

whereas no differential sensitivity was evident in NOAELs between the two groups (Pope and Liu, 1997)—a fact repeated with several chemicals in our series. Thus, we also considered the comparison of LOAELs among the newborn and young animal studies. Unfortunately, the traditional comparison of LOAELs has frequently suffered from a disparity in severities among studies, a situation that continues today with comparison of BMDLs.

This lead us again to employ a new terminology; the presumed unequivocally toxic level (pUETL) and defined it as the clear toxic dose giving similar severity for both newborn and young rats (at the same endpoints as far as possible). However, this was not simple to apply because the toxicity profile differed from chemical to chemical and also from newborn to young rats, the number per dose setting usually only being three in each group. Therefore, the most practical analytical strategy had to be a case by case approach. In most cases, the appropriate pUETL for either newborn or young rats was chosen first, thereafter the matching toxic dose or the range of doses was estimated giving similar severity for either group of rats, considering the whole data balance. Again, data from the dose-finding studies were also taken into account, especially considering the kinds of toxicity which appeared and the limits to be used. It should be noted that pUETL is not an absolute value, being different from pNOAEL/BMDL, but useful nevertheless to compare toxic responses between newborn and young rats at sufficient exposure.

This unique approach using two original definitions, with additional data from dose-finding studies concerning limitations, was fully supported by peer-reviewers of toxicology journals. On the other hand, the BMD approach for our whole data, including the dose-finding studies, was first conducted for this article.

#### 4. Comparison of sensitivity of newborn and young rats to chemicals

##### 4.1. Toxicity profiles

Critical toxicity data and the preliminary evaluation for 18 chemical studies have already been published in the literature. Table 1 shows a summary of the major findings for toxicities from the newborn and young studies. Fourteen chemicals commonly induced similar types of toxicities in both ages with the data considered in the pNOAEL or BMD approaches. With 3-ethylphenol and 1,1,2,2-tetrabromoethane, the toxicity profiles of both ages were not similar. In the case of 3-ethylphenol, the toxic similarity or difference between newborn and young rats cannot be predicted because of inadequate high dose setting in the newborn study. For 1,1,2,2-tetrabromoethane, hepatotoxicity in newborn rats can be speculated to appear at higher doses because a remarkable increase of relative liver weight was observed in the dose-finding study, although pathological examination was not conducted. In contrast, 2,4,6-trinitrophenol demonstrated a completely different profile of the major toxicities between the differently aged rats; also, tetrabromobisphenol A demonstrated unique toxicity in newborn rats.

##### 4.1.1. Specific toxicity to reproductive organs in newborn rats

Although specific developmental parameters such as preputial separation and vaginal opening were carefully examined in newborn studies, no significant changes for any chemicals were observed.

In the case of *p*-( $\alpha,\alpha$ -dimethylbenzyl) phenol, ovary weights were lowered at the end of the dosing as well as the recovery-maintenance periods and increased numbers

Table 1  
Major types or symptoms of toxicities of 18 industrial chemicals in newborn and young studies

Chemical name	Newborn studies	Young studies	References
4-Nitrophenol	Convulsions	Hypoactivity, convulsions	Koizumi et al. (2001)
2,4-Dinitrophenol	Hypoactivity, convulsions	Hypoactivity, convulsions	Koizumi et al. (2001)
3-Aminophenol	Tremors, thyroid hypertrophy	Tremors, thyroid hypertrophy, anemia	Koizumi et al. (2002)
2-Chlorophenol	Tremors, renal toxicity	Tremors, hypoactivity	Hasegawa et al. (2005)
4-Chlorophenol	Tremors	Tremors, tachypnea	Hasegawa et al. (2005)
2- <i>tert</i> -Butylphenol	Hypoactivity, ataxia	Hypoactivity, ataxia	Hirata-Koizumi et al. (2005b)
2,4-Di- <i>tert</i> -butylphenol	Hepatic and renal toxicity	Hepatic and renal toxicity	Hirata-Koizumi et al. (2005b)
3-Methylphenol	Tremors, hyperactivity	Tremors	Koizumi et al. (2003)
3-Ethylphenol	Low BW	Ataxia, forestomach lesions	Takahashi et al. (2006)
4-Ethylphenol	Hypoactivity, delayed reflexes	Ataxia, forestomach lesions	Takahashi et al. (2006)
<i>p</i> -( $\alpha,\alpha$ -Dimethylbenzyl) phenol	Renal toxicity, ovarian lesions	Renal toxicity, forestomach lesions	Hasegawa et al. (2005)
1,3,5-Trihydroxybenzene	Thyroid hypertrophy	Thyroid hypertrophy	Hasegawa et al. (2005)
2,4,6-Trinitrophenol	Low BW	Anemia, testicular atrophy	Takahashi et al. (2004)
(Hydroxyphenyl)methyl phenol	Low BW	Low BW, forestomach lesions	Hasegawa et al. (2005)
Trityl chloride	Low BW, hepatotoxicity	Low BW, hepatotoxicity	Hasegawa et al. (2005)
1,3-Dibromopropane	Low BW, hepatotoxicity	Low BW, hepatotoxicity, anemia	Hirata-Koizumi et al. (2005a)
1,1,2,2-Tetrabromoethane	Low BW	Hepatotoxicity	Hirata-Koizumi et al. (2005a)
Tetrabromobisphenol A	Renal toxicity	None	Fukuda et al. (2004)

BW: body weight.

of atretic follicles at the end of the dosing period. Most females continued to show various changes after the recovery-maintenance period, such as decreased numbers of corpora lutea in the ovaries, and hypertrophy of endometrial epithelium in the uteri. Therefore, further studies on this chemical should be conducted to elucidate the underlying mechanisms.

With (hydroxyphenyl)methyl phenol, some estrogenic effects were expected because it consists of bisphenol D, E and F isomers, and bisphenol F is reported to have estrogenic potential on the evidence of several *in vitro* and *in vivo* experiments (Hashimoto et al., 2001; Yamasaki et al., 2002; Stroheker et al., 2003). Some phenols such as nonylphenol, *p-tert*-octylphenol, bisphenol A and diethylstilbestrol have already been reported to induce morphological alteration of sex organs on early phase exposure after birth although the administration routes were either intraperitoneal or subcutaneous (Lee, 1998; Katsuda et al., 2000; Khan et al., 1998; Suzuki et al., 2002). The negative result in our study may be related to an insufficient component level of bisphenol F to induce such action.

#### 4.1.2. Other specific toxicity in newborn rats

There was one exceptional case of toxicity limited to newborn rats. Tetrabromobisphenol A induced polycystic kidneys at 200 and 600 mg/kg in newborn rats but not in doses up to 1000 mg/kg in the main young study and 6000 mg/kg for 18 days exposure in an additional young study. Such specific renal toxicity in newborn rats has also been described for other chemicals such as chlorambucil (Kavlock et al., 1987), tetrachloro-1,4-dibenzodioxine (Couture-Haws et al., 1991) and difluoromethylornithine (Gray and Kavlock, 1991). Kidney nephrons of rats are formed in the period of the advanced stage of pregnancy until 2 weeks after birth (Chevalier, 1998), only 10% of nephrons are present at birth (Merlet-Benichou et al., 1994). It is possible that developing renal tubules in newborn rats may be sensitive to induction of hyperplasia of the tubular epithelium in response to cellular damage, leading to polycystic lesions. Although this toxicity is unusual—at least in newborn rats—it seems reasonable to consider similar unusual potential effects in newborn humans for some chemicals.

#### 4.1.3. Specific toxicity in young rats

2,4,6-Trinitrophenol induced anemia and atrophy of seminiferous tubules of testes in young rats but only slight lowering of body weights in the main newborn study. Higher doses in the dose-finding newborn study induced severe suppression of body weight gain and death but not anemia or testicular toxicity. Sertoli cells in rats proliferate rapidly from day 19 of gestation to postnatal Day 15, then slow down and cease multiplying by approximately postnatal Day 20 (Orth, 1982, 1984; Toppari et al., 1996); 2,4,6-trinitrophenol seems unlikely to affect this stage rather affecting the maturation of spermatids. For anemia, the same

pattern, of anemia only in young rats, was found for 3-aminophenol and 1,3-dibromopropane. Although methemoglobin levels were not determined in this study, it was reported that methemoglobin reductase levels in newborn rats are distinctly higher than in young animals (Gruener, 1976; Lo and Agar, 1986), which could be a reason for higher susceptibility in the latter. Another possible explanation is that major metabolites such as picramic acid may damage seminiferous tubules as well as induce hemolytic anemia but the metabolic rate may be very slow in newborn rats because of low P450 content (Rich and Boobis, 1997).

Hyperplasia of squamous cells in forestomach was observed for 3-ethylphenol, 4-ethylphenol, *p*-( $\alpha,\alpha$ -dimethylbenzyl) phenol and (hydroxyphenyl)methyl phenol only at high toxic doses in young rats. Generally, phenols have similar toxicological effects due to their actions as extremely corrosive protoplasmic poisons (Manahan, 2003; Bloom and Brandt, 2001). The fact that the epithelium of the gastrointestinal tract of newborn rats may be more quickly renewed than that of young rats because of more active body metabolism in developing newborn rats, as well as a low capacity for gastric acid secretion, could explain any lower sensitivity in this regard.

#### 4.2. Comparison of pNOAELs and pUETLs

pNOAELs for newborn and young rats with all chemicals were re-evaluated as shown in Table 2. Single pNOAELs for newborn and young rats were estimated for most chemicals on the basis of careful analyses of the results from the dose-finding and main studies. In two cases we judged that specification of a single value was not appropriate and therefore ranges were adopted. In case of 3-methylphenol for newborn rats, tremors only with contact stimuli were noted in three males on single days at the medium dose of 100 mg/kg in the main study. Thus the overt NOAEL became the low dose of 30 mg/kg, but the realistic NOAEL was considered to be slightly lower than 100 mg/kg, supported by overt NOAEL at 100 mg/kg in the dose-finding study. Therefore, the pNOAEL was established in the range of 60–80 mg/kg/day for more accurate comparison with data from the young study. The second case concerned the value for 2,4,6-trinitrophenol for newborn rats because they showed only a slight lowering of the body weight at 61.5 mg/kg and the low dose of 16.1 mg/kg was not considered appropriate as the pNOAEL; we adopted the range of 40–50 mg/kg/day instead. It should be noted that the pNOAEL of 1000 mg/kg/day of young rats for tetrabromobisphenol A is also not realistically appropriate because it was the highest limit dose indicated in the Test Guideline. As for estimation of pUETL, 8 values were given as ranges based on the definition of matching toxic dose ranges to induce clear toxicity at similar severity as described earlier. There were two chemicals without matches: 3-ethylphenol and tetrabromobisphenol A. For the former case, a dose in

Table 2  
Summary of pNOAELs and pUETLs for 18 industrial chemicals in newborn and young rats

Chemical name	Newborn studies		Young studies		Young/Newborn	
	pNOAEL	pUETL	pNOAEL	pUETL	pNOAEL	pUETL
	(mg/kg/day)		(mg/kg/day)			
4-Nitrophenol	110	230	400	600–800	3.6	2.6–3.5
2,4-Dinitrophenol	10	30	20	80	2.0	2.7
3-Aminophenol	80	240	240	720	3.0	3.0
2-Chlorophenol	40	200–250	200	1000	5.0	4.0–5.0
4-Chlorophenol	100	300	100	500	1.0	1.7
2- <i>tert</i> -Butylphenol	20	100–150	100	500	5.0	3.3–5.0
2,4-Di- <i>tert</i> -butylphenol	5	100	20	500	4.0	5.0
3-Methylphenol	60–80	300	300	1000	4.0–5.0	3.3
3-Ethylphenol	100	—	300	—	3.0	—
4-Ethylphenol	30	200–250	100	1000	3.3	4.0–5.0
<i>p</i> -( $\alpha,\alpha$ -Dimethylbenzyl) phenol	30	300	100	700–800	3.3	2.3–2.7
1,3,5-Trihydroxybenzene	100	500	300	1000	3.0	2.0
2,4,6-Trinitrophenol	40–50	65	20	100	0.4–0.5	1.5
(Hydroxyphenyl)methyl phenol	100	140–160	40	1000	0.4	6.3–7.1
Trityl chloride	60	400–500	12	300	0.2	0.6–0.8
1,3-Dibromopropane	50	150	10	250	0.2	1.7
1,1,2,2-Tetrabromoethane	50	200	6	300–400 <sup>a</sup>	0.1	1.5–2.0
Tetrabromobisphenol A	40	—	1000 <sup>b</sup>	—	25 <sup>b</sup>	—

—: Appropriate values were not able to be given.

<sup>a</sup> These range values were estimated on the basis of all relevant toxicity data, including single dose toxicity data in young rats (the lowest mortality dose was 722 mg/kg for males and 852 for females) (MHLW, 2003).

<sup>b</sup> No accurate values for pNOAEL and pNOAEL ratio could be generated because 1000 mg/kg/day is the highest dose indicated in the Test Guideline.

newborn rats matching the toxic dose in young rats could not be predicted because the high dose in newborn rats did not induce any clear toxicity. The latter is that the high dose in young rats did not induce any toxicity.

The last column in Table 2 shows ratios for the young/newborn pNOAELs, and young/newborn pUETLs. Among the pNOAEL ratios for all 18 chemicals, newborn rats were less or nearly equal in sensitivity (less than 2-fold) to 6 chemicals (33%), clearly more sensitive (2–5-fold) to 11 chemicals (61%) and more than 25-fold for one exceptional case (6%). The mean ratio was 3.9 for all 18 chemicals or 2.5 for all but the exceptional case. Among the available pUETL ratios for 16 chemicals, 5 were less or nearly equal in newborn rats (less than 2-fold change) (31%) and 11 chemicals were clearly more toxic (2–8-fold) (69%). The mean ratio was 3.1 for the 16 chemicals.

Based on reliable calculated ratios for our two endpoints, approximately 94% of values (32 out of 34 ratios) demonstrated differences of 5-fold or less, one chemical had a 6–8-fold variation, and in the case of a 25-fold ratio of tetrabromobisphenol A, the nephrotoxicity in newborn rats is a specific toxicity rather than a higher susceptibility to the same toxic endpoint in young rats. These same ratios can be used to state that a higher susceptibility (more than 2-fold) in newborn rats was found for 62% of all tested chemicals in terms of pNOAELs and pUETLs, via oral repeated administration.

To appraise correlations between pUETL and pNOAEL ratios (young/newborn rats), available values were plotted on a logarithmic scale in a correlation diagram. As shown in Fig. 2, two separate groups became

apparent, group 1 has the same or lower pNOAELs for newborn than young rats, and group 2 has higher pNOAELs for newborn than young rats. The mechanistic speculation for the differences is discussed next.

#### 4.3. Speculation on differences in responses between low and high doses in newborn and young rats

Immature functions of organs (especially the liver and kidneys), in newborn rats may contribute to the difference of response. There were at least two types of dose response curve shifts between newborn and young rats, as illustrated in Fig. 3. The first was a parallel shift from right (young) to left (newborn) for 12 phenolic chemicals (group 1). The other 5 chemicals demonstrated a steeper shaped curve in newborn than young rats but young rats were clearly more sensitive around the pNOAEL doses (group 2).

Group 1 chemicals may primarily have direct actions on their target organs such as the central nervous system, kidneys or thyroid. They may be detoxified by the formation of conjugates, for example, glucuronidation of 4-nitrophenol (Robinson et al., 1951) and 3-methylphenol (Bray et al., 1950). UDP-glucuronyltransferase activity at birth in the rat liver is known to be comparable to that in adults but nearly 50% lower during nursing (Watkins and Klaassen, 1985; Rachmel and Hazelton, 1986). Therefore, a low capacity for glucuronidation may be one of the major causes of higher susceptibility of newborn rats to these phenols. This may also occur in human infants since immature hepatic glucuronidation and low activity of bilirubin glucuronidation at birth have been shown in human infants

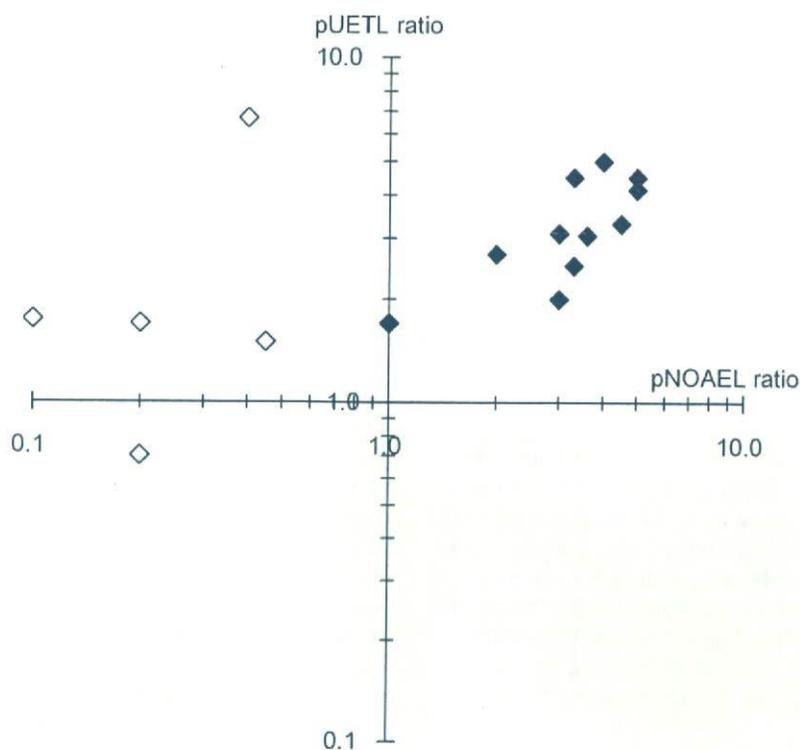


Fig. 2. Correlations of pUETL and pNOAEL ratios (young/newborn). Each point is plotted on a logarithmic scale from the ratios for young/newborn pUETLs and pNOAELs. Closed and open diamonds indicate group 1 and 2 chemicals, respectively.

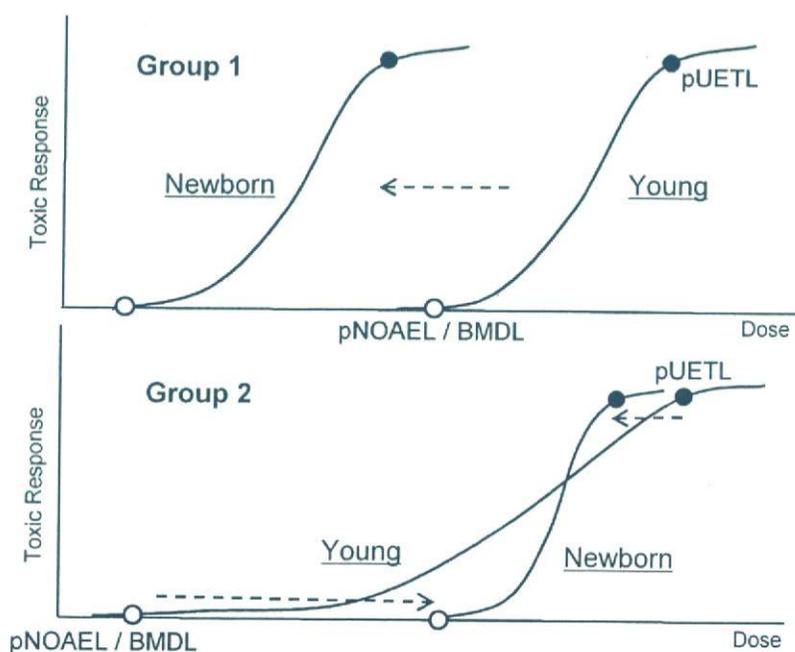


Fig. 3. Illustration of two patterns of shift of dose response curves from young to newborn rats.

(Gow et al., 2001; Kawade and Onishi, 1981). In addition, there is a possibility that high susceptibility may be due to a low capacity for hepatic cytochrome P450 (Rich and Boobis, 1997) and renal excretion (Horster, 1977), and

increased permeability of the blood-brain barrier (Cremer et al., 1979).

Group 2 chemicals did not demonstrate as many effects at the low dose but nearly the same or higher

number of effects at the high dose in newborn compared to young rats. These chemicals may need metabolic activation to exert toxic effects. Newborn rats have been shown to have a low content of hepatic cytochrome P450 (Rich and Boobis, 1997) and a drop of glutathione-S-transferase activity in the early days after birth (Tee et al., 1992). Therefore, production of active metabolites may be significantly lower in newborn rats. In fact, it has been suggested that 1,1,2,2-tetrabromoethane requires an oxidative biotransformation to produce active intermediates (Kennedy et al., 1993) and 1,3-dibromopropane is conjugated with glutathione before or after oxidative biotransformation (James et al., 1981) as is common for dihaloalkanes or dihaloalkenes (Zoetemelk et al., 1986; Trevisan et al., 1989). However, pUETLs for newborn rats for 4–5 chemicals were in approximately the same ranges as in young rats. Although major reasons for variation in susceptibility are unclear, one possible explanation might be a low capacity for protection against deleterious oxidative stress in the newborn when the toxic chemical burden crosses a threshold in the liver, which has a low activity of catalase and glutathione peroxidase during the nursing period (Yoshida et al., 1982).

#### 4.4. Application trial of the BMD approach

We attempted to derive BMDLs as sensitive and appropriate endpoints in each study in addition to pNOAELs whenever possible. These calculated values are shown in

Table 3  
Summary of BMDL values and ratios for 18 industrial chemicals in newborn and young rats

Chemical name	Newborn studies (mg/kg/day)	Young studies (mg/kg/day)	Young/newborn
4-Nitrophenol	141	392	2.8
2,4-Dinitrophenol	11	14	1.3
3-Aminophenol	54	254	4.7
2-Chlorophenol	31	126	4.1
4-Chlorophenol	79	63	0.8
2- <i>tert</i> -Butylphenol	43	130	3.0
2,4-Di- <i>tert</i> -butylphenol	7.5	48	5.1
3-Methylphenol	50	397	7.9
3-Ethylphenol	276	376	1.4
4-Ethylphenol	53	173	3.3
<i>p</i> -( $\alpha,\alpha$ -Dimethylbenzyl) phenol	28	42	1.5
1,3,5-Trihydroxybenzene	63	206	3.3
2,4,6-Trinitrophenol	41	15	0.4
(Hydroxyphenyl)methyl phenol	108	42	0.4
Trityl chloride	34	6.8	0.2
1,3-Dibromopropane	32	6.1	0.2
1,1,2,2-Tetrabromoethane	82	3.1	0.04
Tetrabromobisphenol A	45	—	—

—, Appropriate values could not be generated because no toxicity was apparent in the young study.

Table 3. Most BMDLs seem to be relatively close to the corresponding pNOAELs but there are some cases in which BMDLs were lower than the probable values from toxicity profiles. One major reason may be the nature of the toxicity data used for the BMDL calculations. For example, no changes were observed with histopathological data in the young study for trityl chloride at 12 mg/kg, only slight changes in 3 of 6 animals at 60 mg/kg, and 4 mild and 2 moderate levels of change in 6 animals at 300 mg/kg. For the BMDL estimation from these data we input an incidence of 3 animals in 6 at 60 mg/kg and 6 animals in 6 animals at 300 mg/kg, even though the severities of these changes were different. So an actual dose response curve was obviously steeper than the input data curve, leading to a lower BMDL of 6.8 mg/kg/day, compared to the pNOAEL of 12 mg/kg/day. Nonetheless, Fig. 4 shows a good relationship between pNOAEL and BMDL since the correlation coefficient was 0.904 (calculated without logarithmic conversion). The BMDL ratios in Table 3 are slightly lower than pNOAEL ratios in Table 2, with 9 chemicals (53%) demonstrating less or nearly equal sensitivity in newborn rats (less than 2-fold) and 8 chemicals (47%) demonstrating more sensitivity (2–8-fold) in newborn rats. However, a correlation diagram of the pUETL ratios versus the BMDL ratios also showed the same profile as Fig. 2 (not shown here). Therefore, the BMD approach can be considered very useful for the present purposes and somewhat easier than our pNOAEL estimation because extensive experience in toxicology is necessary for the latter estimations.

#### 5. Discussion of pediatric susceptibility

Major uncertainty exists in the derivation of human safety doses from animal experimental data. This uncertainty consists primarily of toxicokinetic and toxicodynamic differences between experimental animals and humans and among humans, and is addressed through the use of two factors, inter-species differences and human variability (intra-species differences). For either factor, a value of 10-fold has generally been applied for most assessments.

The aim of risk assessment is to derive the estimated no adverse toxic response level in sensitive humans. Thus, NOAELs or BMDLs are used as the starting point values, and not higher doses exhibiting toxicity, although descriptions of such toxicity provide critical information on risk assessment. Human variability implies appreciable differences of NOAELs or BMDLs between average populations and sensitive subpopulations as indicated by Dourson et al. (2002). Since the general human population or a more uniform experimental animal population is typically the focus group for toxicity evaluation, risk assessment needs to include sensitive subpopulations, such as infants, children, the elderly, and specific subgroups with minor diseases or relevant genetic polymorphisms. However, some hypersusceptible individuals might be excluded, for example, patients with severe hepatic or renal dysfunction should

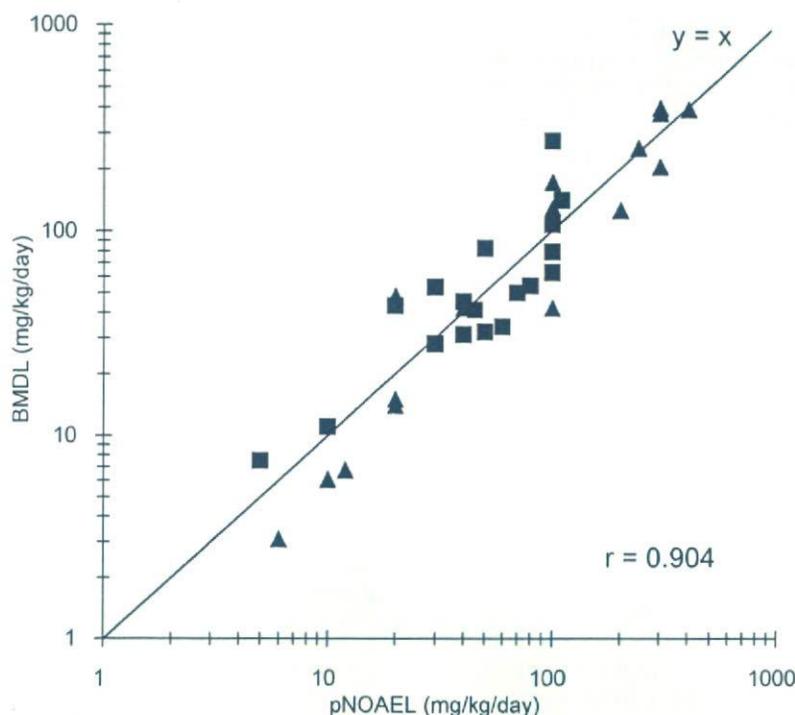


Fig. 4. Correlation between pNOAELs and BMDLs from both newborn (closed squares) and young (triangles) studies.

be excluded because they may be extremely sensitive to chemicals due to their impaired capacity for detoxification or excretion. Pregnant women and fetuses are also sensitive subpopulations, but the toxicity to these groups is routinely tested by reproductive and developmental toxicity studies.

Recently, Dourson et al. (2002) summarized considerations on adequacy of uncertainty factors of human variability for children. With human data, Glaubiger et al. (1981) reported that overall differences in sensitivity between children and adults are quite small on the basis of comparing maximum tolerated doses for 17 anticancer drugs. Using animal data analyses, Charnley and Putzrath (2001) demonstrated that younger animals appear to be less susceptible to 47% and more susceptible to 40% of the tested chemicals in carcinogenesis studies. Sheehan and Gaylor (1990) showed newborn mammals to be more sensitive than adults (86% within 10-fold) by comparing  $LD_{50}$  ratios for 238 chemicals. Calabrese (2001) also reported that younger animals have a greater sensitivity than older animals in 54% of cases (more than 10-fold in 14%) with  $LD_{50}$  ratio analyses for 313 chemicals. All these reports suggest the degree of variation in sensitivity of infants/younger animals as compared to adults for most chemicals may be within 10-fold, so that a 10-fold uncertainty factor may be sufficient to cover the variation (Dourson et al., 2002). However, only Sheehan and Gaylor (1990) targeted newborn rather than young animals and the report was a meeting abstract.

Concerning the methodology for risk assessment with repeated exposure, NOAELs or BMDLs from repeated-dose toxicity studies are starting values to derive risk values such

as acceptable daily intake (ADI) or tolerable daily intake (TDI). These studies might be as short as 28 days or as long as 2 years, but invariably dosing generally starts around 6 weeks of age for rodents. These animals are referred to as “young” in this article rather than adult because their growth is still vigorous. Therefore, toxicity responses of young animals, equivalent to late childhood in humans, may already be covered by the general repeated-dose toxicity studies (see also Table 2 of Dourson et al., 2002 which summarized work by Scheuplein et al., 2002, on this point).

However, only limited data exist for animals from birth to 5 or 6 weeks of age. During these initial few weeks after birth, susceptibility to toxic insult might be expected to be greater than at later periods because organ growth rates are higher. Moreover, metabolism and elimination pathways are not yet mature (see for example the discussion of kinetic comparisons of newborn, infants and children as compared with adults by Rane, 1992 and Renwick, 1998 in Dourson et al., 2002). Although for some chemicals this lack of maturation in metabolism and elimination might serve to protect the newborn, it is clearly very important to clarify newborn sensitivity versus young animal sensitivity. Thus we have designed our newborn rat study protocol (18 day newborn study) to follow the conditions of the 28 day repeated-dose toxicity study (onset of administration at 5–6 weeks old) as closely as possible using 18, mostly phenolic, compounds. In addition to the unique design of the 18 day newborn study, new clarifying terminology has been developed, pNOAELs and pUETLs, in order to more appropriately determine ratios between newborn and young studies.

Our analyses of 18 such pNOAEL ratios or 17 BMDL ratios revealed less or nearly equal sensitivity in newborn animals (less than 2-fold) in 33–53%, clearly greater sensitivity (2–8-fold) in 47–61% and one exceptional case of more than 25-fold sensitivity in the newborn. In the case of 16 pUETL ratios, 31% of chemicals showed less or nearly equal toxicity in newborns (less than 2-fold) and 69% more toxicity (2–8-fold) in newborns. This distribution and the extent of newborn susceptibility in toxicity are in line with the conclusions of several investigators summarized previously by Dourson et al. (2002), but evidence presented here is more direct because of careful design of the comparative studies and comprehensive toxicological analyses and judgments. In addition, two kinetic analyses showing newborns to be more sensitive than adults, with a 3.5 arithmetic average difference in elimination half life (Rane, 1992) or a 4-fold longer average half life (Ginsberg et al., 2002), support relatively similar degrees of average susceptibility as we have found.

Collectively, all of this work suggests that studying the early life stage sensitivity to toxic insult is important. When such studies determine the critical effect, then ADIs or TDIs should be based on their findings. When such studies do not determine the critical effect, then the ADI or TDI is appropriately based on a critical effect found in a different study and the newborn is protected. It is when such studies have not been conducted that uncertainty factors must be invoked to protect the newborn, and other potential sensitive subpopulations, and several investigators have looked at the adequacy of such factors (e.g., Burin and Saunders, 1999; Dourson et al., 2002). Based on our results and those of other investigators, we suggest that an uncertainty factor of 10-fold for human variability and an uncertainty factor of between 3- and 10-fold for database completeness can be considered appropriate for risk assessment unless knowledge of particular toxicity in newborn or infants is present, or if not present is discountable due to other credible information on the chemical.

In conclusion, newborn rats are clearly more susceptible than young animals (at most 8-fold) to two thirds of the present series of 18 chemicals, mostly phenolic substances, and less or nearly equal sensitive to the others for oral repeated exposure. However, it should be noted that there was one exceptional case in which the toxicity appeared only in newborn rats. These repeated oral exposure newborn studies are unique for this limited group of chemicals, and perhaps for other chemicals as well.

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## ORIGINAL ARTICLE

## Comparative susceptibility of newborn and young rats to six industrial chemicals

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**ABSTRACT** To elucidate the comparative susceptibility of newborn rats to chemicals, newborn and young animals were administered six industrial chemicals by gavage from postnatal days (PND) 4 to 21, and for 28 days starting at 5–6 weeks of age respectively, under the same experimental conditions as far as possible. As two new toxicity endpoints specific to this comparative analysis, presumed no-observed-adverse-effect-levels (pNOAELs) were estimated based on results of both main and dose-finding studies, and presumed unequivocally toxic levels (pUETLs) were also decided. pNOAELs for newborn and young rats were 40 and 200 for 2-chlorophenol, 100 and 100 for 4-chlorophenol, 30 and 100 for p-( $\alpha,\alpha$ -dimethylbenzyl) phenol, 100 and 40 for (hydroxyphenyl)methyl phenol, 60 and 12 for trityl chloride, and 100 and 300 mg/kg/day for 1,3,5-trihydroxybenzene, respectively. To determine pUETLs, dose ranges were adopted in several cases because of the limited results of experimental doses. Values for newborn and young rats were thus estimated as 200–250 and 1000 for 2-chlorophenol, 300 and 500 for 4-chlorophenol, 300 and 700–800 for p-( $\alpha,\alpha$ -dimethylbenzyl) phenol, 140–160 and 1000 for (hydroxyphenyl)methyl phenol, 400–500 and 300 for trityl chloride, and 500 and 1000 mg/kg/day for 1,3,5-trihydroxybenzene, respectively. In most cases, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL. An exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.

**Key Words:** industrial chemicals, newborn rats, susceptibility

## INTRODUCTION

In risk assessment of chemicals, the no-observed-adverse-effect-level (NOAEL) determined with repeated dose toxicity studies is generally divided by uncertainty factors (UFs) to obtain the tolerable daily intake (TDI) (Hasegawa *et al.* 2004). UFs include inter- and intraspecies differences, lack of data quality and the nature of observed toxicity. As TDI is an allowable lifetime exposure level for a chemical, at which no appreciable health risk would be expected over a lifetime, the NOAEL must be derived from lifetime exposure studies and appropriate reproductive/developmental studies, or their equivalents. Administration generally starts at the prepubertal stage (4–5 weeks old) or with young adults (10–12 weeks old) in rodent studies. Therefore, the suckling phase is the major remaining period where animals are not directly administered to chemicals. If susceptibility of infant animals to chemicals via direct

exposure was evidenced by appropriate comparative studies, the results would preferably be incorporated into the UF as one justification for lack of data quality.

In the latest decade, infant and child health has become a major focus (Landrigan *et al.* 2004), especially since endocrine disruptors became a contentious issue around the world (IPCS 2002). Since there are distinct differences in characteristics from the adult case (Dourson *et al.* 2002), particular attention must be paid to infant and child health. The Japanese government has therefore incorporated the newborn rat study (newborn study) into Existing Chemical Safety Programs as an especial project to comparatively determine susceptibility to 18 industrial chemicals. As the core of this program is to conduct 28-day repeated dose toxicity studies using young rats (young study) with untested chemicals from the existing list, chemicals for newborn studies were selected among the chemicals scheduled for young studies in the same year for the best comparison of data. Furthermore, we have had to newly establish a newborn rat study protocol because of the lack of any standard testing guidelines. Major differences of newborn from young studies are a shorter administration period (18 days only for the suckling phase) and additional examination of early functional, external and sexual development (Koizumi *et al.* 2001). Studies were conducted from 1995 to 1998 and we have already reported the results of comparative analysis for eight chemicals, showing newborn rats to be generally 2–4 fold more susceptible than young rats in most cases on basis of NOAEL and the unequivocally toxic level (UETL), the latter being uniquely defined in this program as doses inducing clear clinical toxic signs, death or critical histopathological damage (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005).

The purpose of this study is to obtain additional information on susceptibility of newborn rats to other chemicals. Here we selected the following six industrial chemicals, mostly phenolic compounds: 2-chlorophenol, 4-chlorophenol, p-( $\alpha,\alpha$ -dimethylbenzyl) phenol (hydroxyphenyl)methyl phenol, trityl chloride and 1,3,5-trihydroxybenzene, because of structural similarity to endocrine-disrupting phenols, bisphenol A (Takahashi & Oishi 2001), and nonylphenol (Lee 1998). These chemicals have been used as an intermediate in dyes and an ingredient in pesticides (2-chlorophenol), an intermediate in dyes, bactericides and an ingredient in cosmetics (4-chlorophenol), an ingredient in surfactants, bactericides, an intermediate in pesticides and plasticizers (p-( $\alpha,\alpha$ -dimethylbenzyl) phenol), an ingredient in resins ((hydroxyphenyl)methyl phenol), an intermediate in medicines (trityl chloride) and an ingredient in medicines, a stabilizer of synthetic rubbers and an adhesive of rubbers (1,3,5-trihydroxybenzene) (Chemical Products' Handbook 2004). Under the same experimental conditions as far as possible, we have examined the repeated dose toxicity of these chemicals in newborn and young rats and compared susceptibility for each. Previously we had applied NOAEL and UETL as estimated doses

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or ranges of doses for comparison of chemical susceptibility, but we have decided to employ the new terminology of presumed NOAEL (pNOAEL) and presumed UETL (pUETL) in their place. As a result, in most cases newborn rats were more susceptible to these industrial chemicals than young rats in terms of both pNOAEL and pUETL.

## MATERIALS

2-Chlorophenol (CAS no. 95-57-8, Lot no. OJL-15, purity: 99.49%) was obtained from Inui Corporation and prepared in olive oil; 4-chlorophenol (CAS no. 106-48-9, Lot no. PJF-3, purity: 99.29%) from Inui Corporation and in corn oil; p-( $\alpha,\alpha$ -dimethylbenzyl) phenol (CAS no. 599-64-4, Lot no. 101002, purity: 99.88%) from Sun TechnoChemical Inc. in olive oil; (hydroxyphenyl)methyl phenol (CAS no. 1333-16-0, Lot no. S980013, purity: 99.0% [2,2' isomer 14–18%, 2,4' isomer 44–48%, 4,4' isomer 26–32%]) from Mitsui Chemicals, Inc. in 0.5% CMC-Na solution containing 0.1% Tween 80; trityl chloride (CAS no. 76-83-5, Lot no. 1038, purity: 99.5%) from Kurogane Kasei Co. Ltd. in olive oil; and 1,3,5-trihydroxybenzene (CAS no. 108-73-6, Lot no. OS-12074, purity: 99.9%) from Ishihara Sangyou Co., Ltd. in olive oil. Test solutions were prepared at least once a week and were kept cool and in the dark until dosing. The stability was confirmed to be at least seven days under these conditions. All other reagents used in this study were specific purity grade.

## METHODS

All animal studies were performed in five testing laboratories contracted to the Japanese Government, after we approved the test protocol.

### Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at  $24 \pm 2^\circ\text{C}$  with a relative humidity of  $55 \pm 15\%$ , a ventilation rate of more than 10 times per hour, and a 12:12 h light/dark cycle. For the studies of newborns, 20 pregnant rats (shipped in at gestation day 14) were allowed to deliver spontaneously. All newborns were separated from dams on postnatal day (PND) 3 and groups of 12 males and 12 females were selected and assigned to each of the four dose groups, including the controls. Twelve foster mothers were selected based on health and nursing conditions, and suckled the four males and four females assigned to each group up to weaning on PND 21 (termination of dosing and autopsy for half of the animals). After weaning, the rest of the animals for the recovery-maintenance group (see Study Design) were individually maintained for nine weeks. In the studies of young, four-week-old male and female rats were obtained and used at ages of 5–6 weeks after acclimation. All animals were allowed free access to a basal diet and water.

### Study design (time schedule as described previously [Koizumi et al. 2001])

#### 1. 18-day repeated dose study in newborn rats (newborn study)

In a dose-finding study, chemicals were administered by gastric intubation to newborn male and female rats on PNDs 4–21. Animals were examined for general behavior and body weights during the dosing period, and sacrificed at PND 22 for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, newborn rats (12/sex/dose) were administered chemicals by gastric intubation on PNDs 4–21, the dosage being set on the basis of results of the dose-finding study. On PND 22, half of the animals were sacrificed and the rest were maintained for nine weeks without chemical treatment, and then sacrificed at 12 weeks of age (the recovery-maintenance group). During the study, general behavior and body weight were examined at least once a day and each week, respectively. In addition, developmental parameters were assessed, such as surface righting and visual placing reflex for reflex ontogeny, fur appearance, incisor eruption and eye opening for external development, and preputial separation, vaginal opening and estrous cycle for sexual development. Urinalysis (color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, volume of the urine and osmotic pressure) was conducted in the late recovery-maintenance period.

At weaning age PND 22 after the last treatment, blood was collected under anesthesia from the abdomen of all animals in the scheduled-sacrifice group. In the recovery-maintenance group, this was conducted at 85 days of age after overnight starvation. Blood was examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count, and for biochemistry (total protein, albumin, albumin/globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase, alanine aminotransferase (ALT), alkaline phosphatase,  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP), calcium, inorganic phosphorus, sodium, potassium and chlorine). Prothrombin time and activated thromboplastin time were examined only in the recovery-maintenance group. The brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, ovaries and uterus were weighed, and these, with other macroscopically abnormal organs, were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides). Paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. All studies were conducted in compliance with the Good Laboratory Practice Act of the Japanese Government.

#### 2. 28-day repeated dose study in young rats (young study)

In a dose-finding study, chemicals were administered by gastric intubation to five-week-old male and female rats for 14 days. The general behavior, body weight and food consumption were examined, and the animals were sacrificed the day after the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, 5–6 week old male and female rats were given chemicals by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

### Statistical analysis

Quantitative data were analyzed by Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recog-

nized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups. If not homogeneous, the data were analyzed using Steel's multiple comparison test (Steel 1959) or the mean rank test of the Dunnett type (Hollander & Wolfe 1973). For qualitative data such as histopathological findings, the Mann-Whitney's *U*-test (Mann & Whitney 1947) or the Fisher's exact test (Fisher 1973) were performed.

#### Adoption of pNOAEL and pUETL

NOAEL is a measure used in toxicity studies for the greatest dose at which no adverse effects are observed. No toxicologically meaningful changes are excluded for any grounds, including increase of relative organ weights without any other related changes. As the present purpose was to elucidate susceptibility of newborn rats to chemicals as compared with young rats as accurately as possible, simple application of NOAELs obtained from newborn and young main studies was considered not to be necessarily appropriate even though the dose setting is pertinent. Therefore, we newly defined a pNOAEL as the most likely estimated no-adverse-effect-dose on the basis of data from both main and dose-finding studies. As urinalysis and histopathological examination were not conducted in both dose-finding studies, and the administration period in young dose-finding study was half of the main study, we carefully weighed how the results from the dose-finding study should be taken into account, especially concerning the type of toxicity. In order to consider equivalently toxic intensity doses for newborn and young rats, we also newly defined a pUETL, although this is not without problems given the limited dose points. Therefore, in the most cases, the appropriate pUETL for either newborn or young rats was chosen first, thereafter the matching pUETL or the range of pUETL was speculated to assess equivalent toxicity, considering the entire body of data.

## RESULTS

### 2-Chlorophenol (Table 1)

The newborn investigation was conducted at doses of 0, 20, 100, and 500 mg/kg for the dose-finding and 0, 8, 50, and 300 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 200, and 500 mg/kg for the dose-finding and 0, 8, 40, 200, and 1000 mg/kg for the main study.

Major toxic effects on the central nervous system (CNS) were found in both sexes of newborn and young rats. In the newborn study, tremors appeared within five minutes and disappeared within four hours in most animals at 300 mg/kg. Hypoactivity and an abnormal gait were also observed in a few cases. The histopathological examination showed slight to moderate basophilic renal tubules in more than half the animals of both sexes, without relative kidney weight changes (increase by 8% for males, 4% for females). In addition to these effects, the body weights of both sexes at this dose were transiently decreased. At 50 mg/kg, only one female showed tremors once from 15 to 30 minutes on day nine after the dosing start. There were no chemical-related changes in developmental parameters. In the young study, most animals of both sexes sporadically showed various effects on the CNS such as tremors, hypoactivity, and an abnormal gait within three hours after dosing at 1000 mg/kg. Most animals also exhibited slight centrilobular hypertrophy of hepatocytes, suggesting a compensatory response to a requirement for hepatic metabolism. In the dose-finding study, no toxic signs were observed, but the information was limited because of the small number of animals, the short administration period, and the lack of histopathological examination. There were no chemical-related abnormalities at 200 mg/kg in the main study.

Although the NOAEL was 8 mg/kg/day for newborn rats based on the main study results, this value was concluded to be too low

Table 1 Toxicity findings for 2-chlorophenol in the newborn and young rat main studies

	Newborn study (mg/kg)					Young study (mg/kg)			
	0	20†	50	100†	300	0	200	500†	1000
Male									
General behavior									
Tremors	0/12	0/4	0/12	0/4	11/12	0/12	0/12	0/3	4/12
Hypoactivity	0/12	0/4	0/12	0/4	2/12	0/12	0/12	0/3	8/12
Abnormal gait	0/12	0/4	0/12	0/4	1/12	0/12	0/12	0/3	4/12
Histopathology									
Renal tubules, basophilic	0/6	no data	0/6	no data	4/6	0/6	0/6	no data	0/6
Centrilobular hypertrophy	0/6	no data	0/6	no data	0/6	0/6	0/6	no data	6/6
Female									
General behavior									
Tremors	0/12	0/4	1/12	0/4	12/12	0/12	0/12	0/3	5/12
Hypoactivity	0/12	0/4	0/12	0/4	3/12	0/12	0/12	0/3	5/12
Abnormal gait	0/12	0/4	0/12	0/4	1/12	0/12	0/12	0/3	7/12
Histopathology									
Renal tubules, basophilic	0/6	no data	0/6	no data	5/6	0/6	0/6	no data	0/6
Centrilobular hypertrophy	0/6	no data	0/6	no data	0/6	0/6	0/6	no data	5/6

Only data for items showing change are included in this table. Data are numbers of animals with the change of the total examined. †indicates dose and data from the dose-finding study. All newborn animals died by the 9th dosing day at 500 mg/kg in the dose-finding study. Body weights of both sexes were only transiently, but not finally reduced, at 300 mg/kg in the newborn main study. Clinical signs in newborn rats were not observed at doses of 20 and 100 mg/kg in the dose-finding study.

because of the absence of clinical signs at 20 and 100 mg/kg in the dose-finding study, and only one female showed tremors once at 50 mg/kg in the main study. The pNOAEL for newborn rats was therefore estimated to be 40 mg/kg/day, a little below the 50 mg/kg. For young rats, the pNOAEL can be considered to be 200 mg/kg/day because of the limited information at 500 mg/kg in the dose-finding study. The toxicity at 300 mg/kg for newborn rats seemed to be slightly higher than that at 1000 mg/kg for young rats, because of the transient depression of body weight found limited to the former cases, although the toxicity profile regarding the CNS was very similar in newborn and young rats. The dose for newborn rats showing the same toxic intensity, as that for young rats at 1000 mg/kg, is considered to be slightly lower than 300 mg/kg, at 200–250 mg/kg/day. Therefore, pUETLs of 200–250 and 1000 mg/kg/day may be considered equivalent doses for newborn and young rats, respectively.

#### 4-Chlorophenol (Table 2)

The newborn investigation was conducted at doses of 0, 20, 100, and 500 mg/kg for the dose-finding and 0, 12, 60, and 300 mg/kg for the main study. With young rats doses of 0, 20, 100, and 500 mg/kg were applied in both dose-finding and main studies.

Toxic effects on the CNS were observed in both sexes of newborn and young rats. Most newborn rats at 500 mg/kg in the dose-finding study showed tremors, hypoactivity, bradypnea and hypothermia, and died. All newborn rats at 300 mg/kg exhibited tremors, mostly within 15 minutes to one hour, but these completely disappeared within four hours after dosing. There were no abnormalities at 100 mg/kg in the dose-finding, and 60 and 12 mg/kg in the main study. No developmental abnormalities were observed at any dose in the newborn dose-finding and main studies. In the young study, tremors, tachypnea and salivation were observed from five to 30 minutes after dosing in most animals in

both sexes at 500 mg/kg. There were no other dose-dependent changes at any dose.

The pNOAEL for newborn rats is considered to be 100 mg/kg/day, because CNS toxicity was not observed at 100 mg/kg in the dose-finding study. The pNOAEL for young rats must be set at 100 mg/kg/day, because there were no doses set between 100 and 500 mg/kg. Although the toxicity profile regarding the CNS differed to some extent between newborn rats at 300 mg/kg and young rats at 500 mg/kg with respect to symptom appearance and duration, the same level can be concluded, considering the specific characteristics of the newborn body. Thereby, pUETLs of 300 and 500 mg/kg/day were estimated as appropriate for newborn and young rats, respectively.

#### p-( $\alpha,\alpha$ -Dimethylbenzyl) phenol (Table 3)

The newborn investigation was conducted at doses of 0, 30, 100, and 300 mg/kg for both dose-finding and main studies. The young investigation was conducted at doses of 0, 250, 500, and 1000 mg/kg for dose-finding and 0, 100, 300, and 1000 mg/kg for the main study.

No newborn animals died although the body weights of both sexes were transiently lowered at 300 mg/kg (8% maximum decrease). General behavior, functional parameters and urinalysis, hematology and biochemistry data were all within normal ranges except for high urinary volume in males and high BUN in females at 300 mg/kg. The relative kidney weights were increased more than double at 300 mg/kg in both sexes, and dilation of tubules and papillary ducts was observed at relatively high grades in kidneys of both sexes, with no complete recoveries even after a nine-week recovery-maintenance period. Such histopathological change in kidneys was also slightly observed at 100 mg/kg in both sexes. In addition, there were effects on the endocrine systems, despite no effects on sexual differentiation. Absolute testicular weights were reduced by 16% at 300 mg/kg and ovary weights by 26% at 100

Table 2 Toxicity findings for 4-chlorophenol in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)		
	0	60	100†	300	0	100	500
<b>Male</b>							
General behavior							
Tremors	0/12	0/12	0/4	12/12	0/12	0/6	12/12
Tachypnea	0/12	0/12	0/4	0/12	0/12	0/6	11/12
Salivation	0/12	0/12	0/4	0/12	0/12	0/6	9/12
Histopathology							
Kidney	0/6	0/6	no data	0/6	0/6	0/6	0/6
Liver	0/6	0/6	no data	0/6	0/6	0/6	0/6
<b>Female</b>							
General behavior							
Tremors	0/12	0/12	0/4	12/12	0/12	0/6	11/12
Tachypnea	0/12	0/12	0/4	0/12	0/12	0/6	9/12
Salivation	0/12	0/12	0/4	0/12	0/12	0/6	8/12
Histopathology							
Kidney	0/6	0/6	no data	0/6	0/6	0/6	0/6
Liver	0/6	0/6	no data	0/6	0/6	0/6	0/6

Data are numbers of animals with the change of the total examined. All newborn males and 3/4 females died at 500 mg/kg in the dose-finding study. †indicates dose and data from the dose-finding study.

Table 3 Major toxicity findings for p-( $\alpha,\alpha$ -dimethylbenzyl) phenol in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	30	100	300	0	100	300	1000
<b>Male</b>								
Dead or moribund	0/12	0/12	0/12	0/12	0/14	0/7	0/7	3/14
ALT, $\gamma$ -GTP	/	-	-	-	/	-	-	↑
BUN, Creatinine	/	-	-	-	/	-	-	↑
Relative liver weight	/	-	-	-	/	-	↑	↑
Relative kidney weight	/	-	-	↑	/	-	-	↑
Stomach, hyperplasia	0/6	0/6	0/6	0/6	0/7	0/7	0/7	1/6
Liver, proliferation bile ducts	0/6	0/6	0/6	0/6	0/7	0/7	0/7	6/6
Kidney, regeneration	0/6	0/6	0/6	0/6	3/7	3/7	5/7	6/6
Kidney, dilatation	0/6	0/6	1/6	6/6	0/7	0/7	0/7	6/6
<b>Female</b>								
Dead or moribund	0/12	0/12	0/12	0/12	0/14	0/7	0/7	1/14
ALT, $\gamma$ -GTP	/	-	-	-	/	-	-	↑
BUN, Creatinine	/	-	-	↑, -	/	-	-	-
Relative liver weight	/	-	-	-	/	-	-	↑
Relative kidney weight	/	-	-	↑	/	-	-	↑
Stomach, hyperplasia	0/6	0/6	0/6	0/6	0/7	0/7	0/7	3/7
Liver, proliferation bile ducts	0/6	0/6	0/6	0/6	0/7	0/7	0/7	7/7
Kidney, regeneration	0/6	0/6	0/6	0/6	0/7	1/7	0/7	7/7
Kidney, dilatation	0/6	0/6	2/6	6/6	0/7	0/7	0/7	4/7

Only critical data are shown in this table. Data are numbers of animals with the change of the number examined. Slashes and bars mean no statistical significance as compared to controls. † indicates significant increase at  $P < 0.05$ . Relative kidney weights were increased 2.5- and 2.1-fold for males and females at 300 mg/kg in the newborn study. For the young study, 14 males and 14 females (half for examination of recovery) were assigned to each group but 6 males and 7 females at 1000 mg/kg were re-assigned for 28-day examination because of deaths.

and 300 mg/kg. The absolute ovary weights were still lowered by 32% at 300 mg/kg after the recovery-maintenance period. Increased numbers of atretic follicles were found in ovaries of half of the females at 300 mg/kg at the end of the dosing period, and most females continued to show various changes such as decreased numbers of corpora lutea in the ovaries and hypertrophy of endometrial epithelium in the uteri, after the recovery-maintenance period.

In the young study, two males and one female died, and one male was killed in a moribund condition at 1000 mg/kg. The final body weights were reduced by 18%, limited to males. On urinalysis, both sexes showed irregularly sized particles of a black substance, accompanied by 2-4 fold elevation of urine volume. Clear changes of several biochemical parameters such as ALT,  $\gamma$ -GTP, BUN, and creatinine, increases of relative liver and kidney weights, and histopathological changes in the forestomach (squamous hyperplasia), liver (bile duct proliferation), and kidney (regeneration of tubular epithelium and dilatation of tubules) were also observed at 1000 mg/kg. A dose of 300 mg/kg was considered to cause slight toxicity, because the abnormal urinary contents described above were found in half of both sexes and a slightly elevated incidence of mild regeneration of the tubular epithelium was noted in male kidneys. After the two-week recovery period, the pathological changes in male kidneys at 1000 mg/kg continued to be evident. There were no signs of toxicity at 250 and 500 mg/kg in the dose-finding study although the administration period was only half and urinalysis and histopathological examinations were not performed.

The pNOAEL of 30 mg/kg/day for newborn rats is clear and one of 100 mg/kg/day for young rats is reasonable because of slight toxicity at 300 mg/kg in the main study and limited information at 250 mg/kg in the dose-finding study. Toxicity for newborn rats was evident at 300 mg/kg as all animals of both sexes showed histopathological changes in kidneys, with increased relative weights. However, the degree of toxicity for young rats at 1000 mg/kg was obviously much stronger than that of newborn rats at 300 mg/kg, which appeared to be equivalent to doses of 700-800 mg/kg in young rats. Therefore, pUETLs of 300 and 700-800 mg/kg/day may be appropriate for newborn and young rats, respectively. It should be specially noted that this chemical may have endocrine disrupting properties, especially against females, when given only during the suckling phase.

#### (Hydroxyphenyl)methyl phenol (Table 4)

The newborn investigation was conducted at doses of 0, 20, 60, and 200 mg/kg for dose-finding and 0, 16, 40, and 100 mg/kg for the main study. The young study was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and 0, 8, 40, 200, and 1000 mg/kg for the main study.

Common changes were limited to depression of body weight and death at high doses in newborn and young rats. The highest dose of 100 mg/kg in the newborn main study did not cause any changes, but half the animals at 200 mg/kg in the newborn dose-finding study died, without accompanying liver weight changes in surviving

Table 4 Major toxicity findings for (hydroxyphenyl)methyl phenol in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)			
	0	100	200†	0	40	200	1000
<b>Male</b>							
Dead or moribund	0/12	0/12	3/6	0/12	0/12	0/12	0/12
Final body weight	/	-	↓	/	-	-	↓
Total cholesterol	/	-	↑	/	-	-	↓
Relative liver weight	/	-	-	/	-	-	↑
Stomach, hyperplasia	0/6	0/6	no data	0/6	0/6	0/6	6/6
Liver, centrilobular hypertrophy	0/6	0/6	no data	0/6	0/6	2/6	4/6
<b>Female</b>							
Dead or moribund	0/12	0/12	3/6	0/12	0/12	0/12	1/12
Final body weight	/	-	(↓)	/	-	-	(↓)
Total cholesterol	/	-	-	/	↓	↓	↓
Relative liver weight	/	-	-	/	-	↑	↑
Stomach, hyperplasia	0/6	0/6	no data	0/6	0/6	0/6	6/6
Liver, centrilobular hypertrophy	0/6	0/6	no data	0/6	0/6	0/6	4/6

Only critical data are shown in this table. † indicates a dose from the dose-finding study. Numbers are for animals with the feature in the total examined. Slashes and bars mean no statistical significance as compared with controls. ↑ indicates significant increase  $P < 0.05$ . ↓ indicates significant decrease at  $P < 0.05$ . () indicates that statistical significance was not obtained. Final body weights of surviving newborn males at 200 mg/kg in the dose-finding study were reduced by 30% (14% for females, not significant), respectively. Final body weights of young male rats at 1000 mg/kg in the main study were decreased by 11.8% (5.7% for females, not significant). Increase of relative liver weights was 13% in females at 200 mg/kg, and 16 and 27% in males and females at 1000 mg/kg in the young main study.

animals. There were no chemical-related changes with other examinations, including developmental parameters. In the young study, one female became moribund and the final body weights of males were decreased at 1000 mg/kg. All animals of both sexes at this dose showed squamous hyperplasia of the forestomach or limiting ridge with ulceration, and two-thirds of the animals featured centrilobular hypertrophy of hepatocytes with decrease of total cholesterol (29–51% drop) and increase of relative liver weight. At 200 mg/kg, low incidences of centrilobular hypertrophy in the livers of males and slight increase of liver weights in females with low total cholesterol (45% drop) were found. No toxicity was apparent at 40 mg/kg in the main study. No toxicity was also found at 100 mg/kg in the dose-finding study, but a histopathological examination was not conducted. There were no abnormalities on hematological examination and urinalysis at any dose.

The pNOAEL is considered to be 100 mg/kg/day for newborn rats and 40 mg/kg/day may be appropriate for young rats because of the limited information at 100 mg/kg in the dose-finding study. Although toxicity at 1000 mg/kg for young rats was evident, the dose inducing the same effects in newborn rats was clearly less than 200 mg/kg, because half of the animals died at this dose. We speculate that the dose range for one death in 12 newborn rats would be within 140–160 mg/kg. It is clear that the dose-response curve is much steeper for newborn than young rats. Based on our consideration, pUETLs of 140–160 and 1000 mg/kg/day may be equivalent for newborn and young rats, respectively.

#### Trityl chloride (Table 5)

The newborn investigation was conducted at doses of 0, 20, 60, 200, and 600 mg/kg for dose-finding and 0, 12, 60, and 300 mg/kg for the main study. The young investigation was conducted at doses

of 0, 30, 100, 300, and 1000 mg/kg for dose-finding and 0, 12, 60, and 300 mg/kg for the main study.

Common effects were observed in livers of newborn and young rats. In the newborn study, increase of relative liver weights were shown at 60 mg/kg and more in both sexes and centrilobular hypertrophy of hepatocytes was noted in 300 mg/kg females. In the dose-finding newborn study, one female died and increase of relative liver weights of both sexes at 600 mg/kg was more evident with low body weights (11.3% drop for males, 13.8% for females). There were no chemical-related changes with other examinations, including developmental parameters. In the young study, both sexes at 60 mg/kg showed a high incidence of centrilobular hypertrophy of hepatocytes with limited increases of relative liver weights (10–14%). At 300 mg/kg, soft feces and mucosal thickening of cecum in most animals were observed in addition to more extensive hepatic changes. Although relative kidney weights were increased at 300 mg/kg in males and 60 and 300 mg/kg in females, there were no renal histopathological findings. Hematological and blood chemical examinations revealed several slight to moderate changes (56% as the maximum) in fibrinogen, ALT, total cholesterol and glucose, as well as prolongation of prothrombin and activated thromboplastin times, at 300 mg/kg.

pNOAELs of 60 and 12 mg/kg/day for newborn and young rats appear appropriate because of the lack of information at higher doses in the dose-finding study, which showed no toxicity but without histopathological examination. The dose of 300 mg/kg in the young main study was a clear toxic level, but intensity was much stronger than that at 300 mg/kg in the newborn main study, while less than that at 600 mg/kg in the dose-finding study. Based on these data, the toxicity with 300 mg/kg for young rats is considered to be within the range with 400–500 mg/kg for newborn rats.

Table 5 Major toxicity findings for trityl chloride in the newborn and young rat main studies

	Newborn study (mg/kg)				Young study (mg/kg)			
	0	60	300	600†	0	12	60	300
<b>Male</b>								
Death	0/12	0/12	0/12	0/6	0/12	0/6	0/12	0/12
Final body weight	/	-	-	↓	/	-	-	↓
ALT, Total cholesterol	/	-	-	-	/	-	-	↑
Relative liver weight	/	↑	↑	↑	-	-	↑	↑
Relative kidney weight	/	-	-	-	-	-	-	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	0/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	0/6	no data	0/6	0/6	3/6	6/6
<b>Female</b>								
Death	0/12	0/12	0/12	1/6	0/12	0/6	0/12	0/12
Final body weight	/	-	-	↓	/	-	-	-
ALT, Total cholesterol	/	-	-	-	/	-	-	-,↑
Relative liver weight	/	↑	↑	↑	-	-	↑	↑
Relative kidney weight	/	-	-	-	-	-	↑	↑
Cecum, thickening	0/6	0/6	0/6	no data	0/6	0/6	2/6	5/6
Liver, centrilobular hypertrophy	0/6	0/6	4/6	no data	0/6	0/6	5/6	6/6

Only critical data are shown in this table. † indicates a dose from the dose-finding study. Numbers are for animals with the feature in the total examined. Slashes and bars mean no statistical significance as compared to controls. ↑ indicates significant increase  $P < 0.05$ . ↓ indicates significant decrease at  $P < 0.05$ . Relative liver weights were increased by 11% for males and 8% for females at 60 mg/kg, and 29% for both sexes at 300 mg/kg in the newborn main study and by 44% for males and 46% for females at 600 mg/kg in the newborn dose-finding study. Body weight depression in males (13%) and an increase of relative liver weights (32% for males, 40% for females) were observed at 300 mg/kg in the young main study.

Therefore, pUETLs of 400–500 and 300 mg/kg/day are proposed as appropriate for newborn and young rats, respectively.

### 1,3,5-Trihydroxybenzene (Table 6)

The newborn investigation was conducted at doses of 0, 100, 500, and 1000 mg/kg for dose-finding and at 0, 20, 100, and 500 mg/kg for the main study. The young investigation was conducted at doses of 0, 100, 250, 500, and 1000 mg/kg for dose-finding and at 0, 30, 100, 300, and 1000 mg/kg for the main study.

Common changes were observed in the thyroids and liver. The only toxic change in newborn main study was hypertrophy of thyroid follicular cells with increase in relative thyroid weights in both sexes at 500 mg/kg. Increased relative liver weights in females were not accompanied by any histopathological changes. Although decrease of adrenal weight and histopathological alterations such as vacuolization and pigmentation were noted at the end of the dosing and recovery-maintenance periods, these were always slight and not dose-dependent. There were no chemical-related changes with other examinations, including developmental parameters, in newborn rats. In the young study, similar effects on the thyroids and liver were found at 1000 mg/kg, but the incidence of thyroid histopathological changes was slightly less than in newborn animals at 500 mg/kg.

pNOAELs of 100 and 300 mg/kg/day for newborn and young rats can be considered appropriate because of the lack of data with dose settings between 100 to 500 mg/kg in the newborn, and no histopathological examination at 500 mg/kg in the young dose-finding study. The degree of toxicity at 1000 mg/kg for young rats was almost equal to that at 500 mg/kg for newborn rats. Therefore,

pUETLs of 500 and 1000 mg/kg/day are proposed as equivalents for newborn and young rats, respectively.

## DISCUSSION

More than 100 000 industrial chemicals are now in use around the world and sufficient toxicity information is available for only a small proportion. The Japanese government started the Existing Chemical Safety Program to obtain minimal toxicity data sets from 28-day toxicity studies using young rats for high production volume chemicals lacking toxicity information. For the present six targeted chemicals, we found toxicity information for only two chemicals by literature search. Daniel *et al.* (1993) reported no toxic effects of 2-chlorophenol on oral administration to male and female Sprague Dawley rats at up to 257 mg/kg for 10 days or 150 mg/kg for 90 days. Our results were consistent with their data, as we found no toxicity at 500 mg/kg in young dose-finding study (14 days administration) and at 200 mg/kg in the young study (28 days), while further providing information on CNS effects at higher doses. As for (hydroxyphenyl)methyl phenol, consisting of bisphenol D, E, and F isomers, bisphenol F has been reported to have estrogenic potential evidenced by several *in vitro* and *in vivo* experiments (Hashimoto *et al.* 2001; Yamasaki *et al.* 2002; Stroheker *et al.* 2003). However, we could not establish any such activity in this study. Our results are reasonable because oral administration of bisphenol F increased relative uterus weights only at more than 100 mg/kg, but not 50 mg/kg given during PNDs 22–25 (Stroheker *et al.* 2003), while our highest dose of (hydroxyphenyl)methyl phenol was equivalent to 30 mg/kg of bisphenol F.

**Table 6** Major toxicity findings for 1,3,5-trihydroxybenzene in the newborn and young rat main studies

	Newborn study (mg/kg)			Young study (mg/kg)		
	0	100	500	0	300	1000
<b>Male</b>						
Relative organ weight						
Liver	/	-	-	/	-	↑
Thyroids	/	-	↑	/	-	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	4/6	0/6	0/6	2/6
<b>Female</b>						
Relative organ weight						
Liver	/	-	↑	/	-	↑
Thyroids	/	-	(↑)	/	-	(↑)
Histopathology						
Liver	0/6	0/6	0/6	0/6	0/6	0/6
Thyroids, hypertrophy	0/6	0/6	5/6	0/6	0/6	4/6

Only critical data are shown in this table. Slashes and bars mean no statistical significance as compared with controls. ↑ indicates significant increase  $P < 0.05$  (except in parentheses where statistical significance was not attained). Numbers are for animals with the feature in the total examined. Increase of relative organ weights at 500 mg/kg in the newborn main study was observed for thyroids (39% for males, 24% for females) and liver (9% for females). Increase of relative organ weights at 1000 mg/kg in the young main study was observed for thyroids (14% for males, 19% for females) and liver (23% for males and 9% for females).

**Table 7** Comparative susceptibility of newborn and young rats to the six chemicals

	Newborn study		Young study		pNOAEL Young/Newborn	pUETL Young/Newborn
	pNOAEL mg/kg/day	pUETL	pNOAEL mg/kg/day	pUETL		
2-Chlorophenol	40	200-250	200	1000	5.0	4.0-5.0
4-Chlorophenol	100	300	100	500	1.0	1.7
p-( $\alpha,\alpha$ -Dimethylbenzyl) phenol	30	300	100	700-800	3.3	2.3-2.7
(Hydroxyphenyl) methyl phenol	100	140-160	40	1000	0.4	6.3-7.1
Trityl chloride	60	400-500	12	300	0.2	0.6-0.8
1,3,5-Trihydroxybenzene	100	500	300	1000	3.0	2.0

Although there has been no reports for p-( $\alpha,\alpha$ -dimethylbenzyl) phenol, it causes endocrine disruption and possible antiestrogenic activity, when administered to newborn female rats in this study. Therefore, further studies on this chemical should be conducted to elucidate the mechanisms, because the present investigation did not indicate any effects on sexual differentiation such as preputial separation, vaginal opening and the estrous cycle.

For our focus on the comparative sensitivity of newborn and young rats to chemicals, two toxicity endpoints, pNOAEL and pUETL, were newly defined as appropriate, considering the entire data sets from both main and dose-finding studies. We believe that this alternative assessment approach allowed us to make more realistic comparisons between newborn and young rats under the same experimental conditions as far as possible.

The ratios of pNOAELs for chemicals between newborn and young rats may provide an additional UF value in risk assessment according to susceptibility of newborn rats, because regulatory limit values for chemicals to protect public health of humans,

including infants, are derived from the division of NOAEL by UFs. The data in Table 7 indicate newborn rats to be 1-5 times more susceptible to four of the tested chemicals, 2- and 4-chlorophenols, p-( $\alpha,\alpha$ -dimethylbenzyl) phenol and 1,3,5-trihydroxybenzene, than young rats in terms of the pNOAELs, similar to the results of previous analyzes of five phenolic chemicals, 4-nitro-, 2,4-dinitro-, 2,4,6-trinitro-, 3-methyl- and 3-amino-phenols (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004). Immaturity in the detoxification potential of phase 1 and phase 2 enzymes in newborn animals may be the major cause of higher toxicity in newborn rats (Rich & Boobis 1997; Gow *et al.* 2001), because these chemical classes are probably direct toxicants. In the case of (hydroxyphenyl)methyl phenol, the pNOAEL (100 mg/kg/day) for newborn rats was 2.5 times higher than that (40 mg/kg/day) for young rats, but it can be speculated that values are in practice rather similar because the toxicity for young rats at the high dose, 200 mg/kg, was only slight (Table 4). As for trityl chloride, newborn rats were obviously less susceptible (0.2 for the pNOAEL ratio). Similar results were

also reported from our previous analysis for bromoalkanes (Hirata-Koizumi *et al.* 2005) and may be explained by mechanisms of action and metabolic characteristics of newborn rats. As this class of chemicals possibly requires metabolism to act as toxicants, the relatively mature metabolic enzyme status of young rats would be expected to provide toxic intermediates by metabolic activation to a greater extent than in newborn rats, as evidenced by data for previously reported chemicals (Onkenhout *et al.* 1986; Kennedy *et al.* 1993). Other compounds such as acetaminophen, bromobenzene, and carbon tetrachloride have also been shown to not produce liver injury in neonatal animals at doses that are hepatotoxic to adults (Gregus & Klaassen 1998).

The ratios of pUETLs, doses inducing the same degree of toxicity in newborn and young rats, were almost the same as for pNOAELs with the direct toxicants, as shown in Table 7. However, newborn rats were considerably more susceptible to (hydroxyphenyl)methyl phenol when considering the pUETL, due to the much steeper dose-response curve in newborn rats, with a 100 mg/kg/day pNOAEL and half the animals dying at 200 mg/kg, compared with a 40 mg/kg/day pNOAEL and only one death in 12 animals at 1000 mg/kg for young rats. Although young rats showed stomach hyperplasia in addition to hepatotoxicity at 1000 mg/kg, the cause of newborn deaths at 200 mg/kg was unclear. With regard to trityl chloride, the pUETL for young rats was almost the same as for newborn although the latter were less susceptible. Such an anomaly has also been found for bromoalkanes previously analyzed. Another example of a chemical for which susceptibility differs at low and high doses is chlorpyrifos, the maximum tolerated dose in 17-day-old rats being reported to be five times less than that in adults following oral exposure (Moser & Padilla 1998), but the differential sensitivity not appearing in low-dose exposure (Pope & Liu 1997). Thus as there are several chemicals of which dose-response curve in newborn rats was obviously steeper than that in young rats, pUETL ratios should be also taken into account for the susceptibility of newborn rats as the second endpoint marker.

In conclusion, newborn rats were 2–5 times more susceptible than young rats in terms of both the pNOAEL and the pUETL in most cases. One exception was that young rats were clearly more susceptible than their newborn counterparts for trityl chloride.

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## In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals

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### Abstract

Genotoxicity is one of the important endpoints for risk assessment of environmental chemicals. Many short-term assays to evaluate genotoxicity have been developed and some of them are being used routinely. Although these assays can generally be completed within a short period, their throughput is not sufficient to assess the huge number of chemicals, which exist in our living environment without information on their safety. We have evaluated three commercially available *in silico* systems, i.e., DEREK, MultiCASE, and ADMEWorks, to assess chemical genotoxicity. We applied these systems to the 703 chemicals that had been evaluated by the Salmonella/microsome assay from CGX database published by Kirkland et al. [1]. We also applied these systems to the 206 existing chemicals in Japan that were recently evaluated using the Salmonella/microsome assay under GLP compliance (ECJ database). Sensitivity (the proportion of the positive in Salmonella/microsome assay correctly identified by the *in silico* system), specificity (the proportion of the negative in Salmonella/microsome assay correctly identified) and concordance (the proportion of correct identifications of the positive and the negative in Salmonella/microsome assay) were increased when we combined the three *in silico* systems to make a final decision in mutagenicity, and accordingly we concluded that *in silico* evaluation could be optimized by combining the evaluations from different systems. We also investigated whether there was any correlation between the Salmonella/microsome assay result and the molecular weight of the chemicals: high molecular weight (>3000) chemicals tended to give negative results. We propose a decision tree to assess chemical genotoxicity using a combination of the three *in silico* systems after pre-selection according to their molecular weight.

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**Keywords:** *In silico*; (Quantitative) structure-activity relationship; (Q)SAR; Chemical genotoxicity; Decision tree

### 1. Introduction

It is said that more than 20,000 chemicals are in use in Japan. Among them, only approximately 10% are thought to have been assessed for human hazard based

on data from *in vitro* and *in vivo* bioassays. According to the “Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc.” [2], the Salmonella/microsome (Ames) assay, *in vitro* chromosomal aberration assay (or alternatively mouse lymphoma TK assay), and 28-day repeat dose toxicity test in rodents are obligatory to notify new chemicals for production/import at a level of more than 10 t per year.

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To screen the remaining 18,000 chemicals for human hazard by application of this three-test battery is not realistic from the time and economical point of view. We need a much higher-throughput system to assess these chemicals, at least for prioritization of those chemicals that should be submitted to biological testing. To assess human hazard for regulatory purposes, *in silico* systems are now beginning to be used [3]. Here, we evaluated three commercially available *in silico* (quantitative) structure-activity relationship ((Q)SAR) systems and tried to construct a decision tree for prioritization of which chemicals need *in vitro* and/or *in vivo* testing. Also, within the drug discovery process, integrated computational analysis has been proposed to be incorporated as a toxicity prediction tool [4].

Kirkland et al. [1] published a database (CGX database, see <http://www.lhasalimited.org/cgx>) for nearly 1000 carcinogens and non-carcinogens with results of representative *in vitro* genotoxicity assays, i.e., Salmonella/microsome assay (Ames), mouse lymphoma TK assay using L5178Y cells (MLA), and *in vitro* chromosomal aberration assay or *in vitro* micronucleus assay (CA/MN). We used 703 chemicals that had been assessed in the Ames assay for evaluation of the three *in silico* systems, i.e., DEREK, MultiCASE (MCase), and ADMETWorks (AWorks). We also used a database (the ECJ database) that we constructed from chemicals existing in Japan that had recently been assessed in the Ames assay, *in vitro* chromosomal aberration assay, and 28 day repeat dose rodent toxicity test and/or reproductive and developmental toxicity test for their safety evaluation under GLP compliance. The ECJ database consisted of 206 chemicals but only 26 chemicals were positive by the Ames assay. Initially we evaluated both sensitivity and specificity of these three systems using the ECJ database of 206 chemicals [5].

We selected these three *in silico* systems because of their different modes of analysis. DEREK is a rule-based system [6], MCase [7] is a database/substructure based system, and AWorks is a QSAR. We applied these systems individually to assess gene-mutation induction on the 703 and 206 chemical sets described above and evaluated their sensitivity, specificity, concordance, and applicability (how many chemicals could be assessed), independently.

It is known that high molecular weight polymers tend not to induce gene mutation and chromosomal aberrations mainly because they cannot enter the target cells to react with DNA, or other bio-molecules necessary for genetic stability. We analyzed 194 Ames positive chemicals (confidential source) for the effect of molecular weight.

## 2. Materials and methods

### 2.1. Data sources for chemicals assessed

Of about 1000 chemicals, 703 that had been assessed in the Ames test were chosen from the CGX database published by Kirkland et al. [1]. All chemical structures were re-drawn using Chemdraw Ultra (Cambridge Soft Corporation, USA) and converted to MOL files before application to each system. We also used the database of 206 chemicals evaluated in the MHLW project "Safety Examination of Existing Chemicals and Safety Programmes in Japan" (ECJ database). The test summary for each of these chemicals can be seen at <http://www.db.mhlw.go.jp/ginc/html/db1.html>. In addition, we collected 194 Ames positive chemicals from a confidential source and investigated the relationship between gene mutation induction and molecular weight, with identification of any active side chain that might have contributed to the positive result in the Ames assay.

### 2.2. *In silico* systems used and definition of positive and negative responses

We used DEREK (Lhasa Ltd., UK) version 8.0.1. When the system gave an evaluation as "certain", "probable" or "plausible" we considered this as "positive", and when the system gave "equivocal", "doubted", "improbable", "impossible", or "no alert" we considered this as "negative". We used MCase (Multicase Co. Ltd.) version mc4pc. When the system gave "active" or "marginal" we considered this as "positive", and when the system gave "inactive" we considered this as "negative". In the case of AWorks (Fujitsu Kitakyushu, Co. Ltd., version 2.0), we considered as "positive" when system evaluation was "positive", and considered as "negative" when the system evaluation was "negative". We excluded chemicals from further analysis when DEREK or AWorks gave no answer, or the evaluation was "inconclusive" by MCase.

### 2.3. Definition of sensitivity, specificity, concordance, and applicability

We calculated sensitivity, specificity, concordance, and applicability as follows:

$$\text{sensitivity} = \frac{N_{A+S+}}{N_{A+}} \times 100, \quad \text{specificity} = \frac{N_{A-S-}}{N_{A-}} \times 100,$$

$$\text{concordance} = \frac{N_{A+S+} + N_{A-S-}}{N_{\text{eval}}} \times 100,$$

$$\text{applicability} = \frac{N_{\text{eval}}}{N_{\text{all}}} \times 100$$

where  $N_{A+}$  is number of chemicals revealing positive in Ames assay;  $N_{A-}$  is number of chemicals negative in Ames assay;  $N_{A+S+}$  is number of chemicals revealing positive by both Ames assay and *in silico* evaluation;  $N_{A-S-}$  is number of chemicals negative in both Ames assay and *in silico* evaluation;  $N_{\text{eval}}$  is