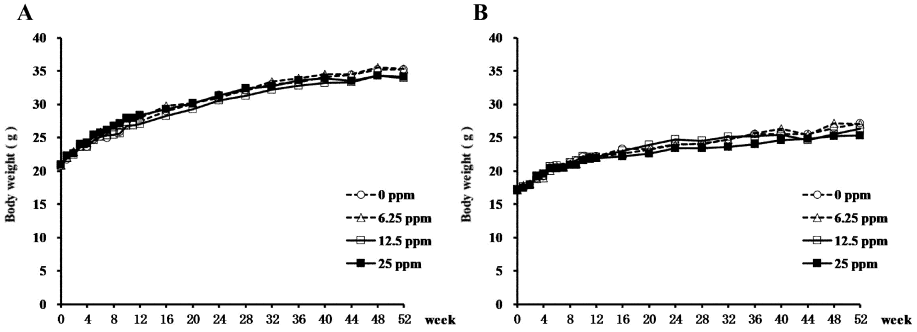

Fig. 1. Body weight curves in male (A) and female (B) mice.

the DPAA-treated mice throughout the study. There were no significant differences in final body weights between the DPAA-treated groups and the control groups; slight body weight suppression was noted in the 12.5 and 25 ppm male and female groups.

The intake of DPAA was approximately proportional to the doses administered in the drinking water. There were no apparent differences in food consumption or water intake between the DPAA treatment groups and control groups.

Serum biochemistry

Data for serum biochemistry are shown in Table 2. ALB in the male 25 ppm group was significantly decreased compared with the control group. There were no significant differences in other parameters between the DPAA-treated groups and the control groups.

Organ weights

Organ weights are shown in Table 3. In the liver, the relative, but not absolute, weights were significantly increased in males treated with 25 ppm DPAA, and the absolute, but not relative, weights were significantly decreased in the female 25 ppm DPAA group. The absolute and relative kidney weights were both significantly increased in the male 25 ppm DPAA group. There were no DPAA treatment-related changes in other organs.

Histopathology

Histopathological data are shown in Table 4. The incidences of cholangitis and simple bile duct hyperplasia were significantly increased in the female 25 ppm group: cholangitis was characterized as degeneration of the biliary epithelium, mononuclear inflammatory cell (neutrophil and lymphocyte) infiltration, and fibrosis (Fig. 2A) and was observed in the liver of one female mouse in the 12.5 ppm

Table 2. Serum Biochemistry of C57BL/6J Mice Treated with Diphenylarsinic Acid (DPAA) in Their Drinking Water for 52 Weeks

	Male				Female			
DPAA (ppm)	0	6.25	12.5	25	0	6.25	12.5	25
No. of mice	10	10	10	10	9	9	9	7
AST (IU/l)	76 ± 53	74 ± 46	69 ± 18	75 ± 13	97 ± 20	88 ± 23	108 ± 36	100 ± 18
ALT (IU/l)	25.3 ± 5.6	27.0 ± 4.2	29.6 ± 12.4	26.3 ± 4.6	35.9 ± 9.1	32.9 ± 10.4	43.2 ± 23.8	29.0 ± 3.9
ALP (IU/l)	276 ± 25	284 ± 31	273 ± 30	245 ± 26	390 ± 116	406 ± 65	405 ± 92	473 ± 87
γ-GPT (IU/l)	3.2 ± 3.3	2.9 ± 3.8	1.6 ± 0.5	1.7 ± 1.3	1.9 ± 2.0	2.0 ± 1.1	2.2 ± 2.3	1.3 ± 0.5
T-BIL (mg/dl)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
TP (g/dl)	5.5 ± 0.2	5.6 ± 0.2	5.5 ± 0.3	5.3 ± 0.2	5.3 ± 0.5	5.4 ± 0.1	5.3 ± 0.5	5.4 ± 0.2
ALB (g/dl)	4.0 ± 0.2	4.1 ± 0.2	4.1 ± 0.1	3.8 ± 0.2 [a]	3.8 ± 0.6	4.0 ± 0.2	3.8 ± 0.8	4.0 ± 0.1
TG (mg/dl)	19 ± 3	23 ± 7	19 ± 6	21 ± 7	15 ± 3	13 ± 3	13 ± 4	13 ± 2
T-Cho (mg/dl)	81 ± 4	87 ± 5	75 ± 8	79 ± 8	64 ± 6	62 ± 4	67 ± 9	61 ± 9
BUN (mg/dl)	26.7 ± 3.0	27.8 ± 4.5	27.5 ± 5.1	26.7 ± 3.0	42.8 ± 39.9	27.1 ± 9.4	36.5 ± 30.2	34.5 ± 4.9

[a]Significantly different from the male 0 ppm group.

Table 3. Organ Weights of C57BL/6J Mice Treated with Diphenylarsinic Acid (DPAA) in Their Drinking Water for 52 Weeks

		Male				Female			
DPAA (ppm)		0	6.25	12.5	25	0	6.25	12.5	25
No. of mice		10	10	10	10	10	9	9	8
Liver	Absolute (g)	1.21 ± 0.09	1.24 ± 0.06	1.17 ± 0.09	1.24 ± 0.07	1.06 ± 0.07	1.02 ± 0.06	1.03 ± 0.08	0.95 ± 0.08[b]
	Relative (%)	3.43 ± 0.17	3.52 ± 0.13	3.45 ± 0.18	3.65 ± 0.16[a]	3.91 ± 0.35	3.80 ± 0.21	3.93 ± 0.38	3.72 ± 0.30
Kidney	Absolute (g)	0.36 ± 0.02	0.37 ± 0.02	0.37 ± 0.03	0.40 ± 0.04[a]	0.34 ± 0.04	0.31 ± 0.01	0.34 ± 0.05	0.31 ± 0.04
	Relative (%)	1.02 ± 0.06	1.06 ± 0.07	1.09 ± 0.08	1.16 ± 0.09[a]	1.27 ± 0.18	1.16 ± 0.07	1.31 ± 0.25	1.24 ± 0.14
Spleen	Absolute (g)	0.06 ± 0.01	0.08 ± 0.02[a]	0.06 ± 0.01	0.06 ± 0.01	0.10 ± 0.06	0.07 ± 0.01	0.09 ± 0.05	0.07 ± 0.01
	Relative (%)	0.16 ± 0.01	0.22 ± 0.07[a]	0.17 ± 0.02	0.18 ± 0.02	0.36 ± 0.21	0.26 ± 0.04	0.33 ± 0.23	0.26 ± 0.05
Adrenal	Absolute (g)	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Relative (%)	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01[b]	0.04 ± 0.01
Heart	Absolute (g)	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
	Relative (%)	0.40 ± 0.03	0.41 ± 0.05	0.41 ± 0.03	0.44 ± 0.04[a]	0.45 ± 0.07	0.44 ± 0.05	0.46 ± 0.09	0.46 ± 0.04
Brain	Absolute (g)	0.46 ± 0.01	0.46 ± 0.01	0.45 ± 0.02	0.46 ± 0.02	0.47 ± 0.02	0.46 ± 0.01	0.47 ± 0.02	0.47 ± 0.02
	Relative (%)	1.30 ± 0.05	1.31 ± 0.06	1.34 ± 0.09	1.36 ± 0.07	1.71 ± 0.06	1.72 ± 0.10	1.79 ± 0.04	1.84 ± 0.07[b]
Thymus	Absolute (g)	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
	Relative (%)	0.08 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.10 ± 0.03	0.12 ± 0.02	0.10 ± 0.04	0.13 ± 0.03
Testis	Absolute (g)	0.19 ± 0.02	0.18 ± 0.03	0.21 ± 0.04	0.21 ± 0.04	-	-	-	-
	Relative (%)	0.53 ± 0.05	0.51 ± 0.10	0.62 ± 0.13	0.60 ± 0.10	-	-	-	-

[a]Significantly different from the male 0 ppm group. [b]Significantly different from the female 0 ppm group.

DPAA group and six female mice in the 25 ppm DPAA group, and simple bile duct hyperplasia (Fig. 2B) was observed in two female mice in the 12.5 ppm DPAA group and eight female mice in the 25 ppm DPAA group. In addition, focal necrosis of hepatocytes (Fig. 2B) was observed in the two female mice that died at weeks 40 and 45 in the 25 ppm DPAA group. There were no DPAA treatment-related changes in the livers of male mice.

In the spleen, a histiocytic sarcoma was found in one male mouse in the 6.25 ppm DPAA group. In the lung, an adenoma was found in one male mouse in the 12.5 ppm DPAA group. Lymphoma was observed in one female mouse in the control group.

There were no DPAA treatment-related changes in the brains or sciatic nerves of male or female mice.

Identification of differentially expressed proteins by proteomic comparison analysis of the 25 ppm and control groups

Proteomic analysis was conducted for 3 female mice in the 25 ppm group and 3 female mice in the control group by QSTA Elite LC/MS/MS. A total of 188 proteins were differentially overexpressed in the 25 ppm groups compared with the control groups (Supplementary Table 1: online only); no underexpressed proteins were identified. Of the 188 proteins differentially overexpressed in the livers of mice administered 25 ppm DPAA, 18 are coded by genes categorized as hepatotoxicity related proteins by Ingenuity Pathway Analysis (Table 5).

Ingenuity Pathway Analysis predicted that 52 pathways were activated and 2 pathways were inactivated in the livers of female mice administered 25 ppm DPAA. Ingenu-

Table 4. Histopathology Data of C57BL/6J Mice Treated with Diphenylarsinic Acid (DPAA) in Their Drinking Water for 52 Weeks

Organ/finding	Male				Female			
DPAA (ppm)	0	6.25	12.5	25	0	6.25	12.5	25
No. of mice	10	10	10	10	10	9 [a]	10	10
Liver								
Fatty change	10	10	10	10	10	9	10	10
Cholangitis	0	0	0	0	0	0	1	6
Simple bile duct hyperplasia	0	1	0	0	0	0	2	8[b]
Focal necrosis, hepatocyte	0	0	0	0	0	0	0	2[c]
Kidney								
Dilatation, distal tubule	0	0	0	0	1	3	5	2
Cast	0	0	0	0	2	5	4	6
Calcification	0	0	0	0	0	0	0	2
Nephropathy	0	0	0	0	0	0	1	0
Heart								
Inflammatory cell infiltrate, cardiomyocyte	0	0	0	0	0	0	1	0
Pancreas								
Focal infiltration, inflammatory cell	1	0	0	1	1	0	3	1
Salivary gland (submaxillary gland)								
Focal infiltration, inflammatory cell	9	8	9	7	8	7	7	6
Fibrosis and atrophy	0	0	0	1	0	0	0	1

[a]One mouse was excluded for analysis due to postmortem changes. [b]Significantly different from the female 0 ppm group. [c]Dead at week 40 or 45.

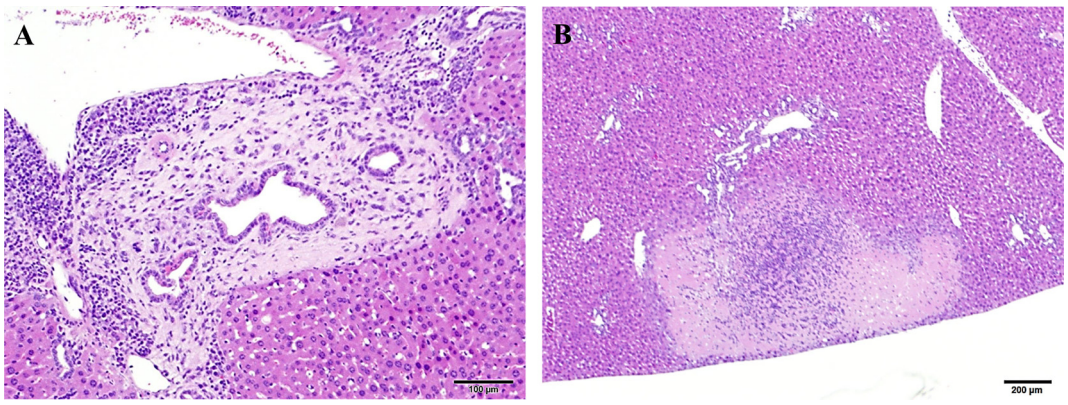

Fig. 2. Cholangitis (A) and focal necrosis of hepatocytes and bile duct hyperplasia (B) in the liver were observed in the dead female mice administered 25 ppm diphenylarsinic acid (DPAA) (HE staining). Bar = 100 µm (A) or 200 µm (B).

Table 5. Date for Overexpressed Proteins Related to Hepatotoxicity from Proteomic Comparison Analysis for Female Mice in the 25 ppm Diphenylarsinic Acid (DPAA) and Control Groups

Molecules	Gene name	GI number	Fold change	Location
Gstt1	glutathione S-transferase, theta 1	160298219	2.0	Cytoplasm
ALB	albumin	163310765	1.5	Extracellular space
RGN	regucalcin	6677739	1.4	Nucleus
GSTM5	glutathione S-transferase mu 5	6754084	1.4	Cytoplasm
Gsta4	glutathione S-transferase, alpha 4	160298217	1.4	Cytoplasm
GSTA3	glutathione S-transferase alpha 3	31981724	1.4	Cytoplasm
GSTZ1	glutathione S-transferase zeta 1	6754092	1.3	Cytoplasm
SLC25A5	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5	22094075	1.3	Cytoplasm
GNMT	glycine N-methyltransferase	6754026	1.3	Cytoplasm
CYP2E1	cytochrome P450 family 2 subfamily E member 1	11276065	1.3	Cytoplasm
FABP1	fatty acid binding protein 1	8393343	1.3	Cytoplasm
CAT	catalase	157951741	1.3	Cytoplasm
ALDH1A1	aldehyde dehydrogenase 1 family member A1	85861182	1.3	Cytoplasm
HSPD1	heat shock protein family D (Hsp60) member 1	183396771	1.3	Cytoplasm
POR	cytochrome p450 oxidoreductase	6679421	1.3	Cytoplasm
FAH	fumarylacetoacetate hydrolase	240120112	1.3	Cytoplasm
GPX1	glutathione peroxidase 1	84871986	1.3	Cytoplasm
ADK	adenosine kinase	339895909	1.2	Nucleus

ity Pathway Analysis data are summarized in Supplementary Table 2 (online only). The top 15 activated pathways are shown in Fig. 3, including glutathione-mediated detoxification.

Immunohistochemistry of CYP2E1 in the liver

One of the overexpressed proteins is the phase I metabolic enzyme CYP2E1, and five are phase II glutathione S-transferases. Since CYP enzymes are the enzymes primarily responsible for the metabolism of xenobiotics and since CYP2E1 is the only CYP enzyme upregulated in DPAA-treated mice, we confirmed its overexpression by immunostaining. As shown in Fig. 4, expression of CYP2E1 was increased in female mice in the 25 ppm group compared with the control group. In contrast, expression of CYP2E1 was not increased in male mice in the 25 ppm group compared with the control group (Supplementary Fig. 1: online only).

Discussion

This study evaluated the chronic toxicity of DPAA in mice. Our results demonstrate that DPAA is toxic to the bile duct epithelium and hepatocytes in female mice as evidenced by bile duct hyperplasia, cholangitis, and necrosis of hepatocytes. The no-observed-adverse-effect level for mice administered DPAA in their drinking water were 12.5 ppm for males and 6.25 ppm for females under the conditions of this study as evidenced by decreased ALB and increased liver and kidney weight in the 25 ppm male group, simple bile duct hyperplasia and cholangitis in the 12.5 and 25 ppm female groups, and necrosis of hepatocytes in the 25 ppm female group.

Similar to the findings in our previous rat chronic toxicity study[13], simple bile duct hyperplasia was observed in the female 25 ppm group. DPAA was not carcinogenic in male or female rats in our previous 104-week carcinogenicity study[14]; however, it induced bile duct hyperplasia, dilation of the common bile duct outside the papilla of Vater, and stenosis of the papilla of Vater in male and female rats in our rat chronic toxicity study[13]. Our currently ongoing 2-year carcinogenicity study in mice will clarify the potential carcinogenicity of DPAA in mice.

Metabolism of xenobiotics is generally divided into two phases[16]. In phase I, enzymes such as cytochrome P450 oxidases introduce reactive or polar groups into the xenobiotic molecules. These modified compounds are then conjugated to polar compounds such as glutathione in phase II reactions catalyzed by transferase enzymes such as glutathione S-transferases. It has been reported that glutathione conjugation plays important roles in the toxicity of DPAA[17–19]. DPAA-GS(III), a GSH adduct of DPAA, is highly toxic to human HepG2 cells, and the potency is about 1000 times that of DPAA. However, the phase I enzymes involved in the metabolism of DPAA remain unclear.

Proteomic comparison analysis of the livers of female mice administered 25 ppm DPAA and control mice revealed five glutathione S-transferases are overexpressed as a result of DPAA treatment. These results are in line with the findings of the role of DPAA-GS(III) in the hepatotoxicity of DPAA and implicate glutathione in the hepatotoxicity of DPAA. Importantly, CYP2E1 is the only CYP enzyme differentially overexpressed in female mice administered DPAA. Immunohistochemistry analysis confirmed the increased expression of CYP2E1 in the livers of female mice administered 25 ppm DPAA. These results suggest that CYP2E1 is the phase I metabolic enzyme involved in the hepatotoxicity of DPAA.

In addition to glutathione-mediated detoxification, Ingenuity Pathway Analysis predicted that multiple pathways were activated by DPAA. Fatty acid oxidation, LPS/

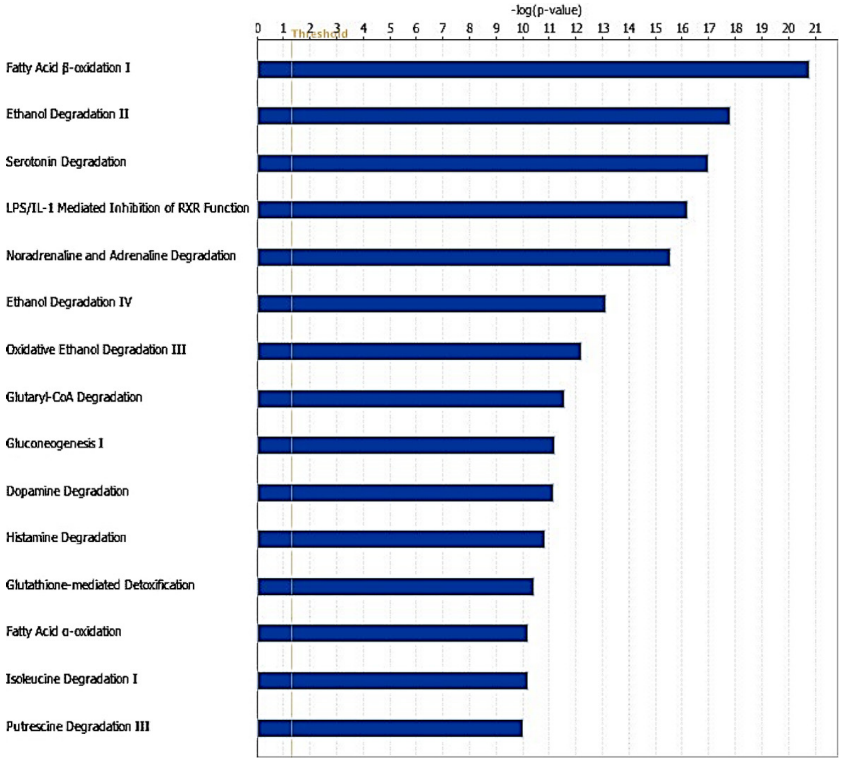

Fig. 3. The top 15 pathways activated in the livers of female mice administered 25 ppm diphenylarsinic acid (DPAA): predicted by Ingenuity Pathway Analysis.

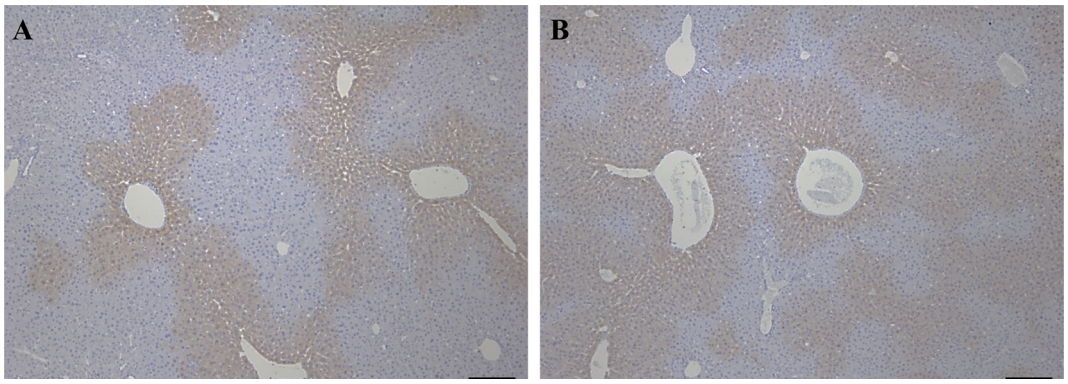

Fig. 4. Immunohistochemistry of CYP2E1 in the livers of female mice. A: a mouse in the control group. B: a mouse in the 25 ppm diphenylarsinic acid (DPAA) group. Bar = 200 μm.

IL-1-mediated inhibition of RXR function, and NRF2-mediated oxidative stress response pathways are involved in the hepatotoxicity of nitrotoluene compounds in rats[20], and gluconeogenesis and retinol biosynthesis pathways are involved in the hepatotoxicity of CCl_4 in rats[21]. Moreover, it was reported that liver disease can alter nutritional status and amino acid metabolism; therefore, isoleucine, tryptophan, histidine, valine, and aspartate degradation, and arginine biosynthesis pathways may be involved in the hepatotoxicity. This study suggests that multiple pathways may contribute to the hepatotoxicity of DPAA in mice.

In the female 25 ppm group, cholangitis and necrosis of hepatocytes were observed. Severe cholangitis and necrosis of hepatocytes were observed in dead mice. No significant change was observed in serum AST, ALT, or γ-GPT in surviving mice, possibly due to the fact that only mild cholangitis was observed.

In the male 25 ppm group, a significant decrease in ALB and a significant increase in absolute and relative kidney was observed; however, no histopathological changes were noted in the kidneys. A significant decrease in ALB indicates functional toxicity, and although the toxicological significance of an increase in absolute and relative kidney weight is unknown, the decrease in ALB suggests that these

changes are a consequence of DPAA toxicity.

In conclusion, the present study demonstrated that DPAA exhibits biliary and liver toxicity, and it suggests that CYP2E1 is involved in the metabolism and toxicity of DPAA in female mice. The no-observed-adverse-effect levels of DPAA were 12.5 ppm (1.6 mg/kg bw/day) for males and 6.25 ppm (1.1 mg/kg bw/day) for females under the conditions of this study.

Disclosure of Potential Conflict of Interest: The authors declare that there is no conflict of interest.

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