

SEMI-QUANTITATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF MALE RAT-SPECIFIC α_{2u} -GLOBULIN ACCUMULATION FOR CHEMICAL TOXICITY EVALUATION

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ABSTRACT — We purified male rat urinary α_{2u} -globulin, prepared the antibody in rabbits, and improved an immunohistochemical detection method using this antibody for male rat-specific α_{2u} -globulin accumulation appearing as hyaline droplets in the kidneys. Our prepared antibody reacted specifically with α_{2u} -globulin in both immunohistochemical and Western blotting analyses, furthermore, and the graded immuno-reactivities on the slide were well associated with computational image analyzing results. Using this method, we retrospectively analyzed the renal sections from the toxicity studies of 12 nephrotoxic chemicals, which had already been conducted under the Japanese Existing Chemicals Survey Program. We demonstrated that the hyaline droplets induced by treatment with 10 chemicals (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene and 3,5,5-trimethylhexan-1-ol) were directly associated with α_{2u} -globulin accumulation. This immunohistochemical method is convenient for applying, even retrospectively, paraffin sections from general toxicity studies and could be useful for qualifying male rat-specific hyaline droplets consisting of α_{2u} -globulin and renal risk in humans.

KEY WORDS: α_{2u} -globulin, Immunohistochemistry, Hyaline droplet, Nephrotoxicity

INTRODUCTION

For risk assessment of chemicals, the most critical data are derived from animal toxicity studies because of a general lack of information on humans. Although all available results from animal studies have been applied to human risk assessment, in principle, exclusion of some specific toxicities, which might not occur in humans, should be taken into account. Among laboratory animals, the rat has been commonly used for toxicity studies, especially sub-acute, long-term or carcinogenicity studies. Nephropathy with hyaline droplets and renal tubular neoplasia caused by chemicals inducing α_{2u} -globulin accumulation (CIGA) are con-

sidered to be a male rat-specific toxicity, not occurring in female rats or other animals, including primates. Although low molecular proteins homologous to α_{2u} -globulin can be detected in other species, including mice and humans, none of these proteins have been confirmed to bind to CIGA, followed by accumulation of the protein-CIGA complex as in the case of α_{2u} -globulin. It is therefore believed that renal toxicity induced by CIGA in male rats is unlikely to occur in humans (Hard *et al.*, 1993).

α_{2u} -Globulin was first identified in male rat urine (Roy and Neuhaus, 1966), and had been reported to be a male rat-specific protein with a molecular weight of 18 to 20 kDa. The major source of urinary α_{2u} -globulin

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is the liver, where α_{2u} -globulin mRNA constitutes approximately 1% of the total hepatic mRNA (Sippel *et al.*, 1976; Kurtz and Feigelson, 1977). Neither α_{2u} -globulin nor its mRNA is detectable in the female liver (Sippel *et al.*, 1975, 1976; MacInnes *et al.*, 1986). The blood α_{2u} -globulin secreted from the liver is freely filtered through the glomerulus, and in mature rats, about two-thirds of the filtered protein is reabsorbed by tubules and the remainder is excreted through the urine (Neuhaus *et al.*, 1981). CIGA binds noncovalently to α_{2u} -globulin, and the resulting complex shows less degradability with proteolytic enzymes, causing an accumulation of the complex that is detectable as hyaline droplets with a light microscope. Various chemicals have been suspected of being CIGA based on detection of the evidence for exacerbation of hyaline droplets in renal proximal tubules in male rats, though not in females. Direct evidence for increasing α_{2u} -globulin levels has been demonstrated for only a few of these chemicals, however, including 2,2,4-trimethylpentane (Stonard *et al.*, 1986; Charbonneau *et al.*, 1987; Lock *et al.*, 1987), decalin (Kanerva *et al.*, 1987), d-limonene (Lehman-McKeeman *et al.*, 1989; Webb *et al.*, 1989), 1,4-dichlorobenzene (Charbonneau *et al.*, 1989), isophorone (Strasser *et al.*, 1988), lindane (Dietrich and Swenberg, 1990), tri- or per-chloroethylene and pentachloroethane (Goldsworthy *et al.*, 1888).

A number of initial safety assessments has so far been conducted for industrial chemicals, including both new and existing chemicals by the Japanese government or the OECD high production volume chemicals programs. Certain chemicals among these industrial chemicals have been suspected of being CIGA. In some cases, however, renal changes in male rats have been assessed as the endpoint for extrapolation to human health risk owing to a lack of direct evidence caused by α_{2u} -globulin accumulation, because no antibody against α_{2u} -globulin is commercially available for general toxicity studies. Some immunohistochemical α_{2u} -globulin analysis methods had already been developed (Burnett *et al.*, 1989; Hashimoto and Takaya, 1992; Caldwell *et al.*, 1999). As these methods required glycolmethacrylate embedding or specific computational analysis, they would be inappropriate for confirming α_{2u} -globulin accumulation in routinely conducted guideline-based toxicity studies. We therefore improved an immunohistochemical α_{2u} -globulin detection system using paraffin sections, which are generally used for standard toxicity studies. We evaluated the several chemicals suspected of being CIGA, moreover, and indicated the direct evidence caused by

α_{2u} -globulin accumulation.

MATERIALS AND METHODS

Preparation of anti α_{2u} -globulin antibody

α_{2u} -globulin as an antigen was obtained from the urine collected from aged male rats, pooled, and used to immunize rabbits. The immunization procedures, including the amount of antigen and immunizing intervals, were determined from the results of a preliminary test referring to the methods of Kurtz *et al.* (1976). The antigen was injected under the skin at a dose of 1 mg/animal (1st injection) or 0.5 mg/animal (2nd and subsequent injections) once at two weeks. Blood sampling was conducted periodically and the antibody titer measured. When the antibody titer level reached a plateau, whole blood was collected and antiserum was obtained from the blood. The antiserum was used for immunohistochemistry and immuno-electron microscopy. For measurement of the α_{2u} -globulin content in the urine and tissues, the antibody was purified from the antiserum using a DEAE ionic exchange column after ammonium sulfate precipitation. The singularity of the antibody was confirmed as a single diffuse band of approximately 19 kDa by Western blotting analysis. This study and the following study were carried out in accordance with the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management, etc. of Experimental Animals in Japan.

Experiment 1 Confirmation of specific reactivity of the antibody to α_{2u} -globulin

1. Preparation of α_{2u} -globulin nephropathy rats

To confirm the specific reactivity of the anti- α_{2u} -globulin antibody, we prepared α_{2u} -globulin nephropathy rats as follows. Male and female Crj:CD(SD)IGS rats were obtained from Charles River Japan Inc. and used at the age of 11 weeks. d-Limonene (Nacalai Tesque Inc.), a well-known α_{2u} -globulin nephropathy inducer, was administered to the rats, consisting of 4 males and 4 females each, for 10 days at doses of 0, 150 and 300 mg/kg/day by gavage using corn oil as a vehicle. The rats were housed individually in stainless steel wire cages in an animal room with a controlled temperature of $24 \pm 2^\circ\text{C}$, humidity of $55 \pm 10\%$ and a 12-hr light/dark cycle (lighting from 7:00 to 19:00) and allowed access to food and water ad libitum.

Pooled urine was collected for 24 hr on the day before the start of administration and on Day 9 of administration. After the 10-day administration period,

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the rats were anesthetized with intraperitoneal injection of 30 mg/kg of sodium pentobarbital and perfused with physiological saline-added lactose (Lactec, Otsuka Pharmaceutical Factory Inc.) through the sinus aortae, after which the liver and kidneys were removed. The urine and a part of the liver and kidneys were used for measurement of their α_{2u} -globulin content and the remainder of the liver and kidneys for histopathology, immunohistochemistry and immuno-electron microscopy. The samples for histopathology and immunohistochemistry were embedded in paraffin following fixation with 10% neutral buffered formalin solution for about two weeks. The samples for immuno-electron microscopy were dehydrated with an ascending series of ethanol and embedded in spurr resin following pre- and post-fixation with 2.5% glutaraldehyde and 1% osmium tetroxide solutions, respectively.

2. Histopathology and immunohistochemistry

The serial paraffin sections were prepared, deparaffinized and then stained with hematoxylin and eosin (HE) accompanied by Azan-Mallory staining and periodic acid Schiff (PAS) reaction.

For immunohistochemistry, the paraffin sections were deparaffinized and incubated with 0.25% pronase E for 20 min at 37°C, after which they were washed 3 times in Tween-PBS (PBS containing 0.1% Tween 20, pH 7.6). The specimens were incubated with 0.3% H_2O_2 in methanol at room temperature for 30 min to inactivate the endogenous peroxidase activity, and then washed 3 times in Tween-PBS. After blocking against nonspecific immuno-reactions with 10% FCA was conducted at room temperature for 20 min, the sections were incubated overnight with rabbit anti- α_{2u} -globulin antiserum at 4°C at a dilution of 1:80000 in PBS containing 1% BSA. Negative controls were incubated with an equivalent volume of diluent solution alone. The sections were washed 3 times in Tween-PBS and incubated with biotinylated secondary antibody (goat anti-rabbit and goat anti-mouse immunoglobulins, Dako, LSAB2 kit) at room temperature for 30 min. After they were washed 3 times in Tween-PBS, the sections were incubated with horseradish peroxidase (HRP)-labelled streptavidin (Dako, LSAB2 kit) at room temperature for 30 min. The sections were then washed 3 times in PBS and reacted with 3,3'-diaminobenzidine (DAB) for 5 min. The reactions were quenched by placement in running tap water, and the sections were then counterstained lightly with methylgreen, dehydrated in n-butanol, cleaned in xylene, and mounted.

3. Immuno-electron microscopy

Ultra-thin sections were prepared and reacted overnight with the anti- α_{2u} -globulin antiserum at a dilution of 1:5000 at 4°C. Protein A-colloidal Gold (10 nm, British Bio Cell International Inc.) was used at a dilution of 1:10, after which the sections were double stained with uranyl acetate and lead citrate.

4. Measurement of α_{2u} -globulin content in the liver, kidneys and urine

The α_{2u} -globulin content was measured in the liver and kidneys in all males in all the groups of α_{2u} -globulin nephropathy rats, and in the urine in two males each in the control and highest dose groups. The liver and kidneys were homogenized with phosphate buffer weighing 4 times their tissue weights and centrifuged at 105,000 g for one hour. The protein content of the supernatant thus obtained was measured for every molecular weight and the urine was measured similarly as is. Western blotting was then conducted using purified anti- α_{2u} -globulin antibody and the content of the protein showing a positive reaction was regarded as α_{2u} -globulin content.

Experiment 2 α_{2u} -globulin analysis for industrial chemicals

The selected chemicals are listed in Table 1. We selected 10 chemicals, which are suspected of being CIGA, among all the chemicals in the Japanese Existing Chemicals Survey Program (JECSP). In addition, two chemicals which caused renal toxicity without hyaline droplet accumulation were selected as negative controls. We used paraffin-embedded renal specimens originating from the JECSP toxicity studies conducted in several laboratories and stored for four to seven years in each. For each toxicity study, three groups (the control and low- and high-dose groups for 11 chemicals) or two groups (the control and high-dose groups for the other) were selected. The low-dose group has the dose showing the lowest effect for hyaline droplets in tubules or other renal changes, and the high-dose group has the highest dose administered in each toxicity study. The doses selected for each chemical are described in Table 1. Three male specimens were arbitrarily selected for each dose group based on the results obtained from HE-stained sections in the original studies.

The serial paraffin sections were prepared, deparaffinized and then stained with HE accompanied by Azan-Mallory staining and PAS reaction. The sections were also stained immunohistochemically using anti-

Table 1. Chemical name and effect dose derived from the general toxicity studies.

Chemical	Test type	Original study doses (mg/kg/day)	Effect doses (mg/kg/day) ^{a)}			Original reported NOEL (mg/kg/day) ^{a)}	The selected doses for analyzing (contr./low/high) (mg/kg/day)
			AN	Other	Non histopathological observations		
1,4-Dibromobenzene	RD	0/ 4/ 20/100/500	20≤ / -	100≤	100≤ / 20≤	0/ 20/500	
Dicyclopentadiene	RT	0/ 4/ 20/100	4≤ / -	20≤ / 100	20≤ / 100	0/ 4/100	
3,4-Dimethylaniline	RD	0/10/ 50/250	50≤ / -	250	250 / 50≤	0/ 50/250	
1,4-Dicyanobenzene	RD	0/ 1.25/ 5/ 20/ 80	5≤ / -	20≤ / -	20≤	0/ 5/ 80	
Tetrahydrothiophene-1,1- dioxide	RD	0/60/ 200/700	200≤ / -	-	700	0/200/700	
1,3-Dicyanobenzene	RD	0/ 8/ 40/200	8≤ / -	40≤ / 200	40≤	0/ 8/200	
Acenaphthene	RD	0/12/ 60/300	60≤ / -	300	300 / 60≤	0/ 60/300	
3,4-Dichloro-1-butene	RT	0/ 0.4/ 2/10/ 50	10≤ / -	50	10≤ / 50	0/ 10/ 50	
3a,4,7a-Tetrahydro-1H- indene	RT	0/ 67/200/600	67≤ / -	600	67≤ / 200≤	0/ 67/600	
3,5,5-Trimethylhexan-1-ol	RT	0/ 12/ 60/300	12≤ / -	60≤	60≤	0/ 12/300	
2,4-di- <i>tert</i> -butylphenol	RD	0/ 5/ 20/ 75/300	- / -	300	300 / 75≤	0/ - /300	
4-aminophenol	RD	0/ 4/ 20/100/500	- / -	100≤	100≤	0/100/500	

^{a)} The data were described in a pattern of male/female when the data were different between the male and female.
RD, 28-day Repeat Dose Toxicity Test; RT, Combined Repeat Dose and Reproductive/Developmental Toxicity Test.
AN, α_2 -globulin nephropathy including hyaline droplets and subsequent tubular alteration.

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α_{2u} -globulin antiserum by the above-mentioned protocol. HE-stained sections were used to examine the degree of hyaline droplets and to determine whether or not other findings were present. The degree of occurrence of hyaline droplets was divided into five grades, including none (-), minimal (\pm , barely detectable minimal appearance), slight (+, multifocal but not dispersed appearance), moderate (++, dispersed appearance over the cortex) and severe (+++, diffused appearance over the whole cortex). The staining sections with PAS, Azan-Mallory and anti- α_{2u} -globulin reaction were also graded similarly for positive-stained droplets. In addition, computational image analysis was carried out to verify the above-mentioned grading criteria using three typical immuno-stained samples for each grade. Images including almost all the renal superficial cortex were captured using a light microscope (Olympus BHS) and a digital camera (Olympus DP12). The captured images were measured for positive area using an image analyzing system (C-Imaging System, Compix Inc.), and the positive area (%) was then calculated from the data.

RESULTS

Experiment 1 Specific reactivity of the antibody to α_{2u} -globulin

On the HE-stained sections of the kidneys, hyaline droplets with round to irregular shapes were observed in the renal proximal tubular epithelium only in males administered d-limonene (Photo. 1a). The hyaline droplets were negative for PAS reaction (Photo 1b) but stained positively with Azan-Mallory staining (Photo 1c). With immuno-staining with the anti- α_{2u} -globulin antibody, the hyaline droplets were more clearly stained and more distinguishable than with Azan-Mallory staining (Photo 1d). The hyaline droplets showed a dose-dependent increase on the HE-stained sections (Photo 2, a-c) and positive reactions for hyaline droplets showed a correlational increase with immuno-staining (Photo 2, d-f). Very fine positive granules were also detected on the immuno-stained sections for all the males as background, but no positive reactions were observed in other tissue components. This background was observed generally in male kidneys and was, therefore, excluded from the grading in experiment 2. In the liver, all the males showed a positive reaction for the antibody in centrilobular hepatocytes. The degree of intensity was weaker than in the kidneys, and there was no clear intensification by d-limonene. No positive reaction for

the anti- α_{2u} -globulin antibody was detected in the liver or kidneys in any females.

With electron microscopy, electron-dense and irregular-shaped inclusions surrounded by a single membrane were observed as changes corresponding to the hyaline droplets in the renal proximal tubular epithelium, and positive reactions were observed for the antibody with post-embedding method in the inclusions (Photo 3). A similar positive reaction was observed in the lysosomes of the renal tubule epithelium, but no positive reaction was detected in the hepatocytes.

The α_{2u} -globulin content in the kidneys of the males was increased dose-dependently by administration with d-limonene (Fig. 1). A dose-dependent but mild increase in α_{2u} -globulin content was also observed in the liver of the males. While no dose-dependent increase in the urine was noticeable, a lower molecular type of α_{2u} -globulin appeared in the males in the highest dose group, with the α_{2u} -globulin type reported as an early marker for α_{2u} -globulin nephropathy (Saito *et al.* 1991).

Experiment 2 α_{2u} -globulin analysis for industrial chemicals

Table 2 indicates the grades of all the samples with respect to hyaline droplets, positive droplets and immunological positive droplets analyzed with HE, Azan-Mallory and anti- α_{2u} -globulin antibody staining, respectively. In the controls there was a minimal to moderate amount of hyaline droplets in some animals and consequent variation for Azan-Mallory and anti- α_{2u} -globulin reaction. This variation was due to the arbitrary sampling of specimens, or probably related to the lot of the animals or to the difference of food used in each study. Dose-dependent increases of hyaline droplets in the renal proximal tubular epithelium were, however, confirmed for HE-staining of 10 chemicals suspected of being CIGA (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene, 3,5,5-trimethylhexan-1-ol). This was described in the original reports (Toxicity Testing Reports of Industrial Chemicals), although the occurrence of hyaline droplets varied in shape, size and number/cell with chemicals and showed no clear common features. In the highest dose groups of these chemicals, basophilic tubules, granular casts in the tubules and/or tubular dilatation were intensified or occurred as in the original reports. These changes

showed similar features in spite of the various severity and incidence with the chemicals. In serial sections prepared simultaneously, Azan-Mallory-positive reactions for hyaline droplets were detected dose-dependently in these 10 chemicals. No PAS-positive reaction was detected in any chemical. These staining behaviors of the hyaline droplets were the same as those in the case of d-limonene described above. Immunohistochemical staining using the anti- α_{2u} -globulin antibody revealed thoroughly dose-dependent positive reactions for hyaline droplets in all these chemicals. The resulting grades from three types of analysis were the same, demonstrating that a highly positive correlation exists among the three staining methods. As for the remainder not suspected of being CIGA (2,4-di-tert-butylphenol, 4-aminophenol), there was no increase of hyaline droplets or positive immunohis-

tochemical reactions in any dose groups, as well as no stain in either PAS or Azan-Mallory staining. In addition, computational image analysis using three typical immuno-stained sections for each grade (Photo 4) showed a close correlation between the quantitative analysis and semi-quantitative grading (Fig. 2).

DISCUSSION

Many toxicity studies using laboratory animals have been conducted on environmental and industrial chemicals to ensure their safety or toxicity levels concerning human health. On extrapolating the results to humans, toxic mechanisms that are unlikely to occur in humans should be taken into account. A typical example of such toxicities is α_{2u} -globulin-related nephropathy and the consequent renal tumorigenesis in repeated

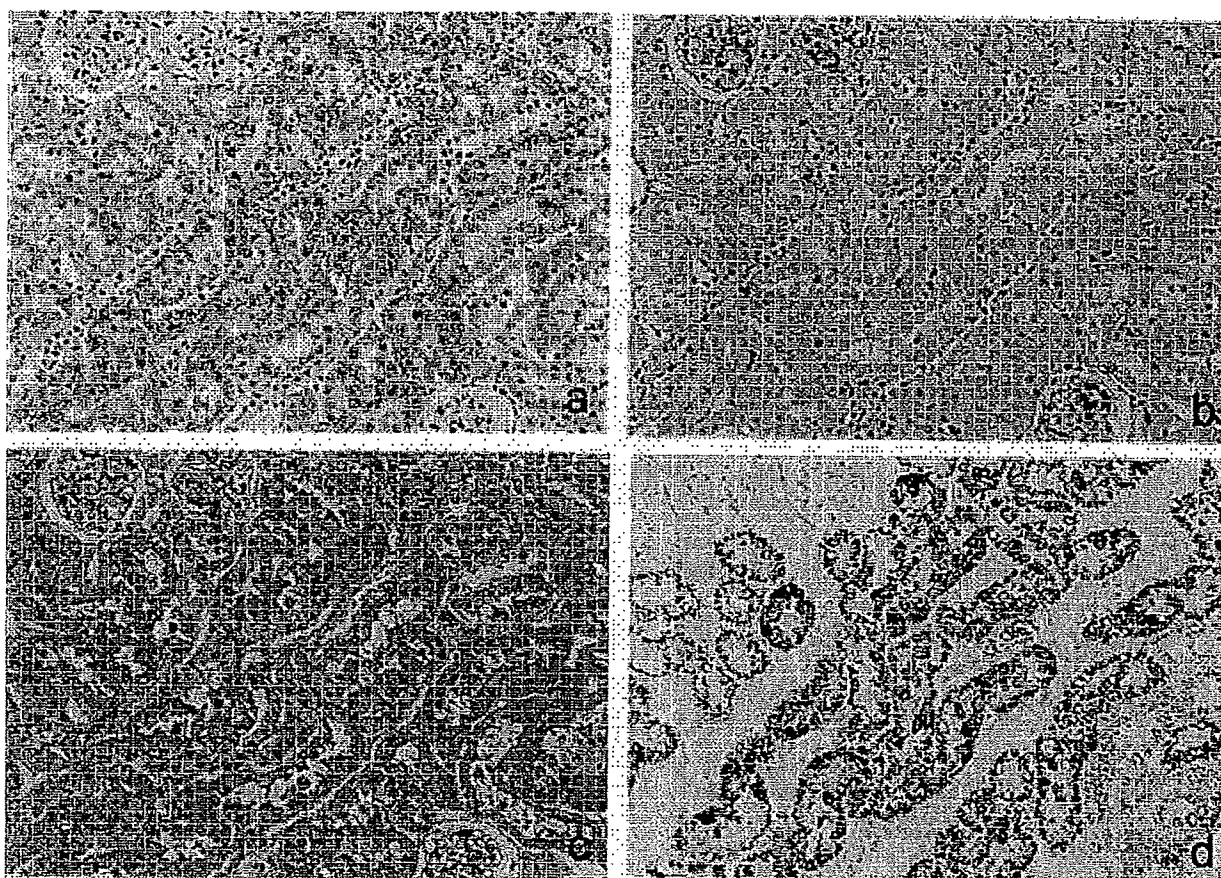


Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti- α_{2u} -globulin antibody showed a clear positive reaction consistent with the hyaline droplets (d). Original magnification, $\times 66$.

Semi-quantitative immunohistochemical analysis of male rat-specific α_{2u} -globulin accumulation.

dose toxicity studies using male rats. This male rat-specific nephrotoxicity is not considered to occur in humans (Hard *et al.*, 1993). To exclude this male rat-specific toxicity from chemical risk assessment, it is necessary to demonstrate properly that such renal tox-

icity results from α_{2u} -globulin-CIGA complex accumulation. Detection analysis of α_{2u} -globulin in the nephrotoxicity has not been conducted in most conventional toxicity studies, however, especially in sub-acute toxicity screening studies for industrial chemicals. As

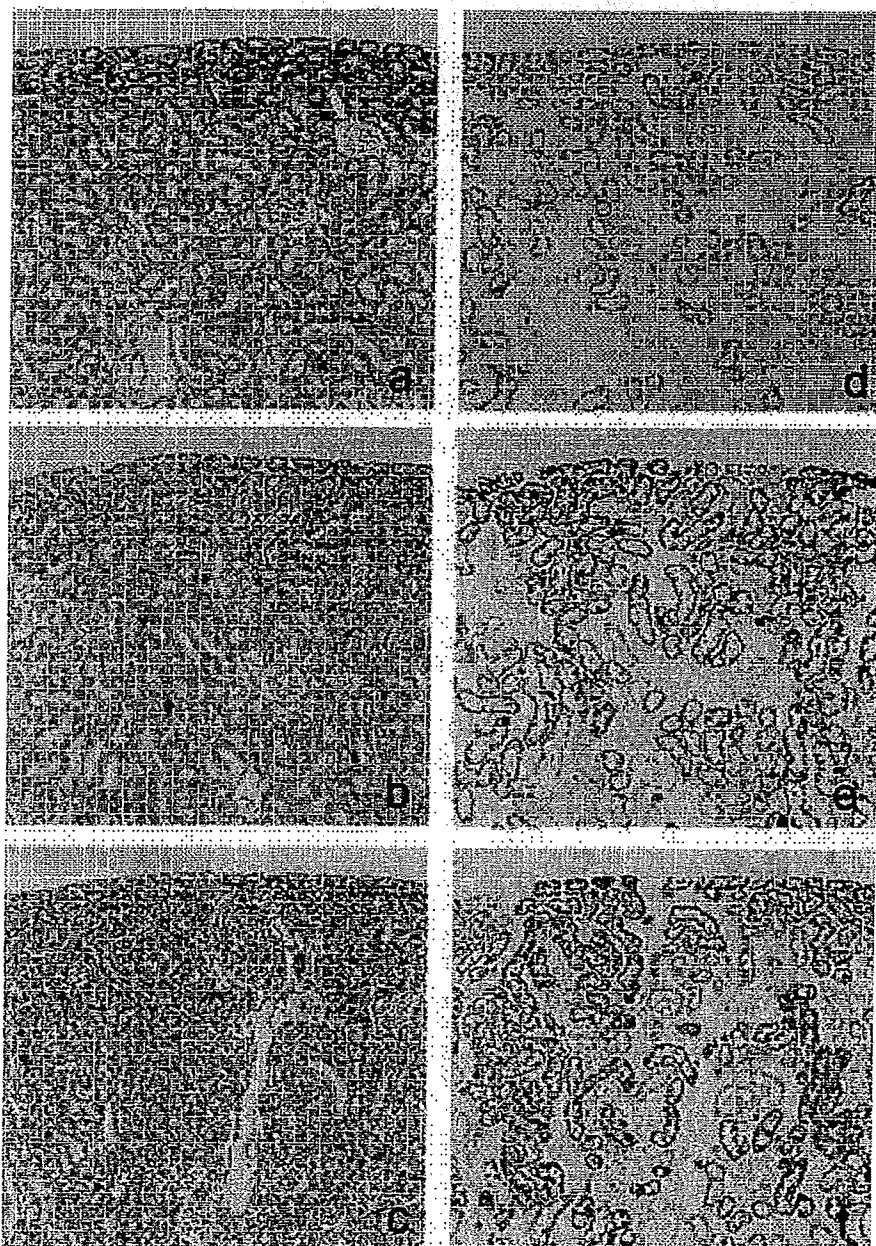


Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of *d*-limonene(HE, a - c). Positive reaction for the anti- α_{2u} -globulin antibody also increased with similar dose dependency (d - f). Original magnification, $\times 33$.

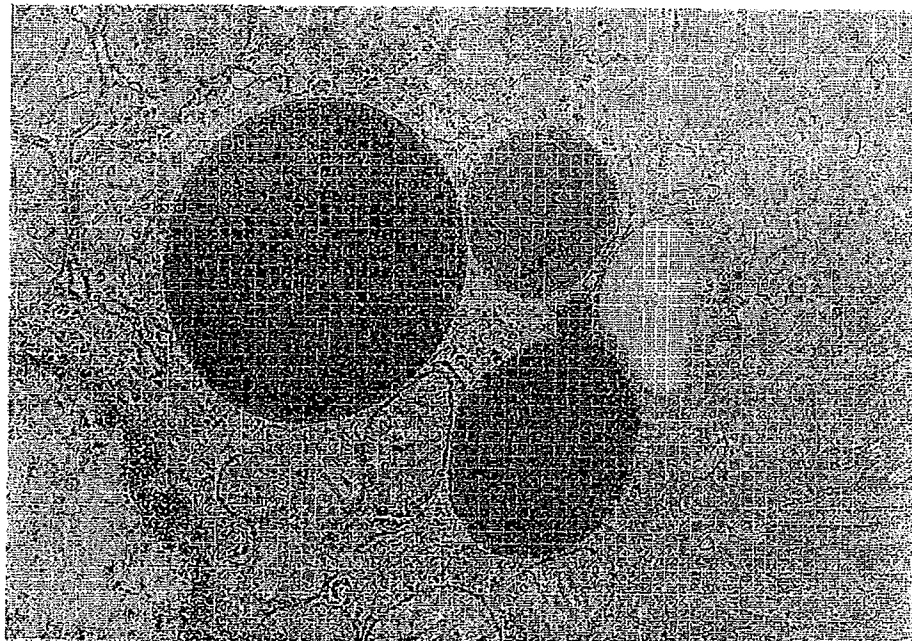


Photo 3. Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, $\times 10,000$.

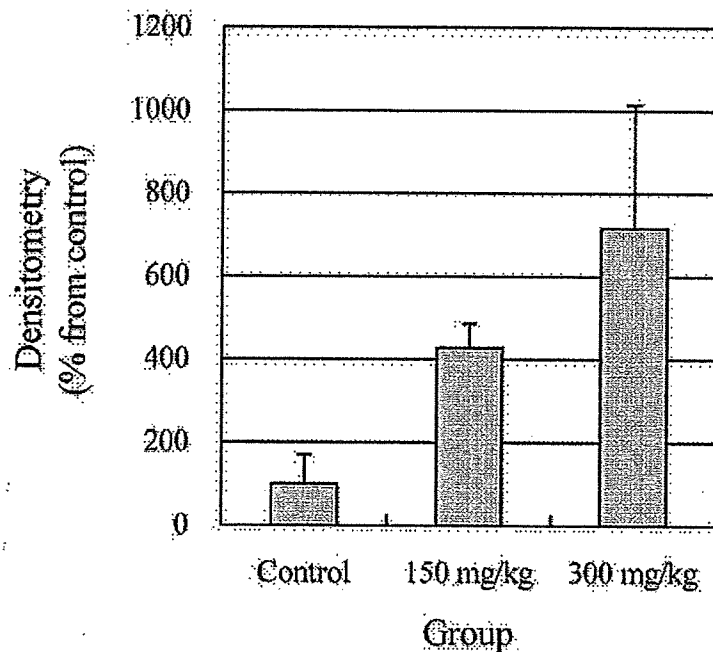


Fig. 1. Western blot analysis of α_{2u} -globulin in kidney from male rats treated with *d*-limonene. Results are expressed as mean \pm SD (n=4).

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an alternative detection method, it is well known that α_{2u} -globulin droplets in the kidneys are negative for PAS reaction, but that they are stained positively by Azan-Mallory staining (U.S. EPA, 1991; Alden *et al.*, 1984). Although these additional stainings can distin-

guish hyaline droplets resulting from α_{2u} -globulin accumulation from those resulting from other causes, these analyses provide only indirect evidence. Direct evidence of α_{2u} -globulin accumulation in renal hyaline droplets could be required for appropriate risk assess-

Table 2. Grading results of histological/histochemical examination.

Chemical	Staining	Results		
		Control	Low dose	High dose
1,4-Dibromobenzene	HE ¹⁾	-/-±	+/+/+	+/+/+/+
	Azan-Mallory ²⁾	-/-±	+/+/+	+/+/+/+
	Anti- α_{2u} -globulin ²⁾	-/-±	+/+/+	+/+/+/+
Dicyclopentadiene	HE	-/-/-	+/+/+/+	+/+/+/+/+
	Azan-Mallory	-/-/-	+/+/+/+	+/+/+/+/+
	Anti- α_{2u} -globulin	-/-/-	+/+/+/+	+/+/+/+/+
3,4-Dimethylaniline	HE	-/-/-	-/-±	±/±/+
	Azan-Mallory	-/-/-	-/-±	±/±/+
	Anti- α_{2u} -globulin	-/-/-	-/-±	±/±/+
1,4-Dicyanobenzene	HE	-/-/-	±/+/+	+/+/+/+/+
	Azan-Mallory	-/-/-	±/+/+	+/+/+/+/+
	Anti- α_{2u} -globulin	-/-/-	±/+/+	+/+/+/+/+
Tetrahydrothiophene-1,1-dioxide	HE	+/-/-	+/+/+	+/+/+/+
	Azan-Mallory	+/-/-	+/+/+/+	+/+/+/+
	Anti- α_{2u} -globulin	+/-/-	+/+/+/+	+/+/+/+
1,3-Dicyanobenzene	HE	-/-±	+/±/±	+/+/+/+/+
	Azan-Mallory	-/±/±	+/±/±	+/+/+/+/+
	Anti- α_{2u} -globulin	-/±/±	+/±/±	+/+/+/+/+
Acenaphthene	HE	±/-/+	+/-/+	+/+/+
	Azan-Mallory	±/-/+	+/±/+	+/+/+
	Anti- α_{2u} -globulin	±/-/+	+/±/+	+/+/+
3,4-Dichloro-1-butene	HE	-/-/++	+/+/±	+/+/+/+
	Azan-Mallory	-/-/++	+/+/+	+/+/+/+
	Anti- α_{2u} -globulin	-/-/++	+/+/+	+/+/+/+
3a,4,7,7a-Tetrahydro-1H-indene	HE	+/+/+	+/+/+/+	+/+/+/+/+
	Azan-Mallory	+/+/+	+/+/+/+	+/+/+/+/+
	Anti- α_{2u} -globulin	+/+/+	+/+/+/+	+/+/+/+/+
3,5,5-Trimethylhexan-1-ol	HE	-/-±	+/+/+	+/+/+/+/+
	Azan-Mallory	±/-±	+/+/+	+/+/+/+/+
	Anti- α_{2u} -globulin	±/-±	+/+/+	+/+/+/+/+
2,4-Di- <i>tert</i> -butylphenol	HE	-/-/-	-/-/-	-/-/-
	Azan-Mallory	-/-/-	-/-/-	-/-/-
	Anti- α_{2u} -globulin	-/-/-	-/-/-	-/-/-
4-Aminophenol	HE	-/±/-	-/-/-	-/-/-
	Azan-Mallory	-/±/-	-/-/-	-/-/-
	Anti- α_{2u} -globulin	-/±/-	-/-/-	-/-/-

¹⁾ Grading for hyaline droplets.

²⁾ Grading for positive droplets.

No PAS-positive reaction for the hyaline droplets was observed in any sample.

Low dose for 2,4-di-*tert*-butylphenol was not examined.

ment, and a reliable detection method for the existence of α_{2u} -globulin is therefore necessary.

Using both immunochemical staining for paraffin-embedded sections and the immuno-electron microscopy technique, we demonstrated that our prepared antibody reacted specifically to α_{2u} -globulin in renal hyaline droplets in the male rats administered d-limonene, a well-known α_{2u} -globulin nephropathy inducer. The dose-dependent positive immuno-reaction of the antibody in both the tissue sections and the homogenates from d-limonene-treated rat kidneys indicated that the antibody could be applicable for semi-quantitative analysis. In addition, computational image analysis revealed that classical visual microscopic grading was also useful for semi-quantitative analysis of α_{2u} -globulin accumulation.

Although immunohistochemical α_{2u} -globulin analysis of the glycolmethacrylate-embedded sections

had already been reported by Burnett *et al.* (1989), our method was advantageous from the standpoint of applicability to the paraffin-embedded sections. The paraffin-embedded specimens were usually prepared and stored for the general toxicity studies. In fact, all the sections used in experiment 2 in this study originated from study specimens which were prepared in the Japanese Existing Chemicals Survey Program conducted previously and stored for a long time. It indicated that our method is applicable to specimens derived directly from ordinary toxicology studies retrospectively. Hashimoto and Takaya (1992) previously investigated the application of α_{2u} -globulin immunostaining to paraffin sections by modifying the protocol of Burnett *et al.* (1989). The protocol includes pronase E treatment owing to enhancement of the antigen reactivity and removal of the non-specific reaction. Our method also includes the pronase E treatment, but

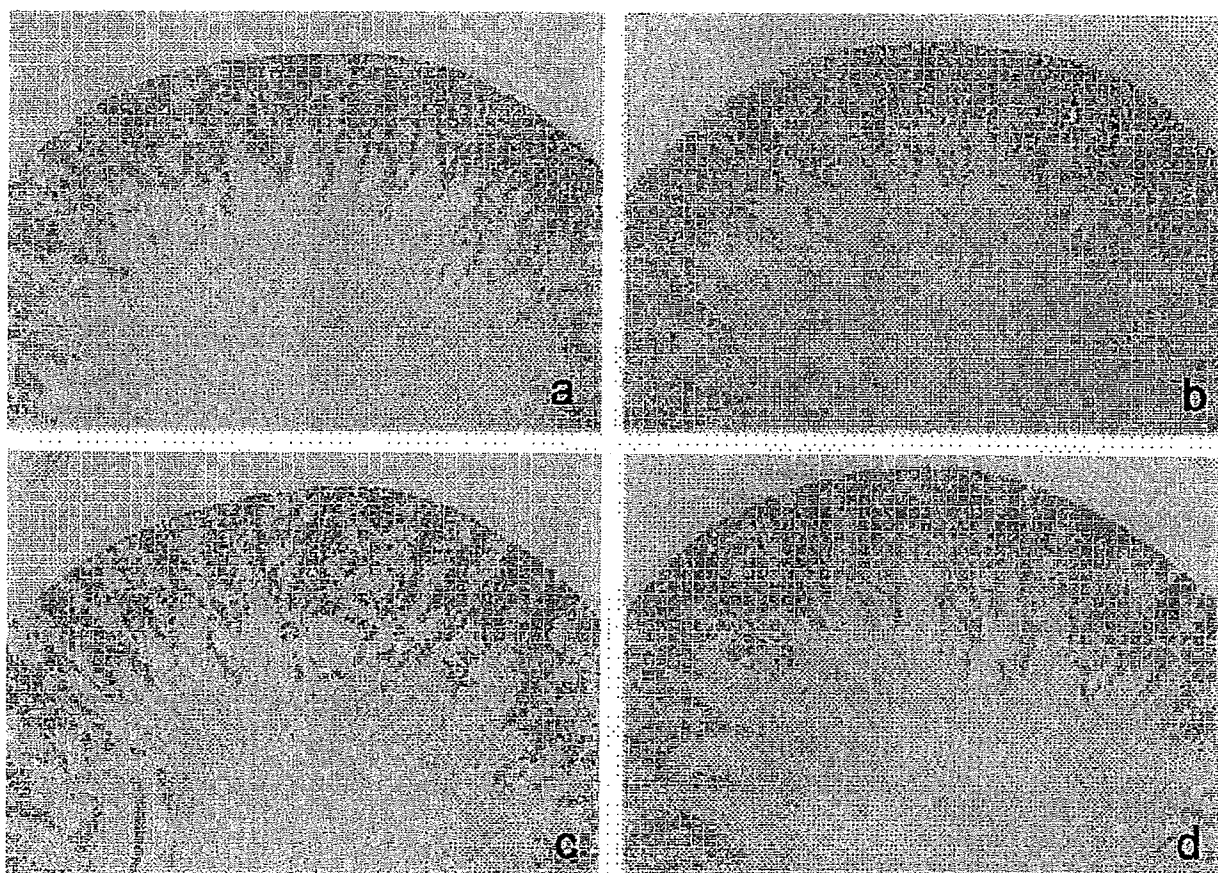


Photo 4. Immunohistochemical features of the anti- α_{2u} -globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, $\times 5$.

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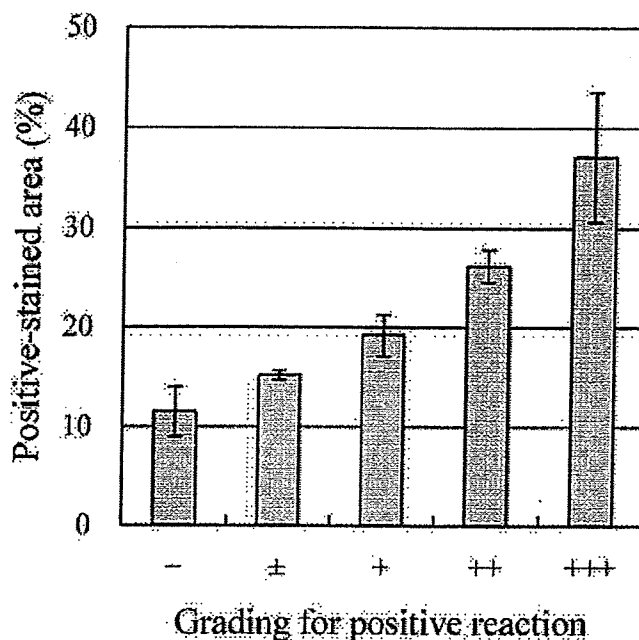


Fig. 2. Correlation between semi-quantitative and quantitative analyses for immuno-stained sections. Results are expressed as mean \pm SD (n=3).

the treatment is performed only in order to enhance the antigen activity and not to remove the non-specific reaction. This may suggest that our prepared antibody has a high specificity for α_{2u} -globulin. Caldwell *et al.* (1999) had conducted a similar quantitative immunohistochemical α_{2u} -globulin analysis, but it seems that the actual analyzed area was limited to narrower fields than in our study.

Urinary immunochemical analysis for detection of α_{2u} -globulin accumulation in male rat kidneys has been developed by Saito *et al.* (1996). Although the convenient urinary analysis is sufficient for detecting CIGA, the detectability is weaker than with kidney soluble protein analysis. The aim of the present analysis is not only to detect CIGA, but also to exclude the α_{2u} -globulin-induced nephrotoxic effects from risk assessment of chemicals. For 10 chemicals suspected of being CIGA, the occurrence of hyaline droplets in the kidneys with treatment was the lowest endpoint. In the process of evaluating chemical toxicity, if the most sensitive nephrotoxicity is concluded to be a neglected effect for human health, the NOAEL could be set based on other kinds of toxicological effects.

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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
<i>Body weight gain during pregnancy (g)^a</i>				
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**
<i>Food consumption during pregnancy (g)^a</i>				
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**
<i>Water consumption during pregnancy (ml)^a</i>				
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
<i>Body weight of live fetuses (g)^a</i>				
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**
<i>Fetal crown-rump length (mm)^a</i>				
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
<i>Placental weight (g)</i>				
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 toxic-

ity, 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
<i>External examination</i>				
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
<i>Skeletal examination</i>				
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**
<i>Internal examination</i>				
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, sternebra 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 pre-mating 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*-methyilmorpholine (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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