Table 6. Summary of histopathology of chronic toxicity study

Tissue	Findings	Males					Females				
		Catechin					Catechin				
	Dose (%)	0	0.02	0.3	1	3	0	0.02	0.3	1	3
Number of rats examined		10	10	10	10	10	10	10	10	10	10
Neoplastic lesions											
Pituitary	Adenoma of anterior lobe	0	0	0	0	0	1	1	0	3	0
Thyroid	C-cell adenoma	1	0	2	0	0	0	0	0	0	0
	Follicular cell adenoma	0	0	0	0	0	1	0	0	0	0
Spleen	Hemangioma	0	0	0	1	0	0	0	0	0	0
Skin	Papilloma	0	0	0	1	0	0	0	0	0	0
Testis	Leydig cell tumor	0	1	0	0	0	-	-	-	-	-
Uterus	Endometrial stromal polyp	-	-	-	-	-	3	1	1	1	0
	Duciduoma	-	-	-	-	-	1	0	0	0	0
Non-neoplastic lesions											
Liver	Centrilobular hypertrophy of hepatocytes	0	0	0	0	8*	0	0	0	0	0
	Altered foci, basophilic	0	2	2	2	0	3	1	0	4	2
	Altered foci, eosinophilic	0	0	1	0	0	0	0	0	0	0

*, Significantly different from the control value ($p < 0.05$). -, Not examined.

hepatocytes in males at 3% (Fig. 6), and any other changes except CYP3A2 were not detected. In the carcinogenicity study, similar mild hepatocellular hypertrophy was also observed in males at the same dose (Table 7). In both studies, a number of non-neoplastic changes were detected, but their incidences and severities were similar to those in the control groups, and therefore no dose-related non-neoplastic changes except liver hypertrophy were detected.

In the chronic toxicity study, several tumors were detected in all groups including the controls in both sexes. Their incidences were comparable between the control and treated groups in both sexes (Table 6). In the carcinogenicity study, a number of tumors were found in many organs and/or tissues in both sexes in all groups (Table 8). All of the incidences in the treated groups were comparable to those in controls in both sexes. There were several types of tumors showing high incidences such as C-cell adenoma in the thyroid, islet cell adenoma, endometrial stromal polyp or mammary tumor (fibroadenoma and adenocarcinoma). The most common tumor was the anterior adenoma in the pituitary, which was also major cause of death in the present study. In addition thymoma and/or malignant thymoma with lymphocyte proliferation were detected in all groups including controls with similar incidences.

DISCUSSION

The present examination of the chronic toxicity and carcinogenicity of catechin mixture, used as a food additive, in Wistar Hannover GALAS rats showed no effects at concentrations of 0, 0.02, 0.3, 1 or 3% except with regard to body weights, food consumption and hepatocellular hypertrophy at the highest dose. No changes indicating carcinogenic potential were detected.

The food consumption increased or showed a tendency of increase in both sexes of the chronic toxicity and carcinogenicity studies at 3%. In the same dose group depressed body weight gain was observed in females of chronic toxicity study, and both sexes in carcinogenicity study. As for nutritional effects, the caloric contribution of catechin mixture in feed might be low because consistent dose-dependent increase of body weights was lacking. No obvious toxicities were detected in terms of other parameters. In addition, the pellet of 3% was more frangible, but long-term effects of catechin mixture on feeding behavior with continuous dietary administration were not observed. Therefore, the body weight depressions at 3% are considered not to be a toxic effect, but rather a secondary effect resulting from decreased caloric intake. Namely, these decreases were because the diet at the highest concentration was frangible and nominal food consumption may not have reflected the actual food consumption resulting in decrease in caloric intake, rather than toxic effects. Similar suppression of body weights was also noted in males of the 5% group in the subchron-

Table 7. Final and liver weights at the termination and liver histopathology in carcinogenicity study

Group	No of rats examined (At termination)	Body weight (g)	Liver weights		Histoapthology in the liver (Incidence/number of all rats examined)		
			Absolute (mg)	Relative [(a)]	Hepatocellular hypertrophy	Altered foci	
						Eosinophilic	Basophilic
Male							
Catechin 0%	32	692.32 ± 93.74 [(b)]	21242 ± 4305.7	30.717 ± 5.1845	5/50	5/50	2/50
Catechin 0.02%	31	680.19 ± 88.480	20691 ± 4037.1	30.524 ± 5.0252	3/50		
Catechin 0.3%	34	665.94 ± 104.19	20197 ± 4470.8	30.322 ± 5.2846	4/50		
Catechin 1%	37	646.73 ± 84.77	19135 ± 4330.7	29.617 ± 5.5515	8/50		
Catechin 3%	39	627.92 ± 71.32	17957 ± 2999.2	28.762 ± 50.339	18/50*	4/50	2/50
Females							
Catechin 0%	36	441.81 ± 71.34	12884 ± 3092.5	28.334 ± 7.6226	1/50	5/50	1/50
Catechin 0%	31	438.35 ± 55.84	12252 ± 2398.6	27.981 ± 4.180			
Catechin 0.3%	36	436.86 ± 78.29	13607 ± 3511.5	31.002 ± 4.867			
Catechin 1%	35	447.91 ± 68.80	12529 ± 2492.5	28.021 ± 4.206			
Catechin 3%	35	393.8 ± 50.248	11673 ± 2022.3	29.744 ± 4.136	0/50	4/50	7/50

*, Significantly different from the control value at $p < 0.01$. [(a)], Liver weight (mg) / body weight (g). [(b)], Mean ± S.D.

ic study (Takami *et al.*, 2008).

Histopathologically slight centrilobular hypertrophy of hepatocytes associated with induction of CYP3A2 was found at 3% in males of both studies, and relative weights of the liver increased were limited to the chronic toxicity study. However, no signs indicative of hepatotoxicity were seen in the serum biochemical and histopathological examinations in either study. Therefore, the changes observed in the liver were considered to be treatment-related but not adverse in nature. They might be regarded as adaptation. A joint FAO and WHO meeting of pesticides, residues concluded that in the absence of histopathological damage and relevant clinical chemistry changes at a dose inducing only hepatocellular hypertrophy and/or liver size/weight changes, hypertrophy should not be identified as an adverse effects as a general principle in the final assessment of the liver hypertrophy (JMPR, 2006). Large doses of many dietary supplements including green tea are reported to induce cytochrome P450s in rats (Jang *et al.*, 2004). In an earlier study administration of 2% green tea supplement in drinking water for 3 days induced CYP1A2 but not 3A2 (Jang *et al.*, 2004). Although the reasons for the discrepancy with the present study are not clear, the ingredients of the supplement in the previous study might have differed from those in the present study, because details were not given (Jang *et al.*, 2004).

In the present carcinogenicity study, none of the treated groups showed a significant increase in the incidence of any specific tumors compares with the corresponding control ones, and also there were no positive trend in both sexes, clearly indicating no carcinogenic potential of long-term treatment with catechin mixture in rats. All the tumors observed in the present study are known to occur spontaneously in aged rats.

Green tea has been consumed as a popular beverage in Japan and throughout the world for many years. During the past decade, epidemiological studies have shown tea catechin intake to be associated with lower risk of cardiovascular disease (Hertog *et al.*, 1993), or protective effects against some common types of cancer including in the prostate (Adhami *et al.*, 2003; Doss *et al.*, 2005; Jian *et al.*, 2004; Kumar *et al.*, 2007; Siddiqui *et al.*, 2006; Sim and Cheng, 2005; Stuart *et al.*, 2006). These experimental and epidemiological data might indicate that intake of tea catechin is favorable to human health under appropriate consumption conditions. Many studies reported that EGCG is responsible for the majority of the potential health benefits attributed to green tea consumption (Khan *et al.*, 2008) and this was in fact contained as a major component in the test material in the present study.

Based on the results in the present study, we conclude that catechin mixture has no appreciable toxicity in Wistar Hannover GALAS rats. The no observed adverse effect levels estimated form the chronic toxicity study were 3% in both sexes equal to 1922.9 and 2525.7 mg/kg/day in males and females, respectively. No carcinogenic potential was found in male and female rats fed catechin mixture for 2 years.

Table 8. Incidence of neoplastic lesions in the carcinogenicity study

Organ	Findings	Male Catechin 0%	0.02%	0.3%	1%	3%	Female Catechin 0%	0.02%	0.3%	1%	3%
	No. of rats examined	50	50	50	50	50	50	50	50	50	50
Brain	(B) Astrocytoma	0	0	1	0	1	0	0	0	0	0
	(M) Malignant meningioma	0	0	0	0	0	0	0	0	1	0
Pituitary	(M) Anterior adenocarcinoma	0	4	3	3	0	1	7	4	1	3
	(B) Anterior adenoma	12	21	16	19	12	22	27	25	31	16
	(B) Medium lobe adenoma	0	1	0	0	0	0	0	0	0	0
Zymbal gland	(M) Squamous cell carcinoma	0	1	0	0	0	0	0	0	0	0
Eye	(M) Malignant Schwannoma	0	0	1	0	0	0	0	0	0	0
Thyroid	(B) C-cell adenoma	3	6	2	2	6	3	1	4	4	3
	(B) Follicular cell adenoma	3	5	2	1	1	1	2	2	1	0
	(M) Follicular cell adenocarcinoma	0	1	1	1	0	0	0	0	0	0
Parotid gland	(M) Adenocarcinoma	0	0	0	0	0	0	0	0	0	1
Salivary gland	(M) Mixed tumor, malignant	0	0	1	0	0	0	0	0	0	0
Thymus	(B) Thymoma	0	0	2	0	2	1	5	1	2	2
	(M) Malignant thymoma	1	0	1	0	1	2	2	3	2	1
Lung	(B) Alveolar/broncholar adenoma	0	0	1	0	0	0	0	0	0	0
	(M) Alveolar/broncholar adenocarcinoma	0	0	0	1	0	0	0	0	0	0
Heart	(B) Endocardial schwannoma	0	1	0	0	2	0	0	0	0	0
Liver	(M) Angiosarcoma	1	0	0	0	0	0	0	0	0	0
	(B) Adenoma	0	0	1	0	0	1	0	2	1	0
Kidney	(B) Papilloma	0	1	0	0	0	0	0	0	0	0
	(M) Liposarcoma	1	0	0	0	0	0	0	0	0	0
	(M) Renal cell carcinoma	0	0	1	0	0	0	0	0	0	0
Pancreas	(M) Islet cell adenocarcinoma	2	1	0	0	3	1	0	0	0	0
	(B) Islet cell adenoma	1	3	7	4	2	1	1	1	0	1
	(B) Acinar cell adenoma	0	0	1	1	1	0	0	0	0	0
Adrenal	(B) Pheochromocytoma	2	3	3	3	0	1	2	0	0	1
	(B) Adenoma of cortex	0	0	1	0	0	0	1	1	1	1
	(B) Ganglioneuroma	0	0	0	0	0	1	0	0	0	0
	(M) Malignant pheochromocytoma	1	0	1	0	0	0	0	0	0	0
	(M) Pheochromocytoma complex	0	0	2	0	0	0	0	0	0	0
Lymph node	(M) Malignant lymphoma, systermic	0	1	0	0	1	0	0	0	1	0
	(B) Hemangioma	1	0	1	0	1	0	0	0	1	0
Spleen	(B) Hemangioma	0	0	0	0	0	0	0	0	1	0
	(M) Histiocytic sarcoma	0	0	0	0	0	0	0	0	1	0
Stomach	(M) Fibrosarcoma in forestomach	1	0	0	0	0	0	0	0	0	0
Intestine	(B) Fibroadenoma	0	0	0	0	0	1	0	0	0	0
	(B) Fibroma in muscle layer	0	0	0	0	0	1	0	0	0	0
	(B) Leiomyosarcoma	0	1	0	0	0	0	0	0	0	0
	(M) Mucinous carcinoma	0	0	1	0	0	0	0	0	0	0
Muscle	(B) Granular cell tumor	0	0	0	0	1	0	0	0	0	0
Urinay bladder	(B) Papilloma	0	1	0	0	0	0	0	0	0	0

Table 8. (Continued).

Organ	Findings	Male Catechin 0%	0.02%	0.3%	1%	3%	Female Catechin 0%	0.02%	0.3%	1%	3%
Testis	(B) Leydig cell tumor	0	4	3	3	2					
Prostate	(B) Adenoma of ventral lobe	1	1	1	2	0					
	(M) Squamous cell carcinoma	0	1	0	0	0					
Seminal vesicle	(B) Adenoma	0	0	0	2	0					
Ovary	(B) Granulosa-theca cell tumor						1	0	0	0	1
	(B) Granulosa cell tumor						0	0	1	0	0
	(B) Luteoma						0	0	0	0	1
	(B) Cystadenoma						0	1	0	0	0
Uterus	(B) Endmetrial stromal polyp						7	4	6	11	5
	(M) Stromal sarcoma						1	0	1	0	0
	(M) Endmetrial adenocarcinoma						0	1	0	1	1
Preputial gland	(B) Adenoma	1	0	0	0	0					
	(M) Adenocarcinoma	0	0	0	1	0					
	(M) Squamous cell carcinoma	0	1	0	0	0					
Nasal cavity	(B) Adenoma	0	0	0	1	0	0	0	0	0	0
	(B) Chondroma	0	0	0	0	0	0	0	0	1	0
	(M) Osteosarcoma	1	0	0	0	0	0	0	0	0	0
Oral cavity	(M) Squamous cell carcinoma	0	0	0	1	0	0	0	0	0	0
Abdominal cavity	(M) Angiosarcoma	1	0	0	0	0	0	0	0	0	0
	(B) Paraganlioma	0	1	0	0	0	0	0	0	0	0
Spinal cord	(B) Osteoma	0	0	0	0	0	1	0	0	0	0
Skin	(B) Keratoacanthoma	0	0	0	0	1	0	0	0	0	0
	(B) Trichoepithelioma	0	0	0	1	0	0	0	0	0	0
	(B) Papilloma	0	0	0	0	0	0	1	0	0	0
	(M) Squamous cell carcinoma	0	0	1	4	0	1	0	0	0	0
	(M) Basal cell carcinoma	0	0	0	0	0	0	0	0	1	0
	(M) Fibrosarcoma	0	1	0	0	0	0	0	0	0	0
Mammary gland	(B) Adenoma	0	0	0	0	0	5	6	1	3	1
	(B) Fibroadenoma	1	0	0	1	0	9	10	17	7	4
	(M) Adenocarcinoma	0	0	0	0	0	5	1	5	5	2
Subcutis	(B) Fibroma	2	3	2	1	0	0	1	0	2	0
	(B) Lipoma	0	0	0	0	0	1	0	0	0	0
	(B) Hemangioma	0	0	0	2	0	0	0	0	0	0
	(M) Fibrosarcoma	0	0	1	0	0	0	0	0	0	0
	(M) Leiomyosarcoma	0	0	1	0	0	0	0	0	0	0
	(M) Malignant Schwannoma	0	1	0	0	1	0	0	0	0	0
	(M) Angiosarcoma	0	1	1	0	0	0	0	0	0	0
	(M) Liposarcoma	0	0	0	0	0	0	0	0	1	0
	(M) Histiocytic sarcoma	0	0	0	0	1	0	0	0	0	0
	(M) Sarcoma NOS	0	0	0	1	0	0	0	0	0	0
Scrotum/abdomen	(M) Mesothelioma	1	1	0	1	1	0	0	0	0	0
Bone	(B) Schwannoma	0	0	0	1	0	0	0	0	0	0

(B), Benign; (M), Malignant

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REFERENCES

Adhami, V.M., Ahmad, N., Mukhtar, H. and Ahmad, N. (2003): Molecular targets for green tea in prostate cancer prevention. J. Nutr., **133**, 2417S-2424S.

de Whalley, C.V., Rankin, S.M., Hoult, J.R., Jessup, W. and Leake, D.S. (1990): Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. Biochem. Pharmacol., **39**, 1743-1750.

Doss, M.X., Potta, S.P., Hescheler, J. and Sachinidis, A. (2005): Trapping of growth factors by catechins: a possible therapeutical target for prevention of proliferative diseases. J. Nutr. Biochem., **16**, 259-266.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B. and Kromhout, D. (1993): Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet, **342**, 1007-1011.

Jang, E., Park, Y. and Chung, W.G. (2004): Effects of dietary supplements on induction and inhibition of cytochrome P450s protein expression in rats. Food Chem. Toxicol., **42**, 1749-1756.

Jian, L., Xie, L.P., Lee, A.H. and Binns, C.W. (2004): Protective effect of green tea against prostate cancer: a case-control study in southeast China. Int. J. Cancer, **108**, 130-135.

Joint FAO/WHO Meeting of Pesticide Residues (JMPR) (2006): Guidance on the interpretation of hepatocellular hypertrophy. Summary report.

Khan, N., Adhami, V.M. and Mukhar, H. (2010): Apoptosis by dietary agents for prevention and treatment of prostate cancer. Endocr. Relat. Cancer, **17**, R39-R52.

Khan, N., Afaq, F. and Mukhtar, H. (2008): Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid. Redox Signal., **10**, 475-510.

Khan, N., Afaq, F., Saleem, M., Ahmad, N. and Mukhtar, H. (2006): Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. Cancer Res., **66**, 2500-2505.

Kumar, N., Shibata, D., Helm, J., Coppola, D. and Malafa, M. (2007): Green tea polyphenols in the prevention of colon cancer. Front. Biosci., **12**, 2309-2315.

MHLW (Ministry of Health Labor Welfare of Japan) (1996): Guidelines for Designation of food additives and for revision of Standard for Use of Food Additives. Japan Food Additive Association.

Shimizu, M., Shirakami, Y. and Moriwaki, H. (2008): Targeting receptor tyrosine kinases for chemoprevention by green tea catechin, EGCG. Int. J. Mol. Sci., **9**, 1034-1049.

Siddiqui, I.A., Adhami, V.M., Saleem, M. and Mukhtar, H. (2006): Beneficial effects of tea and its polyphenols against prostate cancer. Mol. Nutr. Food Res., **50**, 130-143.

Sim, H.G. and Cheng, C.W. (2005): Changing demography of prostate cancer in Asia. Eur. J. Cancer, **41**. 834-845.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989): Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med., **320**, 915-924.

Stuart, E.C., Scandyln, M.J. and Rosengren, R.J. (2006): Role of epigallocatechin gallate (EGCG) in the treatment of breast and prostate cancer. Life Sci., **79**, 2329-2336.

Takami, S., Imai, T., Hasumura, M., Cho, Y.M. and Hirose, M. (2008): Evaluation of toxicity of green tea catechins with 90-day dietary administration to F344 rats. Food Chem. Toxicol., **46**, 2224-2229.

Verschovle, R.D., Steward, W.P. and Gescher, A.J. (2007): Putative cancer chemopreventive agents of dietary origin - how safe are they? Nutr. Cnacer, **59**, 152-162.

Verschovle, R.D., Steward, W.P. and Gescher, A.J. (2007): Putative cancer chemopreventive agents of dietary origin - how safe are they? Nutr. Cnacer, 59, 152-162.Yang, C.S., Maliakal, P. and Meng, X. (2002): Inhibition of carcinogenesis by tea. Annu. Rev. Pharmacol. Toxicol., **42**, 25-54.

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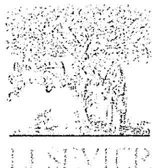

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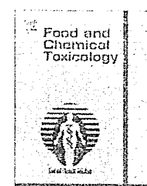

Validation of the (Q)SAR combination approach for mutagenicity prediction of flavor chemicals

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ABSTRACT

Most exposure levels of flavor in food are considered to be extremely low. If at all, genotoxic properties should be taken into account in safety evaluations. We have recently established a (quantitative) structure–activity relationship, (Q)SAR, combination system, which is composed of three individual models of mutagenicity prediction for industrial chemicals. A decision on mutagenicity is defined as the combination of predictive results from the three models. To validate the utility of our (Q)SAR system for flavor evaluation, we assessed 367 flavor chemicals that had been evaluated mainly by JECFA and for which Ames test results were available. When two or more models gave a positive evaluation, the sensitivity was low (19.4%). In contrast, when one or more models gave a positive evaluation, the sensitivity increased to 47.2%. The contribution of this increased sensitivity was mainly due to the result of the prediction by Derek for Windows, which is a knowledge-based model. Structural analysis of false negatives indicated some common sub-structures. The approach of improving sub-structural alerts could effectively contribute to increasing the predictability of the mutagenicity of flavors, because many flavors possess categorically similar functional sub-structures or are composed of a series of derivatives.

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1. Introduction

Many flavor chemicals in current food use have been evaluated under the Threshold of Toxicological Concern concept at the FAO/WHO Joint Expert Committee on Food Additives (JECFA). Most exposure levels of flavor in food are considered to be extremely low. In such cases, genotoxic properties should be taken account in safety evaluations in addition to the empirical threshold. Recently, (quantitative) structure–activity relationship ((Q)SAR) systems have been used to quickly assess the human hazards of chemicals for regulatory purposes (Cronin et al., 2003).

We had developed (Q)SAR models for assessment of chemical genotoxicity, which was optimized for application to industrial chemicals using three commercially available (Q)SAR systems, Derek for Windows and MultiCase, which are used widely by regulatory agencies, and ADMEWorks, which we customized. The results of previous evaluations of our (Q)SAR models using industrial chemical sets independent of the chemicals used for the model development indicated that the sensitivity, specificity and concordance rates were increased when we combined the three (Q)SAR systems to make a definitive decision on mutagenicity. Accordingly, we concluded that the (Q)SAR evaluation could be optimized by combining the evaluations from different systems (Hayashi et al., 2005).

Currently, about 3000 synthetic flavors are distributed commercially in Japan. About 900 of these originate from Japan and have not yet been assessed for their effect on human health. The Japan Flavor and Fragrance Materials Association (JFFMA) has been revaluating these flavor compounds, based on the safety assessment processes of the JECFA; however, for a number of these compounds there is insufficient information on their genotoxicity to be able to follow the JECFA process. It is not realistic for all of the flavor chemicals already used widely in Japan to be examined for genotoxicity because they are so numerous. Therefore, if we could make a reliable prediction of their genotoxicity (the results of the Ames

Abbreviations: JECFA, FAO/WHO Joint Expert Committee on Food Additives; (Q)SAR, (quantitative) structure–activity relationship; JFFMA, Japan Flavor and Fragrance Materials Association; FAS, WHO Food Additives Series; JFSC, Japan Food Safety Commission.

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Table 1
Performances of each (Q)SAR model.

	Ames results	(Q)SAR		
		+	–	Total
Derek for Windows	Positive	10	14	24
	Equivocal	4	8	12
	Negative	22	309	331
	Total	36	331	367
MultiCASE	Positive	6	18	24
	Equivocal	3	9	12
	Negative	19	312	331
	Total	28	339	367
ADMEWorks	Positive	4	20	24
	Equivocal	1	11	12
	Negative	28	303	331
	Total	33	334	367

test or chromosomal aberration test) based on their chemical structures *in silico*, it would be useful in the assessment of flavor chemicals originally used in Japan.

The purpose of our study was to develop an *in silico* system in order to define the priorities for conducting genotoxicity studies of many existing flavors unevaluated and/or flavors newly synthesized; furthermore, in future, to enable exemption from actual genotoxicity studies for evaluating specific chemical groups. In the present study, we applied our previously developed (Q)SAR combination system for predicting the Ames test results of flavors, which we selected from the series of JECFA reports. The prediction performance was not so high, because our system had been customized for industrial chemicals, but the results of this study indicated that our system is capable of improving the predictability of Ames test results for flavors.

2. Materials and methods

2.1. Set of chemicals for validation

The WHO Food Additives Series (FAS) from 1965 to 2008 and evaluation reports published by the Japan Food Safety Commission (JFSC) were used to select a set of flavor chemicals with information from the Ames test.

There were 367 flavor items with information on their activity in the Ames assay. We considered optical and geometrical isomers to be the same compound because sometimes isomers are not distinguished in Ames tests. The 2D structures of chemicals prepared by JFFMA were used for *in silico* evaluation. Moreover, because the results of the (Q)SAR models were not considered by the differences in strains, and with or without S9 mix in Ames tests on a training set, we did not consider their differences in the validation set of 367 compounds.

In the current study, flavors were defined as positive if at least one positive result had been reported. In order to confirm the positive results, we reviewed the corresponding reports in detail, and justified the positive results according to the following criteria. In the case of results obtained by standard methods, a positive result was assigned when a revertant count that exceeded twice the background revertant count was obtained. However, for results by typical methods that were slightly greater than twice or, in the case of positive results obtained by atypical methods, experts reviewed the data of a report, did not consider the report to have clear positive data and judged the report equivocal. If a flavor has reports only with Ames-equivocal results other than Ames-negative results, we considered that flavor to be equivocal. As a result, the judgment consists of "positive," "equivocal," and "negative." Among these 367 flavors, 24 were positive, 12 were equivocal, and 331 were negative compounds in the Ames assay. Overall, 367 flavoring compounds evaluated, and their Ames test and (Q)SAR results are shown in Appendix A. To move closer to our current purpose, we put weight on the findings of Ames-positive alerts, and then considered Ames-equivocal flavors to be positive.

2.2. (Q)SAR programs and in silico definition of positive and negative responses

In silico evaluation of potential mutagenicity was carried out using three commercially available (Q)SAR programs. Derek for Windows (version 10.0.2; Lhasa Ltd., UK) is a specialized or toxic chemical sub-structure rules-based system (Greene et al., 1999). When the system gave an evaluation as "certain", "probable", "plausible" or "equivocal", we considered this as "positive", and when the system gave "doubted", "improbable", "impossible" or "no alert", we considered this as "negative." MultiCase (version 1.90; Multicase Co. Ltd., Japan) is a hybrid system of 2D chemical descriptors based (Q)SAR and known toxic sub-structure identification (Rosenkranz et al., 1999). When the system showed "active", "borderline" or "probably inactive", we considered this as positive, and only when the system showed "inactive" did we consider this as negative. ADMEWorks (version 4.0; Fujitsu Kyushu Systems Ltd., Japan) is a system based mainly on 2D (sometimes 3D) descriptors, such as topological, topographical, physicochemical, and sub-structural parameters. When the system showed "positive," we considered this as "positive", and when the system showed "negative" we considered this as "negative". We selected these systems for the combined prediction system because of their different modes of analysis. In this study, *in silico* prediction of the mutagenicity of 367 flavor chemicals was performed using prediction models developed in our previous study (Hayashi et al., 2005), and compared with the reported experimental results.

2.3. Definitions in (Q)SAR models

We calculated sensitivity, specificity, concordance, applicability, false positive, and false negative as follows:

$$\text{Sensitivity (\%)} = N_{AS+}/N_{A+} \times 100,\ \text{Specificity (\%)} = N_{AS-}/N_{A-} \times 100,$$

$$\text{Concordance (\%)} = (N_{AS+} + N_{AS-})/N_{eval} \times 100,\ \text{Applicability (\%)} = N_{eval}/N_{all} \times 100,$$

$$\text{False positive (\%)} = (N_{A-} - N_{AS-})/N_{S+} \times 100,\ \text{False negative (\%)} = (N_{A+} - N_{AS+})/N_{S-} \times 100,$$

where N_{A+} is the number of chemicals that are positive in an *in vitro* assay (Ames test); N_{A-} is the number of chemicals negative in an *in vitro* assay (Ames test); N_{AS+} is the number of chemicals positive by both the Ames test and (Q)SAR evaluation; N_{AS-} is the number of chemicals negative in both the Ames test and (Q)SAR evaluation; N_{all} is the total number of chemicals analyzed; N_{eval} is the number of chemicals evaluated; N_{S+} is the number of chemicals positive in (Q)SAR evaluation; and N_{S-} is the number of chemicals negative in (Q)SAR evaluation.

3. Results

The predictions were performed by the single (Q)SAR model, the performances of each (Q)SAR model are shown in Table 1 and the results of their evaluations are summarized in Table 2. The predictions were performed also by combined evaluation of the three (Q)SAR models in three different ways: combination-1, -2 and -3. In combination-1, *in silico* mutagenicity evaluated using (Q)SAR systems was considered to be positive (or negative) only when all three models gave unanimous evaluations. In combination-2, *in silico* mutagenicity was considered to be positive (or negative) when two or more models gave the same evaluations. In combination-3, *in silico* mutagenicity was considered to be positive when one or more models gave a positive evaluation and to be negative when all three models gave negative evaluations. Performances of each combination of three (Q)SAR modes are shown in Table 3 and results of their evaluations are summarized in Table 4.

Table 2
Results of evaluation of each (Q)SAR model.

	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	False positive (%)	False negative (%)
Derek for Windows	38.9	93.4	88.0	100.0	61.1	6.6
MultiCase	25.0	94.3	87.5	100.0	67.9	8.0
ADMEWorks	13.9	91.5	83.9	100.0	84.8	9.3

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Table 3
Performance of each combination of three (Q)SAR models.

Ames results	(Q)SAR				
	Combination-3(+)			Combination-3(-)	
	Combination-2(+)		Combination-2(-)		
	Combination-1(+)			Combination-1(-)	
	3+	2+, 1-	1+, 2-	3-	Total
Positive	3	3	5	13	24
Equivocal	1	0	5	6	12
Negative	1	5	56	269	331
Total	5	8	66	288	367

The highest sensitivity with the Ames results was provided by Derek for Windows (38.9%), followed by MultiCase (25.0%). ADMEWorks provided the lowest sensitivity (13.9%), the specificities and concordances provided by three all models were more than 90% and 80%, respectively, and the applicability of all three (Q)SAR models was 100%. The applicability of each (Q)SAR model used depends on the system of the model; however, all compounds were evaluated by all three (Q)SAR models. The false positives and false negatives were 61–85% and 6–10%, respectively. In combinatorial (Q)SAR evaluation, sensitivity was 17.4% (combination-1) to 47.2% (combination-3), specificity 81.3% (combination-3) to 99.6% (combination-1), concordance 77.9% (combination-3) to 93.2% (combination-1), and applicability 79.8% (combination-1) to 100.0% (combination-2 and 3). For combination-1, some compounds could not be judged based on three (Q)SAR outcomes, such as two positives with one negative ("2+,1−") and one positive with two negatives ("1+,2−"), shown in Table 3, and so the applicability was less than 100% in this case.

4. Discussion

Our previous (Q)SAR models were developed especially to be customized for application to industrial chemicals, and the sensitivities of the previous combinatorial (Q)SAR systems were 73–99% (Hayashi et al., 2005). The sensitivities in the current study were lower, probably because the chemical structure domains in the data set specialized in flavors would be much different from those of the model training data set consisting of general industrial chemicals. The number of positives was very low compared with negatives, and the percentage of positive chemicals was about 7.3% (24/331). If a chemical had some positive results, most of the results indicated weak mutagenicity. This suggested that most of them are expected to not have genotoxicity, because the chemicals tested in the present study were evaluated as safe for use as food additive flavors by JECFA; however, according to our definition of Ames-positive in the present study, some flavors suspected as negative were judged as positive. For example, methylsulfinylmethane, phenol and eugenol, etc., were defined as Ames-positive based on only one positive result, while many other results for those chemicals indicated negative.

In combination-2, 325 Ames-negative chemicals were correctly judged as negative from 331 Ames-negatives and the specificity was 98.2%; however, only 7 Ames-positive chemicals were correctly judged as positive from 36 Ames-positives, including equivocal flavors, and the sensitivity was low (19.4%). In contrast, 79 chemicals were judged as positive in combination-3, and the sensitivity increased to 47.2%. The model with the highest sensitivity (38.9%) among three single models was Derek for Windows, as indicated in Table 2. The contribution of this increased sensitivity in combination-3 was mainly due to the result of the prediction by Derek

Table 5
False negative flavors in all three (Q)SAR models.

JECFA No.	Compound	CAS No.
217	*trans*-Anethole	4180-23-8
408	Diacetyl	431-03-8
429	Menthone	89-80-5
507	Methylsulfinylmethane (DMSO)	67-68-5
712	Resorcinol	108-46-3
735	2-Phenylphenol	90-43-7
767	2,6-Dimethylpyrazine	108-50-9
1032	Thiazole	228-47-1
1307	Methyl 2-pyrrolyl ketone	1072-83-9
1346	Cadinene	29350-73-0
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	3658-77-3
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF)	27538-09-6
1480	Maltol	118-71-8

These compound names are used in JECFA. These 13 flavors have one or some report(s) that are Ames-positive, but they were negative with three (Q)SAR models (Derek for Windows, MultiCase, ADMEWorks) in our present study.

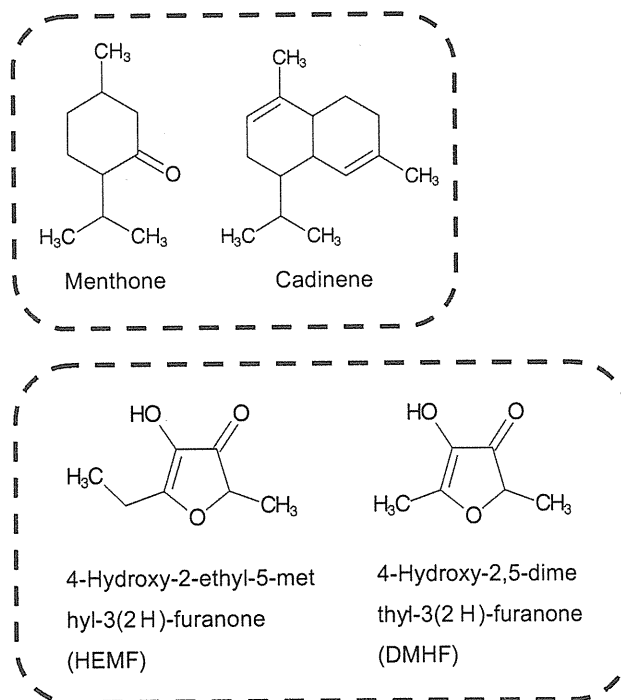

Fig. 1. Structures of false negative chemicals with a common sub-structure.

Table 4
Results of evaluation of each combination of three (Q)SAR models.

	Sensitivity (%)	Specificity (%)	Concordance	Applicability (%)	False positive (%)	False negative (%)
Combination-1	17.4	99.6	93.2	79.8	20.0	6.6
Combination-2	19.4	98.2	90.5	100.0	46.2	8.2
Combination-3	47.2	81.3	77.9	100.0	78.5	6.6

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Appendix A
Flavoring compounds evaluated and their Ames and (Q)SAR results.

JECFA No.	Flavor chemicals	Ames result	Derek for Windows	MultiCASE	ADMEWorks
1175	*trans,trans*-2,4-Hexadienal	Positive	+	+	+
1302	6-Methylquinoline	Positive	+	+	+
937	Pyruvaldehyde	Positive	+	+	+
739	Furfuryl acetate	Positive	+	+	–
1147	1-Penten-3-one	Positive	+	+	–
1353	2-Hexenal	Positive	+	–	+
656	*trans*-cinnamaldehyde	Positive	+	–	–
1364	2-Pentenal	Positive	+	–	–
1503	2-Furyl methyl ketone	Positive	+	–	–
1576	Ethyl 3-phenylglycidate	Positive	+	–	–
820	4-Phenyl-3-buten-2-one	Positive	–	+	–
217	*trans*-Anethole	Positive	–	–	–
408	Diacetyl	Positive	–	–	–
429	Menthone	Positive	–	–	–
507	Methylsulfinylmethane (DMSO)	Positive	–	–	–
712	Resorcinol	Positive	–	–	–
735	2-Phenylphenol	Positive	–	–	–
767	2,6-Dimethylpyrazine	Positive	–	–	–
1032	Thiazole	Positive	–	–	–
1307	Methyl 2-pyrrolyl ketone	Positive	–	–	–
1346	Cadinene	Positive	–	–	–
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Positive	–	–	–
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF)	Positive	–	–	–
1480	Maltol	Positive	–	–	–
1560	Allyl isothiocyanate	Equivocal	+	+	+
738	Furfuryl alcohol	Equivocal	+	–	–
744	Furfural	Equivocal	+	–	–
1561	Butyl isothiocyanate	Equivocal	+	–	–
1563	Phenethyl isothiocyanate	Equivocal	+	–	–
1168	3-Propylidenephthalide	Equivocal	–	+	–
1450	4-Hydroxy-5-methyl-3(2*H*)-furanone	Equivocal	–	+	–
252	Isobutyraldehyde	Equivocal	–	–	+
690	Phenol	Equivocal	–	–	–
836	Benzoin	Equivocal	–	–	–
1172	6-Methylcoumarin	Equivocal	–	–	–
1342	δ-3-Carene	Equivocal	–	–	–
1481	Ethyl maltol	Equivocal	–	–	–
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Equivocal	–	–	–
1209	2-Methyl-2-pentenal	Negative	+	+	+
686	alpha-Hexylcinnamaldehyde	Negative	+	+	–
689	*para*-Methoxy-alpha-methylcinnamaldehyde	Negative	+	+	–
973	*para*-Mentha-1,8-dien-7-al	Negative	+	+	–
977	2,6,6-Trimethylcyclohexa-1,3-dienyl methanal	Negative	+	+	–
1225	Citral	Negative	+	–	+
683	alpha-Methylcinnamaldehyde	Negative	+	–	–
685	alpha-Amylcinnamaldehyde	Negative	+	–	–
688	*ortho*-Methoxycinnamaldehyde	Negative	+	–	–
745	5-Methylfurfural	Negative	+	–	–
1185	2,4-Nonadienal	Negative	+	–	–
1186	Nona-2-*trans*-6-*cis*-dienal	Negative	+	–	–
1190	2-*trans*,4-*trans*-Decadienal	Negative	+	–	–
1360	2-Heptenal	Negative	+	–	–
1362	2-Nonenal	Negative	+	–	–
1363	2-Octenal	Negative	+	–	–
1487	2-Methylfuran	Negative	+	–	–
1488	2,5-Dimethylfuran	Negative	+	–	–
1497	3-(2-Furyl)acrolein	Negative	+	–	–
1562	Benzyl isothiocyanate	Negative	+	–	–
1577	Ethyl methylphenylglycidate	Negative	+	–	–
1716	Dihydroxyacetone	Negative	+	–	–
42	Isoamyl formate	Negative	–	+	–
413	3,4-Hexanedione	Negative	–	+	–
492	Methylthio 2-(acetyloxy)propionate	Negative	–	+	–
493	Methylthio 2-(propionyloxy)propionate	Negative	–	+	–
521	Allyl mercaptan	Negative	–	+	–
526	Benzyl mercaptan	Negative	–	+	–
841	Benzyl formate	Negative	–	+	–
1002	Phenylacetaldehyde	Negative	–	+	–
1023	*para*-Tolylacetaldehyde	Negative	–	+	–
1356	Methyl 2-nonynoate	Negative	–	+	–
1357	Methyl 2-octynoate	Negative	–	+	–
1681	Allyl thiohexanoate	Negative	–	+	–
1687	3,6-Diethyl-1,2,4,5-tetrathiane	Negative	–	+	–
1774	*N*-Lactoyl ethanolamine	Negative	–	+	–

(continued on next page)

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Appendix A (*continued*)

JECFA No.	Flavor chemicals	Ames result	Derek for Windows	MultiCASE	ADMEWorks
83	Propionaldehyde	Negative	–	–	+
258	3-Methylbutyraldehyde	Negative	–	–	+
301	4-Methyl-2-pentanone	Negative	–	–	+
349	2,6-Dimethyl-5-heptenal	Negative	–	–	+
405	Acetoin	Negative	–	–	+
410	2,3-Pentanedione	Negative	–	–	+
532	1,2-Ethanedithiol	Negative	–	–	+
564	Dimethyl disulfide	Negative	–	–	+
761	2-Methylpyrazine	Negative	–	–	+
798	5-Methylquinoxaline	Negative	–	–	+
857	Isoamyl benzoate	Negative	–	–	+
884	Methyl anisate	Negative	–	–	+
899	Methyl salicylate	Negative	–	–	+
909	Glycerol	Negative	–	–	+
1013	Isobutyl phenylacetate	Negative	–	–	+
1120	6-Methyl-5-hepten-2-one	Negative	–	–	+
1131	4-Methyl-3-penten-2-one	Negative	–	–	+
1135	(*E*)-7-Methyl-3-octen-2-one	Negative	–	–	+
1268	Isoeugenyl benzyl ether	Negative	–	–	+
1534	Methyl anthranilate	Negative	–	–	+
1535	Ethyl anthranilate	Negative	–	–	+
1537	Isobutyl anthranilate	Negative	–	–	+
1543	Phenylethyl anthranilate	Negative	–	–	+
1545	Methyl *N*-methylanthranilate	Negative	–	–	+
1549	Methyl *N*-formylanthranilate	Negative	–	–	+
1654	α,α-Dimethylphenethyl formate	Negative	–	–	+
3	Allyl hexanoate	Negative	–	–	–
7	Allyl isovalerate	Negative	–	–	–
19	Allyl cinnamate	Negative	–	–	–
22	Benzaldehyde	Negative	–	–	–
23	Benzyl acetate	Negative	–	–	–
24	Benzyl benzoate	Negative	–	–	–
25	Benzyl alcohol	Negative	–	–	–
52	Isoamyl alcohol	Negative	–	–	–
58	Geranyl acetate	Negative	–	–	–
79	Formic acid	Negative	–	–	–
80	Acetaldehyde	Negative	–	–	–
81	Acetic acid	Negative	–	–	–
82	Propyl alcohol	Negative	–	–	–
84	Propionic acid	Negative	–	–	–
85	Butyl alcohol	Negative	–	–	–
86	Butyraldehyde	Negative	–	–	–
87	Butyric acid	Negative	–	–	–
88	Amyl alcohol	Negative	–	–	–
92	Hexanal	Negative	–	–	–
93	Hexanoic acid	Negative	–	–	–
95	Heptanal	Negative	–	–	–
96	Heptanoic acid	Negative	–	–	–
97	1-Octanol	Negative	–	–	–
98	Octanal	Negative	–	–	–
99	Octanoic acid	Negative	–	–	–
101	Nonanal	Negative	–	–	–
104	Decanal	Negative	–	–	–
105	Decanoic acid	Negative	–	–	–
107	Undecanal	Negative	–	–	–
109	Lauryl alcohol	Negative	–	–	–
111	Lauric acid	Negative	–	–	–
113	Myristic acid	Negative	–	–	–
114	1-Hexadecanol	Negative	–	–	–
116	Stearic acid	Negative	–	–	–
125	Methyl acetate	Negative	–	–	–
127	Butyl acetate	Negative	–	–	–
139	Acetone	Negative	–	–	–
184	Butyl stearate	Negative	–	–	–
196	Ethyl isovalerate	Negative	–	–	–
219	4-Hydroxybutyric acid lactone (gamma-Butyrolactone)	Negative	–	–	–
225	gamma-Heptalactone	Negative	–	–	–
229	gamma-Nonalactone	Negative	–	–	–
233	gamma-Undecalactone	Negative	–	–	–
239	omega-Pentadecalactone	Negative	–	–	–
249	1,4-Dodec-6-enolactone	Negative	–	–	–
251	Isobutyl alcohol	Negative	–	–	–
253	Isobutyric acid	Negative	–	–	–
254	2-Methylbutyraldehyde	Negative	–	–	–
260	2-Methylpentanal	Negative	–	–	–

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Appendix A (*continued*)

JECFA No.	Flavor chemicals	Ames result	Derek for Windows	MultiCASE	ADMEWorks
267	2-Ethyl-1-hexanol	Negative	–	–	–
273	2,6-Dimethyloctanal	Negative	–	–	–
277	Isopropyl alcohol	Negative	–	–	–
278	2-Butanone	Negative	–	–	–
302	2,6-Dimethyl-4-heptanone	Negative	–	–	–
305	Isopropyl acetate	Negative	–	–	–
311	Isopropyl myristate	Negative	–	–	–
333	Oleic acid	Negative	–	–	–
346	Methyl linoleate	Negative	–	–	–
356	Linalool	Negative	–	–	–
359	Linalyl acetate	Negative	–	–	–
366	alpha-Terpineol	Negative	–	–	–
374	β-Teroineol	Negative	–	–	–
380	Carvone	Negative	–	–	–
381	Carveol	Negative	–	–	–
382	Carvyl acetate	Negative	–	–	–
388	alpha-Ionone	Negative	–	–	–
389	β-Ionone	Negative	–	–	–
398	Methyl-alpha-ionone	Negative	–	–	–
400	Methyl-delta-ionone	Negative	–	–	–
418	Methylcyclopentenolone	Negative	–	–	–
424	2-Hydroxy-2-cyclohexen-1-one	Negative	–	–	–
427	Menthol	Negative	–	–	–
443	(-)-Menthol ethyleneglycol	Negative	–	–	–
444	(-)-Menthol 1- and 2-propylene glycol carbonate	Negative	–	–	–
446	(±)-Menthone 1,2-glycerol ketal	Negative	–	–	–
458	Allyl sulfide	Negative	–	–	–
525	Benzenethiol	Negative	–	–	–
551	2-Mercaptopropionic acid	Negative	–	–	–
572	Allyl disulfide	Negative	–	–	–
578	Phenyl disulfide	Negative	–	–	–
579	Benzyl disulfide	Negative	–	–	–
595	Ethyl acetoacetate	Negative	–	–	–
610	Hydroxycitronellol	Negative	–	–	–
611	Hydroxycitronellal	Negative	–	–	–
612	Hydroxycitronellal dimethyl acetal	Negative	–	–	–
614	Diethyl malonate	Negative	–	–	–
616	Dimethyl succinate	Negative	–	–	–
618	Fumaric acid	Negative	–	–	–
619	*l*-Malic acid	Negative	–	–	–
623	Adipic acid	Negative	–	–	–
625	Dibutyl sebacate	Negative	–	–	–
626	Ethylene brassylate	Negative	–	–	–
627	Aconitic acid	Negative	–	–	–
645	3-Phenylpropionaldehyde	Negative	–	–	–
647	Cinnamyl alcohol	Negative	–	–	–
657	Cinnamic acid	Negative	–	–	–
659	Ethyl cinnamate	Negative	–	–	–
667	Cyclohexyl cinnamate	Negative	–	–	–
670	Benzyl cinnamate	Negative	–	–	–
674	alpha-Amylcinnamyl alcohol	Negative	–	–	–
691	*ortho*-Cresol	Negative	–	–	–
692	*meta*-Cresol	Negative	–	–	–
693	*para*-Cresol	Negative	–	–	–
694	*para*-Ethylphenol	Negative	–	–	–
706	2,5-Xylenol	Negative	–	–	–
707	2,6-Xylenol	Negative	–	–	–
708	3,4-Xylenol	Negative	–	–	–
709	Thymol	Negative	–	–	–
713	Guaiacol	Negative	–	–	–
721	2,6-Dimethoxyphenol	Negative	–	–	–
727	2-Hydroxyacetophenone	Negative	–	–	–
733	4-(1,1-Dimethyl)ethylphenol	Negative	–	–	–
736	Phenyl salicylate	Negative	–	–	–
753	Pulegone	Negative	–	–	–
758	Menthofuran	Negative	–	–	–
762	2-Ethylpyrazine	Negative	–	–	–
765	2,3-Dimethylpyrazine	Negative	–	–	–
766	2,5-Dimethylpyrazine	Negative	–	–	–
768	2-Ethyl-3-methylpyrazine	Negative	–	–	–
774	2,3,5-Trimethylpyrazine	Negative	–	–	–
775	2-Ethyl-3,5-dimethylpyrazine and 2-Ethyl-3,6-dimethylpyrazine	Negative	–	–	–
780	2,3,5,6-Tetramethylpyrazine	Negative	–	–	–
788	2-Methoxy-(3, 5 or 6)-methylpyrazine	Negative	–	–	–

(*continued on next page*)

Appendix A (*continued*)

JECFA No.	Flavor chemicals	Ames result	Derek for Windows	MultiCASE	ADMEWorks
799	alpha-Methylbenzyl alcohol	Negative	–	–	–
806	Acetophenone	Negative	–	–	–
811	Methyl beta-naphthyl ketone	Negative	–	–	–
812	4-Acetyl-6-*tert*-butyl-1,1-dimethylindan	Negative	–	–	–
818	4-(*para*-Methoxy-phenyl)-2-butanone	Negative	–	–	–
819	4-Phenyl-3-buten-2-ol	Negative	–	–	–
824	Propiophenone	Negative	–	–	–
825	alpha-Propylphenethyl alcohol	Negative	–	–	–
826	1-(*para*-Methoxyphenyl)-1-penten-3-one	Negative	–	–	–
831	Benzophenone	Negative	–	–	–
833	1-Phenyl-1,2- propanedione	Negative	–	–	–
834	Ethyl benzoylacetate	Negative	–	–	–
850	Benzoic acid	Negative	–	–	–
851	Methyl benzoate	Negative	–	–	–
864	Isopropylbenzyl alcohol	Negative	–	–	–
867	Tolualdehydes (mixed *ortho, meta, para*)	Negative	–	–	–
868	Cuminaldehyde	Negative	–	–	–
870	Butyl *para*-hydroxybenzoate	Negative	–	–	–
871	Anisyl alcohol	Negative	–	–	–
877	Veratraldehyde	Negative	–	–	–
878	*para*-Methoxybenzaldehyde	Negative	–	–	–
879	*para*-Ethoxybenzaldehyde	Negative	–	–	–
888	Vanillyl butyl ether	Negative	–	–	–
889	Vanillin	Negative	–	–	–
893	Ethyl vanillin	Negative	–	–	–
894	Piperonyl acetate	Negative	–	–	–
896	Piperonal	Negative	–	–	–
897	Salicylaldehyde	Negative	–	–	–
918	Glyceryl monostearate	Negative	–	–	–
925	Propylene glycol	Negative	–	–	–
930	Lactic acid	Negative	–	–	–
931	Ethyl lactate	Negative	–	–	–
935	Butyl butyryllactate	Negative	–	–	–
936	Pyruvic acid	Negative	–	–	–
938	Ethyl pyruvate	Negative	–	–	–
951	Pyrazine	Negative	–	–	–
953	Ethyl vanillin isobutyrate	Negative	–	–	–
987	Phenethyl alcohol	Negative	–	–	–
1007	Phenylacetic acid	Negative	–	–	–
1009	Ethyl phenylacetate	Negative	–	–	–
1014	Isoamyl phenylacetate	Negative	–	–	–
1027	Ethyl (*para*-tolyloxy)acetate	Negative	–	–	–
1028	2-Phenoxyethyl isobutyrate	Negative	–	–	–
1029	Sodium 2-(4-methoxyphenoxy)propanoate	Negative	–	–	–
1035	4,5-Dimethylthiazole	Negative	–	–	–
1043	4-Methylthiazole	Negative	–	–	–
1050	5-Methyl-2-thiophenecarboxyaldehyde	Negative	–	–	–
1094	Cyclohexyl butyrate	Negative	–	–	–
1100	Cyclohexanone	Negative	–	–	–
1101	Cycopentanone	Negative	–	–	–
1106	2-Hexylidene cyclopentanone	Negative	–	–	–
1108	2,2,6-Trimethylcyclohexanone	Negative	–	–	–
1111	Tetramethyethylcyclohexanone (mixure of isomers)	Negative	–	–	–
1112	Isophorone	Negative	–	–	–
1124	3-Penten-2-one	Negative	–	–	–
1134	6-Methyl-3,5-heptadien-2-one	Negative	–	–	–
1153	1-Decen-3-ol	Negative	–	–	–
1164	(+/-)-(2,6,6-Trimethyl-2-hydroxycyclohexylidene)acetic acid γ-lactone	Negative	–	–	–
1166	Octahydrocoumarin	Negative	–	–	–
1171	Dihydrocoumarin	Negative	–	–	–
1193	Ethyl 2,4,7-decatrienoate	Negative	–	–	–
1199	2-Methylbutanol	Negative	–	–	–
1219	*dl*-Citronellol	Negative	–	–	–
1220	Citronellal	Negative	–	–	–
1223	Geraniol	Negative	–	–	–
1230	Farnesol	Negative	–	–	–
1234	Eucalyptol	Negative	–	–	–
1241	Anisole	Negative	–	–	–
1243	*p*-Methylanisole	Negative	–	–	–
1244	*p*-Propylanisole	Negative	–	–	–
1248	1,2-Dimethoxybenzene	Negative	–	–	–
1249	*m*-Dimethoxybenzene	Negative	–	–	–
1250	*p*-Dimethoxybenzene	Negative	–	–	–
1255	Diphenyl ether	Negative	–	–	–
1256	Dibenzyl ether	Negative	–	–	–

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Appendix A (*continued*)

JECFA No.	Flavor chemicals	Ames result	Derek for Windows	MultiCASE	ADMEWorks
1257	β-Naphthyl methyl ether	Negative	–	–	–
1258	β-Naphthyl ethyl ether	Negative	–	–	–
1259	β-Naphthyl isobutyl ether	Negative	–	–	–
1260	Isoeugenol	Negative	–	–	–
1263	Isoeugenyl phenylacetate	Negative	–	–	–
1264	Propenylguaethol	Negative	–	–	–
1289	*Erythro*- and *threo*-3-mercapto-2-methylbutanol	Negative	–	–	–
1301	Indole	Negative	–	–	–
1303	Isoquinoline	Negative	–	–	–
1304	Skatole	Negative	–	–	–
1314	Pyrrole	Negative	–	–	–
1315	3-Ethylpyridine	Negative	–	–	–
1316	3-Acetylpyridine	Negative	–	–	–
1323	Camphene	Negative	–	–	–
1324	β-Caryophyllene	Negative	–	–	–
1325	*p*-Cymene	Negative	–	–	–
1326	*d*-Limonene	Negative	–	–	–
1327	Myrcene	Negative	–	–	–
1329	α-Pinene	Negative	–	–	–
1330	β-Pinene	Negative	–	–	–
1332	Biphenyl	Negative	–	–	–
1334	4-Methylbiphenyl	Negative	–	–	–
1335	1-Methylnaphthalene	Negative	–	–	–
1340	*p*-Mentha-1,4-diene	Negative	–	–	–
1351	Ethyl acrylate	Negative	–	–	–
1371	(*E*)-2-Butenoic acid	Negative	–	–	–
1385	Borneol	Negative	–	–	–
1391	Isobornyl propionete	Negative	–	–	–
1395	*d*-Camphor	Negative	–	–	–
1408	3-*l*-Menthoxypropane-1,2-diol	Negative	–	–	–
1411	3-*l*-Menthoxy-2-methylpropan-1,2-diol	Negative	–	–	–
1413	*d,l*-Menthol 1- and 2-propylene glycol carbonate	Negative	–	–	–
1416	*p*-Menthan-3,8-diol	Negative	–	–	–
1441	2-(3-Phenylpropyl)tetrahydrofuran	Negative	–	–	–
1443	Tetrahydrofurfuryl alcohol	Negative	–	–	–
1445	Tetrahydrofurfuryl propionate	Negative	–	–	–
1459	β-Methylphenethyl alcohol	Negative	–	–	–
1467	2-Phenylpropionaldehyde	Negative	–	–	–
1468	2-Phenylpropionaldehyde dimethyl acetal	Negative	–	–	–
1470	2-Phenylpropyl isobutyrate	Negative	–	–	–
1494	3-Methyl-2-(3-methyl-2-butenyl)furan	Negative	–	–	–
1511	4-(2-Furyl)-3-buten-2-one	Negative	–	–	–
1513	Ethyl 3-(2-furyl)propanoate	Negative	–	–	–
1526	*O*-Ethyl *S*-(2-furylmethyl)thiocarbonate	Negative	–	–	–
1529	Eugenol	Negative	–	–	–
1536	Butyl anthranilate	Negative	–	–	–
1540	Linalyl anthranilate	Negative	–	–	–
1541	Cyclohexyl anthranilate	Negative	–	–	–
1552	*N*-Benzoylanthranilic acid	Negative	–	–	–
1575	beta-Caryophyllene oxide	Negative	–	–	–
1579	Ethylamine	Negative	–	–	–
1581	Isopropylamine	Negative	–	–	–
1582	Butylamine	Negative	–	–	–
1583	Isobutylamine	Negative	–	–	–
1584	*sec*-Butylamine	Negative	–	–	–
1585	Pentylamine	Negative	–	–	–
1592	Acetamide	Negative	–	–	–
1595	2-Isopropyl-*N*,2,3-trimethylbutyramide	Negative	–	–	–
1598	*N*-Isobutyl (*E,E*)-2,4-decadienamide	Negative	–	–	–
1600	Piperine	Negative	–	–	–
1607	Piperidine	Negative	–	–	–
1609	Pyrrolidine	Negative	–	–	–
1610	Trimethylamine	Negative	–	–	–
1611	Triethylamine	Negative	–	–	–
1615	Piperazine	Negative	–	–	–
1649	1-Phenyl-3-methyl-3-pentanol	Negative	–	–	–
1700	Allyl propyl disulfide	Negative	–	–	–
1767	*N*-(Heptan-4-yl)benzo[*d*][1,3]-dioxole-5-carboxamide	Negative	–	–	–
1768	N^1-(2,4-Dimethoxybenzyl)-N^2-(2-(pyridine-2-yl)ethyl)oxalamide	Negative	–	–	–
1772	*N*-Gluconyl ethanolamine	Negative	–	–	–
1777	*N*-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide	Negative	–	–	–

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for Windows, which is a knowledge-based model. In the point of defining the priority of conducting Ames tests on many flavors, a model with higher sensitivity was better, and therefore combination-3 was the best among the current models. In this case, the percentage of false positive increases, we could confirm the actual results by conducting Ames tests for only limited numbers of flavors.

In the present study, 13/24 of chemicals reported as positive without Ames-equivocal were negative by all three (Q)SAR models. These 13 chemicals are shown in Table 5. On the other hand, one chemical, 2-methyl-2-pentenal, was negative in the Ames test but positive according to all three models. Detailed structural analysis of these 13 chemicals indicated that some of these chemicals possessed common sub-structures. The structures of false negatives with various common sub-structures are indicated in Fig. 1, and the chemicals enclosed within the dotted line in the figure have a common sub-structure. The applicability domain of each (Q)SAR model is basically limited within the chemical spaces of training chemical structures. The positive structural alerts for those sub-structures might not have been confirmed in our (Q)SAR models because of the lack of chemicals which have these sub-structures in our database used for the development of current (Q)SAR models. Expansion of the applicability domain of the (Q)SAR models by additional training including those sub-structures and development of sub-structural alerts could effectively contribute to increasing the predictability of mutagenicity for flavors, because many flavors possess categorically similar functional sub-structures or are composed of a series of derivatives.

There is an another possibility for the discrepancy between (Q)SAR prediction and experimental results. 2,5-Dimethyl-4-hydroxy-3(2H)-furanone and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone may cause genotoxicity by indirect mechanisms, of action (in particular, generation of reactive oxygen species) (Hiramoto et al., 1996a,b) and for those such as trans-anethole, an Ames-positive result was reported only under the conditions with metabolic activation. The current (Q)SAR models were mainly developed based on information about typical genotoxic chemicals (Kirkland et al., 2005; Hayashi et al., 2005), and thus might be optimized for the direct mechanism rather than the indirect mechanism. Additional improvement of prediction might be achieved in combination with *in silico* tools which can predict indirect mechanisms.

In conclusion, the *in silico* prediction results from the combination of our (Q)SAR models were validated for priority setting to conduct Ames tests of many unevaluated flavors. The overall performance was lower than expected from the case of industrial chemicals; however, our combination (Q)SAR model approach was suitable for improving the *in silico* prediction and priority setting for Ames tests of flavors by raising the accuracy of each (Q)SAR model with a wider knowledge base for flavor-specific structures.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix A

See Appendix A.

References

Cronin, M.T.D., Jaworska, J.S., Walker, J.D., Comber, M.H.I., Watts, C.D., Worth, A.P., 2003. Use of QSARs in international decision-making frameworks to predict health effects of chemical substances. Environ. Health Perspect. 111, 1391–1401.

Greene, N., Judson, P.N., Langowski, J.J., Marchant, C.A., 1999. Knowledge-based expert systems for toxicity and metabolism prediction: DEREK, StAR and METEOR. SAR QSAR Environ. Res. 10, 299–314.

Hayashi, M., Kamata, E., Hirose, A., Takahashi, M., Morita, T., Ema, M., 2005. In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals. Mutat. Res. 588, 129–135.

Hiramoto, K., Sekiguchi, K., Ayuha, K., Aso-o, R., Moriya, N., Kato, T., Kikugawa, K., 1996a. DNA breaking activity and mutagenicity of soy sauce: characterization of the active components and identification of 4-hydroxy-5-methyl-3(2H)-furanone. Mutat. Res. 359, 119–132.

Hiramoto, K., Aso-o, R., Ni-iyama, H., Hikage, S., Kato, T., Kikugawa, K., 1996b. DNA strand break by 2,5-dimethyl-4-hydroxy-3(2H)-furanone, a fragrant compound in various foodstuffs. Mutat. Res. 359, 17–24.

JECFA. FAO/WHO Joint Expert Committee on Food Additives. WHO Food Additives Series (FAS) in 1965 to 2008.

JFSC. Japan Food Safety Commission. Evaluation Reports of Food Additives (in Japanese).

Kirkland, D., Aardema, M., Henderson, L., Müller, L., 2005. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity. Mutat. Res. 584, 1–256.

Rosenkranz, H.S., Cunningham, A.R., Zhang, Y.P., Claycamp, H.G., Macina, O.T., Sussman, N.B., Grant, S.G., Klopman, G., 1999. Development, characterization and application of predictive-toxicology models. SAR QSAR Environ. Res. 10, 277–298.

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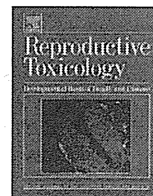

Two-generation reproductive toxicity study of aluminium sulfate in rats

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ABSTRACT

In a two-generation reproductive toxicity study, male and female rats were given aluminium sulfate (AS) in drinking water at 0, 120, 600 or 3000 ppm. AS reduced water consumption in all treatment groups, and body weight was transiently decreased in the 3000 ppm group. In the F1 and F2 pups, preweaning body weight gain was inhibited at 3000 ppm, and the liver and spleen weight was decreased at weaning. At this dose, vaginal opening was slightly delayed. There were no compound-related changes in other reproductive/developmental parameters, including developmental neurobehavioral endpoints. The data indicated that the NOAEL of AS in this two-generation study is 600 ppm for parental systemic toxicity and reproductive/developmental toxicity. The total ingested dose of aluminium from drinking water and food (standard rat diet, containing 25–29 ppm of aluminium) combined for this 600 ppm group was calculated to be 8.06 mg Al/kg bw/day.

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1. Introduction

Aluminium is the most abundant metal on Earth and constitutes 8.13% of the crust [1]. It is released into the environment largely by natural processes, but also due to anthropogenic activities [2]. People engaging in certain occupations, such as welding, aluminium soldering and production of abrasives, could be exposed to aluminium-containing dust particles by inhalation [3–5]; however, aluminium exposure by the general population is considered to occur mainly through food ingestion [1] although the use of aluminium-containing antacids and buffered analgesics may result in much higher aluminium intake [6,7]. While aluminium is inherently contained in most foodstuffs, its salts are artificially added to various food products (acidity regulator, raising agent, anti-caking agent, etc.) [8]. Use of aluminium and aluminium compounds in the processing, packaging and storage of food products is also a significant factor in the increased aluminium levels in foods [8]. On the other hand, aluminium salts are widely used as flocculants in the treatment of drinking water to reduce organic matter, color, turbidity and microorganism levels [9], which may lead to increased aluminium intake by the general public. Total dietary exposure to aluminium, including exposure via drinking water, has been assessed using a duplicate diet, total diet or market basket approach in a number of countries [8]. Based on these data, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimates that the mean total dietary exposure of the adult population ranges from 14 to 280 mg Al/week [8].

In humans, aluminium is regarded as a primary cause of dialysis encephalopathy syndrome, in which various neurological symptoms, such as speech difficulty, myoclonus and dementia, have been observed in patients on chronic hemodialysis [10,11]. For more general exposure, it is suspected that oral aluminium exposure via foods and drinking water may be associated with the risk of Alzheimer's disease and cognitive impairment, but this hypothesis remains controversial [12–14]. The neurotoxicological properties of aluminium have been clearly shown in laboratory animals, and the observed effects include encephalopathy, impairments of cognitive and motor function and neurofibrillary degeneration [15–18]. In animals, aluminium compounds also affect male reproductive systems [19–23], and developmental toxicity, including effects on the developing nervous system, has been reported after maternal exposure [24–32].

Concerning the adverse effects of aluminium on human health, its reference values in food and drinking water should be established based on appropriate toxicological data; however, the available data are insufficient to assess its health effects. As human data, there have been a number of epidemiological studies about

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the neurological effects of aluminium exposure via drinking water, but these studies did not account for aluminium intake from food, which is the most important route of exposure. Epidemiological studies on dietary aluminium exposure are preliminary at this time [8]. As for animal studies, most have focused on the specific endpoints or mechanisms of action, and the dosage is insufficient for dose–response assessment. In addition, considering the low oral bioavailability of aluminium [33,34] and actual human exposure via food and drinking water, many available study results from administration by gavage as well as by the parenteral route are not appropriate to evaluate the risk. In the WHO guidelines for drinking water quality, it was concluded that a health-based guideline value cannot be derived because of limitations in the animal data as a model for humans and the uncertainty surrounding human data [9]. JECFA clearly stated the need for further data on the bioavailability and developmental and multigenerational toxicity while it established a provisional tolerable weekly intake (PTWI) for aluminium of 1 mg/kg bw in food based on the available toxicological information [8].

In the present study, a two-generation reproductive toxicity study was conducted for aluminium sulfate (AS). AS is a water-soluble salt of aluminium, and is primarily used as a flocculant for water purification, paper sizing agent, fire extinguisher materials, etc. [35,36]. The present study was conducted according to OECD test guidelines under GLP. The selected route of administration is via drinking water because it is relevant to human exposure. As for the reproductive toxicity of aluminium, oral exposure studies evaluating sufficient endpoints in both sexes as well as multigenerational studies have not been reported yet; therefore, the data presented would provide useful information to assess the risk to human health from aluminium exposure.

2. Materials and methods

This study was conducted in 2008–2009 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). The study design complied with the OECD guideline 416 "Two-generation reproduction toxicity study" [37], and the Japanese guidelines for the designation of food additives and for revision of standards for the use of food additives [38]. All procedures involving the use and care of animals were performed in accordance with the principles for Good Laboratory Practice [39,40] and applicable animal welfare regulations ["Act on Welfare and Management of Animals" [41,42], "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain" [43] and "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare" [44]].

2.1. Chemical and dosing

AS (CAS No. 10043-01-3) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). The AS (Lot No. 007X1828) used in this study was 98.5% pure, and was kept in a sealed container under cool and dark conditions. The test article was dissolved in ion-exchanged water, and served as drinking water to the animals. Control rats were given the ion-exchanged water alone as drinking water. Before the start of the study, the stability of AS in ion-exchanged water at concentrations of 0.1, 0.6 and 15 mg/mL was confirmed after at least 4-day storage at room temperature following 6-day refrigerated storage; therefore, dosing solutions were prepared at least once every 6 days and kept in a cool place until serving. Fresh drinking water was served at least once every 4 days. During the study, the concentrations of AS in drinking water were analyzed in the first and last preparations and once every 3 months, and confirmed to be 97.5–106.3% of the target by high performance liquid chromatography. AS contained in the drinking water for the control group was less than the quantitation limit (5 μg/mL).

Prior to the present two-generation reproductive toxicity study, a dose-finding study was performed in male and female rats given drinking water containing AS at 0, 1000, 3000, 10,000 or 30,000 ppm. In that study, males were dosed for 7 weeks, beginning 14 days before mating, and females were dosed for 6–8 weeks beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. In the highest dose group, animals were euthanized at the end of the 2nd week of administration because of a marked decrease in body weight as a result of water avoidance. Water consumption also decreased in all other treatment groups. Decreased food consumption and body weight were observed at 3000 ppm and above. At autopsy, thickening of the limiting ridge in the stomach, and atrophy of the thymus and spleen were detected at 10,000 ppm. The relative weights of the liver, thymus and spleen were decreased in females in 3000 and 10,000 ppm groups. Although there were no changes in any reproductive parameters, the body weights of pups on postnatal day (PND) 4 were decreased at 10,000 ppm. Taking into account the results of this dose-finding study, the dose levels of AS in the present study were set as 120, 600 or 3000 ppm.

2.2. Animals and housing conditions

Crl:CD(SD) rats (4 weeks old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). This strain was chosen because they are the most commonly used in reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimated to the laboratory for 7 days, and subjected to treatment at 5 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. The rats were distributed into four groups of 24 males and 24 females each by stratified random sampling based on body weight, and all animals were assigned a unique number and the ear was tattooed prior to the start of the experiment.

Throughout the study, animals were maintained in an air-conditioned room at 21–25 °C, with a relative humidity of 36–59%, a 12-h light/dark cycle (8:00–20:00) and ventilation at 10–15 times/h. They were housed individually, except for the acclimation, mating and nursing periods, in suspended wire-mesh cages. From day 17 of gestation to day 21 after delivery, the wire-mesh floor of the cage was replaced with a stainless-steel tray, and individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc., Yokohama, Japan). All animals were fed *ad libitum* with a standard rat diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan), but were supplied with different drinking water solutions, as mentioned above, through two generations. Aluminium concentration in the standard diet, analyzed by atomic absorption spectrometry for each lot of diet, ranged from 25 ppm to 29 ppm.

2.3. Experimental design

Twenty-four F0 rats (5-week-old males and females)/sex/group were exposed to AS in drinking water at 0, 120, 600 or 3000 ppm. After 10-week administration of AS, each female rat was mated with a male rat of the same dosage group, and pregnant females were allowed to deliver spontaneously and nurse their pups. Administration of AS was continued throughout the mating, gestation and lactation periods. F0 parental male rats were necropsied after the parturition of paired females. F0 females were necropsied after weaning of their pups.

For the second (F1) generation, 24 male and 24 female weanlings in each group were selected as F1 parents on PNDs 21–25 to equalize the mean body weights among groups as much as possible. One male and 1 female F1 weanlings were selected from each of litters born during the 5 days including the day of the largest number of F0 parturition, and if the number of litters was insufficient, a second weanling pup in the litter was selected with care to prevent litter effects. The day on which F1 parental animals were selected was designated as day 0 of dosing for the F1 generation. F1-selected rats were given drinking water with the respective formulation, and were mated, allowed to deliver and nurse their F2 pups, and necropsied in the same manner as described for F0 rats. Unselected F1 weanlings and all F2 weanlings were necropsied on PND 26.

2.4. Mating procedures

Each female was mated with a single male of the same dosage group until successful copulation occurred or the mating period of 2 weeks had elapsed. For F1 matings, cohabitation of siblings was avoided. During the mating period, vaginal smears were examined daily for the presence of sperm, and the presence of sperm in the vaginal smear and/or a vaginal plug were considered as evidence of successful mating. The day of successful mating was designated as day 0 of gestation. Females that did not mate successfully during the 2-week mating period were cohabited with another male from the same group who had been proven to copulate with limits of not less than 7 days.

2.5. Parental data

Throughout the study, all parental animals were observed for clinical signs of toxicity at least twice a day. The body weight and food consumption were measured weekly. For females exhibiting evidence of successful mating, body weight and food consumption were recorded on gestational days 0, 7, 14 and 20 of gestation and days 0, 7, 14 and 21 of lactation (and additionally day 4 of lactation for body weight). Water consumption was recorded twice a week, and on days 0, 4, 7, 11, 14, 17 and 20 of gestation and days 0, 4, 7, 11, 14, 17, 19 and 21 of lactation. The intake of test substance was calculated based upon mean values for body weight and water consumption in each group.

For each female, daily vaginal lavage samples were evaluated for estrous cyclicity throughout the last 2 weeks of the premating period and during cohabitation until evidence of copulation was detected. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles.

2.6. Litter data

Once insemination was confirmed, female rats were checked at least three times daily on days 21–25 of gestation to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21 (the day of weaning). The day on which dams held their pups under the abdomen in the nest by 13:00 was designated as day 0 of lactation or PND 0. On PND 0, all live and dead pups were counted, and live pups were sexed and examined grossly. They were observed daily for clinical signs of toxicity, and the body weight of live pups was recorded on PNDs 0, 4, 7, 14 and 21. On PND 4, litters were randomly adjusted to eight pups of four males and four females. No adjustment was made for litters of fewer than eight pups. Pups were assigned a unique number and limb tattooed on PND 4.

2.7. Developmental landmarks

All F1 and F2 live pups were observed for pinna unfolding from PND 1 to PND 4. Body weight was recorded daily during this period. The anogenital distance (AGD) was measured using calipers on PND 4 in all F1 and F2 pups, and the normalized value of AGD to body weight, AGD/cube root of the body weight ratio, was calculated. One male and one female F1 and F2 pup selected from each dam were evaluated for incisor eruption beginning on PND 8 and eye opening beginning on PND 12, and continued until each pup fulfilled the criteria. The body weight of the respective F1 and F2 pups was recorded on the day the criteria were fulfilled. Surface righting reflex, negative geotaxis and mid-air righting reflex were assessed on PND 5, 8 and 18, respectively, for one male and one female F1 and F2 pup selected from each dam. All F1 offspring selected as F1 parents were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25 until completion. The body weight of the respective F1 rats was recorded on the day of completion of these pubertal landmarks.

2.8. Behavioral test

Spontaneous locomotor activity was measured at 4 weeks of age in 10 male and 10 female F1 rats randomly selected from each group, using a multi-channel activity monitoring system (SUPERMEX; Muromachi Kikai Co., Ltd., Tokyo, Japan). Rats were placed individually in transparent polycarbonate cages [285 (W) mm × 450 (D) mm × 210 (H) mm, CL-0108-1; CLEA Japan, Inc., Tokyo, Japan], which were placed under an infrared sensor that detects thermal radiation from animals, and spontaneous motor activity was determined at 10-min intervals and for 60 min.

A test in a water-filled multiple *T*-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The apparatus was similar to that described by Biel [45]. The water temperature of the maze was kept 20.5–22 °C. As a preliminary swimming ability test, each rat was allowed to swim three times in a straight channel on the day before the maze trial, and then tested in the maze with three trials per day for the next three consecutive days. The elapsed time between entry into the water at the starting point and touching the goal ramp, and the number of errors were recorded. To prevent the exhaustion of the rats, no animal was allowed to remain in the water for more than 3 min in any trial.

2.9. Termination/necropsy (adults)

All surviving parental male rats were euthanized by exsanguination under ether anesthesia after the parturition of paired females. All female rats showing successful reproductive performance were evaluated for estrous cycle stage by examination of the vaginal smear after weaning of pups, and euthanized at the proestrous stage by exsanguination under ether anesthesia. Females that did not copulate or had not completed parturition and dams with total litter loss were euthanized in the same way around the same time as females with successful reproduction. For all parental animals, the external surfaces were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Major organs were removed and the number of uterine implantation sites was recorded for each female. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol, and the other organs were stored in 10% neutral-buffered formalin. The brain, pituitary, thyroids, thymus, liver, kidneys, spleen, adrenals, testes, epididymides, seminal vesicles (with coagulating glands and their fluids), ventral prostate, uterus and ovaries were weighed before fixation. The thyroid and seminal vesicle were weighed after fixation.

Histopathological evaluations were performed in all animals of the control and highest dose groups, in females with abnormal estrous cycles, abnormal delivery or totally dead pups, in males and females without evidence of copulation or insemination, and in all animals with grossly abnormal reproductive organs. Of these animals, the testes, epididymides, seminal vesicles, ventral prostate, coagulating gland, ovaries, uterus and vagina, which were fixed as mentioned above, were embedded in paraffin by a routine procedure. They were sectioned, stained with hematoxylin–eosin and examined histopathologically under a light microscope. If treatment-related histopathological changes were found in the highest dose group, were the same tissues from the next lower dose group then examined.

In 10 F1 females, randomly selected from the control and highest dose groups, the number of primordial follicles was counted as follows. The right ovary, fixed in 10% neutral-buffered formalin, was dehydrated and then embedded in paraffin in longitudinal orientation by routine procedures. Sections were cut serially at 5 μm and every 20th section was serially mounted on a slide and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.10. Termination/necropsy (pups)

Following the adjustment of litter size on PND4, culled pups were euthanized by inhalation of carbon dioxide and subjected to a gross external and internal observation. Grossly abnormal organs/tissues were removed and stored in 10% neutral-buffered formalin. All pups found dead before weaning were necropsied immediately, and the whole body was stored in 10% neutral-buffered formalin.

F1 weanlings not selected to become parents and all F2 weanlings were euthanized and necropsied on PND 26, as described for adults. For one male and one female F1 and F2 weanlings selected from each dam, the brain, thymus, liver, kidneys, spleen, adrenals, testes, epididymides, ventral prostate, uterus and ovaries were removed and the organ weights were measured. Major organs, including the weighed organs, were stored in 10% neutral-buffered formalin.

Since test substance-related organ weight changes were found in the liver and spleen of the highest dose group, they were histopathologically examined for 10 male and 10 female F1 and F2 weanlings in the control and highest dose groups. The examined animals were randomly selected from animals whose organs were stored. If treatment-related histopathological changes were observed in the highest dose group, were the same tissues from the next lower dose group then examined. For the histopathological examination, paraffin sections were routinely prepared and stained with hematoxylin and eosin.

2.11. Sperm parameters

Sperm parameters were determined for all F0 and F1 male adults on the day of the scheduled sacrifice. The right testis was used to count testicular homogenization-resistant spermatid heads. The right epididymal cauda was weighed and used for sperm analysis. For sperm motility, the percentage of motile sperm and progressively motile sperm, and the swimming speed and pattern were determined using a computer-assisted cell motion analyzer (TOX IVOS; Hamilton Thorne Bioscience, Beverly, MA, USA). After recording sperm motion, the cauda epididymal fluid was diluted and the sperm were enumerated with a hemacytometer under a light microscope. Sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. The sperm was stained with eosin and mounted on a slide glass. Two hundred sperm in each sample were examined under a light microscope, and the percentage of morphologically abnormal sperm was calculated.

2.12. Statistical analysis

Parametric data, such as body weight, food and water consumption, length of the estrous cycle and gestation, precoital interval, the number of implantations and pups born, delivery index, reflex response time, age at sexual maturation, parameters of behavioral tests, organ weight and sperm parameters, were analyzed by Bartlett's test for homogeneity of distribution. For preweaning pups, body weight, AGD, viability, and age at the completion of developmental landmarks were similarly analyzed using the litter as the experimental unit. When homogeneity was recognized, one-way analysis of variance was performed. If a significant difference was detected, Dunnett's test was conducted for comparisons between control and individual treatment groups. Data without homogeneity were analyzed using the Kruskal–Wallis rank sum test. If significant differences were found, the Mann Whitney's *U* test was conducted for comparison between the control and each dosage group. The incidence of parental animals with clinical signs, and autopsy and histopathological findings, the incidence of females with normal estrous cycles, incidence of weanlings with histopathological findings, copulation, fertility and gestation index, neonatal sex ratio and completion rate of negative geotaxis were compared between the AS and control group using Fisher's exact test. The incidence of pups with clinical signs or autopsy findings per litter, the completion rate of pinna unfolding in each litter, and the success rate of surface and mid-air righting reflex were analyzed by the Wilcoxon rank sum test. The number of primordial follicles in the control and highest dose groups was compared by Student's *t*-test because the homogeneity of variance was indicated by the *F*-test. All of these statistical analyses were conducted using the 5% level of probability as the criterion for significance.

3. Results

3.1. Clinical observations, water consumption, food consumption and body weight during the pre-mating, mating, gestation and lactation periods (F0 and F1)

In the 120 ppm group, one F1 male was found dead at 9 weeks of dosing. In this animal, soiling of periocular and perinasal fur and decreased locomotor activity were observed before death. At

autopsy, various changes, including accumulation of ascitic and pleural fluid and dark purple discoloration of the liver and kidneys, were found. In the 600 ppm group, a subcutaneous mass was observed in the abdominal region of one F0 female from the beginning of 5 weeks of dosing, and this animal was found dead at 2 weeks of gestation. One F1 male at 3000 ppm was also found dead at 12 weeks of dosing without any clinical signs of toxicity. In these two animals, no abnormality was found on gross internal examination. No significant difference was seen between control and AS-treated groups in the incidence of clinical signs of toxicity in either male or female F0 and F1 rats (data not shown).

Water consumption, food consumption and the body weight of F0 parental animals are shown in Figs. 1–3, respectively. In F0 males and females of all AS-treated groups, water consumption was significantly lower than in controls almost throughout the dosing period. In F0 males, there were significant decrease in food consumption in the first week of dosing at 600 and 3000 ppm, and during week 8 and weeks 13–14 of dosing at 3000 ppm. Food consumption of F0 females showed a significantly lower value during week 1 of dosing at 3000 ppm and during week 3 of lactation at 600 and 3000 ppm. The body weight of F0 males and females was significantly lowered in the first 2 or 3 weeks of dosing at 3000 ppm.

Figs. 4–6 show the water and food consumption, and body weight of F1 parental animals, respectively. Water consumption was significantly decreased through the dosing period in 600 ppm and 3000 ppm treated males, and during weeks 3–6, week 8 and week 10 of dosing in 120 ppm treated males. In F1 females, significant reductions in water consumption were found almost throughout the dosing period at 3000 ppm, during week 10 of dosing and week 3 of lactation at 600 ppm, and during weeks 9–10 of dosing at 120 ppm. Food consumption was significantly decreased during week 10 of dosing in F1 males of the 600 and 3000 ppm groups, and during week 3 of lactation in F1 females of the same groups. There was also a transient significant increase in food consumption during week 6 of dosing in F1 females of the 120 ppm group. The body weight of F1 males and females exhibited no significant differences between the control and AS-treated groups, except that F1 females of the 120 ppm group had significantly higher body weight during weeks 6–8 of dosing.

Based on water consumption and body weight, daily AS intakes during the premating and postmating periods in males and during the premating, gestation and lactation periods in females were calculated for each of the AS-treated groups. Calculated mean AS intakes during the whole of these period were 8.6, 41.0 and 188 mg/kg bw/day in F0 males, 14.4, 71.5 and 316 mg/kg bw/day in F0 females, 10.7, 50.2 and 232 mg/kg bw/day in F1 males, and 15.3, 74.2 and 338 mg/kg bw/day in F1 females, in the 120, 600 and 3000 ppm groups, respectively. The total ingested dose of aluminium from drinking water and food combined was estimated from the water and food consumption and body weight. Average aluminium intake was 1.62, 2.96, 8.06 and 31.2 mg Al/kg bw/day in F0 males, 2.29, 4.50, 13.5 and 52.0 mg Al/kg bw/day in F0 females, 1.93, 3.55, 9.78 and 38.5 mg Al/kg bw/day in F1 males, and 2.35, 4.72, 14.0 and 55.6 mg Al/kg bw/day in F1 females for control through high-dose groups.

3.2. *Reproductive effects (F0 parents/F1 offspring and F1 parents/F2 offspring)*

During the premating period, AS produced no significant deviations in the estrous cycle of F0 and F1 females although a few control and AS-treated rats had persistent diestrus. The incidence of females with a normal estrous cycle also did not change significantly in either generation (data not shown).

The reproductive performance of F0 and F1 parental animals are summarized in Table 1. During the mating period, copulation was not observed in two males each in the control, 120 ppm and 3000 ppm groups and in one female of the control group in the F0 generation. In the F1 generation, one male in the control group, two males and one female in the 120 ppm group, one male in the 600 ppm group, and three males and one female in the 3000 ppm group did not copulate. Among females with successful copulation, one female each in the control and 3000 ppm group and two females at 120 ppm in the F0 generation and two females each in the control, 600 ppm and 3000 ppm groups, and four females at 120 ppm in the F1 generation were not impregnated. In addition, one pregnant F0 female each at 120, 600 and 3000 ppm and one pregnant F1 female at 120 ppm did not deliver live pups; however, there were no significant differences in the copulation, fertility or gestation index, and the precoital interval or gestation length between the control and AS-treated groups in F0 and F1 generation. No significant changes were observed in the number of implantations or pups delivered, and delivery index in either generation.

As for the sperm parameters examined for scheduled-sacrificed adults, in F0 generation, the absolute number of cauda epididymal sperm was significantly decreased at 3000 ppm ($253.8 \pm 61.3 \times 10^6$/cauda versus $286.3 \pm 40.3 \times 10^6$/cauda in the control); however, no significant changes were found in the number per gram of tissue. No such change was observed in F1 adults. There were no significant differences in the number of testis sperm, the percentage of motile sperm and progressively motile sperm, the swimming speed and pattern, and the percentage of morphologically abnormal sperm between control and AS-treated groups in either F0 or F1 adults (data not shown).

3.3. *Developmental effects (F1 and F2)*

Gross examination of delivered pups revealed one F1 pup with trauma in the perianal region and tail in the control group and one F1 pup with hemimelia and oligodactyly in the 120 ppm group, but no significant difference was found in the incidence between the control and AS-treated groups. No malformed F2 pups were found in any groups.

Table 2 shows sex ratio of delivered pups, and the viability and body weight during the preweaning period. No significant changes were found in the sex ratio of pups and the viability index in either generation. In the 3000 ppm group, the body weight of male and female F1 pups was significantly lower than the control on PND 21. Body weights of F2 female pups were also significantly lower than controls on PND 21 at 3000 ppm. There were no significant differences in the body weight of male F2 pups between the control and AS-treated groups during the preweaning period.

For the physical development of male and female F1 pups and male F2 pups, there was no significant difference in the completion rate of pinna unfolding, and the age at completion of incisor eruption and eye opening between the control and AS-treated groups. In female F2 pups, the completion rate of pinna unfolding on PND 2 was significantly lower in the 600 ppm group (17.0 ± 35.4%, compared with 45.8 ± 46.9 in controls), but no dose dependency was observed in this change. No significant changes were found in the completion rate of pinna unfolding on PND 1, 3 or 4 and in other physical developmental landmarks in female F2 pups. The AGD and AGD per cube root of the body weight ratio were not significantly different between control and AS-treated groups in male and female F1 and F2 pups (data not shown).

All male and female F1 pups in all groups achieved the surface righting reflex on PND 5, negative geotaxis reflex on PND 8 and mid-air righting reflex on PND 18. No significant changes were observed in the response time of surface righting and negative geotaxis reflex. In F2 pups, one female of the 600 ppm group failed in one of three trials of the mid-air righting reflex on PND 18; however, there was no significant difference in the mean success rate between the

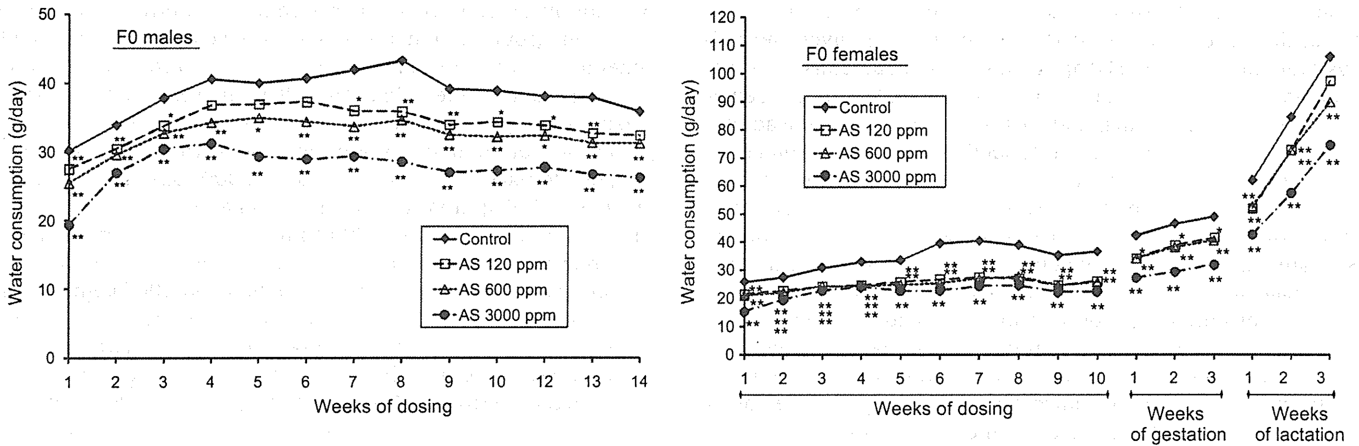

Fig. 1. Water consumption of F0 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

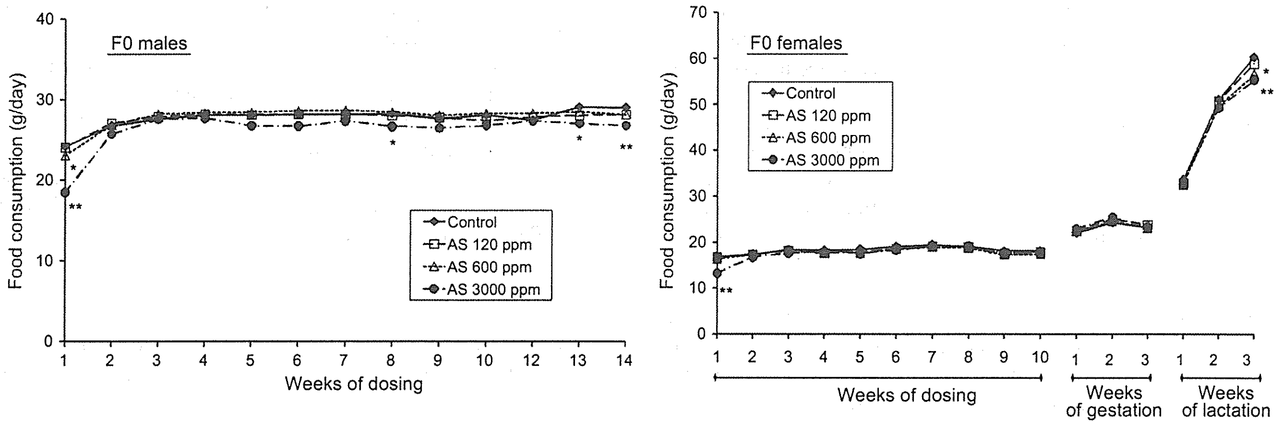

Fig. 2. Food consumption of F0 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

control and 600 ppm groups (100 ± 0.0% versus 98.4 ± 7.3%). The surface righting reflex on PND 5 and negative geotaxis reflex on PND 8 were achieved in all male and female F2 pups in all groups, and no significant changes were found in the response time (data not shown).

As for the sexual development of F1 male and female animals, vaginal opening was significantly delayed at 3000 ppm (31.4 ± 1.7, compared to 29.5 ± 2.1 in control). At this dose, body weight at the time of vaginal opening was slightly heavier than the control (119.0 ± 13.3 g versus 109.6 ± 11.6 g) although not statistically significant. No significant differences between control and AS-treated groups were noted in the age at preputial separation or body weight at the time of completion in males.

3.4. Behavioral effects (F1)

Spontaneous locomotor activity at 10-min intervals and for 60 min was not significantly different between control and AS-treated groups in male and female F1 rats. In the water-filled *T*-maze test, pre-test swimming trials in the straight channel

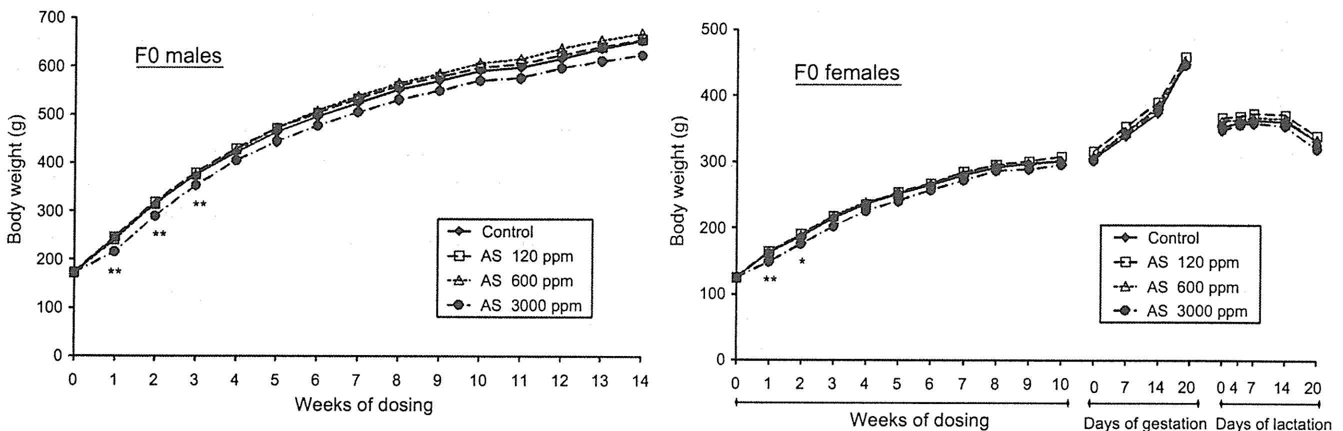

Fig. 3. Body weight of F0 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

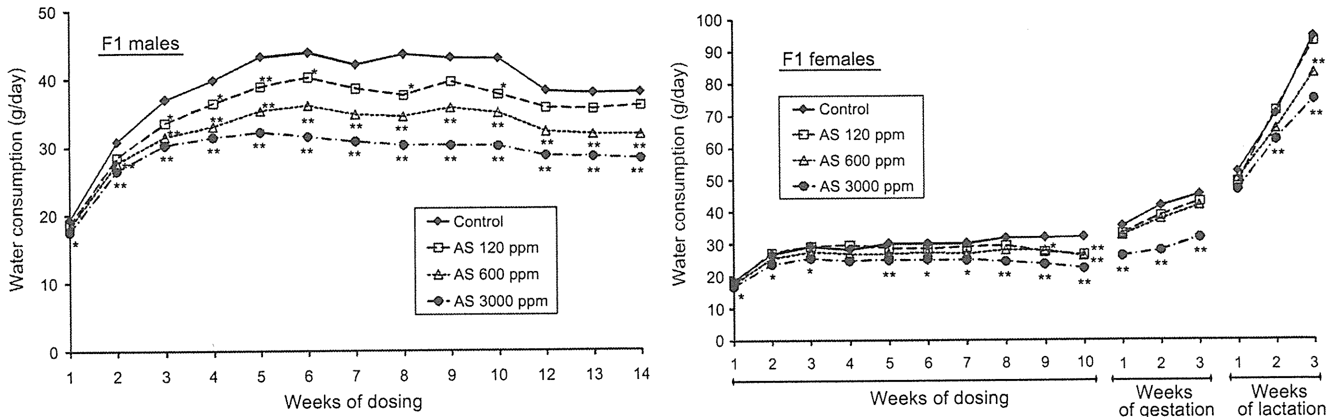

Fig. 4. Water consumption of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

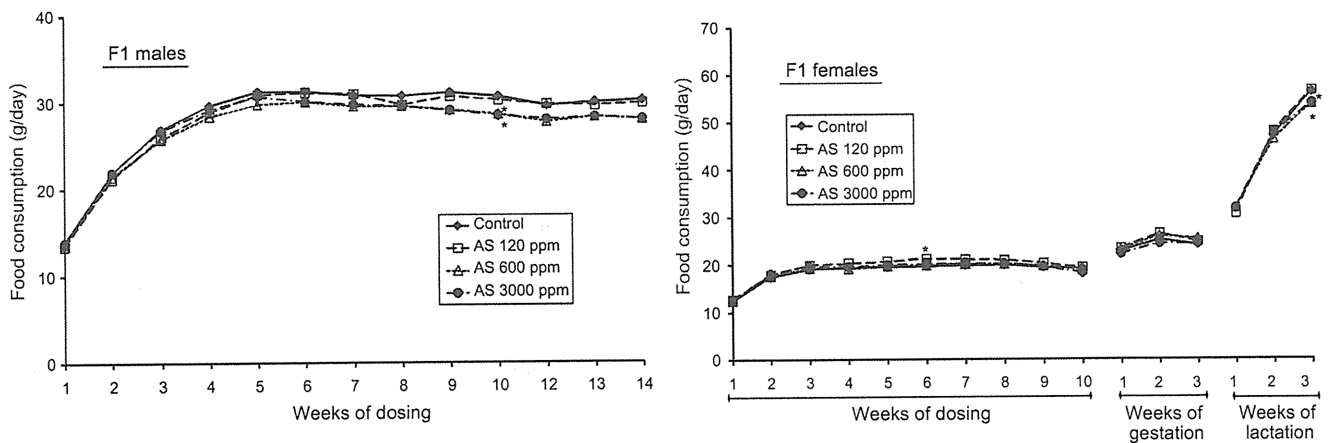

Fig. 5. Food consumption of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

revealed that all male and female F1 rats in each group could swim satisfactorily, and no significant changes were observed in the elapsed time to traverse the straight channel. On days 2–4 of the *T*-maze test, no significant changes were observed in the elapsed time and number of errors in males. In females, the elapsed time and the number of errors on day 2 of the *T*-maze was significantly lowered at 600 ppm, but there were no significant differences in the elapsed time or number of errors on days 3 and 4 of the *T*-maze test between control and AS-treated groups (data not shown).

3.5. Necropsy, organ weight and histopathology of adults (F0 and F1)

In F0 males, absolute and relative liver weights were significantly decreased at 3000 ppm. Absolute spleen weight was also

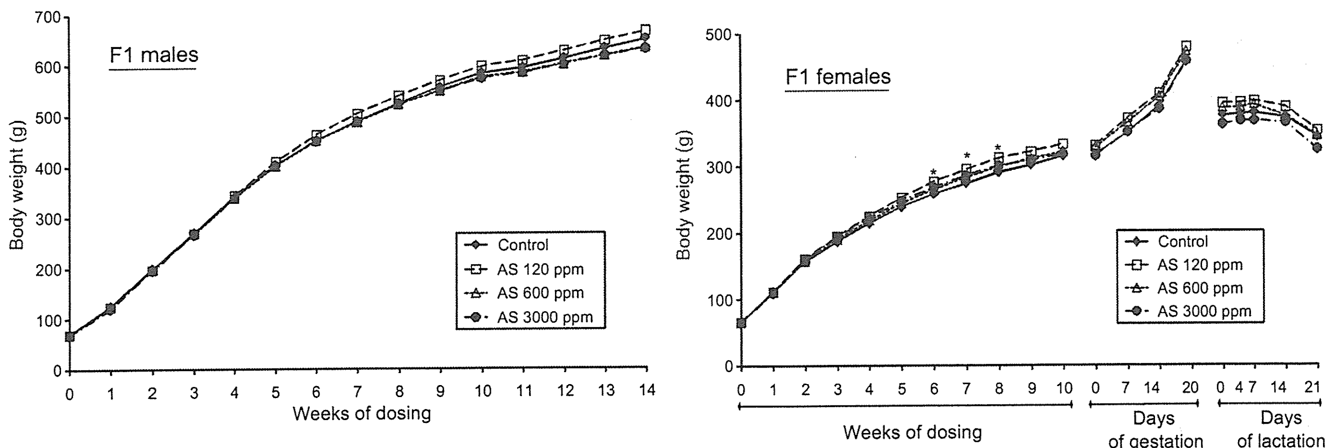

Fig. 6. Body weight of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.