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 (±, 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-limonen 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 immunohistochemical 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

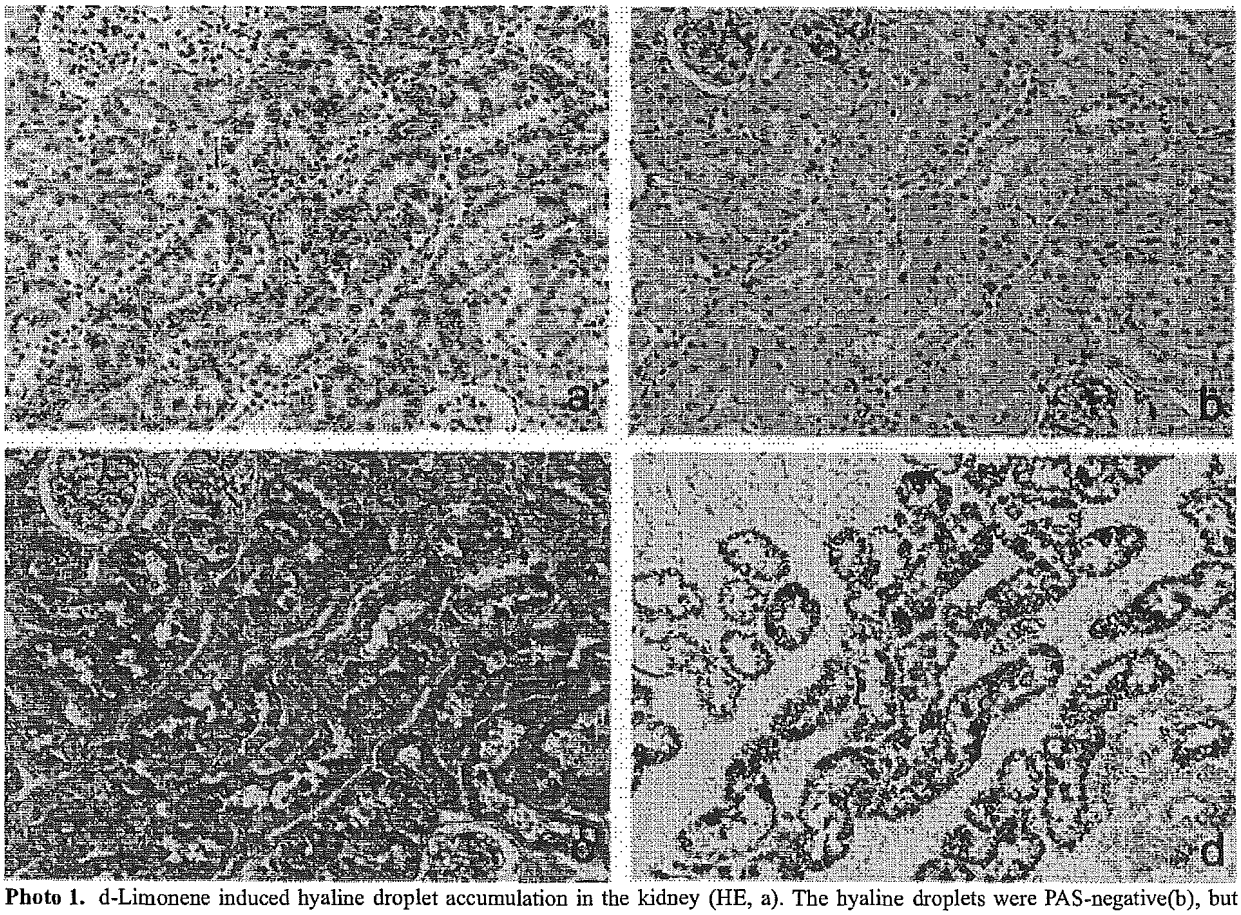

Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti-α_{2u}-globulin antibody showed a clear positive reaction consistent with the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

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 toxicity 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

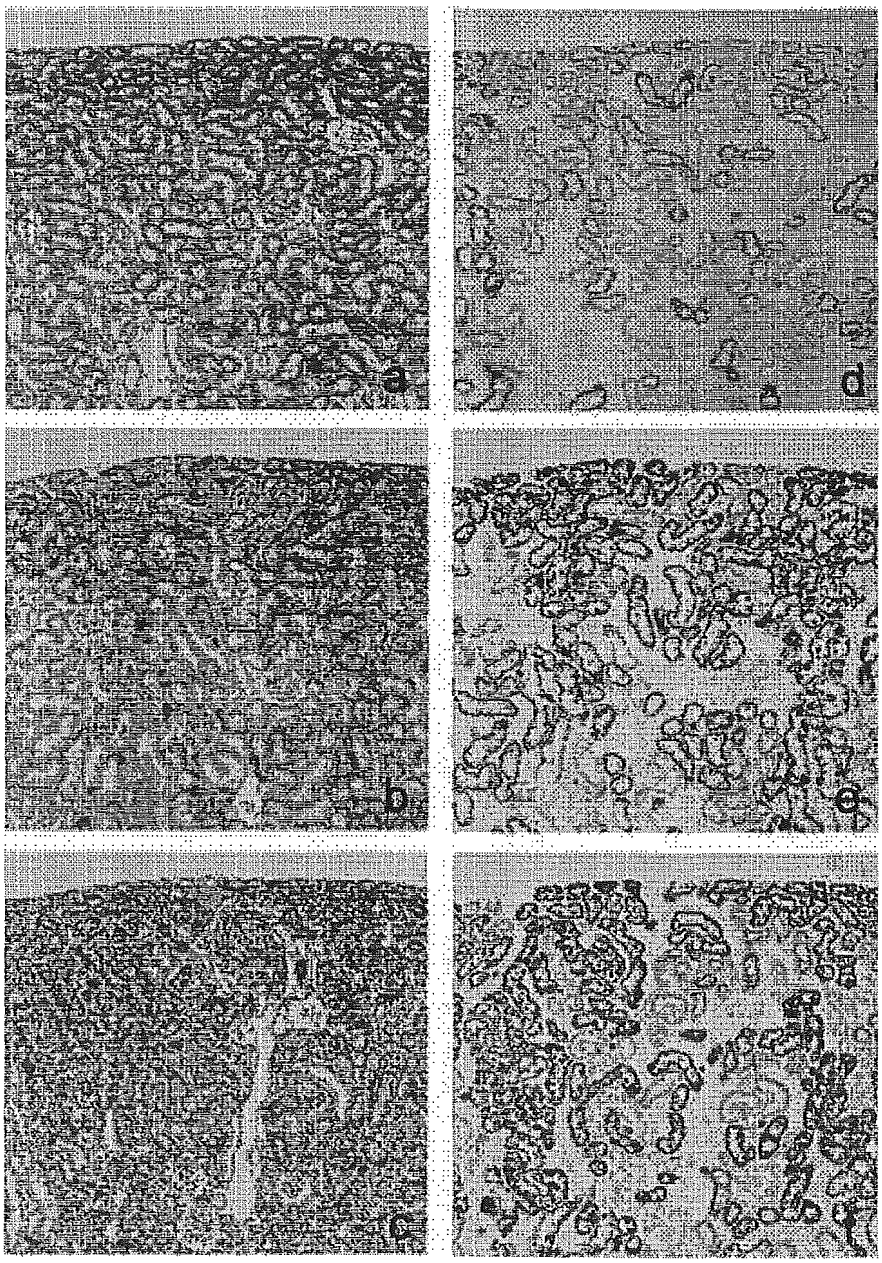

Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of *d*-limonene(HE, a - c). Positive reaction for the anti-α_{2u}-globulin antibody also increased with similar dose dependency (d - f). Original magnification, ×33.

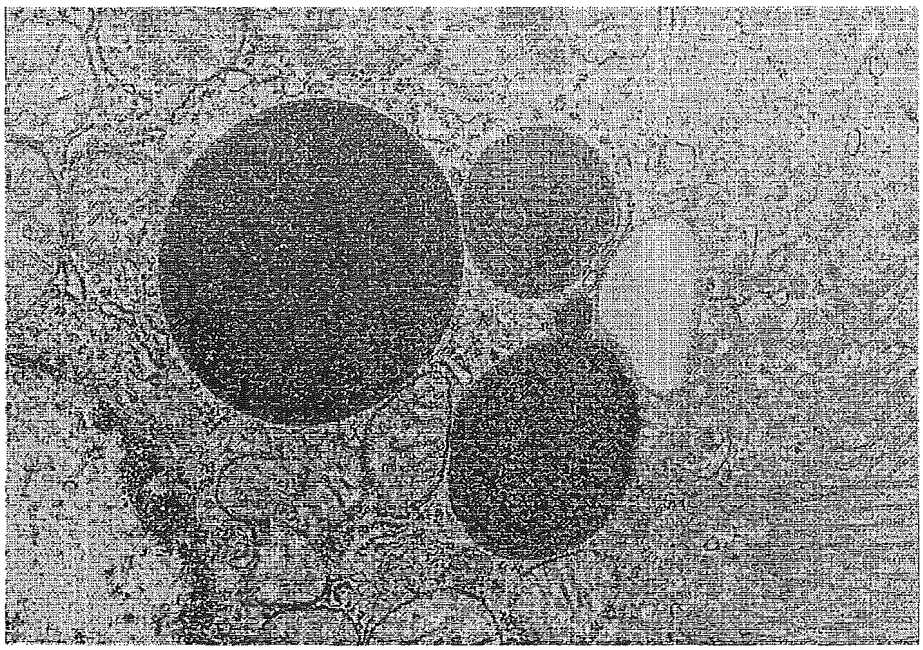

Photo 3. Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, ×10,000.

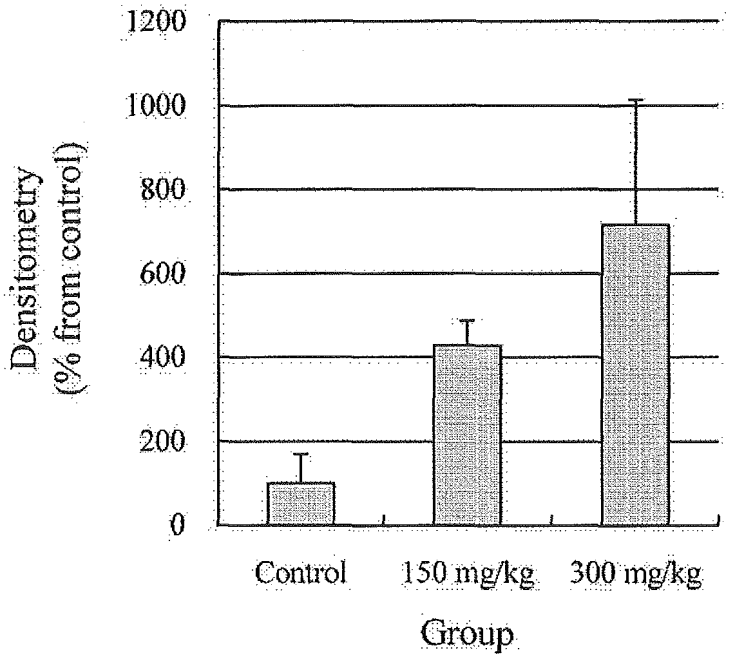

Fig. 1. Western blot analysis of α_{2u}-globulin in kidney from male rats treated with *d*-limonene.
Results are expressed as mean ± SD (n=4).

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 distinguish 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-1*H*-indene	HE	+/+/++	++/++/++	+++/+++/+++
	Azan-Mallory	+/+/++	++/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	+/+/++	++/++/++	+++/+++/+++
3,5,5-Trimethylhexan-1-ol	HE	−/−/±	+/+/++	+++/++/+++
	Azan-Mallory	±/−/±	+/+/++	+++/++/+++
	Anti-α_{2u}-globulin	±/−/±	+/+/++	+++/+++/+++
2,4-Di-tert-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

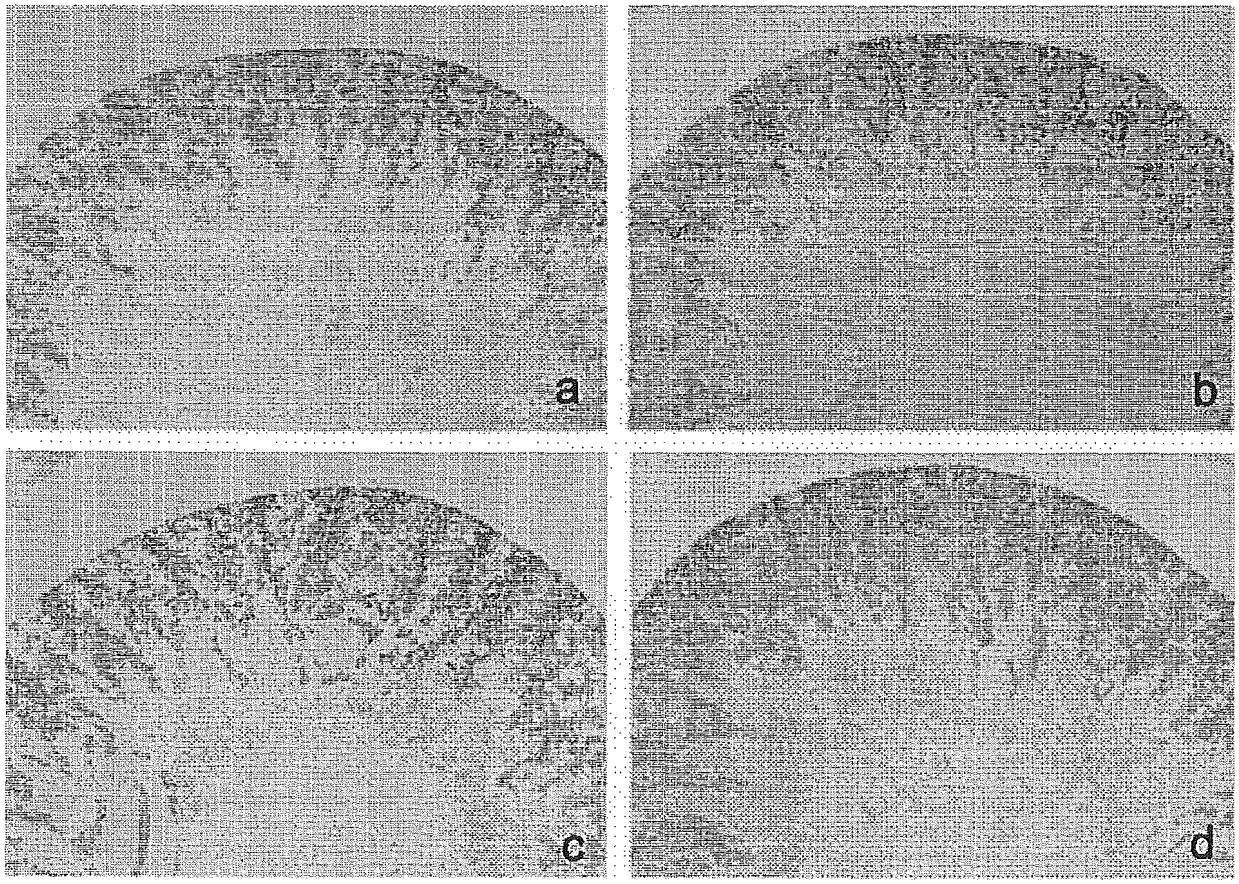

Photo 4. Immunohistochemical features of the anti-α_{2u}-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.

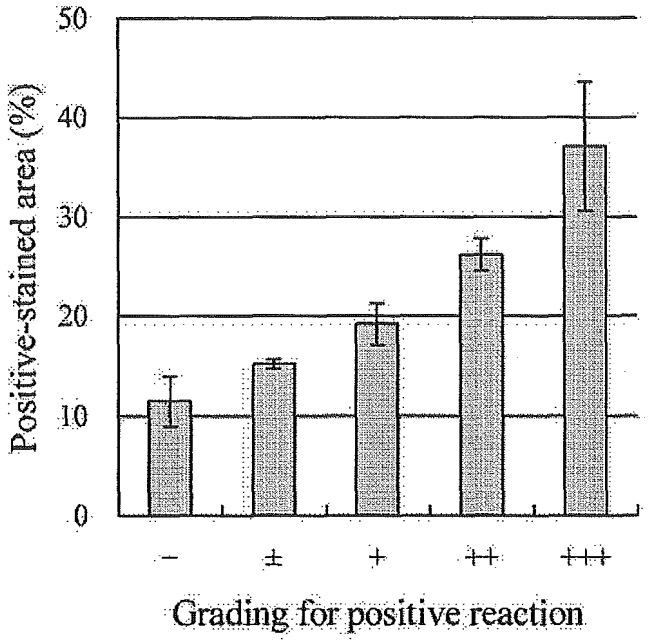

Fig. 2. Correlation between semi-quantitative and quantitative analyses for immuno-stained sections.
Results are expressed as mean ± SD (n=3).

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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REFERENCES

Alden, C.L., Kanerva, R.L., Ridder, G. and Stone, L.C. (1984): The pathogenesis of the nephrotoxicity of volatile hydrocarbons in the male rat. In (Mehlman, M.A., Hemstreet, G.P., Thorpe, J.J. and Weaver, N.K., eds.) Advances in Modern Environmental Toxicology. Vol. VII. pp.107-120. Renal Effects of Petroleum Hydrocarbons. Princeton Scientific Publishers, Inc., Princeton, New Jersey.

Burnett, V.L., Short, B.G. and Swenberg, J.A. (1989): Localization of α_{2u}-globulin within protein droplets of male rat kidney: Immunohistochemistry

using perfusion-fixed, GMA-embedded tissue sections. J. Histochem. Cytochem., **37**, 813-818.

Caldwell, D.J., Eldridge, S.R., Lington, A.W. and McKee, R.H. (1999): Retrospective evaluation of α_{2u}-globulin accumulation in male rat kidneys following high doses of diisononyl phthalate. Toxicol. Sci., **51**, 153-160.

Charbonneau, M., Lock, E.A., Strasser, J., Cox, M.G., Turner, M.J. and Bus, J.S. (1987): 2,2,4-Trimethylpentane-induced nephrotoxicity. I. Metabolic disposition of TMP in male and female Fischer 344 rats. Toxicol. Appl. Pharmacol., **91**, 171-181.

Charbonneau, M., Strasser, J.Jr., Lock, E.A., Turner, M.J.Jr. and Swenberg, J.A. (1989) Involvement of reversible binding to α_{2u}-globulin in 1,4-dichlorobenzene-induced nephrotoxicity. Toxicol. Appl. Pharmacol., **99**, 122-132.

Dietrich, D.R. and Swenberg, J.A. (1990): Lindane induces nephropathy and renal accumulation of alpha 2u-globulin in male but not in female Fischer 344 rats or male NBR rats. Toxicol. Lett., **53**, 179-181.

Goldsworthy, T.L., Lyght, O., Burnett, V.L. and Popp, J.A. (1988): Potential role of α_{2u}-globulin, protein droplet accumulation, and cell replication in the renal carcinogenicity of rats exposed to trichloroethylene, perchloroethylene, and pentachloroethane. Toxicol. Appl. Pharmacol., **96**, 367-379.

Hard, G.C., Rodgers, I.S., Baetcke, K.P., Richards, W.L., McGaughy, R.E. and Valcovic, L.R. (1993): Hazard evaluation of chemicals that cause accumulation of α_{2u}-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ. Health Perspect., **99**, 313-349.

Hashimoto, N. and Takaya, O. (1992): Immunohistochemical staining method for hyaline droplets (α_{2u}-globulin) observed in male rat kidney. Japan. J. Histotechnology, **1**, 83-86 (in Japanese).

Kanerva, R.L., Ridder, G.M., Lefever, F.R. and Alden, C.L. (1987): Comparison of short-term renal effects due to oral administration of decalin or d-limonene in young adult male Fischer-344 rats. Food Chem. Toxicol., **25**, 345-353.

Kurtz, D.T. and Feigelson, P. (1977): Multihormonal induction of hepatic α_{2u}-globulin mRNA as measured by hybridization to complementary DNA. Proc. Natl. Acad. Sci. USA, **74**, 4791-4795.

Kurtz, D.T., Sippel, A.E. and Feigelson, P. (1976): Effect of thyroid hormones on the level of the hepatic mRNA for α_{2u}-globulin. Biochemistry, **15**, 1031-1036.

Lehman-McKeeman, L.D., Rodriguez, P.A., Takigiku, R., Caudill, D. and Fey, M.L. (1989): d-Limonene-induced male rat-specific nephrotoxicity: Evaluation of the association between d-limonene and α_{2u}-globulin. Toxicol. Appl. Pharmacol., **99**, 250-259.

Lock, E.A., Stonard, M.D. and Elcombe, C.R. (1987): The induction of omega and beta-oxidation of fatty acids and effect on α_{2u}-globulin content in the liver and kidney of rats administered 2,2,4-trimethylpentane. Xenobiotica, **17**, 513-522.

MacInnes, J.I., Nozik, E.S. and Kurtz, D.T. (1986): Tissue-specific expression of the rat alpha$_{2u}$-globulin gene family. Mol. Cell. Biol., **6**, 3563-3567.

Neuhaus, O.W., Flory, W., Biswas, N. and Hollerman, C.E. (1981): Urinary excretion of α_{2u}-globulin and albumin by adult male rats following treatment with nephrotoxic agents. Nephron, **28**, 133-140.

Roy, A.K. and Neuhaus, O.W. (1966): Identification of rat urinary protein by zone and immunoelectrophoresis. Proc. Soc. Exp. Biol. Med., **121**, 894-899.

Saito, K., Uwagawa, S., Kaneko, H. and Yoshitake, A. (1991): Behavior of α_{2u}-globulin accumulating in kidneys of male rats treated with d-limonene: Kidney-type α_{2u}-globulin in the urine as a marker of d-limonene nephropathy. Toxicology, **79**, 173-183.

Saito, K., Uwagawa, S., Kaneko, H., Shiba, K., Tomigahara, Y. and Nakatsuka, I. (1996): α_{2u}-Globulins in the urine of male rats: A reliable indicator for α_{2u}-globulin accumulation in the kidney. Toxicology, **106**, 149-157.

Sippel, A.E., Feigelson, P. and Roy, A.K. (1975): Hormonal regulation of the hepatic messenger RNA levels for α_{2u}-globulin. Biochemistry, **14**, 825-829.

Sippel, A.E., Kurtz, D.T., Morris, H.P. and Feigelson, P. (1976): Comparison of *in vivo* translation rates and messenger RNA levels of α_{2u}-globulin in rat liver and Morris hepatoma 5123D. Cancer Res., **36**, 3588-3593.

Stonard, M.D., Phillips, P.G., Foster, J.R., Simpson, M.G. and Lock, E.A. (1986): α_{2u}-Globulin: Measurement in rat kidney following adminis-

tration of 2,2,4-trimethylpentane. Toxicology, **41**, 161-168.

Strasser, J., Charbonneau, M., Borghoff, S.J., Turner, M.J. and Swenberg, J.A. (1988): Renal protein droplet formation in male Fischer 344 rats after isophorone (IPH) treatment [abstracts]. Toxicologist, **8**, 136.

U.S. EPA (1991): Alpha2u-globulin: Association with chemically induced renal toxicity and neoplasia in the male rat. Risk Assessment Forum, U.S. Enivironmental Protection Agency, Washington, D.C.

Webb, D.R., Ridder, G.M. and Alden, C.L. (1989): Acute and subchronic nephrotoxicity of d-limonene in Fischer 344 rats. Food Chem. Toxicol., **27**, 639-649.

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ORIGINAL ARTICLE

Susceptibility of newborn rats to 3-ethylphenol and 4-ethylphenol compared with that of young rats

Mika Takahashi[1], Mutsuko Hirata-Koizumi[1], Nobuo Nishimura[2], Yoshihiko Ito[3], Masao Sunaga[4], Sakiko Fujii[4], Eiichi Kamata[3], Ryuichi Hasegawa[1] and Makoto Ema[1]

[1]National Institute of Health Sciences, Tokyo, [2]Gotemba Laboratory, Bozo Research Center Inc., Gotemba, [3]Research Institute for Animal Science in Biochemistry and Toxicology, Saganihara, and [4]Safety Research Institute for Chemical Compounds Co., Ltd, Sapporo, Japan

ABSTRACT **Newborn rat studies were conducted with oral administration of 3-ethylphenol (3EP) and 4-ethylphenol (4EP) from postnatal days (PD) 4–21 to allow comparison of no observed adverse effect level (NOAEL) and unequivocally toxic level (UETL) with those from 28-day studies of young rats starting at 5–6 weeks of age. In the newborn rat studies, slightly lowered body weight was observed after 3EP treatment, and deaths, hypoactivity, Straub tail, deep respiration and delayed righting reflex were clearly observed after 4EP treatment. In the young rat studies, salivation, staggering gait, changes in the liver including high values of liver weight and alanine aminotransferase or total cholesterol and the lesions in the forestomach were clearly observed after 3EP and 4EP treatments. NOAELs of 3EP and 4EP in the newborn rat studies appeared to be almost 3 times lower than those in the young rat studies. As a clear toxicity of 3EP was not observed in newborn rats, UETLs were not established for 3EP. Regarding 4EP, UETL of young rats was 4–5 times higher than that of newborn rats. In conclusion, newborn rats were 3–5 times more susceptible to 3EP and 4EP than young rats.**

Key Words: 3-ethylphenol, 4-ethylphenol, newborn rats, repeated-dose toxicity, young rats

INTRODUCTION

The possible toxic effect of chemical substances on the development of fetuses and newborns has aroused great concern among the public and the protection of fetuses and newborns has become a major scientific and political issue. In the EPA children's environmental health yearbook, US EPA (1998) has already stated comprehensively that children have their special vulnerability to certain toxic substances such as drugs and environmental chemicals. The special vulnerability in children to toxic substances may results from a combination of toxicokinetic, toxicodynamic and exposure factors, and kinetic factors are of importance mainly in the early postnatal period, largely as the result of immature elimination systems, i.e. metabolizing enzymes and/or renal function (Schwenk *et al.* 2002). There is much less information about differences between children and adults with regard to toxicodynamics (Schwenk *et al.* 2002). Regarding exposure factors, children play close to the ground and are constantly licking their fingers or mouthing toys or objects. As a result, mouthing becomes a potentially significant exposure route (US EPA 2002).

The potential toxic effects of chemicals cannot be anticipated from data on adults, and a data set on exposed children is essential for assessment of children's health. In this context, we have determined the toxicity of chemicals in newborn rats after direct dosing and compared it with that in young rats. We have already reported the differences in the susceptibility to toxicities of chemicals between newborn and young rats for 4-nitrophenol and 2,4-dinitrophenol (Koizumi *et al.* 2001), for 3-aminophenol (Koizumi *et al.* 2002), for 3-methylphenol (Koizumi *et al.* 2003), for tetrabromobisphenol A (Fukuda *et al.* 2004), for 2,4,6-trinitrophenol (Takahashi *et al.* 2004), for 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005). With regard to the no observed adverse effect level (NOAEL), these reports showed that the toxic response in newborn rats was at most 3–4 times (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol and 3-methylphenol) higher than that in young rats. On the other hand, the toxic response in newborn rats was 5 times (1,3-dibromopropane) and 8 times (1,1,2,2-tetrabromoethane) lower than that in young rats. The toxicological profiles of 4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane and 1,1,2,2-tetrabromoethane were similar between newborn rats and young rats. The nephrotoxicity of tetrabromobisphenol A was specific for newborn rats. We also reported that the toxicity profiles induced by 2,4,6-trinitrophenol were markedly different between newborn and young rats.

3-Ethylphenol (3EP) is a photographic chemical intermediate and an intermediate for the cyan coupler of photographic paper (Horikawa *et al.* 1998). 4-Ethylphenol (4EP) is a chemical compound widely used as a source material of reactive polymers, antioxidants, drugs, agricultural chemicals and dyes (Chemical Products' Handbook 2004). These chemicals are listed in the 2004 OECD list of high production volume (HPV) chemicals (OECD 2004a). The HPV chemicals list contains those chemicals that are produced at levels greater than 1000 tons per year in at least one member country/region of OECD. Regarding the toxicity information on these two chemicals, only a few studies are available. Thompson *et al.* (1995) showed that 4EP was metabolized to a reactive quinone methide intermediate by rat liver enzymes and that this oxidation mechanism played a significant role in the cytotoxic effect of 4EP. This intermediate was subsequently trapped with glutathione to produce two diastereomeric conjugates. Recently, 28-day repeated dose oral toxicity studies of 3EP and 4EP in young rats were conducted as part of the Japanese Existing Chemical Safety Program and published in the annual toxicity

Correspondence: Makoto Ema, DVM, PhD, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.
Email: ema@nihs.go.jp

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testing report (MHLW 2001a,b), in which no observed effect level was evaluated.

In the present paper, we re-evaluated the toxicity of 3EP (MHLW 2001a) and 4EP (MHLW 2001b) in young rats in terms of NOAEL and unequivocally toxic level (UETL). We considered that the findings in the main test of repeated dose study and the dose-finding study were useful for characterizing the toxicity of chemicals. NOAEL is the highest tested dose in a study that did not produce any observable adverse effects and is expressed in terms of the weight of a test substance given daily per unit weight of a test animal. UETL has been used only for our comparative toxicity analysis as a clear toxic dose. It is generally not readily definable because it depends on the type of toxicity (Hirata-Koizumi *et al.* 2005). We determined the toxicity of 3EP and 4EP in newborn rats, compared and discussed NOAELs and UETLs of 3EP and 4EP for young and newborn rats.

MATERIALS AND METHODS

Chemicals

3EP (3-ethylphenol, CAS no. 620-17-7, purity 96.2%) was obtained from Taoka Chemical Co., Ltd. (Osaka, Japan) and 4EP (4-ethylphenol, CAS no. 123-07-9, purity 98.4% for the newborn rat study and 98.3% for the young rat study) was obtained from Maruzen Petrochemical Co., Ltd. (Tokyo, Japan) and they were dissolved in olive oil.

Animals

In the newborn rat study, pregnant SPF Crj:CD(SD)IGS rats (gestation day 14–15) were purchased from Atsugi Breeding Center, Charles River Japan (Yokohama, Japan) and allowed to deliver spontaneously. The day on which parturition was completed was designated as postnatal day (PND) 0. Pups (newborn rats) were separated from dams on PND 3 and were suckled by foster mothers. In the young rat study, four-week old males and females of the same strain were purchased from the same farm as in the newborn rat study.

The animals were maintained in an environmentally controlled room set at 20–26°C with a relative humidity of 45–65% and a 12:12 h light/dark cycle. All animals in the newborn and young rat studies were allowed free access to a sterilized basal diet (CRF-1, Oriental Yeast, Tokyo, Japan or Laboratory MR Stock, Nosan Corporation, Yokohama, Japan) and water. The animals were euthanized by exsanguination under anesthesia using ether.

Study design

Time schedule for 3EP and 4EP studies is shown in Figure 1.

18-Day repeated dose study in newborn rats

Dose-finding study. Twenty-four male and 24 female newborns for 3EP or 20 male and 20 female newborns for 4EP were randomly selected and assigned to four dose groups, including a control group. Six foster mothers for 3EP and five for 4EP were used. One foster mother suckled four male and four female pups. Newborn rats (6/sex/dose for 3EP, 5/sex/dose for 4EP) were given 3EP at 0, 30, 100 or 300 mg/kg/day or 4EP at 0, 100, 300 or 1000 mg/kg/day by gavage once a day on PNDs 4–21 (for 18 days) and killed on PND 22 after overnight starvation. General condition, body weights, hematology, blood biochemistry, necropsy and organ weights were examined. The similar study design was applied to the main study.

Main study. Forty-eight males and 48 females for each chemical for two autopsy groups (the end of the dosing period and the recovery-maintenance period) were randomly selected and assigned to four dose groups, including a control group. Twelve foster mothers were used for each chemical. One foster mother suckled four male and four female newborn rats up to weaning on PND 21. After weaning, newborn rats of the recovery-maintenance group were individually maintained for 9 weeks. Newborn rats (6/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 30, 100 or 300 mg/kg/day on PNDs 4–21 (for 18 days) and killed on PND 22 after overnight starvation. The dosage levels were determined based on the results of the dose-finding study. Recovery-maintenance groups (6/sex/dose for each chemical) given the same dosage were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age, almost the same age as young rats at the end of the recovery period.

General condition was observed at least once a day for newborn rats during the dosing period (separated from each foster mother) and during the recovery-maintenance period. Body weight was measured before dosing, more than two times per week during the dosing period and at seven-day intervals thereafter. Food consumption was measured about 2 times per week only during the recovery-maintenance period. Some developmental landmarks were assessed (OECD 2004b), such as pilation, incisor eruption, eye opening, testes descent and vaginal opening. All newborn rats were examined for abnormalities of reflex ontogeny; e.g. pupillary

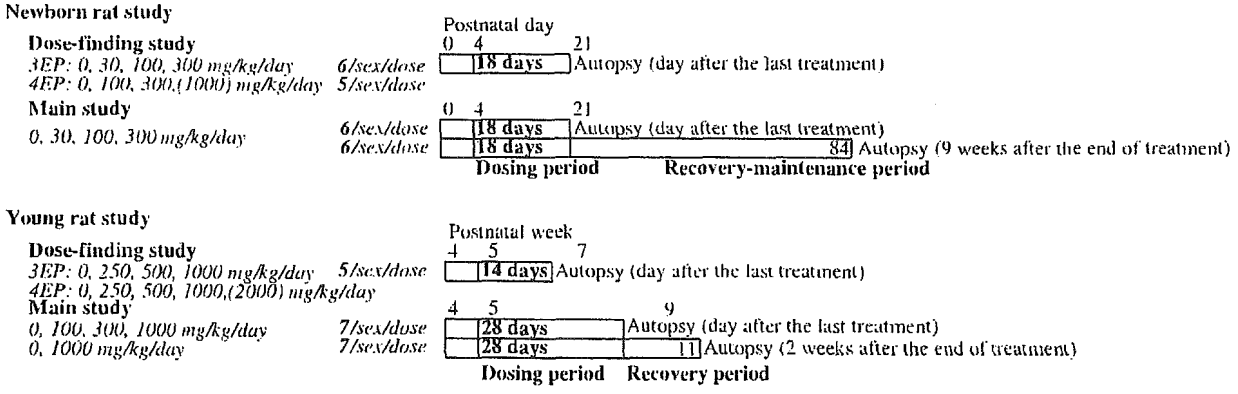

Fig. 1 Time schedule of newborn and young rat studies of 3-ethylphenol (3EP) and 4-ethylphenol (4EP).

reflex, Preyer's reflex, corneal reflex, righting reflex and air righting reflex on PND 20 or 21.

In urinalysis, color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment, specific gravity, osmotic pressure and volume of urine were examined in the late recovery-maintenance period. Newborn rats were killed on PND 22 or 85. On the day of the sacrifice, blood was collected from the abdominal aorta. Hematological parameters, such as the red blood cell count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte ratio, differential leukocyte count, and blood clotting parameters such as prothrombin time and activated thromboplastin time were determined. The blood biochemical parameters, such as the total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase (ALT), γ-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, cholinesterase, phospholipids, calcium, inorganic phosphorus, sodium, potassium and chloride levels in the serum, were also determined. After a gross examination, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes/ovaries and epididymides/uterus were weighed. The organs were fixed with 10% buffered formation-phosphate and paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. The studies using newborn rats were conducted at Gotemba Laboratory, Bozo Research Center Inc. (Gotemba, Japan) for 3EP and at Research Institute for Animal Science in Biochemistry and Toxicology (Saganihara, Japan) for 4EP under Good Laboratory Practice (GLP) conditions (MHW 1988), and accordance with 'Guidelines for Animal Care and Use' of these laboratories.

28-Day repeated dose study in young rats

Dose-finding study. Five-week-old rats (5/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 250, 500, 1000 or 2000 (only for 4EP) mg/kg/day for 14 days and killed the day following the last administration after overnight starvation. General condition, body weights, food consumption, hematology, blood biochemistry, necropsy and organ weights were examined.

Main study. Five-week-old rats (7/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 100, 300 or 1000 mg/kg/day for 28 days and killed after overnight starvation following the last treatment. The dosage levels were determined based on the results of the dose-finding study in young rats. Recovery groups (0 or 1000 mg/kg/day) (7/sex/dose for each chemical) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. The rats were examined for general condition, body weights, food consumption, urinalysis, hematology, blood biochemistry, necropsy findings, organ weights and histopathological findings. The study using young rats was conducted at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) for 3EP and 4EP under GLP conditions (MHW 1988), and accordance with 'Guidelines for Animal Care and Use' of these laboratories.

Statistical analysis

Continuous data were analyzed with Bartlett's test for homogeneity of variance. If the data were homogeneous, one-way analysis of variance and Dunnett's test were conducted for group comparisons between the control and individual chemical-treated groups. If not homogenous or in case of quantitative urinalysis data, analysis was performed using the Kruskal–Wallis test. In consequence, if a significant difference was detected, the Dunnett type test or Mann–Whitney's *U*-test was conducted. In the newborn rat study, categorical data for general appearance and reflex ontogeny were analyzed by Fisher's exact probability test or Mann–Whitney's *U*-test. A probability less than 5% was considered statistically significant.

RESULTS

18-Day study of 3EP in newborn rats

In the dose-finding study, body weights were considerably lowered in males (max. 9% decrease) and females (max. 6% decrease) at 300 mg/kg/day during the dosing period when compared to controls. However, the decreases were not statistically significant due to variations of the data.

Only slight changes were found in the main study as shown in the Table 1 and Figure 2. At 300 mg/kg/day, body weights recorded in males from PND 11–17 (max. 6% decrease) and females from PNDs 11–21 (max. 7% decrease) were significantly lower than controls. Significantly high value of relative liver weight was observed in males at 300 mg/kg/day and in females at 100 and 300 mg/kg/day at the end of the dosing period; however, it was not considered toxicologically significant because of the absence of changes in parameters of blood biochemistry and histopathological findings related to liver damage. There were no effects on the developmental landmarks at any dose. There were no effects of 3EP treatment at the end of the recovery-maintenance period.

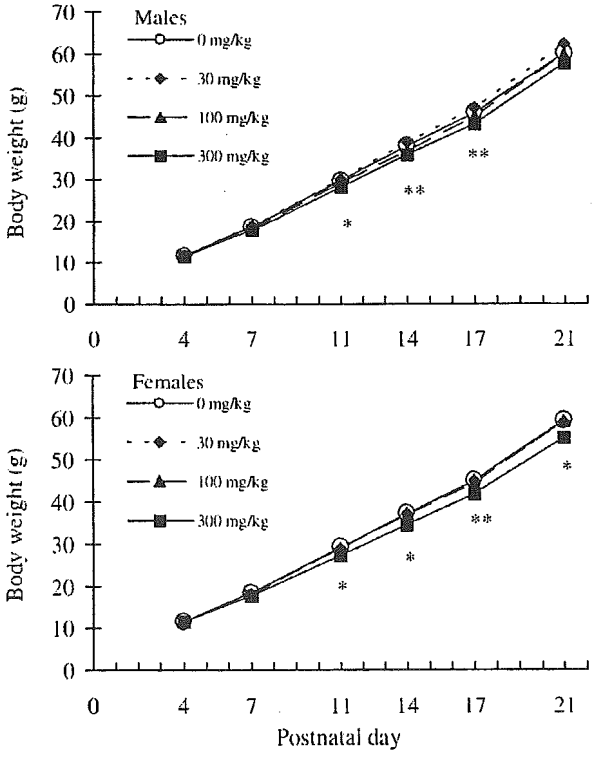

Fig. 2 Body weight curves in 18-day study of 3-ethylphenol (3EP) in newborn rats.

Table 1 Main findings of 3-ethylphenol (3EP) at the end of the dosing in the newborn and the young rat main studies

	Newborn rat study (mg/kg/day)				Young rat study (mg/kg/day)			
	0	30	100	300	0	100	300	1000
Male								
No. of animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	2
No. of animals examined	6	6	6	6	6‡	7	7	7
ALT (IU/L)	36 ± 7	36 ± 4	41 ± 9	35 ± 5	24 ± 2	25 ± 3	27 ± 4	40 ± 2**
Total cholesterol (mg/dL)	85 ± 8	86 ± 17	83 ± 11	99 ± 18	55 ± 8	53 ± 9	59 ± 15	61 ± 7
Relative liver weight (g/100 g BW)	3.00 ± 0.16	3.14 ± 0.10	3.18 ± 0.11	3.42 ± 0.21**	3.11 ± 0.19	2.98 ± 0.14	3.36 ± 0.24	3.62 ± 0.25**
Relative kidney weight (g/100 g BW)	1.10 ± 0.09	1.08 ± 0.03	1.10 ± 0.06	1.05 ± 0.06	0.81 ± 0.02	0.80 ± 0.05	0.80 ± 0.11	0.91 ± 0.06**
Forestomach, hyperplasia	0	0	0	0	0	0	0	7
Female								
No. of animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	5
No. of animals examined	6	6	6	6	7	7	7	7
ALT (IU/L)	34 ± 3	30 ± 4	32 ± 4	30 ± 6	22 ± 4	22 ± 3	22 ± 2	28 ± 6*
Total cholesterol (mg/dL)	89 ± 10	90 ± 21	96 ± 18	94 ± 10	56 ± 15	57 ± 7	61 ± 7	76 ± 15**
Relative liver weight (g/100 g BW)	2.93 ± 0.10	3.03 ± 0.12	3.14 ± 0.10*	3.39 ± 0.17**	3.10 ± 0.14	3.09 ± 0.16	3.28 ± 0.18	3.68 ± 0.25**
Relative kidney weight (g/100 g BW)	1.07 ± 0.07	1.15 ± 0.08	1.13 ± 0.06	1.15 ± 0.05	0.82 ± 0.05	0.83 ± 0.03	0.85 ± 0.07	0.86 ± 0.04
Forestomach, hyperplasia	0	0	0	0	0	0	0	7

Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. BW: body weight.

†Staggering gait, prone/lateral position, tremor or soiled perigenital fur.

‡Data from one animal were excluded because its hard palate was accidentally broken on day 23 of dosing.

28-Day study of 3EP in young rats

In the dose-finding study, one female showed staggering gait and a lateral position for three hours after the first dosing at 1000 mg/kg/day. At this dose, significantly high values of relative liver weight and ALT in males and relative liver weight and total cholesterol in females were observed. At 500 mg/kg/day, significantly high values of ALT in males and relative liver weight in females were observed.

In the main study (Table 1 and Fig. 3), adverse effects as below were found at 1000 mg/kg/day. Clinical signs, such as staggering gait, a prone/lateral position and soiled perigenital fur, were observed in 2/14 males and 5/14 females. Staggering gait and a prone and/or lateral position occasionally occurred 10 min after dosing and lasted one hour. Soiled perigenital fur was also observed in 1/14 males and 3/14 females at this dose. Body weight of males was significantly lowered on days 2 and 7 of dosing. In urinalysis, significantly high volumes of urine and water consumption and significantly low protein were observed in males and females at the end of the dosing period. In blood biochemistry, significantly high values of ALT in males and females and total cholesterol in females were observed. In the necropsy findings, thinning of the limiting ledge in the forestomach in 5/7 males and 2/7 females were observed at the end of the dosing period. Significantly high values of relative liver weight in males and females and relative kidney weight in males were observed at the end of the dosing period. Hyperplasia of the squamous cell in the forestomach was observed in all 7 males and all 7 females at the end of the dosing period. There were no effects of 3EP treatment at the end of the recovery period.

18-Day study of 4EP in newborn rats

In the dose-finding study, deaths occurred at 300 mg/kg/day in one female each on days 6 and 8 of dosing, and at 1000 mg/kg/day in all rats by day 3 of dosing. In these dead rats, hypoactivity was observed and additionally, deep respiration, pale skin and/or dehydration were observed. In the surviving rats, hypoactivity during the dosing period was found in 3/5 males and 1/3 females at 300 mg/kg/day.

The main findings in the main study are shown in Table 2 and Figure 4. Clinical signs, such as hypoactivity, hypothermia, tremor, Straub tail, deep respiration and emaciation, were observed in 10/12 males and all 12 females at 300 mg/kg/day. Hypoactivity in males and females and hypothermia, tremor, Straub tail, deep respiration and emaciation in females were significantly more frequent at this dose and these clinical signs disappeared by day 9 of dosing for males and day 13 of dosing for females. At 300 mg/kg/day, 2/12 females were found dead on days 10 and 12 of dosing. One of them showed dark red lung and congestive edema of the lung and the other showed distention of the gastrointestinal tract and atrophy of the thymic cortex at necropsy. The delay in the righting reflex was observed in 4/12 males at 300 mg/kg/day, in 1/12 females at 100 mg/kg/day and in 1/10 females at 300 mg/kg/day. At 300 mg/kg/day, body weights of males and females were significantly lower on PNDs 7–21. Significantly high relative weight of the liver was observed in males and females at 300 mg/kg/day at the end of the dosing period. There were no changes in the parameters of blood biochemistry or histopathological findings related to liver damage. There were no effects of 4EP treatment at the end of the recovery-maintenance period.

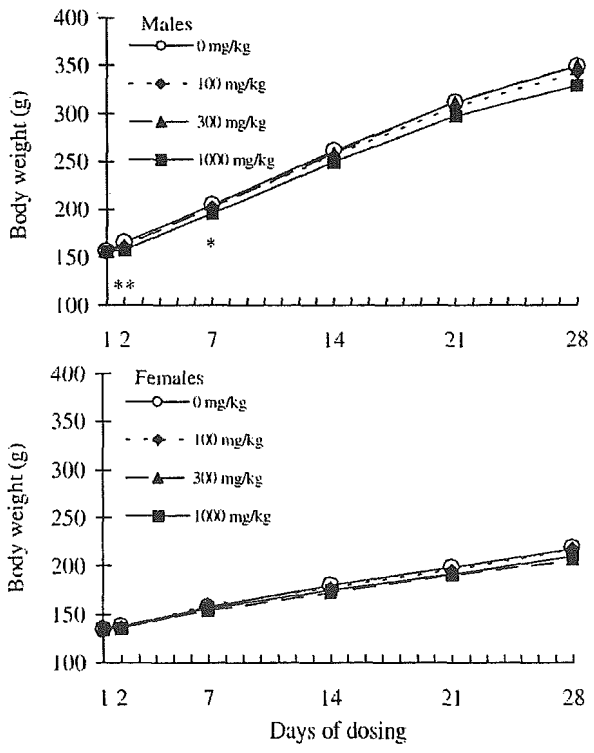

Fig. 3 Body weight curves in 28-day study of 3-ethylphenol (3EP) in young rats.

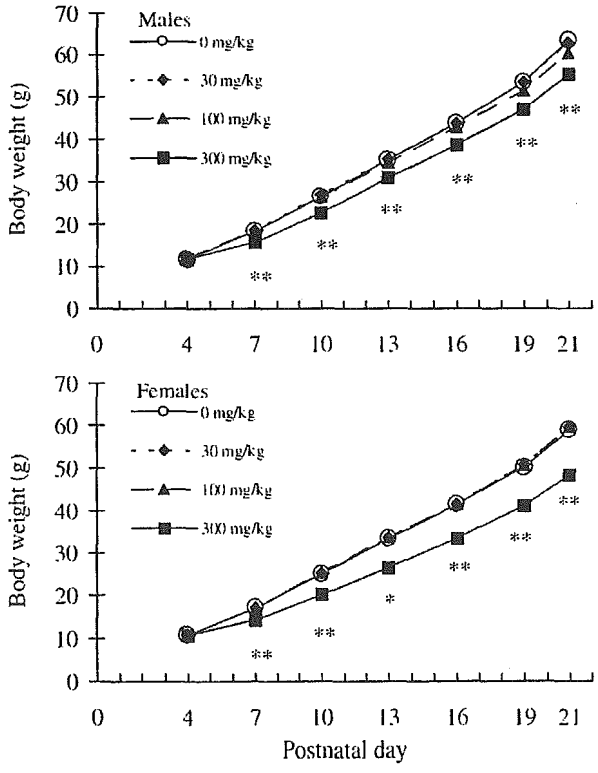

Fig. 4 Body weight curves in 18-day study of 4-ethylphenol (4EP) in newborn rats.

Table 2 Main findings of 4-ethylphenol (4EP) at the end of the dosing in the newborn and the young rat main studies

	Newborn rat study (mg/kg/day)				Young rat study (mg/kg/day)			
	0	30	100	300	0	100	300	1000
Male								
No. of animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	1‡	0	0	10	0	0	0	11
Death	0	0	0	0	0	0	0	0
Delayed righting reflex	0	0	0	4*				
No. of animals examined	6	6	6	6	7	7	7	7
ALT (IU/L)	27 ± 7	21 ± 5	23 ± 2	25 ± 4	24 ± 3	24 ± 1	28 ± 3	41 ± 9**
Total cholesterol (mg/dL)	82 ± 13	83 ± 14	84 ± 8	91 ± 5	66 ± 6	58 ± 8	63 ± 9	68 ± 9
Relative liver weight (g/100 g BW)	3.37 ± 0.14	3.39 ± 0.22	3.40 ± 0.13	3.68 ± 0.16**	3.13 ± 0.18	3.28 ± 0.18	3.46 ± 0.16**	3.58 ± 0.17**
Relative kidney weight (g/100 g BW)	1.18 ± 0.05	1.17 ± 0.08	1.17 ± 0.06	1.22 ± 0.07	0.80 ± 0.05	0.79 ± 0.05	0.79 ± 0.05	0.89 ± 0.03**
Forestomach, hyperplasia	0	0	0	0	0	0	1	7
Female								
No. of animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	12	0	0	0	9
Death	0	0	0	2§	0	0	0	0
Delayed righting reflex	0	0	1	1				
No. of animals examined	6	6	6	5	7	7	7	7
ALT (IU/L)	19 ± 3	20 ± 3	20 ± 2	19 ± 1	22 ± 8	21 ± 2	20 ± 2	27 ± 4
Total cholesterol (mg/dL)	80 ± 11	84 ± 11	85 ± 12	85 ± 23	61 ± 13	69 ± 10	65 ± 5	82 ± 14**
Relative liver weight (g/100 g BW)	3.25 ± 0.12	3.26 ± 0.05	3.37 ± 0.11	3.63 ± 0.23**	3.07 ± 0.17	2.99 ± 0.15	3.12 ± 0.12	3.47 ± 0.21**
Relative kidney weight (g/100 g BW)	1.21 ± 0.11	1.17 ± 0.05	1.20 ± 0.05	1.26 ± 0.07	0.82 ± 0.04	0.84 ± 0.06	0.83 ± 0.05	0.88 ± 0.05
Forestomach, hyperplasia	0	0	0	0	0	0	0	6

Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. BW: body weight.

†Hypoactivity, hypothermia, tremor, straub tail, deep respiration or emaciation for newborn rats and salivation, staggering gait, prone/lateral position or soiled perigenital fur for young rats.

‡Straub tail casually occurred on PND 9.

§Each female died on day 10 and 12 of dosing.

28-Day study of 4EP in young rats

In the dose-finding study, 4/5 males and all 5 females at 2000 mg/kg/day died after the first dosing and the remaining 1/5 males was killed because of moribundity on day 3 of dosing. At 1000 mg/kg/day, 1/5 females showed soiled perineal fur on days 5–7 of dosing and then died on day 8 of dosing. The body weight of females was significantly lower on day 2 of dosing at 1000 mg/kg/day. Significantly high values of ALT and total cholesterol at 1000 mg/kg/day and significantly high value of ALT at 500 mg/kg/day were detected in males. Significantly low value of alkaline phosphatase and significantly high value of potassium at 1000 mg/kg/day were detected in females. In the necropsy findings for rats died during the dosing period, acute changes, such as red coloration of the lung, forestomach and kidney, thinning of the mucosa in the glandular stomach, discoloration of the liver and spleen, blood pooling in the urinary bladder and abdominal dropsy were observed at 2000 mg/kg/day and reddish spots of the glandular stomach and atrophy of the thymus and spleen were detected at 1000 mg/kg/day. For the surviving rats, thickening of the mucosa in the forestomach was observed in 2/5 males and 3/4 females at 1000 mg/kg/day at the end of the dosing period. At 1000 mg/kg/day, significantly high values of the relative liver weight in males and females and a significantly low value of relative spleen weight in females were observed. At 500 mg/kg/day, a significantly low value of relative spleen weight in females was observed.

In the main study (Table 2 and Fig. 5), clinical signs, such as salivation, staggering gait, a lateral position and soiled perigenital fur, were observed in 11/14 males and 9/14 females at 1000 mg/kg/day. At this dose, salivation for males and females was observed within 30 min after dosing daily from day 6 to the end of the dosing period. Staggering gait and a lateral position were occasionally observed in males and females for 1 h from a few minutes after dosing, and soiled perigenital fur was occasionally observed for males and females. Significantly low body weights from days 7–28 of dosing in males and from days 14–28 in females were also observed. In urinalysis, a significantly high volume of urine was observed in females at 1000 mg/kg/day at the end of the dosing period. In the blood biochemistry, significantly high values of ALT in males and total cholesterol in females at 1000 mg/kg/day were observed. In the necropsy findings, thinning of the mucosa in the glandular stomach in 5/7 males and 6/7 females and reddish spots in the glandular stomach in 1/7 females were observed at 1000 mg/kg/day at the end of the dosing period. Significantly high values of relative liver weight at 300 and 1000 mg/kg/day in males and at 1000 mg/kg/day in females were observed at the end of the dosing period. Significantly high value of relative kidney weight at 1000 mg/kg/day in males was observed at the end of the dosing period. Erosion, hyperplasia of squamous cells, degeneration of squamous cells and/or edema of the submucosa in the forestomach was observed in all 7 males at 1000 mg/kg/day. Hyperplasia of squamous cells in the forestomach was observed in 1/7 males at 300 mg/kg/day. Hyperplasia of squamous cells in the esophagus, degeneration of squamous cells, edema of the submucosa, granulation of the submucosa, hyperplasia of squamous cells and/or ulcer in the forestomach were observed in 6/7 females at 1000 mg/kg/day. There were no effects of 4EP treatment at the end of the recovery period except for the lowered body weight of males at 1000 mg/kg/day.

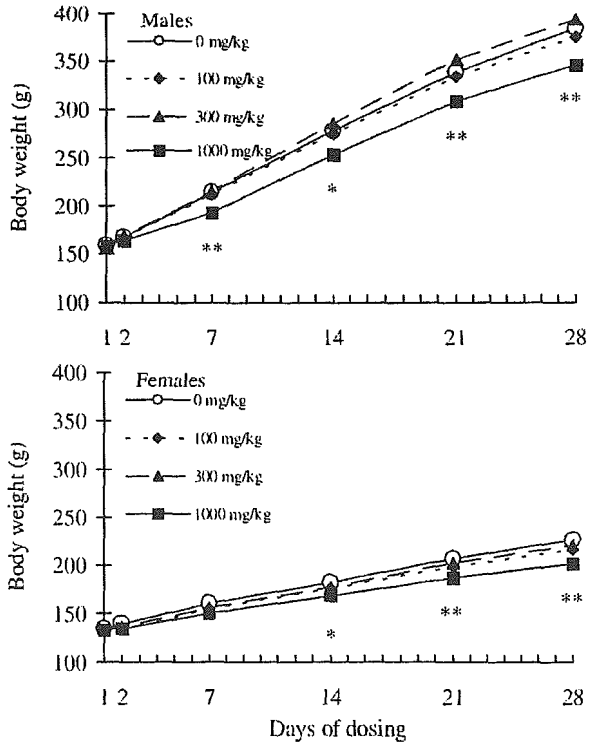

Fig. 5 Body weight curves in 28-day study of 4-ethylphenol (4EP) in young rats.

DISCUSSION

In the present paper, we determined the toxicity of 3EP and 4EP in newborn rats and reevaluated the toxicity of these chemicals in young rats, then compared the susceptibility of newborn rats in terms of NOAEL and UETL with that of young rats.

As for the administration of 3EP, NOAEL in the newborn rat study was concluded to be 100 mg/kg/day based on the lowered body weight at 300 mg/kg/day, although an increase in relative liver weight in females with no histopathological change and no changes in parameters of blood biochemistry related to liver damage was observed at 100 mg/kg/day in the main study. NOAEL in the young rat study was concluded to be 300 mg/kg/day based on the clinical toxic signs (staggering gait, prone/lateral position, tremor and soiled perigenital fur), changes in the liver (high values of weight and ALT or total cholesterol) and lesions in the forestomach at 1000 mg/kg/day. As clear toxicity did not appear in the newborn rat study even at the highest dose, we were not able to estimate UETL for 3EP.

As for the administration of 4EP, NOAEL in the newborn rat study was concluded to be 30 mg/kg/day based on the delay in the development of the righting reflex at 100 mg/kg/day. At 300 mg/kg/day, most animals showed clinical toxic signs and some females died in both the main and dose-finding studies. NOAEL in the young rat study was concluded to be 100 mg/kg/day, based on the lesions in the forestomach at 300 mg/kg/day. At 1000 mg/kg/day, clinical toxic signs were observed in all animals with the lesions in the forestomach. At this dose, no animal died in the main study but 1/5 females died in the dose-finding study (data not shown). When the dose of 1000 mg/kg/day for young rats was presumed as a UETL, which was the minimum lethal dose expecting the possibility of one female death, equivalent UETL for newborn rats was considered to be in the range of 200–250 mg/kg/day because 2/12

and 2/5 females died at 300 mg/kg/day in the main and dose-finding newborn studies, respectively.

In the newborn rat studies, slightly lowered body weight was observed after 3EP treatment, and deaths, hypoactivity, Straub tail, deep respiration and a delayed righting reflex were clearly observed after 4EP treatment. In the young rat studies, salivation, staggering gait, changes in the liver, including high values of liver weight and ALT or total cholesterol and lesions in the forestomach were clearly observed after 3EP and 4EP treatments. As for NOAEL, the susceptibility of newborn rats to 3EP and 4EP was approximately 3 times higher than that of young rats. The reason that newborn rats had higher susceptibility than young rats could be that newborn rats have immature metabolic activity, thus oxidation and conjugation of 3EP or 4EP in their livers would occur less, and toxic effects of the parent chemicals would continue longer.

The change of the mucosa and lesions of the submucosa and squamous cells in the forestomach caused by the corrosiveness of 3EP and 4EP were observed in young rats, but not in newborn rats. Generally, the phenols have similar toxicological effects and phenol is a protoplasmic poison and extremely corrosive (Bloom & Brandt 2001; Manahan 2003). 3EP and 4EP are irritating to the eyes, skin, mucous membranes and upper respiratory tract (Lenga 1985). Histopathological findings were not observed in the newborn rat study at any dose. The fact could be expected from the assumption that the membrane of the gastrointestinal tract of newborn rats would be more quickly renewed than that of young rats because of a higher turnover rate of the gastric membrane in developing newborn rats (Majumdar & Johnson 1982).

Methylphenol is an analog chemical of ethylphenol. Methylphenols or cresols, including three isomers, were reviewed as to their toxicity, and they have strong skin irritation and induce symptoms of poisoning (ASTDR 1992; WHO 1995; Stouten 1998). These reviews show that 4-methylphenol is more toxic than 3-methylphenol on the repeated-dose toxicity. In the present study, severer lesions in the forestomach were found after administration of 4EP than with 3EP in young rats. 4EP was also more toxic than 3EP in the newborn rat study. Deaths occurred after administration of 4EP.

Based on NOAEL, the susceptibility of newborn rats to 3EP and 4EP appeared to be almost 3 times higher than that of the young rats, being consistent with our previous results for four chemicals, 4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol and 3-methylphenol, which showed 2–4 times differences in the toxic response between newborn and young rats. As for 3EP, unequivocal toxicity was not observed in the newborn rat study. As for 4EP, UETL in the young rat study was 4–5 times higher than that in the newborn rat study. In conclusion, newborn rats were 3–5 times more susceptible to 3EP and 4EP than young rats.

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REFERENCES

Agency for Toxic Substances and Disease Registry (ASTDR) (1992) *Toxicological Profile for Cresols*. US Public Health Service. ASTDR, Atlanta.

Bloom JC, Brandt JT (2001) Toxic responses of the blood. In: Klaassen CD (ed.). *Casarett and Doull's Toxicology: the Basic Science of Poisons*. 6th edn. McGraw-Hill, New York. pp. 000–000.

Chemical Products' Handbook (2004) *Chemical Products of 14504 '14504 no Kagakushohin'*. The Chemical Daily, Tokyo (in Japanese).

Fukuda N, Ito Y, Yamaguchi M *et al.* (2004) Unexpected nephrotoxicity induced by tetrabromobisphenol A in newborn rats. *Toxicol Lett* **150**: 145–155.

Hirata-Koizumi M, Kusuoka O, Nishimura N *et al.* (2005) Susceptibility of newborn rats to hepatotoxicity of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, compared with young rats. *J Toxicol Sci* **30**: 29–42.

Horikawa Y, Yamashita M, Morino K, Koyama S, Wada M, Maki S (1998) Industrialization of the process for cyancoupler intermediate production. *Res Dev Rep Sumitomo Chem* **2**: 44–48.

Koizumi M, Yamamoto Y, Ito Y *et al.* (2001) Comparative study of toxicity of 4-nitrophenol and 2,4-dinitrophenol in newborn and young rats. *J Toxicol Sci* **26**: 299–311.

Koizumi M, Nishimura N, Enami T *et al.* (2002) Comparative toxicity study of 3-aminophenol in newborn and young rats. *J Toxicol Sci* **27**: 411–421.

Koizumi M, Noda A, Ito Y *et al.* (2003) Higher susceptibility of newborn than young rats to 3-methylphenol. *J Toxicol Sci* **28**: 59–70.

Lenga RE (ed.). (1985) *The Sigma-Aldrich library of chemical safety data*. Sigma-Aldrich Corp, Milwaukee.

Majumdar APN, Johnson LR (1982) Gastric mucosal cell proliferation during development in rats and effects of pentagastrin. *Am J Physiol* **242**: G135–G139.

Manahan SE (2003) *Toxicological Chemistry and Biochemistry*. 3rd edn. Lewis publishers, Florida.

Ministry of Health, Labour and Welfare, Japan (MHLW) (2001a) Twenty-eight-day repeated dose oral toxicity test of 3-ethylphenol in rats. *Toxicity Testing Reports of Environmental Chemicals* 8: 750–758. MHLW, Japan.

Ministry of Health, Labour and Welfare, Japan (MHLW) (2001b) Twenty-eight-day repeated dose oral toxicity test of 4-ethylphenol in rats. *Toxicity Testing Reports of Environmental Chemicals* 8: 555–566. MHLW, Japan.

Ministry of Health and Welfare Japan (MHW) (1988) *Standard Concerning Testing Facility Provided in Article 4 of Order Prescribing Test Items Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances*. Planning and Coordination Bureau, Environment Agency, no. 39, Environmental Health Bureau, Ministry of Health and Welfare, no. 229, Basic Industries Bureau, Ministry of International Trade and Industry, no. 85, March 31, 1984, and amendments, November 18, 1988. MHW, Japan.

Organisation for Economic Cooperation and Development (OECD) (2004a) *The 2004 OECD List of High Production Volume Chemicals*. OECD, Paris.

Organisation for Economic Cooperation and Development (OECD) (2004b) *Draft Guidance Document on Reproductive Toxicity Testing and Assessment*. OECD Environment, Health and Safety Publications Series on Testing and Assessment No. 43, November 10, 2004 (First Version). OECD, Paris.

Schwenk M, Gundert-Remy U, Heinemeyer G *et al.* (2002) Children as a sensitive subgroup and their role in regulatory toxicology: DGPT workshop report. *Arch Toxicol* **77**: 2–6.

Stouten H (1998) Cresols (o-, m-, p-). DECOS and SCG basis for an occupational standard. *Arbete Och Hälsa* **27**: 1–44.

Takahashi M, Ogata H, Izumi H *et al.* (2004) Comparative toxicity study of 2,4,6-trinitrophenol (picric acid) in newborn and young rats. *Congenit Anom Kyoto* **44**: 204–214.

Thompson DC, Perera K, London R (1995) Quinone methide formation from para isomers of methylphenol (cresol), ethylphenol, and isopropylphenol: Relationship to toxicity. *Chem Res Toxicol* **8**: 55–60.

US Environmental Protection Agency (US EPA) (1998) *The EPA Children's Environmental Health Yearbook*. US EPA, Washington DC.

US Environmental Protection Agency (US EPA) (2002) *Child-Specific Exposure Factors Handbook*. US EPA, Washington DC.

World Health Organization (WHO) (1995) *Cresols, Environmental Health Criteria 168*. International Programme on Chemical Safety. WHO, Geneva.

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Mari S. Golub

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chapter 3

Reproductive and Developmental Toxicity of Organotin Compounds

Makoto Ema and Akihiko Hirose
Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan

Contents

Introduction

Organotin compounds are chemicals widely used in agriculture and industry (Piver 1973, World Health Organization 1980). Tetrasubstituted organotin compounds are mainly used as intermediates in the preparation of other organotin compounds. Trisubstituted organotin compounds have biocidal properties and are used in agriculture as fungicides and acaricides, as rodent repellents, and molluscicides, and are widely used as antifoulants in ship paints and underwater coatings. Especially, triphenyltins (TPTs) and tributyltins (TBTs) have been used extensively in antifouling products such as algaecides and molluscicides. Disubstituted organotin compounds are commercially the most important derivatives, and are mainly used in the plastics industry, particularly as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers. Monosubstituted organotin compounds are used as stabilizers in PVC films. Widespread use of organotin compounds has caused increasing amounts to be released into the environment. The most important nonpesticidal route of entry for organotin compounds into the environment is through leaching of organotin-stabilized PVC in water (Quevauviller et al. 1991), and the use in antifouling agents, resulting in the introduction of organotin into the aquatic environment (Maguire 1991). Data are available regarding the detection of butyltin and phenyltin compounds in aquatic marine organisms (Sasaki et al. 1988, Fent and Hunn 1991, Lau 1991) and marine products (Suzuki et al. 1992, Belfroid et al. 2000, Tsuda et al. 1995, Ueno et al. 1999, Toyoda et al. 2000). Food chain bioamplification of butyltin in oysters (Waldock and Thain 1983), mud crabs (Evans and Laughlin 1984), marine mussels (Laughlin et al. 1986), Chinook salmon (Short and Thrower 1986), and dolphin, tuna, and shark (Kannan et al. 1996), and of phenyltin in carp (Tsuda et al. 1987) and horseshoe crab (Kannan et al. 1995) has been reported. These indicate that organotin compounds accumulate in the food chain and are bioconcentrated, and that humans can be exposed to organotin compounds via seafood. The World Health Organization (WHO) reported in 1980 that the estimated mean total daily intake of tin by humans ranged from 200 μg to 17 mg. Recently, Tsuda et al. (1995) reported that the daily intakes in Shiga prefecture in Japan were 0.7 to 5.4 μg in 1991 and 0.7 to 1.3 μg in 1992 for TPT and 4.7 to 6.9 μg in 1991 and 2.2 to 6.7 μg in 1992 for TBT. Toyoda et al. (2000) also showed that the daily intakes in Japanese consumers, based on analysis with the 1998 total diet samples, were 0.09 μg for TPT, 0 μg for diphenyltin (DPT), 1.7 μg for TBT, and 0.45 μg for dibutyltin (DBT). These values are lower than the acceptable daily intake for TPT according to the JMPR (Joint Meetings of the FAO [Food and Agriculture Organization] and World Health Organization

Panel of Experts on Pesticides Residues), 25 µg (World Health Organization 1992), and the guidance value for oral exposure to tributyltin oxide (TBTO), 18 µg (International Programme on Chemical Safety 1999a). Thus, the levels of organotin compounds in seafood are not considered to be sufficiently high to affect human health (Isuda et al. 1995, Ueno et al. 1999). However, Belfroid et al. (2000) noted that more research on residual TBT levels in seafood is needed before a definitive conclusion on possible health risks can be drawn.

In recent years, adverse effects of environmental chemicals on the reproductive success of wildlife populations have been reported (Colborn et al. 1993). These phenomena may result from interference with the endocrine system. Disturbances of hormonal regulation during pre- and postnatal development may produce deleterious effects on reproduction and development. TPT and TBT are suspected to be endocrine disruptors (Japan Environment Agency 1998). TBT and TPT are known to have strong effects on the development of imposex (imposition of male sex characteristics on females) in the rock shell (Horiguchi et al. 1996, 1997a), and this condition may bring about reproductive failure and a consequent population decline.

Although the toxicity of organotins has been extensively reviewed (World Health Organization 1980, Snoeij et al. 1987, Winship 1988, Boyer 1989, International Programme on Chemical Safety 1999a, b), the reproductive and developmental toxicity of these compounds is not well understood. In this chapter, we summarize the findings of the studies on reproductive and developmental effects of organotin compounds.

Effects on Aquatic Organisms

Imposex on Gastropods

TBT causes reproductive toxic effects in marine gastropods, which were represented by some masculinizing effects including *imposex* or *pseudohermaphodi(ti)sm*. The imposition of male sex organs (a penis and vas deferens) on female mud snails (*Nassarius obsoletus*) was found in near harbors, and the degree of penis development and frequency of imposex were positively correlated to the seawater TBT concentration (Smith 1981a, b). Imposex has been induced experimentally by treatment with 4.5 to 5.5 µg/L of TBT compounds for 60 days. In field studies in southeastern England, imposex has been reported in declining populations of the common dogwhelk (Bryan et al. 1986, 1987, 1989, Gibbs and Bryan 1986, Davies et al. 1987, Gibbs et al. 1987).

Imposex has not just occurred at a regional level, but worldwide on a global scale. Imposex in dogwhelk was not only reported in England, but in Scotland, the Netherlands, and the coastline of the North Sea. Imposex in other whelk species occurred in Canada, West Africa, New Zealand, Australia, Malaysia, Singapole, Indonesia, and Japan (Fent 1996, Horiguchi et al. 1996). Imposex among prosobranchs is known to occur in around 70 species of 50 genera, although some species are less susceptible to TBT compounds (Fioroni et al. 1991, Fent 1996).

TPT also induced imposex in *Thais clavigera* at the same potency as TBT (Horiguchi et al. 1997a). Although, in *Nucwlla lapillus*, TPT did not induce imposex, tripropyltin (TPrT) had a small effect on the development of imposex (Bryan et al. 1988). DBT and monobutyltin (MBT) did not induce imposex in the gastropod species examined. Three trisubstitution compounds (TBT, TPT, TPrT) and monophenyltin (MPT) easily induced imposex in some species, among the eight organotins, i.e., MBT, DBT, TBT, tetrabutyltin (TeBT), MPT, DPT, TPT, and TPrT. (Bryan et al. 1988, Hawkins and Hutchinson 1990, Horiguchi et al. 1997b).

The early studies in the 1980s reached some common conclusions, which are described below (Eisler 2000). Imposex correlated with the body burden of tributyl- and dibutyltin, but not with the tissue concentration of arsenic, cadmium, copper, lead, silver, or zinc. Forty-one percent of females had male characteristics, when the body burden reached to 1.65 mg Sn/kg of dry soft parts, by exposing with 0.02 µg Sn/L for 120 days. Imposex in immature females is caused above the concentration of around 1 ng/L (Sn) in seawater. At higher concentrations of TBT, the oviduct had been blocked, resulting in sterilization. Declining dogwhelk populations could be caused by aborting capsules, sterility, and premature death, which were characterized by a moderate to high degree of imposex, fewer female functions, fewer juveniles, and scarcity of laid egg capsules.

There is also a great variety of gradations of imposex in different species. The intensity is characterized by a classification system, which distinguishes six stages with a few different types, mainly based on a Vas Deferens Sequence (VDS) index (Oehlmann et al. 1991). Imposex development occurred in three variations: (1) a small penis without penis duct, (2) a short distal vas deferens section, or (3) a short proximal vas deferens section (stage 1). At stages 2 and 3 the male sex characteristics of each type are developed continuously. Stage 4 is characterized by a penis with penis duct and a complete vas deferens, and represents the last stage of fertility. The reproductive failure or sterility is induced in later stages. At stage 5 the vagina is replaced with a small prostate gland, the vagina opening is blocked by vas deferens tissue, or the incompletion of the pallial oviduct closure occurs. Abortive egg capsules fill the lumen and vestibulum of the capsule gland and evoke an intense swelling of the gland at stage 6 (Bettin et al. 1996). High TBT exposure in the early stages of life induced gametogenesis or sex changes characterized by a suppression of oogenesis and commencement of spermatogenesis in females (Gibbs et al. 1988, Fioroni et al. 1991, Oehlmann et al. 1991, 1996, Horiguchi et al. 2002). It was thought that the initial phases of imposex corresponding to VDS stages 1 and 2 may be reversible; however, advanced phases of imposex and sterilization with gross morphological changes corresponding to VDS stage 5 and 6 would be irreversible (Fent 1996).

Although many morphological aspects of pseudohermaphodi(ti)sm caused by TBT have been investigated, the biochemical mechanism has been indistinct. It is known that a neurotropic hormone called the penis morphogenic factor (PMF) develops male normal differentiation in mollusks (Féral

and LeGall, 1983). Co-localization of TBT with PMF in ganglia suggested that PMF release through TBT's neurotropic action induced masculinization in females (Bryan et al. 1989). Other studies indicated increased testosterone levels detected in female dogwhelk exposed to TBT, and that testosterone injection without TBT induced penis development in females (Spooner et al. 1991, Stroben et al. 1991). The later studies suggested that TBT disturbed the P-450-dependent aromatization of androgens to estrogen, and a nonsteroidal specific aromatase inhibitor-induced imposex similar to TBT (Bettin et al. 1996). However, the PMF has not been well characterized, and the role of vertebrate sex steroids is not known in gastropods to date. A recent study proposed that the combination of changes in the neuropeptide (APGWamide), which is considered to be a PMF in mud snails, and steroid hormones would lead to imposex induction at extremely low doses of TBT (Oberdörster and McClellan-Green 2002).

Effects on Fish

TBT or TPT exposure in early life stages induces altered embryonic development, and delayed or inhibited hatching in fish. Exposure of TBT or TBT to minnow eggs and larvae at concentrations of 0.2 to 18 µg/L in the water in which the fish lived induced dose-dependent morphological effects on larvae. Marked body axis deformations were observed at more than about 4 µg/L exposure, and incomplete hatching occurred at similar concentrations in 10 to 30% of larvae. At 15.9 µg/L of TPT exposure, hatching was delayed and the hatching rate was reduced significantly (Fent and Meier 1992, 1994). Developmental defects, such as skeletal abnormality and retarded yolk sac resorption, occurred in zebrafish larvae at more than 25 µg/L of triphenyltin acetate (TPTA) exposure, and hatching delay was found at more than 0.5 µg/L (Strmac and Braunbeck 1999). These developmental effects in fish were caused not only by organotin compounds, but also by a variety of contaminants (i.e., heavy metals, chlorinated hydrocarbons, altered pH), suggesting that such alteration would be classified as a nonspecific reaction to organic toxicants (Fent 1996, Strmac and Braunbeck 1999).

Some reproductive effects (i.e., reduced fecundity and sperm counts) in fish were reported. Reproductive success of three-spine stickleback with TBT exposure were examined over a 7-month period; no effects were detected in relation to fecundity, number of hatched fry, or frequency of malformed fry. However, no changes were found in the gonad somatic index (GSI; ovary weight ratio to total body weight); by the 7-month TBT treatment (2 µg/L) despite increasing GSI in controls, which suggested a lack of maturation of egg tissue and consequently a potential reduced fecundity (Holm et al. 1991). In sheepshead minnows, reduction in both total and percent viable eggs was found at more than 1.3 µg/L of TBT exposure, although the reductions were not statistically significant (Manning et al. 1999). TBT exposure to Japanese medaka at 1 mg/kg body weight caused a reduction of the spawning frequency (Nirmala et al. 1999). Additionally, environmentally relevant concentrations of

TBT induced significantly decreased sperm counts in guppies (11.2 to 22.3 ng/L for 21 days), and decreased sperm motility at concentrations less than 1 µg/L (Haubruge et al. 2000, Kime et al. 2001).

Effects on Other Organisms

Despite a great number of studies on imposex in snails and a comparable number of toxicity reports on fish, there is little information on development and reproductive effects on other species by organotin compounds. It was reported that imposex has not only been found in gastropods, but also been induced in Japanese freshwater crabs by TBT (Takahashi et al. 2000). In crabs, imposex has also occurred in males, which is characterized by dual-gender imposex (either a female genital opening or a single ovary occurred in males). Malformations during limb regeneration occurred in fiddler crabs (Weis and Kim 1988) and in axolotl, induced by TBT (Scadding 1990).

Summary of Effects on Aquatic Organisms

TBT or TPT causes the imposition of male sex organs (imposex) on female mud snails above the concentration of about 1 ng/L (Sn) in seawater, but DBT or MPT does not induce imposex. The intensity is characterized by a classification system based on the VDS, and advanced phases of imposex and sterilization with gross morphological changes are irreversible. The biochemical mechanism studies suggested that the induction of either neurotropic hormone or androgen titer would lead to imposex at an extremely low dose of TBT. Also, TBT or TPT exposure in the early life stages of fish causes altered embryonic development, impaired morphological development, and delayed or inhibited hatching, and reduces fecundity and sperm counts. Such reproductive and developmental defects were also found in other species. The impaired reproduction and subsequent population decline in a variety of aquatic organisms by organotins are an important issue in aquatic ecosystems.

Effects on Experimental Animals

Reproductive Toxicity of Phenyltin Compounds

Reproductive Toxicity of Triphenyltins

TPTs have been reported to be insect chemosterilants (Kenaga 1965). Reproductive studies on TPTs are presented in Table 3.1. Several reports on male reproductive toxicity have been published. Male Sharman rats were given a diet containing triphenyltin hydroxide (TPTH) at 50, 100, or 200 ppm and then mated with untreated females repeatedly five times (Gaines and Kimbrough 1968). Reduced fertility, such as decreases in the total number of matings, total number of litters born alive, and ratio of number of litters to number of matings, accompanied by a marked reduction in food consumption

Table 3.1 Reproductive Toxicity of Phenyltin Compounds

Compounds	Animals	Dose	Days of Administration	Route	Reproductive and Developmental Effects	Author(s)
TPTH	Sharman rat	100–200 ppm	64–238 days	Diet	Decreased no. of matings Decreased no. of litters born alive Decreased ratio of no. of litters to no. of matings	Gains and Kimbrough (1968)
TPTA or TPTCl	Holtzman rat	20 mg/kg	19 days	Diet	Decreased testicular size	Pate and Hays (1968)
TPTA or TPTCl	Holtzman rat	20 mg/kg	20 days	Diet	Change in testicular morphology Impairment of spermatogenic process	Snow and Hays (1983)
TPTA	ICR/Ha	2.4–12 mg/kg	1 day	ip	No dominant lethal effect	Epstein et al. (1972)
	Swiss mouse	6 mg/kg	5 days	Gavage	No dominant lethal effect	
TPTH		1.3–8.5 mg/kg	1 day	ip	No dominant lethal effect	
		11 mg/kg	5 days	Gavage	No dominant lethal effect	
TPTA or TPTCl	Holtzman rat	20 mg/kg	4–24 days	Diet	Decreased no. of mature follicles Increased incidence of atresia in early follicle growth Decreased no.of corpora lutea	Newton and Hays (1968)
TPTCl	Wistar rat	4.7–6.3 mg/kg	Days 0–3 of pregnancy	Gavage	Decreased pregnancy rate, decreased fetal wt.	Ema et al. (1997a)
		12.5–25 mg/kg	Days 4–6 of pregnancy	Gavage	Decreased pregnancy rate	
TPTCl	Wistar rat	4.7–6.3 mg/kg	Days 0–3 of pseudopregnancy	Gavage	Suppression of uterine decidualization	Ema et al. (1999a)
DPTCl	Wistar rat	16.5–24.8 mg/kg	Days 0–3 of pregnancy	Gavage	Decreased pregnancy rate, preimplatation loss, decreased fetal wt.	Ema et al. (1999b)
		33.3 mg/kg	Days 4–7 of pregnancy	Gavage	Effects as above, postimplantation loss	
DPTCl	Wistar rat	4.1–24.8 mg/kg	Days 0–3 of	Gavage	Suppression of uterine decidualization	Ema and Miyawaki (2002)

response (DCR) is a model for maternal physiological events that are associated with implantation (Cummings 1990). This technique can distinguish between the adverse effects of chemical compounds in the maternal and fetal compartments, and has been used to evaluate the reproductive toxicity of chemical compounds (Spencer and Sing 1982, Bui et al. 1986, Cummings 1990, Kamrin et al. 1994, Ema et al. 1998). The effects of TPTCl on the reproductive capability of the uterus, as a cause of implantation failure, were evaluated using pseudopregnant rats (Ema et al. 1999a). Female Wistar rats were given TPTCl by gastric intubation at 3.1, 4.7, or 6.3 mg/kg on days 0 to 3 of pseudopregnancy. Between 11:00 and 13:00 on day 4 of pseudopregnancy, induction of DCR was performed via midventral laparotomy under ether anesthesia, and experimental decidualization was initiated by scratching the antimesometrial surface of the endometrium with a bent needle. The uterine weight on day 9 of pseudopregnancy served as an index of the uterine decidualization (De Feo 1963). A decrease in the uterine weight, which indicates suppression of the uterine decidualization, was detected at 4.7 and 6.3 mg/kg. TPTCl at 4.7 and 6.3 mg/kg also produced a decrease in the serum progesterone levels in female rats on day 4 and on day 9 of pseudopregnancy. These doses caused an increase in implantation failure (preimplantation embryonic loss) in female rats given TPTCl on days 0 to 3 of pregnancy (Ema et al. 1997a). These results suggest that TPTCl causes the suppression of uterine decidualization correlated with the reduction in serum progesterone levels, and these participate in the induction of implantation failure due to TPTCl. Protective effects of progesterone against suppression of uterine decidualization and implantation failure induced by TPTCl were examined (Ema and Miyawaki 2001). The hormonal regimen, consisting of progesterone and estorone supported decidual development in ovariectomized rats given TPTCl. The pregnancy rate and number of implantations in groups given TPTCl at 4.7 or 6.3 mg/kg in combination with progesterone were higher than those in the groups given TPTCl alone. These results indicate that the TPTCl-induced suppression of uterine decidualization is mediated, at least partially, by ovarian hormones, and that progesterone protects against TPTCl-induced implantation failure.

Reproductive Toxicity of Diphenyltin Compounds

Oral TPT is metabolized to DPT, MPT, and further to inorganic tin in rats (Kimmel et al. 1977, Ohhira and Matsui 1993 a, b). Reproductive toxicity studies on DPTs are also published (Table 3.1). The adverse effects of diphenyltin dichloride (DPTCl) on the initiation and maintenance of pregnancy, and the role of DPT in the implantation failure of TPT were evaluated. Following successful mating, DPTCl was given to Wistar rats by gavage on days 0 to 3 of pregnancy at 4.1, 8.3, 16.5, or 24.8 mg/kg or on days 4 to 7 of pregnancy at 8.3, 16.5, 24.8, or 33.0 mg/kg (Ema et al. 1999b). The pregnancy rate was decreased after administration of DPTCl on days 0 to 3 at 24.8 mg/kg and on days 4 to 7 at 33.0 mg/kg. The incidence of preimplantation loss was increased at 16.5 mg (equivalent to 48 μmol)/kg on days 0 to 3. In

and weight gain, were observed at 100 or 200 ppm for 64 days. At these doses, food consumption later improved, and with it, fertility. Dietary exposure to triphenyltin acetate (TPTA) or triphenyltin chloride (TPTCl) at 20 mg/kg for 19 days produced marked effects on body weight, testicle size, and testicular structure in male Holtzman rats (Pate and Hays 1968). Microscopic examinations revealed degenerative changes, such as a decrease in the number of layers per tubule, a depletion of the more advanced cell forms from the tubules, and a closing of the tubule lumina. Effects were more pronounced in rats treated with TPTA. TPTA or TPTCl at 20 mg/kg in feed for 20 days was reported to cause an impairment of the spermatogenic process in male Holtzman rats; complete recovery of the spermatogenesis was observed after feeding a normal diet for 70 days (Snow and Hays 1983). No mutagenicity was detected in dominant lethal assay in which male ICR/Ha Swiss mice were given a single intraperitoneal injection of TPTA at 2.4 or 12 mg/kg or TPTH at 1.3 or 8.5 mg/kg, or given TPTA at 6mg/kg or TPTH at 11 mg/kg by gavage on 5 successive days and then mated with untreated females, and pregnancy outcome was determined on day 13 of pregnancy (Epstein et al. 1972).

Adverse effects on female reproductive toxicity were also reported. Dietary TPTA and TPTCl at 20 mg/kg for 4 days produced significant changes in the ovarian tissue, including a decreased number of mature follicles, an increased incidence of atresia in early follicle growth, and a pronounced decrease in the number of corpora lutea in female Holtzman rats (Newton and Hays 1968). These effects were regarded as a decrease in ovulation, and thus decreased fertility. The adverse effects of TPTCl on the initiation and maintenance of pregnancy were determined after administration to the mother during early pregnancy (Ema et al. 1997a). Following successful mating, female Wistar rats were given TPTCl by gavage on days 0 to 3 of pregnancy at 3.1, 4.7, or 6.3 mg/kg or on days 4 to 6 of pregnancy at 6.3, 12.5, or 25.0 mg/kg, and pregnancy outcome was determined on day 20 of pregnancy. TPTCl totally prevented implantation in a dose-dependent manner. The pregnancy rate was decreased after administration of TPTCl on days 0 to 3 at 4.7 and 6.3 mg/kg and on days 4 to 6 at 12.5 and 25.0 mg/kg. Preimplantation loss was increased after administration of TPTCl on days 0 to 3 at 4.7 mg/kg and higher. In females having implantations, the numbers of implantations and live fetuses, and the incidences of pre- and postimplantation embryonic loss in the TPTCl-treated groups were comparable to the controls. These results indicate that TPTCl during early pregnancy causes failure in implantation and has greater antiimplantation effects when administered during the preimplantation period than the periimplantation period.

The function of the uterine endometrium is one of the principle factors in embryonic survival. Uterine decidualization is required for normal implantation, placentation, and therefore normal gestation in rats. The uterine growth induced by endometrial trauma in pseudopregnant animals mimics the decidual response of the pregnant uterus that occurs after embryo implantation (Cummings 1990, Kamrin et al. 1994). The decidual cell

females having implantations, the incidences of pre- and postimplantation embryonic loss in the groups given DPTCl on days 0-3 were comparable to the controls. The incidence of postimplantation embryonic loss was increased after administration of DPTCl on days 4 to 7 at 33.0 mg/kg. These results indicate that DPTCl during early pregnancy causes implantation failure, and that DPTCl has greater effects on reproduction when administered during the preimplantation period rather than the periimplantation period. Following administration on days 0 to 3 of pregnancy, the increased incidence of preimplantation embryonic loss was induced by TPTCl, a parent compound of DPTCl, at 4.7 mg (equivalent to12 μmol)/kg and higher (Ema et al. 1997a), or DPTCl at 16.5 mg (equivalent to 48 μmol)/kg. If, on a mole-equivalent basis, a metabolite is as, or more, effective than the parent compound, this is consistent with the view that the metabolite is the proximate toxicant or at least an intermediate to the proximate toxicant. Thus, it seems unlikely that only DPTCl and/or its further metabolites can be considered the agents responsible for the antiimplantation effects of TPTCl. As for the metabolism of phenyltin, however, Ohhira and Matsui (1993b) showed that TPT compound was formed in the liver of the DPTCl-treated rat by metabolism of DPTCl, and suggested that part of the administered DPT compound has some harmful effect as the TPT compound in rats, and this must be taken into consideration in toxicological research on DPT. Further studies are needed to clarify the difference in the reproductive toxicity induced by TPT and DPT, and to identify the proximate or ultimate toxicant of phenyltins. The effects of DPTCl on the reproductive capability of the uterus were evaluated in pseudopregnant rats according to the procedure described above. Female Wistar rats were given DPTCl by gastric intubation on days 0 to 3 of pseudopregnancy at 4.1, 8.3, 16.5, or 24.8 mg/kg (Ema and Miyawaki 2002). Suppression of uterine decidualization was observed at 16.5 mg/kg and higher. A decrease in the serum progesterone levels in pseudopregnant rats was also found on day 4 and on day 9 of pseudopregnancy at 16.5 mg/kg and higher. These doses induced an increase in preimplantation embryonic loss in female rats given DPTCl on days 0 to 3 of pregnancy (Ema et al. 1999b). No changes in serum estradiol levels in pseudopregnant rats were noted. These results suggest that DPTCl causes the suppression of uterine decidualization correlated with the reduction in serum progesterone levels. These are responsible for the DPTCl-induced implantation failures. The hormonal regimen consisting of progesterone and estrone supported decidual development in ovariectomized rats given DPTCl (Ema and Miyawaki 2002). The pregnancy rate and number of implantations in groups given DPTCl at 16.5 or 24.3 mg/kg in combination with progesterone were higher than those in the groups given DPTCl alone. These results show that the DPTCl-induced suppression of uterine decidualization is mediated, at least partially, by ovarian hormones, and that progesterone protects against the DPTCl-induced implantation failure.