human thalidomide embryopathy have only been observed and replicated in a few strains of rabbits and in primates [1,3,4]. Eight of nine subhuman primates treated with thalidomide showed characteristic limb reduction malformations ranging from amelia to varying degrees of phocomelia at a dosage and timing comparable to those observed in human thalidomide embryopathy [3,5]. Since the first report of thalidomide embryopathy appeared 50 years ago, considerable information regarding the therapeutic applications of this drug has accumulated, but the mechanisms by which thalidomide produce congenital malformations are still not well understood [2,3,5].

The non-human primate *Macaca fascicularis* (cynomolgus monkey) is widely used in prenatal developmental studies because of year-round rather than seasonal breeding behavior [6]. Kalter [5] noted that non-human primates, especially macaques and baboons, are favorable for mechanistic studies; however, only two full reports of the teratogenicity of thalidomide in cynomolgus monkeys are available [7,8]. In those studies, cynomolgus monkeys were given thalidomide by gavage at doses of 5–30 mg/kg-d during gestation days 20–30, and fetuses were examined morphologically. The findings of these studies determined the critical period and doses of thalidomide required for the production of fetal malformations in this macaque species. Although amounts taken were not always accurately recorded in humans, available documents show that typical malformations resulted from the ingestion of as little as 25 mg three times a day or 100 mg/day for 3 days during the sensitive period, equivalent to an astonishingly small dosage of about 1 mg/kg-d [5]. In teratology studies using cynomolgus monkeys, the timing of dosing was comparable to the human one and the doses were estimated to be 5–30 times higher than those which produced typical malformations in humans [5,7,8].

Knowledge of the patterns of altered gene expression in embryonic target organs on a global scale is an important consideration for understanding the mechanisms of teratogenesis [9–13]. The application of cDNA microarray technology, a genome-wide analysis technique, to cynomolgus monkeys facilitates the rapid monitoring of a large number of gene alterations in this species [14]. In order to obtain information about the molecular mechanisms underlying the detrimental effects of thalidomide teratogenicity, the present study has determined the experimental conditions required to produce thalidomide-induced fetal defects that mimicked human abnormalities in cynomolgus monkeys and then profiled altered patterns of gene expression in these embryos during the critical period. The dosing used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams at days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment.

2. Materials and methods

2.1. Teratological evaluation

The teratology study was performed at SNBL USA, Ltd. (Everett, WA, USA) in compliance with the Animal Welfare Act and recommendations set forth in The Guide for the Care and Use of Laboratory Animals [15]. Only females showing 25–32-day menstrual cycles were used in these experiments. Each female monkey was paired with a male of proven fertility for 3 days between days 11 and 15 of the menstrual cycle. When copulation was confirmed, the median day of the mating period was regarded as day 0 of gestation. Pregnancy was confirmed on day 20 or day 25 by ultrasound (SSD-4000, Aloka Co., Mitaka, Japan) under sedation induced by intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, MO, USA). The monkeys were given (±)-thalidomide (Lot no. SEH7050, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 15 or 20 mg/kg-d by oral administration using gelatin capsules (Japanese Pharmacopiae grade) on days 26–28 of gestation. The dosage was adjusted to the body weight on day 25 of gestation. Cesarean section was performed on day 100–102 of gestation under deep anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhalation of isoflurane (0.5–2.0%, Baxter, Liberty Corner, NJ, USA). Salivation was inhibited by atropine (0.01 mg/kg, Phenix Pharmaceutical, St. Joseph, MO, USA). Fetal viability was recorded, and the fetuses were euthanized by intraperitoneal injection of pentobarbital and phenytoin solution (Euthasol®, Virbac Corp., Fort Worth, TX, USA). Fetuses were sexed and examined for external anomalies after confirmation of the arrested heartbeat. After the completion of external examinations, fetuses were examined for internal abnormalities.

2.2. Microarray experiments

The animal experiments were performed at Shin Nippon Biomedical Laboratories (SNBL), Ltd. (Kagoshima, Japan) in compliance with the Guideline for Animal Experimentation (1987), and in accordance with the Law Concerning the Protection and Control of Animals (1973) and the Standards Relating to the Care and Management of Experimental Animals (1980). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the SNBL committee.

Each female monkey was paired with a male of proven fertility for 1 day between day 11 and day 15 of the menstrual cycle. Pregnant females, aged 5–8 years and weighing 2.84–3.76 kg on day 22 of gestation, were allocated randomly to two groups, each with three monkeys, and housed individually. The monkeys were orally dosed with (±)-thalidomide (Lot no. SDH7273/SDJ3347, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 0 or 20 mg/kg by oral administration of a gelatin capsule on day 26 of gestation, which was during the critical period for thalidomide-induced teratogenesis [7,8]. Dosage was adjusted to the body weight on day 22 of gestation. Control monkeys received the capsule only.

2.3. RNA sample collection

Hysterectomy was performed under terminal anesthesia at 6 h after the administration of thalidomide on day 26 of gestation. Whole embryos were rapidly removed from the uterus using a stereomicroscope and immersed in sterilized physiological saline. Three embryos each in the thalidomide-treated and control groups were obtained for RNA analysis and stored at −70 °C until further processing. General factors of maternal age, weight and date of processing these samples are shown in Table 1. Embryos were processed simultaneously, and aside from the blocking factors in Table 1, all six samples were handled concurrently through RNA isolation and hybridization.

2.4. RNA preparation and labeling

Total RNA was isolated from each day-26 embryo, amplified to cRNA, and biotin-labeled for analysis on the Affymetrix NHP GeneChip® Array at Gene Logic Inc. (Gaithersburg, MD, USA) using the TRIzol method and RNeasy columns according to protocols from Affymetrix (Santa Clara, CA, USA). The 28S/18S rRNA ratio of isolated RNA was assessed using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and found to be of sufficiently high quality. Biotinylated cRNA was finally cleaned up and fragmented by limited hydrolysis to a distribution of cRNA fragment sizes below 200 bases.

2.5. Affymetrix NHP GeneChip® Array and hybridization

Biotinylated cRNA samples from control and exposed embryos ($n = 3$ each) were hybridized using Biogen Idec's (NASDAQ: BIIB) proprietary Affymetrix NHP GeneChip® Array platform. This microarray chip contains a comprehensive representation of the Cynomolgus genome derived from Biogen Idec's proprietary sequencing efforts, from which Gene Logic (www.genelogic.com/) subsequently obtained the exclusive rights to provide as a service (personal communication, Jun Mano, Gene Logic). The steps for hybridization followed a protocol described in the Gene Logic GeneChip® Analysis Manual (Gaithersburg, MD, USA). Probe-sets for this analysis consisted of cynomolgus expressed sequence tags (ESTs), published rhesus monkey ESTs, predictive coding sequences from the rhesus genome, and human genes not represented by monkey sequences. Because of the incomplete state of annotation for the cynomolgus genome at the time this study was undertaken, we used human, mouse and rat gene annotations to characterize monkey genes on the NHP GeneChip® Array. This reasonably assumes that most cynomolgus sequences are well-annotated by human ortholog information. After hybridization the GeneChip® Arrays were scanned and raw signal values were subjected to subsequent normalization and processing.

2.6. Microarray data processing and analysis

Probe-level data normalization from the six *.cel files used the robust multi-chip average (RMA) method with perfect-match (PM) but not mismatch (MM) data from the microarrays. RMA returns a single file containing the 51,886 probes in six columns of normalized data, representing the log 2-intensity of each probe. To query differential transcript abundance between sample groups, the log 2 ratio of treated (Q) to reference (R) was computed for all six samples, with R being the average of the three controls. The six columns were centered to MEDIAN = 0.00 and scaled to STDEV = 0.50 [10,12]. These data were loaded to GeneSpring GX7.3 software (Agilent Technologies, Redwood City, CA, USA) for one-way analysis of variance (ANOVA) by treatment group. Due to the small sample size ($n = 3$) and limited annotation of the cynomolgus genome for this preliminary analysis we relaxed the selection criterion

Table 1
Procurement of cynomolgus embryos at SNBL for microarray study.

Group	Embryo	Maternal age in years	Maternal bw in kg (day 22)	Date of embryo collection (day 26)	*.cel filename (NIHS)
Control	001	6	3.76	November 2, 2006	137255bpcyna11.cel
	002	7	2.84	December 2, 2006	137256bpcyna11.cel
	003	8	3.68	December 2, 2006	137257bpcyna11.cel
Thalidomide	101	5	2.97	October 30, 2006	137258bpcyna11.cel
	102	6	3.01	November 6, 2006	137259bpcyna11.cel
	103	8	3.14	November 24, 2006	137260bpcyna11.cel

by not applying a false-discovery rate filter. Genes or probes passing the statistical (ANOVA) filter at a P value of 0.05 were subjected to K-means clustering, with cluster Set 1 and Set 2 that were up-regulated and down-regulated, respectively, in the thalidomide-exposed versus control embryos. Entrez gene identifiers were used for bioinformatics evaluation (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Teratological evaluation

To confirm thalidomide embryopathy in the cynomolgus colony under the conditions used for this study, pregnant dams were given thalidomide at 15 and 20 mg/kg on days 26–28 of gestation. Four fetuses were obtained at each dose for teratological evaluation (Table 2). Although we did not observe a clear dose–response in this limited number of fetuses, we did observe a number of cases with limb defects consistent with human thalidomide embryopathy. Fig. 1 shows external appearance of fetuses of dams exposed to thalidomide on days 26–28 of gestation. Bilateral amelia in the fore-/hindlimbs was noted in one female fetus at 20 mg/kg, and bilateral micromelia in the hindlimbs was observed in four fetuses at 15 mg/kg. Deformities of the paw and/or foot including hyperflexion, ectrodactyly, polydactyly, syndactyly, brachydactyly, and/or malpositioned digits, were observed in all fetuses at 15 mg/kg and in two fetuses at 20 mg/kg. Tail anomalies were found in one fetus at 15 mg/kg and three fetuses at 20 mg/kg. Small penis was noted in one fetus each in both thalidomide-treated groups. No internal abnormalities were noted in any of the thalidomide-treated fetuses examined here. This confirmed the relevant sensitivity of cynomolgus embryos to thalidomide, based on a maternally administered dose of 15–20 mg/kg during days 26–28 of gestation.

3.2. Genes altered by thalidomide

The embryonic transcriptome was evaluated at 6 h after 20 mg/kg maternal thalidomide exposure on day 26. For this analysis, we used a proprietary Non-Human Primate (NHP) microarray having representation of the cynomolgus genome (see Section 2 for details). The NHP array includes 18,293

Table 2
Morphological findings in fetuses of cynomolgus monkeys given thalidomide on days 26–28 of gestation.

Target	Dose	15 mg/kg				20 mg/kg			
Findings	Fetus no.	1	2	3	4	5	6	7	8
	Gender	Female	Male	Female	Female	Male	Male	Male	Female
Forelimb									
Amelia		–	–	–	–	–	–	–	B
Paw									
Hyperflexion		B	–	–	–	–	–	–	–
Ectrodactyly		L	–	–	–	–	–	–	–
	Accessory digit(s)[a]	L	–	–	–	–	–	–	–
Polydactyly[a]		–	R	–	–	–	–	–	–
	Brachydactyly	–	–	–	B	–	–	–	–
Hindlimb									
Micromelia		B	B	B	B	–	–	–	–
Amelia		–	–	–	–	–	–	–	B
Foot									
Hyperflexion		–	B	B	B	–	–	–	–
Ectrodactyly		–	B	R	R	–	–	–	–
Polydactyly		–	–	–	–	B	B	–	–
Syndactyly		R	–	B	–	–	–	–	–
	Brachydactyly	–	–	–	L	–	–	–	–
	Malpositioned digit(s)	–	–	L	–	–	–	–	–
Craniofacial		–	–	–	–	–	–	–	–
Trunk		–	–	–	–	–	–	–	–
Tail									
	Bent or curled tail	–	–	–	+	–	+	+	+
Short tail		–	–	–	–	–	–	+	–
External genital organs									
Small penis		–	+	–	–	+	–	–	–

–: No anomaly was observed.
+: Anomaly was observed.
B: Bilateral anomaly was observed.
R: Unilateral (right side) anomaly was observed.
L: Unilateral (left side) anomaly was observed.
[a] Polydactyly means (almost) complete extra digits existed, and accessory digit incomplete "digit like tissue" attached to a normal digit.

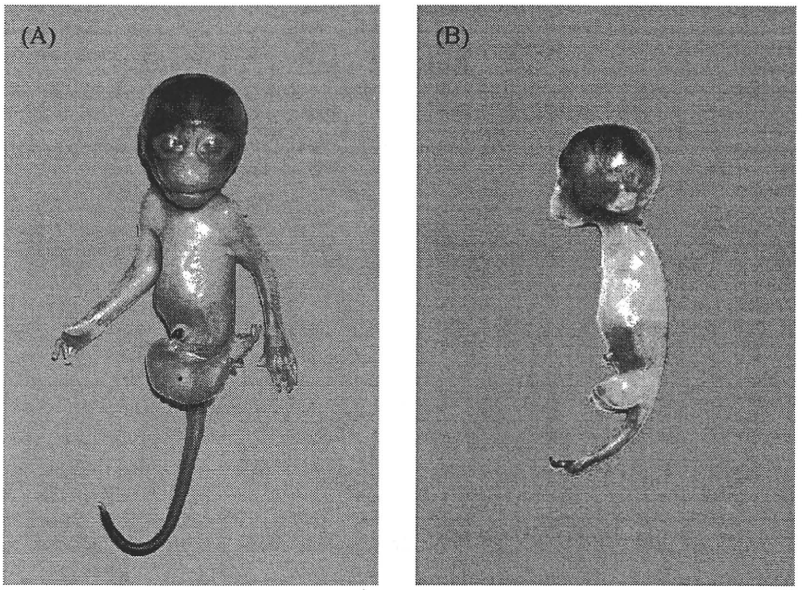

Fig. 1. Malformed fetuses of cynomolgus monkeys exposed to thalidomide on days 26–28 of gestation. (A) The fetus of maternal monkey given thalidomide at 15 mg/kg-d exhibiting brachydactyly in the paw, micromelia in the hindlimb, hyperflexion, ectrodactyly and brachydactyly in the foot and curled tail. (B) The fetus of maternal monkey given thalidomide at 20 mg/kg-d exhibiting amelia in the fore- and hindlimb and bent tail.

cynomolgus genes and 8411 Rhesus genes as well as genes from several other species. The six-array dataset conforming to MIAME standards resides in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/) under platform accession number GPL8393 (series GSM389350–GSM389355). A thalidomide-sensitive subset of genes in the embryonic transcriptome was reflected in the high-percentage of present calls for genes whose expression levels showed ≥1.5-fold difference between thalidomide-treated and control embryos.

Statistical (ANOVA) analysis identified 2362 genes that differed significantly between control and thalidomide groups ($P \leq 0.05$). The heat map for these genes showed a clear pattern (Fig. 2). *K*-means clustering partitioned them into primary sets of up-regulated (1281) genes and down-regulated (1081) genes for thalidomide relative to control embryos.

3.3. Annotation systems

Ranking functional categories of genes in an expression cluster is an important step to unravel the cellular functions and pathways represented in the differentially expressed gene list. To derive the highest ranking biological themes across the up-/down-regulated gene lists, Entrez gene IDs were annotated by Gene Ontology (GO) category using the Database for Annotation, Visualization, and Integrated Discovery (http://apps1.niaid.nih.gov/david/). Table 3 lists the significantly over-represented themes when the 1281 up-regulated genes (Table 3A) and 1081 down-regulated genes (Table 3B) were mapped by GO category. We used level-4 annotation for Biological Processes, Cellular component and Molecular Function as well as curated pathways from the KEGG (Kyoto Encyclopedia of Genes and Genomes) open source pathway resource to obtain categories passing by Fisher exact test ($P \leq 0.05$). For clarity and greater specificity we limited the categories in Table 3 to those having at least 10 hits for sensitivity and no more than 50 hits to improve specificity.

Integrated biological processes evident across the up-regulated categories addressed the regulation of cellular growth, including cell cycle progression, DNA repair and nucleic acid transport. Other up-regulated biological processes addressed the regulation of metabolism, the cytoskeletal cycle, heart development and vesicle transport. Many of these processes were logically reflected in the ontologies for cellular components addressing the nucleo-ribosomal system, the microtubule network, and molecular functions for GTPase activity and actin binding. Up-regulated signaling pathways (KEGG) included several oncogenic growth pathways as well as the TGF-beta, GnRH and insulin signaling pathways.

Integrated biological processes evident across the down-regulated categories addressed ion homeostasis and cellular secretion. These processes were logically reflected in the ontologies for cellular components addressing the endoplasmic reticulum, GTPase activity and transferases. Other down-regulated biological processes addressed cell growth, muscle and vasculature development, and the inflammatory response—consistent with KEGG pathways for hematopoietic cells and antigen processing.

4. Discussion

The results from this study show that a teratogenic dose of thalidomide (20 mg/kg) significantly alters global gene expression profiles in the cynomolgus monkey embryo within 6 h of exposure on day 26 of gestation. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure revealed up-regulation in several signaling pathways with roles in morphogenesis and oncogenesis (e.g., TGF-beta, insulin signaling), and down-regulation of the endoplasmic reticulum and inflammatory response. As might be anticipated, this implies a broad reaction of the embryo to the mechanism of thalidomide and a generalized reprogramming of pathways known to be important in development and teratogenesis.

The dosing scenario used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams on days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment. The teratological exposure induced limb malformations consistent with earlier studies with thalidomide in pregnant macaques. For example, it was previously reported that two fetuses with amelia were obtained from two of four cynomolgus monkeys given thalidomide by gavage at 10 mg/kg-d on days 32–42 after commencement of menses (approximately equivalent to days 20–30 of gestation)

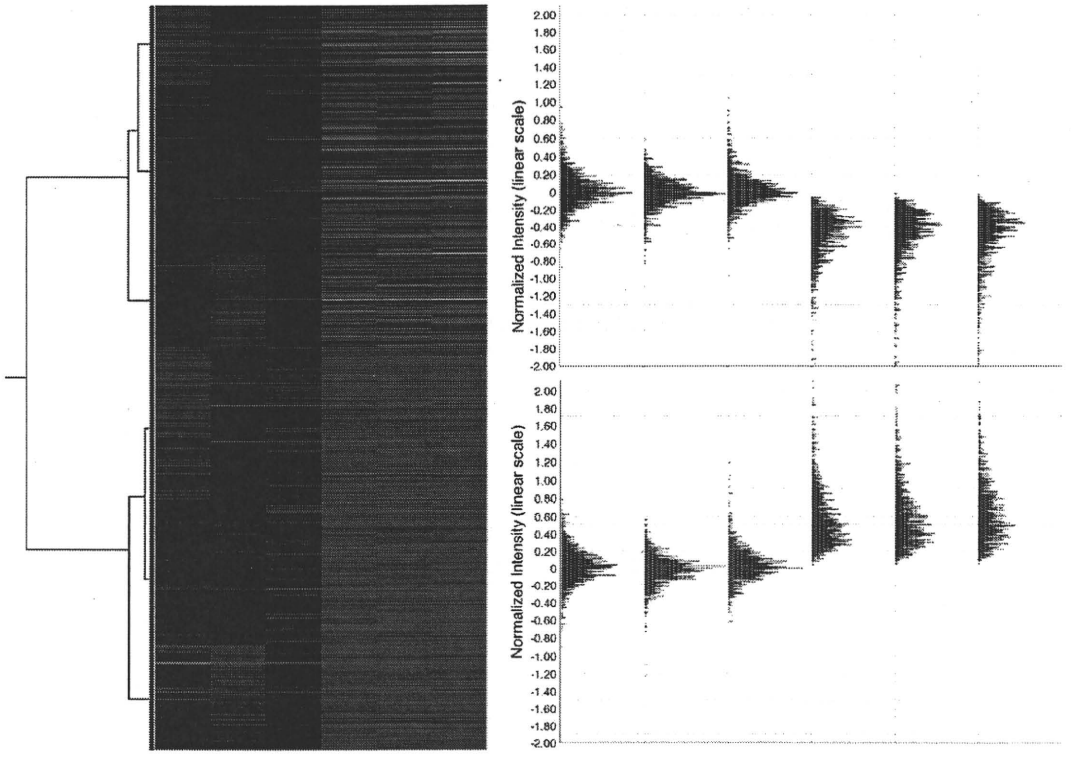

Fig. 2. Molecular abundance profiles of the thalidomide-sensitive genes in the cynomolgus embryonic transcriptome on day 26 of gestation. RNA was isolated from day 26 embryos 6 h after maternal exposure to 20 mg/kg thalidomide or vehicle control. Values represent log2 ratios of treated/reference, where the reference is an average of all three controls for each gene. ANOVA returned 2362 genes that were significantly different between the groups ($n = 3$, $P \leq 0.05$). The heat map visualizes the genes in rows and the embryos in columns, and the histogram shows the distribution of genes in each cluster. Columns left to right: 1–3 from control embryos (#001, #002, #003) and 4–6 from thalidomide embryos (#101, #102, #103). Genes were partitioned by *K*-means clustering into two primary expression clusters with 1281 up-regulated genes (red) and 1081 down-regulated genes (green).

and that the fetal malformations were similar to malformations reported in children whose mothers had taken thalidomide during pregnancy [7]. Forelimb malformations in the cynomolgus fetus were noted following a single oral administration of thalidomide on days 25, 26 or 27 of gestation at 10 and 30 mg/kg and daily administration on days 25–27 of gestation at 5 mg/kg, and both fore- and hindlimb malformations were observed following a single oral administration on day 25 or 28 of gestation at 30 mg/kg [8]. The present study, taken together with the previous studies [7,8], indicate that orally administered thalidomide induces fetal malformations in cynomolgus monkeys similar to human pregnancies and furthermore localizes the vulnerable period to days 25–28 of gestation and the effective doses to 5–30 mg/kg-d.

Given the limitations of working with this species the preliminary application of a custom NHP microarray, the analysis at one dose and time point, and the incomplete state of annotation of the macaque genome, the current study design focused on RNA collected from individual embryos rather than the specific target organ system (forelimb, hindlimb). Ideally a follow-up study on focused gene expression analysis should be performed for specific embryonic limbs in which malformations have been induced with thalidomide; however, the present study is among the first to provide genomic information on the initial changes in gene expression occurring in macaque embryos during the critical events following a teratogenic dose of thalidomide. A total of 43 and 26 functional categories of redundant genes were up- and down-regulated, respectively, based on the GO annotation system for human Locus Link identifiers.

Statistically, the top-ranked 20 up-regulated genes included 4 hits to cell shape and polarity genes: KIAA0992 (twice), FNML2, FMNL3. Palladin, encoded by the KIAA0992 gene, plays a role in cytoskeletal organization, embryonic development, cell motility, and neurogenesis [16]. Formin-related proteins play a role in Rho GTPase-dependent regulation of the actin cytoskeletal cycle and have been implicated in morphogenesis, cell movement and cell polarity [17]. Several genes in the focal adhesion/actin cytoskeleton pathway were up-regulated. Guanine nucleotide exchange factors (GEFs) *DOCK1*, which forms a complex with RhoG, and *VAV2* and *ARHGEF7* that act on Rho family GTPases, play a fundamental role in small G-protein signaling pathways that regulate numerous cellular processes including actin cytoskeletal organization [18–22]. To further understand the mechanisms of thalidomide-induced teratogenicity the regional and developmental stage of expression for these genes and corresponding proteins should be determined; however, these preliminary findings suggest that thalidomide perturbs a general program involving the up-regulation of Rho family GTPases and their GEFs.

One candidate pathway for the control of cytoskeletal remodeling evident in studies of early induction of the Fetal Alcohol Syndrome (FAS) in mouse embryos is the receptor tyrosine kinase (RTK) signaling pathway, mediating insulin-like growth factors [12]. Genes in the RTK insulin signaling pathway were significantly up-regulated by thalidomide treatment as in FAS. AKT1 and GSK3β, which were up-regulated by thalidomide, are key genes in this pathway. AKT1, a serine–threonine protein kinase, is regulated by PDGF and insulin through PI-3 kinase signaling [23–25]. GSK3β, a substrate of AKT, is a proline-directed serine–threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase [26]. IGF-I and IGF-II are expressed in the anterior and posterior mesodermal cells of the developing limbs [27–29]. IGF-I can influence chick limb outgrowth [29–31] and regulate mus-

Table 3A
GO-annotated biological categories for genes up-regulated in the embryo following maternal thalidomide exposure.

Category	Term	Count	*P* value	List Total	Pop Hits	Pop Total	Log 2 Fold Change
GOTERM_BP_4	Biological Process (level 4)						
GO:0015931	Nucleobase, nucleoside, nucleotide and nucleic acid transport	15	0.001	694	100	13,532	+2.92
GO:0050658	RNA transport	13	0.002	694	87	13,532	+2.91
GO:0050657	Nucleic acid transport	13	0.002	694	87	13,532	+2.91
GO:0051236	Establishment of RNA localization	13	0.002	694	87	13,532	+2.91
GO:0051028	mRNA transport	11	0.007	694	79	13,532	+2.71
GO:0045941	Positive regulation of transcription	40	0.000	694	326	13,532	+2.39
GO:0007507	Heart development	15	0.006	694	128	13,532	+2.28
GO:0051276	Chromosome organization and biogenesis	45	0.000	694	394	13,532	+2.23
GO:0006281	DNA repair	28	0.001	694	267	13,532	+2.04
GO:0022618	Protein–RNA complex assembly	12	0.035	694	116	13,532	+2.02
GO:0031325	Positive regulation of cellular metabolic process	42	0.000	694	416	13,532	+1.97
GO:0009893	Positive regulation of metabolic process	44	0.000	694	445	13,532	+1.93
GO:0051169	Nuclear transport	14	0.035	694	145	13,532	+1.88
GO:0016481	Negative regulation of transcription	28	0.003	694	300	13,532	+1.82
GO:0006461	Protein complex assembly	27	0.005	694	295	13,532	+1.78
GO:0045786	Negative regulation of progression through cell cycle	19	0.022	694	209	13,532	+1.77
GO:0009892	Negative regulation of metabolic process	38	0.002	694	436	13,532	+1.70
GO:0031324	Negative regulation of cellular metabolic process	32	0.009	694	387	13,532	+1.61
GO:0000074	Regulation of progression through cell cycle	42	0.005	694	526	13,532	+1.56
GO:0051726	Regulation of cell cycle	42	0.005	694	529	13,532	+1.55
GO:0007010	Cytoskeleton organization and biogenesis	41	0.008	694	526	13,532	+1.52
GO:0016192	Vesicle-mediated transport	39	0.013	694	509	13,532	+1.49
GOTERM_CC_4	Cellular component (level 4)						
GO:0005830	Cytosolic ribosome (sensu Eukaryota)	10	0.017	743	76	14,201	+2.51
GO:0005681	Spliceosome	16	0.004	743	134	14,201	+2.28
GO:0000785	Chromatin	22	0.001	743	194	14,201	+2.17
G O:0031965	Nuclear membrane	15	0.012	743	136	14,201	+2.11
GO:0012506	Vesicle membrane	13	0.030	743	125	14,201	+1.99
GO:0005874	Microtubule	23	0.005	743	233	14,201	+1.89
GO:0005635	Nuclear envelope	18	0.015	743	182	14,201	+1.89
GO:0005768	Endosome	18	0.028	743	196	14,201	+1.76
GO:0005694	Chromosome	32	0.011	743	385	14,201	+1.59
GO:0030529	Ribonucleoprotein complex	41	0.047	743	584	14,201	+1.34
GOTERM_MF_4	Molecular Function (level 4)						
GO:0051427	Hormone receptor binding	10	0.001	578	57	12,599	+3.82
GO:0051020	GTPase binding	11	0.003	578	78	12,599	+3.07
GO:0003712	Transcription cofactor activity	41	0.000	578	311	12,599	+2.87
GO:0003779	Actin binding	27	0.002	578	302	12,599	+1.95
GO:0008234	Cysteine-type peptidase activity	15	0.027	578	172	12,599	+1.90
KEGG_PATHWAY							
hsa05220	Chronic myeloid leukemia	10	0.016	225	74	4,214	+2.53
hsa05222	Small cell lung cancer	11	0.016	225	87	4,214	+2.37
hsa05215	Prostate cancer	11	0.016	225	87	4,214	+2.37
hsa04350	TGF-beta signaling pathway	11	0.020	225	90	4,214	+2.29
hsa04912	GnRH signaling pathway	11	0.026	225	94	4,214	+2.19
hsa04910	Insulin signaling pathway	14	0.025	225	134	4,214	+1.96

cle mass during early limb myogenesis [32]. Although these facts may implicate IGF signals as a potential mediator of thalidomide embryopathy, the present study did not find significant expression or thalidomide-induced alteration in the global pattern of several key transcripts in this signaling pathway, including IGFBPs 13, 5, 6 and 7, IGF1, IGF1R, and IRS14 (data not shown). It is certainly plausible that thalidomide exposure may locally alter upstream events in IGF-1 signaling without necessarily altering the molecular abundance profiles of the pathway in the developing limb of monkey embryos. On the other hand, our preliminary microarray analysis does find evidence for the up-regulation of GSK3β and AKT1 transcripts that are downstream in the insulin signaling pathway. Effects on TGF-beta and WNT signaling may be critical here. Thalidomide-induced oxidative stress in chick embryos can enhance signaling through BMPs (bone morphogenetic proteins), leading to up-regulation of the WNT antagonist Dickkopf1 (Dkk1) and subsequent cell death [33]. We note here a significant up-regulation of genes in the TGF-beta pathway and similarities with genes in the cytoskeletal cycle and WNT pathways for the murine FAS [12].

Some of the responsive genes found in this study are known to play roles in vascular development pathways. For example, vascular endothelial growth factor (VEGF) was down-regulated and platelet-derived growth factor receptor β (PDGFRβ) was up-regulated during early stages in thalidomide embryopathy. VEGF is a key stimulator of vascular cell migration and proliferation and acts directly on endothelial cells, whereas PDGF attracts connective tissue cells that can also stimulate angiogenesis. The reciprocal effect on these transcript profiles, potentially leading to an overall decrease in VEGF/PDGFRβ activities, might be predicted to interfere with vascular cell recruitment and proliferation in the developing embryo or limb. It is well known that thalidomide reduces the activity or production of VEGF and TNF-α, leading to inhibition of angiogenesis [34]. The present microarray data are consistent with this effect. Furthermore, VEGF stimulates PDGFRβ and induces tyrosine phosphorylation [35]. The reciprocal effect that maternal thalidomide exposure had on these transcripts may suggest a key event in the programming or induction of vascular cells or their progenitors has been disrupted within 6 h after exposure. This notion is supported by the study of D'Amato et al. [36] that

Table 3B
GO-annotated biological categories for genes down-regulated in the embryo following maternal thalidomide exposure.

Category	Term	Count	*P* value	List Total	Pop Hits	Pop Total	Log2Fold Change
GOTERM_BP_4	Biological Process (level 4)						
GO:0008284	Positive regulation of cell proliferation	24	0.000	556	240	13,532	−2.43
GO:0007517	Muscle development	16	0.006	556	177	13,532	−2.20
GO:0009889	Regulation of biosynthetic process	18	0.005	556	207	13,532	−2.12
GO:0006417	Regulation of translation	14	0.027	556	174	13,532	−1.96
GO:0032940	Secretion by cell	23	0.004	556	287	13,532	−1.95
GO:0001944	Vasculature development	15	0.026	556	191	13,532	−1.91
GO:0045045	Secretory pathway	18	0.020	556	239	13,532	−1.83
GO:0051246	Regulation of protein metabolic process	23	0.008	556	307	13,532	−1.82
GO:0006873	Cellular ion homeostasis	16	0.031	556	214	13,532	−1.82
GO:0006954	Inflammatory response	22	0.012	556	301	13,532	−1.78
GO:0016192	Vesicle-mediated transport	35	0.004	556	509	13,532	−1.67
GO:0042127	Regulation of cell proliferation	34	0.005	556	499	13,532	−1.66
GO:0019752	Carboxylic acid metabolic process	36	0.012	556	572	13,532	−1.53
GO:0046907	Intracellular transport	40	0.043	556	714	13,532	−1.36
GOTERM_CC_4	Cellular component (level 4)						
GO:0005625	Soluble fraction	21	0.004	602	244	14,201	−2.03
GO:0005768	Endosome	15	0.039	602	196	14,201	−1.81
GO:0005789	Endoplasmic reticulum membrane	28	0.031	602	435	14,201	−1.52
GO:0044432	Endoplasmic reticulum part	30	0.047	602	494	14,201	−1.43
GO:0005624	Membrane fraction	44	0.026	602	749	14,201	−1.39
GO:0005783	Endoplasmic reticulum	46	0.049	602	827	14,201	−1.31
GOTERM_MF_4	Molecular Function (level 4)						
GO:0030594	Neurotransmitter receptor activity	14	0.000	531	99	12,599	−3.36
GO:0051020	GTPase binding	11	0.002	531	78	12,599	−3.35
GO:0016747	Transferase activity, transferring other than amino-acyl groups	15	0.028	531	188	12,599	−1.89
GO:0004175	Endopeptidase activity	31	0.012	531	463	12,599	−1.59
KEGG_PATHWAY							
hsa04640	Hematopoietic cell lineage	12	0.005	223	85	4,214	−2.67
hsa04612	Antigen processing and presentation	10	0.024	223	80	4,214	−2.36

Results for the embryo 6 h after a teratogenic dose of thalidomide (20 mg/kg) on day 26 of gestation for 1281 significantly up-regulated genes (Table 3A) and 1081 significantly down-regulated genes (Table 3B) based on the population of arrayed genes. The annotated system used the NIH/NIAID Database for Annotation, Visualization, and Integrated Discovery (DAVID) at level 4. Count refers to the number of altered genes in the ontology (min = 10 and max = 50). *P* value refers to results from Fisher exact test ($P \leq 0.05$); List Total refers to the number of annotated genes on the array; Pop Hits and Pop Total refers to the number of annotated genes in the database for the category and overall; Log 2 Fold Change is computed as the mean Log 2 (treated/control) for genes in the category.

suggested limb defects caused by thalidomide were secondary to inhibition of blood vessel growth in the developing limb bud. Down-regulation of the vascular development program is consistent with this notion and with the supposition that correct limb bud formation requires a complex interaction of both vasculogenesis and angiogenesis during development [37]. Perhaps these genes might be considered as potential biomarkers of thalidomide-induced teratogenesis in cynomolgus monkeys. A recent study with the teratogenic thalidomide analogue, CPS49, has shown direct evidence for the suppression of endothelial angiogenetic sprouting and failure to establish a normal vascular network as a key event in thalidomide embryopathy [38]. CPS49 mimics the antiangiogenic properties, but not anti-inflammatory properties, of thalidomide.

Finally, the inflammatory response pathway was found to be significantly down-regulated in the early thalidomide embryome. Although down-regulation of the inflammatory response might be anticipated to protect the embryo, studies in laboratory animals have implicated a role for reactive oxygen species (ROS) in thalidomide embryopathy [39]. In that study, thalidomide was found to preferentially increase ROS in embryonic limb cells from a sensitive species (rabbit) but not the insensitive species (rat). Down-regulation of the inflammatory pathways in thalidomide-exposed monkey embryos reinforces this notion.

In conclusion, these findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the cynomolgus monkey embryo. Bioinformatics analysis has now identified many key pathways implicated in thalidomide embryopathy in cynomolgus monkeys, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype. Several pathways, including actin cytoskeleton remodeling and downstream insulin signaling-related genes, in addition to vascular development pathways may provide candidate biomarkers for key events underlying the teratogenicity of thalidomide in primates. To clarify the molecular mechanisms further studies must examine protein expression, phosphorylation, and other modifications in the precursor target organ system.

Conflict of interest statement

None.

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Proposal of new uncertainty factor application to derive tolerable daily intake

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ABSTRACT

We propose new uncertainty factors (UFs) and a new subdivision of default factors in chemical risk assessment using a probabilistic approach based on the latest applicable information. Rounded values of 150 for mice, 100 for hamsters and rats, and 40 for rabbits, monkeys and dogs for inter- and intra-species differences (UF_{AH}) were derived from the probabilistic combination of two log-normal distributions. Further calculation of additional UFs when chronic data (UF_S) or NOAEL (UF_L) are lacking was conducted using available log-normal distribution information. The alternative UF_S and UF_L values of 4 are considered to be appropriate for both cases where data are lacking. The default contributions of inter-species difference (UF_A) and intra-species difference (UF_H) to the UF_{AH} of 100 for hamsters and rats as an example are considered to be 25 and 4, respectively. The UF_A of 25 was subdivided into $25^{0.6}$ (i.e., 7.0) for pharmacokinetics (PK) ($UF_{A,PK}$) and $25^{0.4}$ (i.e., 3.6) for pharmacodynamics (PD) ($UF_{A,PD}$), and the UF_H of 4 was evenly subdivided into $4^{0.5}$ (i.e., 2) ($UF_{H,PK}$ and $UF_{H,PD}$), to account for chemical-specific difference data between humans and laboratory animals for PK and/or PD. These default UFs, which come from actual experimental data, may be more appropriate than previous default UFs to derive tolerable daily intake values.

1. Introduction

Principle uncertainty factors (UFs) consisting of inter-species differences (or extrapolation from laboratory animals to humans, referred to as "UF_A") and intra-species differences (human variability, referred to as "UF_H") have commonly been used when extrapolating from animal experimental data to human risk values in chemical risk assessment. The current combined default UF of 100 ($10_A \times 10_H$) for extrapolation from animal data was introduced in the US in 1954 (Lehman and Fitzhugh, 1954) for food contaminants with a rationale for its suitability for environmental contaminants provided by Dourson and Stara (1983) years later. The physical size of laboratory animals is variable, with animals as small as mice to larger animals like dogs. In some cases the size difference results in more than a 500-fold difference in body weight indicating that some type of variable adjustment might be needed, rather than just a 10-fold factor.

Body surface area correction, (human body weight/animal body weight)$^{1/3}$ was the first data supported size adjustment (Freireich et al., 1966). It has been applied to cancer endpoints in US Environmental Protection Agency (US EPA) assessments and was also used in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) residual solvent guidelines (Connelly et al., 1997). Recently, allometric scaling according to caloric demand or metabolic size, (human body weight/animal body weight)$^{1/4}$ was introduced as a more appropriate adjustment (Schneider et al., 2004) and is currently used by US EPA in cancer risk assessment (US EPA, 1992, 2005a). Size adjustment might be more appropriately based on allometric scaling as discussed by Falk-Filipsson et al. (2007). However, the use of allometric scaling in non-cancer endpoints remains untested by US EPA and other organizational assessments. The caloric demand adjustment factor for a mouse (0.030 kg) or a dog (16 kg) compared to a human (70 kg) based on body weight is 7 or 1.4, respectively, which is significantly lower than the default of 10. However, Schneider et al. (2004) demonstrated that caloric demand scaling was effective for predicting median differences between humans and animals on the basis of body weight in maximal tolerated dose (MTD) ratios of anti-cancer drugs, and also calculated the combined geometric standard deviation (GSD) of the empirical distribution.

Useful experimental data are quite limited for human intra-species differences, specifically variability between different ages (Dourson and Stara, 1983; Dourson et al., 1996, 2002). However, some insights can be gained from experimental animal work. For

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example, a recent comparative investigation of no observed adverse effect levels (NOAELs) in repeat-dose studies of newborn and young rats for 18 chemicals was conducted (Hasegawa et al., 2007). The data provided the median and distribution of NOAEL ratios.

The default UF of 100 for inter- and intra-species differences is typically applied by multiplication of 10_A and 10_H. However, the default UF of 100 is not always appropriate to use. For example, multiplication of two log-normal distributions for inter- and intra-species differences also produces a log-normal distribution, and simple multiplication such as 10×10 causes overestimation if both individual values are in the 95th percentile. Kodell and Gaylor (1999) recommended standard statistical techniques that could be used to estimate the upper tolerance limits on the distribution of sums which can also be used for other UFs (e.g., the ratio of subchronic to chronic NOAELs). Swartout et al. (1998) also addressed this problem and gave hypothetical examples of UF combinations.

Another method for division of the default UF of 100 ($10_A \times 10_H$) for inter- and intra-species differences was proposed by Renwick (1993). He proposed a subdivision of these UFs into two parts, pharmacokinetics (PK) and pharmacodynamics (PD). Based on his analysis of experimental data and physiological parameters between animals and humans, the contribution ratios of PK and PD are 60:40 for inter-species differences and 50:50 for intra-species differences, leading to $10^{0.6}$ (4.0) $\times$ $10^{0.4}$ (2.5) and $10^{0.5}$ ($\sim$3.2) $\times$ $10^{0.5}$ ($\sim$3.2), respectively (IPCS, 1994). When chemical-specific data for the differences between animals and humans for PK and/or PD are available, the data should be used to develop chemical-specific adjustment factors instead of the default PK/PD factors (WHO, 2005). However, the default subdivision factors should be re-estimated if animal size-specific UFs are adopted as inter-species differences.

In this article, we propose new default UFs by a probabilistic approach using appropriate log-normal distribution data, taking animal size into consideration. We also propose development of new default values according to animal size for the subdivision of inter- and intra-species differences.

2. Data for each uncertainty

2.1. Inter-species difference data

Eight publications featuring chemical toxicity comparisons between humans and laboratory animals for anti-cancer drug toxicity were located. The first study by Freireich et al. (1966) showed MTD differences between humans and five animal species (mice, hamsters, rats, monkeys and dogs) in the analysis of 18 drugs. Recently, Schneider et al. (2004) extracted correlated human and animal data sets for 63 anti-cancer drugs from six additional publications (Goldsmith et al., 1975; Schein et al., 1979; Travis and White, 1988; Rozencweig et al., 1981; Grieshaber and Marsoni, 1986; Paxton et al., 1990) to demonstrate that caloric demand scaling was a suitable adjustment factor for the differences of inter-species median MTDs. Schneider et al. (2004) also derived a GSD of 3.23 from the combined distribution of all MTD ratios for humans versus the five animal species stated above.

Alternatively, inter-species differences in susceptibility could be derived based on the differences in NOAELs rather than MTDs. Schneider et al. (2004) also analyzed inter-species differences for pesticide NOAELs between mice/rats, rats/dogs and mice/dogs, providing further support to the caloric demand adjustment. Therefore, the median and GSD derived from MTD ratios of anti-cancer drugs might be equivalent to those based on NOAEL ratios between humans and animals. No other publications featuring an estimated direct comparison of chemical toxicity between humans and animals were identified.

2.2. Intra-species difference data

The NOAEL ratio of a sensitive subpopulation compared to that of the general population is a source of uncertainty for intra-species differences in risk assessment (Dourson et al., 2002). Occasionally the sensitive subpopulations are directly addressed in the risk assessment. For example, the Reference Dose (RfD) for nitrate on the Integrated Risk Information System (IRIS) used methemoglobinemia in children as the critical effect, therefore an intra-species UF may not be needed (US EPA, 2009). However, in most cases, the sensitive subpopulations are only considered protected with the use of an intra-species UF. Generally, infants, pregnant women, the elderly and other specified groups are considered high-susceptibility groups, although exceptions are not uncommon. For example, Tylenol overdose is more of a problem in adults than in children because the toxic metabolite is more readily formed in adults. Effects during pregnancy and gestation are considered to be adequately evaluated in the reproductive/developmental toxicity studies, while lifetime toxicity studies cover the potential for effects to the elderly. For a well-tested chemical, the only remaining sensitive subpopulation to be protected by an intra-species UF are infants. Currently, there are no experimental animal test guidelines intended for direct exposure of neonatal animals to chemicals. Other specified groups may include patients exhibiting hepatic or renal dysfunction and persons with a specific genetic background. These subpopulations need specific risk management and should not be the target population for a chemical risk assessment for public health because it is possible that their susceptibility to specific chemicals may be unexpectedly high owing to significantly reduced metabolism or excretion of toxic substances.

The comparative data between human adults and children/infants was assessed by many scientists. Glaubiger et al. (1981) compared MTDs in patients for 17 anti-cancer drugs demonstrating that children's MTDs were 50% higher than those of adults, indicating that children were less sensitive. Calabrese (1985) investigated the variation in physiological response to exogenous stress in humans, and judged that 80–95% of the variation in a human group for a given agent was less than 10-fold. Hattis et al. (1987) analyzed 101 PK parameter data sets for 49 substances (mostly medications) and showed that 96% of the human variation was also less than 10-fold. Ginsberg et al. (2002) compared PK in adults and children using a database of approximately 45 medications, and showed that the half-lives of medications for 1-week to 2-month old infants were twice as long as the half-lives in adults. Hattis et al. (2003) also showed significantly longer half-lives of medications in infants and children compared to adults.

Animal data has also been reviewed. Dourson and Stara (1983) analyzed acute rat toxicity data for 490 substances reported by Weil (1972). They concluded that the LD_{50}/non-lethal dose ratio for 92% of the chemical substances would be less than 10. In a meeting abstract, Sheehan and Gaylor (1990) stated that the LD_{50} of 238 substances in adult rats was about 2.6 times higher than the LD_{50} in newborn rat pups, and the LD_{50}/LD_{50} ratio for 86% of substances was less than 10. Calabrese (2001) showed that the LD_{50} in younger animals was within a 10-fold range of older animals for 86.3% of 313 substances. Charnley and Putzrath (2001) examined the influence of age on carcinogenesis caused by chemicals, but were unable to reach a clear conclusion. Similarly, the US EPA considered the effect of age in their most recent guidelines for carcinogen risk assessment. They estimated the geometric mean ratio of early-life to adult cancer potencies was 10.4 based on repeated and lifetime exposure data in the available scientific literature for six chemicals acting through a mutagenic mode of action

(US EPA, 2005b; Barton et al., 2005). As for chemicals causing cancer through other modes of action, the ratio was 3.4 for lifetime exposure (5 chemicals) and 2.2 for repeated exposure (6 chemicals).

The quantitative human and experimental animal data for severe endpoints and kinetic parameters are useful. However, a study design similar to the repeat-dose exposure studies used in risk analysis would be ideal to derive an intra-species UF. The UF is applied to the NOAEL derived from the results of repeated dose toxicity studies, therefore a comparative analysis of NOAELs from repeat-dose toxicity studies of newborn and young rats for 18 chemicals was considered more appropriate (Hasegawa et al., 2007). In this study, Hasegawa et al. (2007) strictly compared the NOAEL ratios for newborn and young rats in a repeat-dose study. The NOAEL ratios were log-normally distributed. The ratio median was 3, and 5 was equivalent to 94.4% of the whole data set, from which the GSD can be calculated (see below).

2.3. Data for supplemental uncertainty factors[1]

The appropriate adjustment from short-term NOAEL to lifetime NOAEL for risk assessment was evaluated using 33 data sets of subchronic (3 months)/chronic (2 years) NOAELs in rats and mice reported by Weil and McCollister (1963) and 68 additional data sets from analyses of published reports or papers that we previously summarized (Hasegawa, 1991). Comparison of NOAELs from published 3-month and 2-year repeated dose toxicity studies, unpublished data). The combined data sets yielded a median of 1.7 with a GSD of 3.30. If only a LOAEL was identified, the median LOAEL/NOAEL ratio of 3.5 with a GSD of 1.82 from Abdel-Rahman and Kadry (1995) from other chemicals can be adapted as an UF for this area, with the usual upper bound value of 10. However, it is recognized that the application of the benchmark dose approach is usually more appropriate in cases where only a LOAEL is available, and as such this UF is not used as frequently.

3. Calculation of new uncertainty factors based on experimental data by probabilistic approach, an example of rats

The distribution of both inter- and intra-species differences is log-normal because each component consists of the NOAEL ratios for two groups. If the default values of 10 are used, simple multiplication of 10 by 10, resulting in 100, leads to overestimation for the 95th percentile of the combined distribution, more appropriately it should be 51, as shown by Monte Carlo simulation (Swartout et al., 1998). Generically, the *N*th percentile of a log-normal distribution can be expressed as *N*th percentile = Exp [LN (median) + $\alpha_n \times$ LN (GSD)]. For the 95th percentile, $\alpha_n = 1.645$. The equation for the combination of two log-normal A and B distributions can be shown as follows: 95th percentile of (A × B) = Exp [LN (median$_A$) + LN (median$_B$) + 1.645 × ((LN (GSD$_A$))^2 + (LN (GSD$_B$))^2)$^{0.5}$] (Kodell and Gaylor, 1999).

Inter-species differences were calculated using an analytical method presented by Schneider et al. (2004). A median of 4 was reported for the caloric demand adjustment, rounded from $3.76 = (70/0.35)^{1/4}$ (70 kg human body weight and 0.35 kg that of rats). A GSD of 3.23 was adopted from a combined distribution of MTD ratio for humans versus the 5 animal species previously described. For the 95th percentile, $\alpha_n = 1.645$.

LN (95th percentile) = LN (4) + 1.645 × LN (3.23)

95th percentile = UF (95%) = Exp [1.39 + 1.645 × 1.17] = 27.5.

Intra-species differences were calculated using rat young/newborn NOAEL ratios in repeat-dose toxicity studies (Hasegawa et al., 2007). The median was 3 for 18 data sets and 5 was equivalent to 94.4% of all the data sets. For the 94.4th percentile, $\alpha_n = 1.590$.

LN (5 as 94.4th percentile) = LN (3) + 1.590 × LN (GSD)
Rearranging,
LN (GSD) = (1.61 − 1.10)/1.590 = 0.321
Therefore,
GSD = Exp [0.321] = 1.38
95th percentile = UF (95%) = Exp [1.10 + 1.645 × 0.321] = 5.09.

From the above data for inter- and intra-species differences, the combined UF$_{AH}$ was calculated as follows:

LN (4) + LN (3) + 1.645 × ((LN (3.23))^2 + (LN (1.38))^2)$^{0.5}$ = 1.39 + 1.10 + 1.645 × (1.17^2 + 0.321^2)$^{0.5}$ = 4.48
Exp[4.48] = 88.7.

For adjustment of short-term NOAEL to lifetime NOAEL, all 101 data sets of subchronic NOAEL/chronic NOAEL were used. The median was 1.7 with 10 equivalent to 93.1% of all the data sets. For the 93.1th percentile, $\alpha_n = 1.483$.

LN (10 as 93.1th percentile) = LN (1.7) + 1.483 × LN (GSD)
Rearranging,
LN (GSD) = (2.30 − 0.531)/1.483 = 1.20
Therefore,
GSD = Exp [1.20] = 3.30
95th percentile = UF (95%) = Exp [0.531 + 1.645 × 1.20] = 12.1.

From the above UF calculations, the combined UF$_{AHS}$ was calculated as follows:

1.39 + 1.10 + 0.531 + 1.645 × (1.17^2 + 0.321^2 + 1.20^2)$^{0.5}$ = 5.82
Exp[5.82] = 337.

If a benchmark dose approach cannot be applied, an additional UF should be applied when using LOAEL data. The LOAEL/NOAEL ratio for 24 chemicals was reported by Abdel-Rahman and Kadry (1995). The median was 3.5 and 10 was equivalent to 96% of the whole data. For the 96.0th percentile, $\alpha_n = 1.751$.

LN (10 as 96.0th percentile) = LN (3.5) + 1.751 × LN (GSD)
Rearranging,
LN (GSD) = (2.30 − 1.25)/1.751 = 0.600
Therefore,
GSD = Exp [0.600] = 1.82
95th percentile = UF (95%) = Exp [1.25 + 1.645 × 0.600] = 9.39.

From the above UF calculations, the combined UF$_{AHSL}$ was calculated as follows:

1.39 + 1.10 + 0.531 + 1.25 + 1.645 × (1.17^2 + 0.321^2 + 1.20^2 + 0.6^2)$^{0.5}$ = 7.24
Exp [7.24] = 1400.

4. Summary of combined uncertainty factors for six animal species by probabilistic approach

All fundamental values for the median, GSD and UF (95%) are shown in Table 1. The median for inter-species differences was derived using caloric demand adjustment from the standard human and animal body weights and rounded to a simple value. The

[1] The uncertainty factor used by several organizations for missing certain studies in the database (e.g., Dourson et al., 1992, 2002) was not considered here at this time, as it is being studied for applicability in Japan.

Table 1
Median, GSD and UF (95%) of inter-species differences for 6 animal species and other uncertainties.

	Median	GSD	UF (95%)
Inter-species differences (caloric demand)[a]			
Mice to humans	6.95 → 7		48.2
Hamsters to humans	4.86 → 5		34.4
Rats to humans	3.76 → 4	3.23	27.5
Rabbits to humans	2.04 → 2		13.8
Monkeys to humans	1.77 → 1.8		12.4
Dogs to humans	1.44 → 1.4		9.63
Intra-species differences[b]	3.0	1.38	5.09
Subchronic to chronic[b]	1.7	3.30	12.1
LOAEL to NOAEL[b]	3.5	1.82	9.39

[a] Use of caloric demand and distribution from MTD ratios of 63 anti-cancer drugs between humans and 5 animals given by Schneider et al. (2004). Medians were calculated as caloric demand adjustment ((human body weight/animal body weight)$^{1/4}$) on the bases of body weight: humans = 70 kg, mice = 0.03 kg, hamsters = 0.125 kg, rats = 0.35 kg, rabbits = 4 kg, monkeys = 7 kg and dogs = 16 kg.
[b] Calculation details are shown in the previous section.

GSD for inter-species differences was obtained by combining the distribution of all the MTD data sets. This distribution may contain some additional, but unquantifiable, conservatism since humans are more heterogeneous than laboratory animals; thereby inflating the upper limits. The 95th percentile of UFs for six laboratory animal species ranged from approximately 10–50, a 5-fold difference.

All possible cases of UFs for six laboratory animal species were calculated by a probabilistic approach (Table 2) using the values from Table 1. The UF_{AH} for each animal is calculated by combining inter- and intra-species differences. We propose a rounded UF_{AH} of 150 for mice, 100 for hamsters and rats, and 40 for rabbits, monkeys and dogs. Additional single UFs for either subchronic to chronic (UF_S) or LOAEL to NOAEL (UF_L) extrapolation, resulted in a 3.8-fold increase for the UF_{AHS} from the UF_{AH} and a 4.4-fold increase for the UF_{AHL} from the UF_{AH}, giving UFs approximately 4-fold higher than the UF_{AH} in either case. Finally, the four combined UFs, UF_{AHSL}, when chronic data and NOAEL are lacking, resulted in a 16-fold increase from the UF_{AH}. All the UFs obtained by Monte Carlo simulation, based on the default UF of 10, are slightly lower than our proposed UFs for rats. Simple multiplication of the default value of 10, resulted in much larger values than all three or four combined UFs (UF_{AHS}, UF_{AHL}, UF_{AHSL}) for all animals.

5. Application of subdivision and replacement of uncertainty factors for inter- and intra-species differences (chemical-specific adjustment factors)

In the present article, we propose animal size-specific inter-species UFs and new combined UFs (UF_{AH}) by using probabilistic approaches. For the cases of hamsters and rats, UF_{AH} is set at 100 but the contributions of inter- and intra-species differences are not equal. The application of the same default subdivision factor shown by Renwick (1993) is not appropriate, if the UF_A values of Table 2 are used as the basis of the assessment. However, the concept established by Renwick (1993) is appropriate because we also recommend that actual and reliable experimental data for PK or PD differences should be incorporated into the risk assessment processes wherever possible. Therefore, we subdivided the new UF_A to determine the contribution ratio of inter- and intra-species differences. In the case of hamsters and rats, the average UF_A is approximately 30 (hamsters = 34.4 and rats = 27.5) and the intra-species difference is 5.09, (calculated above from the Hasegawa et al. (2007) data), resulting in a ratio contribution of ~6:1. The UF_{AH} for hamsters and rats is set at 100, which can be divided into factors of 25 and 4, according to the above ratio of 6:1. Considering the contribution ratios of PK and PD as 60:40 for inter-species differences and 50:50 for intra-species differences, 25 will be subdivided into $25^{0.6}$ (7.0) for PK and $25^{0.4}$ (3.6) for PD, and 4 will be evenly subdivided into $4^{0.5}$ (2) (Table 3).

Similar approaches can be used elsewhere. For example, the mice UF_{AH} of 150 can be divided into 38 and 4, then 38 will be subdivided into $38^{0.6}$ (9.0) for PK and $38^{0.4}$ (4.3) for PD. For rabbits, monkeys and dogs, the UF_{AH} of 40 can be divided into 10 and 4, then 10 will be subdivided into $10^{0.6}$ (4.0) for PK and $10^{0.4}$ (2.5) for PD.

If actual data for the difference between humans and animals for PK and/or PD are available, those data can be used as chemical-specific adjustment factors instead of respective default subdivision factors.

Table 2
Combined UFs for six animal species by probabilistic approach (95th percentile), Monte Carlo simulation and simple multiplication of UF 10.

Species	UF_A	UF_{AH}	UF_{AHS}	UF_{AHL}	UF_{AHSL}
Mice	48.2	155	589	684	2440
Hamsters	34.4	111	421	488	1740
Rats	27.5	88.7	337	391	1400
Rabbits	13.8	44.3	168	195	698
Monkeys	12.4	39.9	152	176	628
Dogs	9.63	31.0	118	137	488
All animals					
Monte Carlo[a]	10	51	234	234	1040
Default[b]	10	100	1000	1000	3000

A, inter-species differences; H, intra-species differences; S, subchronic to chronic; L, LOAEL to NOAEL.
[a] Data from Swartout et al. (1998).
[b] Note that US Environmental Protection Agency (USEPA) combines the default values of 4 UFs into 3000, because of the generally conservative nature of combining 10-fold factors that are each somewhat conservative (Dourson, 1994).

6. Discussion

The proposed written document to address chemical safety assessment methodology is needed because officially agreed upon guidelines do not exist in Japan. For this purpose, the latest scientific information has been collected to reduce the uncertainty in the risk assessment process. It would be more reliable for UFs to be estimated on the basis of actual experimental data rather than use conventional default UFs. Furthermore, the values are more representative of the data if they are developed using statistical components such as the median with distribution of differences rather than point estimates. A tolerable daily intake can be derived by probabilistic approaches, using the median or geometric mean (GM) and GSD to combine two or more distributions.

Recently, Falk-Filipsson et al. (2007) reviewed a wide variety of assessment factors in various historical and scientific ranges, including guidelines from national and international bodies. They reported that "over-conservatism" should be avoided by using a probability distribution for the various assessment factors. However, such an approach was only applied to the UF for inter-species

Table 3
Subdivision of uncertainty factors for inter- and intra-species differences.

Species	UF_{AH}	UF_A UF_H	Subdivision PK × PD
Mice	150	38	9.0 × 4.3
		4	2 × 2
Hamsters	100	25	7.0 × 3.6
Rats		4	2 × 2
Rabbits	40	10	4.0 × 2.5
Monkeys		4	2 × 2
Dogs			

Table 4
Median or GM with GSD for each uncertainty in four different methodologies.

	Inter-species differences		Intra-species differences		Subchronic to chronic		LOAEL to NOAEL	
	Median/GM	GSD	Median/GM	GSD	Median/GM	GSD	Median/GM	GSD
Baird et al. (1996) (GM)	AF[a]	4.9	2.7	2.3	2.0	2.1	3.4	1.70
Swartout et al. (1998)	10[b]		10[b]		10[b]		10[b]	
Kodell and Gaylor (1999) (median)	1	5.27	1	5.15	2	3.67	3.5	1.82
Present experiment (median)	AF[c]	3.23	3.0	1.38	1.7	3.30	3.5	1.82

[a] Adjustment factor for each animal on the basis of body surface correction.
[b] Use of 10 for every traditional default factor.
[c] Adjustment factor for each animal on the basis of caloric demand.

differences because appropriate distribution data for intra-species differences could not be located.

This study is the fourth trial following those of Baird et al. (1996), Swartout et al. (1998) and Kodell and Gaylor (1999) to use a probabilistic approach to estimate UFs for chemical risk assessment. Table 4 shows the median/GM and GSD for the four methodologies and Table 5 shows combined UFs for inter- and intra-species differences, and two other uncertainties. Swartout et al. (1998) estimated four UFs by Monte Carlo simulation using a traditional default UF of 10 for each uncertainty. Baird et al. (1996) also performed Monte Carlo simulation with specific software, but used actual data instead of default values. On the other hand, Kodell and Gaylor (1999) used standard statistical techniques, as we do here. Key differences in the three methodologies result from the original data used for inter- and intra-species extrapolation. For inter-species differences, the data used in this assessment are considered appropriate because the data are a direct comparison between humans and animals (Schneider et al., 2004). However, Baird et al. (1996) used comparative data within laboratory animals from pesticide safety studies (Dourson et al., 1992) and Kodell and Gaylor (1999) used toxicity comparisons of marine-life LD_{50} (Calabrese and Baldwin, 1995).

A similar analysis can be done for intra-species differences. This assessment used comparative NOAEL data from newborn and young rat repeat-dose studies as a sensitive subpopulation compared to the general population (Hasegawa et al., 2007). However, the other groups (Baird et al., 1996; Kodell and Gaylor, 1999) used lethality distribution data from acute toxicity studies (Dourson and Stara, 1983).

The different methodologies resulted in similar UF_{AH} values for Kodell and Gaylor (1999) and Baird et al. (1996), but were different from Swartout et al. (1998), as shown in Table 5. However, the Baird et al. (1996) UF_{AH} does not include a scaling adjustment factor, thus the median of inter-species differences of Baird's data was calculated as 1. As presented in this assessment, the body surface area correction factor, such as 13.3 for mice, 5.8 for rats, and 1.6 for dogs, should be used to reduce the uncertainty. This assessment calculated the expected UFs for rats using Baird et al. (1996) data (found in Table 4) and using the standard statistical techniques described in the previous sections of this paper. The results of these calculations are shown as "Baird et al., 1996 Our Calc" in Table 5.

Table 5
Combined UFs at 95% confidence limit by four methodologies.

UFs	Baird et al. (1996)[a]	Baird et al. (1996)[b]Our Calc	Swartout et al. (1998)[c]	Kodell and Gaylor (1999)[c]	Present study[b]	Default[c]
U_{AH}	50	300	51	46	89	100
U_{AHS}	126	764	234	161	337	1000
U_{AHL}	192	1156	234	184	400	1000
U_{AHSL}	484	2920	1040	629	1400	3000

[a] Not including inter-species scaling.
[b] Specific to rats.
[c] For all laboratory animals.

The calculated values were almost six times larger for each UF than those without the scaling adjustment factor (Baird et al., 1996 in Table 5). The calculated UFs in this assessment are relatively similar to Swartout et al. (1998) and much smaller than the default UF values.

The actual data used for our probabilistic estimation of the four UFs are considered suitable at this moment, and the combined UF_{AH} values for several commonly used laboratory animal species were given by standard statistical techniques (Table 2). However, as a rounded value is preferred for risk assessment, we propose size-specific UFs of 150 for mice, 100 for hamsters and rats, and 40 for rabbits, monkeys and dogs. As for other UFs such as UF_{AHS}, UF_{AHL} and UF_{AHLS}, the average uncertainty values for each (UF_{AHS}/UF_{AH}, UF_{AHL}/UF_{AH} and UF_{AHLS}/UF_{AH}) were 3.8, 4.4 and 15.7, respectively. Therefore, we propose to uniformly use a factor of 4 when a NOAEL (UF_L) and/or chronic data (UF_S) is lacking.

The application of an alternative subdivision of UFs should be considered in order to address the new concept of including animal size-specific UFs in the contribution of inter- and intra-species differences. The values of the new subdivision described in this study may be too precise, but this is inevitable, because the contribution of inter- and intra-species differences is definitively different. When further data on human and animal PK/PD differences are available, a more practical risk assessment can be implemented.

7. Conclusions

We propose an animal size-specific UF for UF_{AH} of 150 for mice, 100 for hamsters and rats, and 40 for rabbits, monkeys and dogs, for inter- and intra-species differences using a probabilistic approach. An additional default factor of 4 could be applied for either lack of chronic data or lack of a NOAEL. In addition to the proposed animal size-specific UFs, new subdivided PK/PD default factors for each animal are also proposed according to the different contribution of inter- and intra-species differences.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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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.

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 ($17.0 \pm 35.4\%$, 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

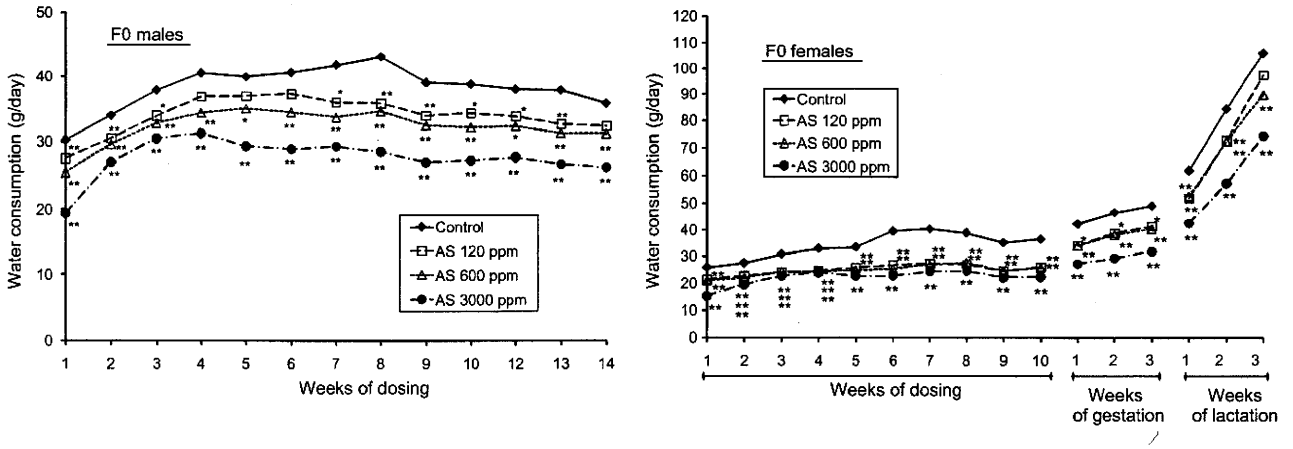

Fig. 1. Water consumption of F0 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

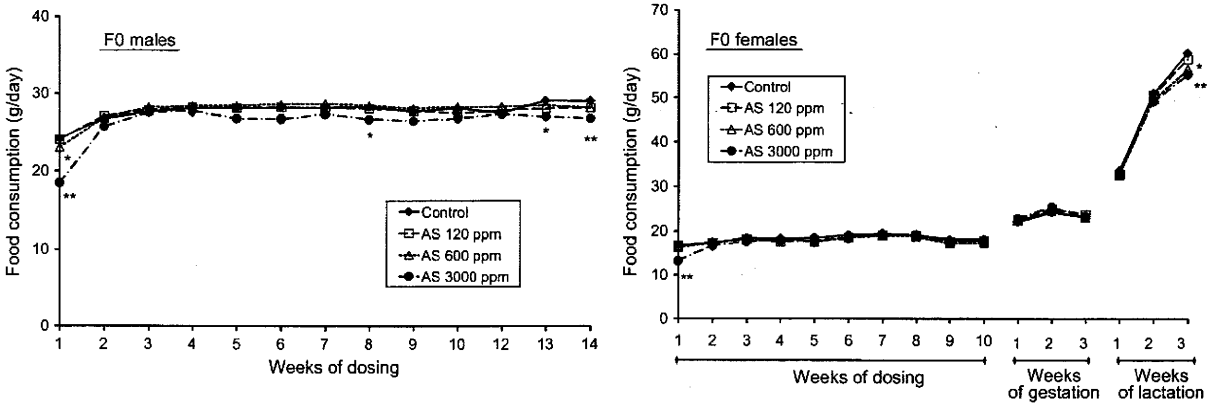

Fig. 2. Food consumption of F0 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

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

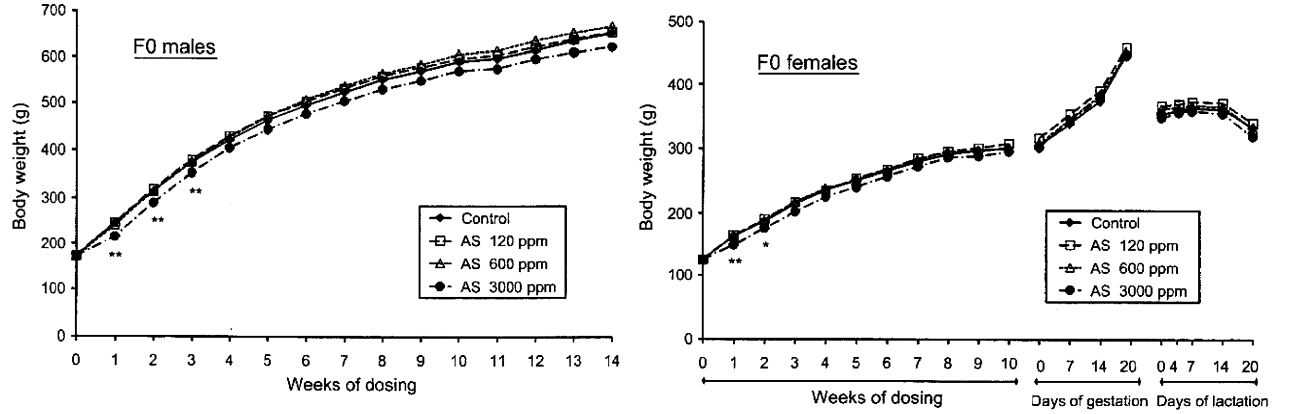

Fig. 3. Body weight of F0 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

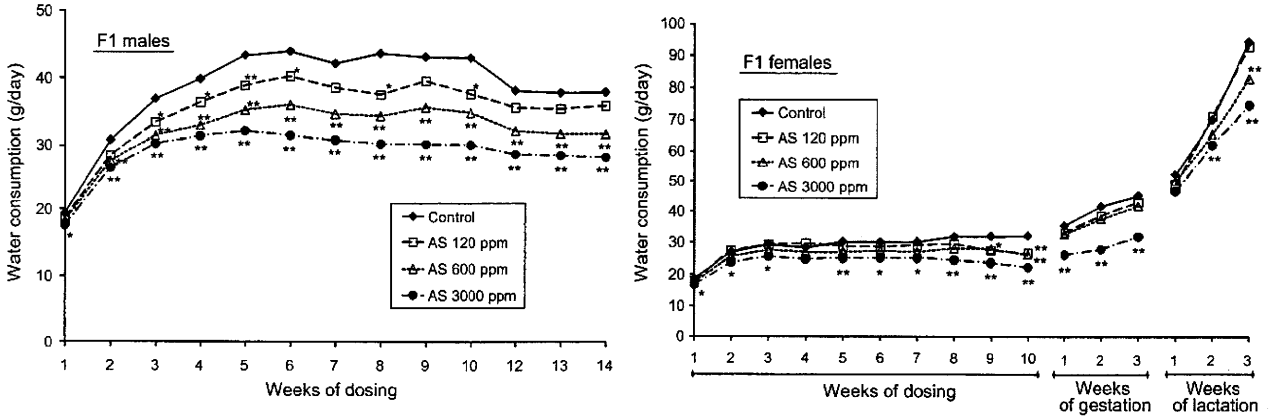

Fig. 4. Water consumption of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

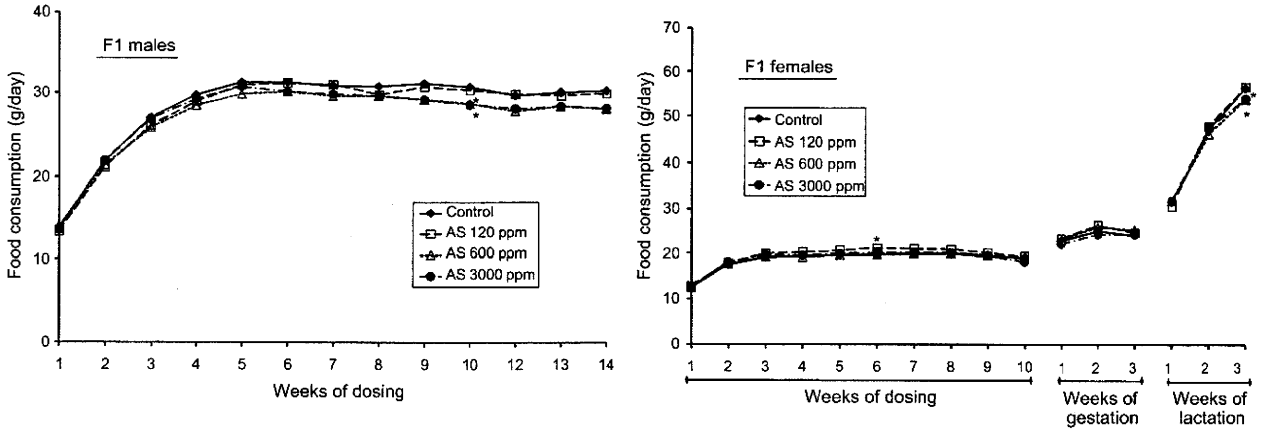

Fig. 5. Food consumption of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

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

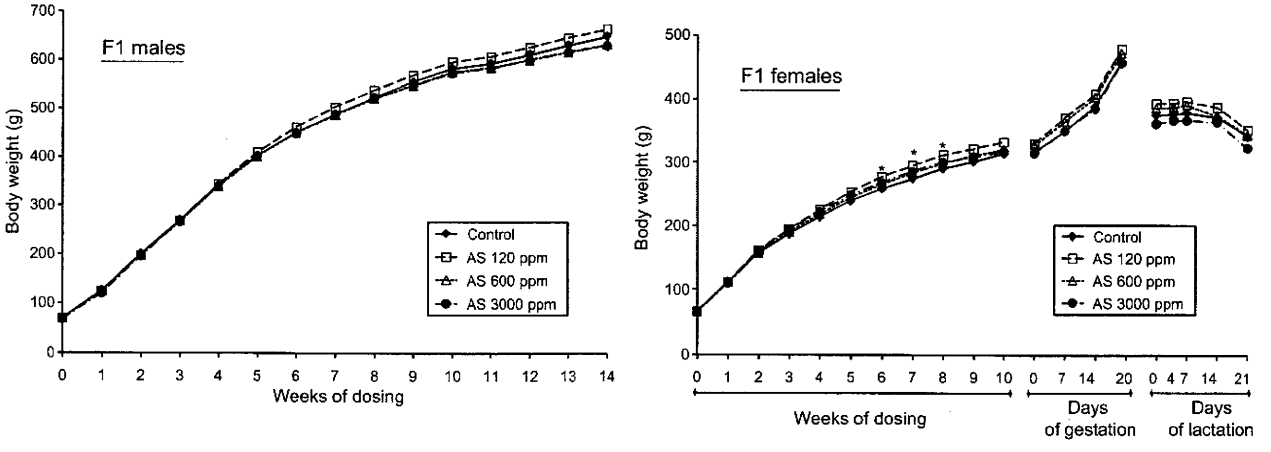

Fig. 6. Body weight of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

Table 1
Reproductive performance of F0 and F1 parental animals.

AS (ppm)		0 (control)	120	600	3000
F0 generation					
No. of rats (male/female)		24/24	24/24	24/24	24/24
Copulation index (%)[a]	Males	91.7	91.7	100	91.7
	Females	95.8	100	100	100
Precoital interval (days)[b]		3.2 ± 1.1	3.2 ± 1.8	2.9 ± 1.3	2.8 ± 1.6
Fertility index (%)[c]	Males	95.5	90.9	100	95.5
	Females	95.7	91.7	100	95.8
Gestation index (%)[d]		100	95.5	95.7	95.7
Gestation length (days)[b]		22.4 ± 0.5	22.5 ± 0.6	22.1 ± 0.4	22.3 ± 0.5
Delivery index (%)[b,e]		94.3 ± 5.6	88.6 ± 21.0	90.7 ± 20.8	92.0 ± 20.5
F1 generation					
No. of rats (male/female)		24/24	23/24	24/24	24/24
Copulation index (%)[a]	Males	95.8	91.3	95.8	87.5
	Females	100	95.8	100	95.8
Precoital interval (days)[b]		3.3 ± 3.2	3.0 ± 2.0	2.7 ± 1.5	2.3 ± 1.1
Fertility index (%)[c]	Males	91.3	81.0	91.3	95.2
	Females	91.7	82.6	91.7	91.3
Gestation index (%)[d]		100	94.7	100	100
Gestation length (days)[b]		22.4 ± 0.5	22.3 ± 0.5	22.2 ± 0.4	22.2 ± 0.4
Delivery index (%)[b,e]		94.0 ± 9.9	87.5 ± 22.6	91.4 ± 10.7	94.6 ± 6.8

[a] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[b] Values are given as the mean ± S.D.
[c] Fertility index (%) = (no. of animals that impregnated a female or were pregnant/no. of animals with successful copulation) × 100.
[d] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.

decreased significantly in this group, but no significant change was found in the relative weight. In F1 males, the absolute weights of the adrenals at 3000 ppm and the testes at 600 ppm were significantly decreased without significant changes in the relative weight. There were no significant changes in the absolute and relative weights of any organ in F0 and F1 female adults (data not shown).

No dose-related gross lesions were found in F0 or F1 adults. Histopathological examination of the reproductive organs revealed no compound-related alterations. There was no significant difference in the number of primordial follicles in the ovary of F1 females between control and 3000 ppm groups (data not shown).

3.6. Necropsy, organ weight and histopathology of weanlings (F1 and F2)

Absolute and relative organ weights of male and female F1 weanlings are shown in Table 3. The 3000 ppm treated males and females had a significantly lower body weight at scheduled sacrifice than the controls. In this group, absolute and relative liver weights were significantly lower than the controls. Absolute spleen weight was also decreased significantly in both sexes of the 3000 ppm group, accompanied by a significant decrease in the relative weight in males. In addition, significant decreases in the absolute weight were found for the thymus in both sexes and for the kidneys, testes and epididymides in males at 3000 ppm, and for the uterus in females at 600 and 3000 ppm. Relative brain weight was significantly increased in both sexes of the 3000 ppm group.

Table 4 presents absolute and relative organ weights of male and female F2 weanlings. The mean body weight at scheduled sacrifice was significantly lowered in both sexes of the 3000 ppm group. In males, the absolute and relative weights of the thymus and spleen were significantly decreased in the 3000 ppm group. Significant decreases were also found in the absolute weight of the liver and epididymides at 3000 ppm. The relative brain weight was significantly increased at this dose. At 120 ppm, the only significant change was a non-dose-related decrease in the relative thymus weight. In F2 females, there were significant decreases in the absolute and relative weights of the liver, and the absolute weight of the spleen, ovary and uterus, and a significant increase in the relative brain weight at 3000 ppm. In addition, a significant decrease in the absolute brain weight was observed only in the 600 ppm group.

External and internal gross observations revealed no compound-related alterations either in F1 and F2 weanlings or in pups found dead during the preweaning period. There were no dose-related histopathological changes in the liver and spleen of male and female F1 and F2 weanlings.

4. Discussion

AS administered via the drinking water to male and female rats resulted in decreased water consumption for both sexes in all treatment groups. Since the dosing solution containing AS was pH 3.57–4.20, the acidity would decrease the palatability of drinking water in AS-treated groups. Decreased water consumption was associated with decreased food consumption by F0 and F1 males and females in the 600 and 3000 ppm groups and decreased body weight in F0 male and females in the 3000 ppm group. Since water-deprived animals typically reduce their levels of feed consumption and consequently lower their body weight [46], decreased food consumption and body weight observed in the present study could be considered secondary to the decreased water consumption. In the present study, food consumption and body weight fell notably during the early dosing period in F0 males and females. Food consumption also decreased in F0 and F1 females at the end of the lactation period, when F1 or F2 pups would commence eating and drinking for themselves [37]. Campbell et al. [46] reported that animals have a certain amount of "buffering" capacity in the form of physiological mechanisms acting to reduce fluid loss. This might explain notable changes around the time when rats start drinking AS-containing water.

Continuous drinking of AS-contained water for two generations did not result in changes in copulation, fertility or gestation indices, pre-coital or gestation length, the number of implantations or pups delivered, or the incidence of pups with malformations or variations. In addition, adverse effects were not found in estrous cyclicity or sperm parameters, and the histopathology of reproductive tissues in male and female parental animals. Previous studies have demonstrated that parenterally administered aluminium affected