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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.

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 ADMEWorks (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://wwwdb.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:

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

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

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

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_{eval} is

Table 1
Performance of in silico systems

	Ames result	+	–	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	–	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	–	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	–	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	–	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	–	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	–	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

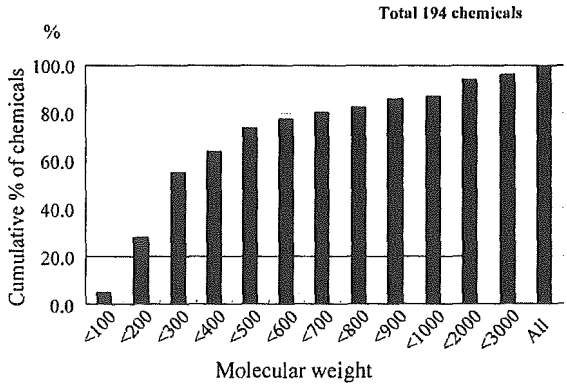

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

In silico / Ames	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	**279**	**40**	319				
-	**42**	**249**	291	**87.8**	**85.6**	**86.7**	**86.8**
Total	321	289	610				
	+++	---					
+	**166**	**1**	167				
-	**3**	**127**	130	**99.4**	**97.7**	**98.7**	**42.2**
Total	168	129	297				

ECJ database

In silico / Ames	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	**19**	**7**	26				
-	**23**	**147**	170	**73.1**	**86.5**	**84.7**	**95.1**
Total	42	154	196				
	+++	---					
+	**13**	**2**	15				
-	**5**	**94**	99	**86.7**	**94.9**	**93.9**	**55.3**
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Reference
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative and Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Source[a]
Bupropion	34911-55-2	+	–	–	–	1
Citalopram	59729-33-8	+	–	–	–	1
Naloxone	465-65-6	+	–	–	–	1
Oxcarbazepime	28721-07-5	+	–	–	–	1
Quetiapine	111976-69-7	+	–	–	–	1
Rabeprazole	117976-89-3	+	–	–	–	1
Zolmitriptan	139264-17-8	+	–	–	–	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	–	–	–	2
2-Chloropyridine	109-09-1	+	–	–	–	2
Pyrogallol	87-66-1	+	–	–	–	2
o-Phenylphenol	90-43-7	+	–	–	–	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	–	–	–	3
2-Vinylpyridine	100-69-6	+	–	–	–	3
Fosfomycin	23155-02-4	–	+	+	+	1
Toremifene	89778-26-7	–	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	–	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	–	+	+	+	3
Methidathion	950-37-8	–	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	–	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive Ames results but were negative in all three in silico systems (DEREK, MCase, TOPKAT/AWorks), and those that were negative in the Ames test but gave positive evaluations in all three systems, are summarized in Table 4. This table, which includes data from Synder et al. [11] and White et al. [13] shows there are 19 exceptional chemicals from both drug and non-drug families. Although it would be unrealistic to expect zero exceptions using this approach, further improvement of the prediction systems is needed. We do not have good reasons to explain the discordance, therefore we will verify the results from both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decision tree (Fig. 2), in order to evaluate chemical induction of gene mutation. We may use the decision tree to prioritize chemicals to be assayed by in vitro and/or in vivo tests. A final goal being that eventually, chemical mutagenicity will be evaluated by in silico systems alone for regulatory use. The decision tree consists of three steps; namely to assess the molecular weight, the existence of epoxy groups, and the in silico evaluation for genotoxicity. Based on the purpose of the in silico evaluation, the tree might be altered by the different final call of the in silico evaluation, i.e., regarding as positive (negative) all three systems show positive (negative). The choice of definition for final call applying to the decision tree should be based on the balance between accuracy of eval-

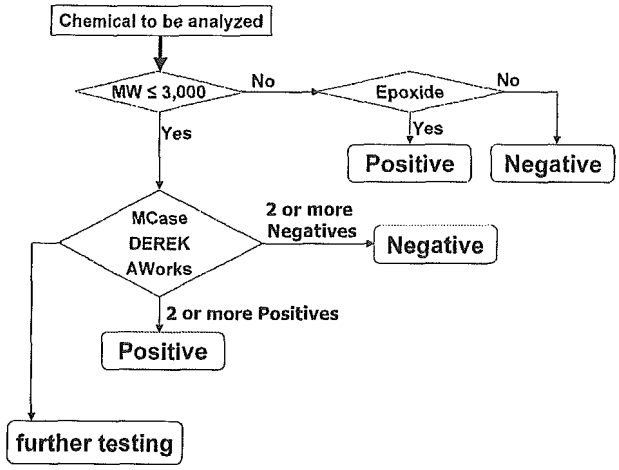

Fig. 2. Decision tree. In in silico evaluation, when two or more give positive then the final call is "positive" and two or more negative then call "negative".

uation and applicability, which are especially important for regulatory purpose. The decision should be made on a case-by-case basis depending upon the purpose of the decisions to be made.

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ORIGINAL ARTICLE

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

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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 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 piliation, incisor eruption, eye opening, testes descent and vaginal opening. All newborn rats were examined for abnormalities of reflex ontogeny; e.g. pupillary 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 (Sagamihara, 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.

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.

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.

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

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

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.

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

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.

ORIGINAL ARTICLE

Comparative susceptibility of newborn and young rats to six industrial chemicals

Ryuichi Hasegawa, Mutsuko Hirata-Koizumi, Mika Takahashi, Eiichi Kamata, and Makoto Ema
National Institute of Health Sciences, Tokyo, Japan

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-(α,α-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-trihydroxybenezene, 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-(α,α-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 disrupters 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-(α,α-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-(α,α-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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ORIGINAL ARTICLE

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In the latest decade, infant and child health has become a major focus (Landrigan *et al.* 2004), especially since endocrine disrupters 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).

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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-(α,α-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 ± 2°C with a relative humidity of 55 ± 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 animotransferase (ALT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-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 homogenous, 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-(α,α-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
Male							
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
Female							
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.