Table 2
Sex ratio, viability and body weight for F1 and F2 pups.

AS (ppm)	0 (control)	120	600	3000
F1 offspring				
No. of litters	22	21	22	22
No. of pups delivered[a]	13.9 ± 1.7	12.4 ± 4.7	13.1 ± 4.1	13.1 ± 3.4
Sex ratio of pups[b]	0.503	0.462	0.513	0.536
Viability index of pups (%)[a]				
On PND 0[c]	100.0 ± 0.0	99.3 ± 2.3	99.7 ± 1.6	99.5 ± 2.4
On PND 4[d]	98.7 ± 2.9	95.2 ± 21.8	98.8 ± 2.6	98.0 ± 5.4
On PND 21[e]	99.4 ± 2.7	100.0 ± 0.0	100.0 ± 0.0	99.4 ± 2.7
Male pup weight during lactation (g)[a]				
On PND 0	7.05 ± 0.61	7.25 ± 0.99	6.74 ± 0.69	6.96 ± 0.76
On PND 4	11.04 ± 0.85	11.41 ± 1.99	10.86 ± 1.37	11.00 ± 1.06
On PND 7	18.91 ± 1.29	19.36 ± 2.77	18.59 ± 1.71	18.47 ± 1.35
On PND 14	37.70 ± 2.63	37.97 ± 3.08	37.39 ± 2.59	36.34 ± 2.41
On PND 21	62.48 ± 4.50	62.63 ± 6.14	60.77 ± 4.01	57.34 ± 4.86**
Female pup weight during lactation (g)[a]				
On PND 0	6.61 ± 0.55	6.89 ± 0.83	6.35 ± 0.57	6.60 ± 0.64
On PND 4	10.46 ± 0.89	11.06 ± 1.71	10.27 ± 1.33	10.43 ± 0.83
On PND 7	18.03 ± 1.27	18.56 ± 2.31	17.69 ± 1.61	17.61 ± 1.21
On PND 14	36.29 ± 2.71	36.94 ± 3.03	35.67 ± 2.60	35.31 ± 2.24
On PND 21	60.17 ± 4.16	60.87 ± 5.68	57.68 ± 4.33	55.60 ± 4.34**
F2 offspring				
No. of litters	22	18	22	21
No. of pups delivered[a]	13.1 ± 3.6	13.2 ± 3.8	12.6 ± 3.9	14.0 ± 1.9
Sex ratio of pups[b]	0.528	0.502	0.536	0.457
Viability index of pups (%)[a]				
On PND 0[c]	99.68 ± 1.51	99.49 ± 2.14	98.42 ± 3.57	98.69 ± 3.60
On PND 4[d]	94.72 ± 14.54	98.07 ± 5.45	99.07 ± 3.15	99.01 ± 2.49
On PND 21[e]	100.00 ± 0.00	98.61 ± 4.04	100.00 ± 0.00	100.00 ± 0.00
Male pup weight during lactation (g)[a]				
On PND 0	6.97 ± 0.68	6.92 ± 0.81	6.87 ± 0.74	6.89 ± 0.60
On PND 4	10.73 ± 1.62	10.53 ± 1.27	11.27 ± 1.81	10.52 ± 1.15
On PND 7	17.96 ± 2.05	17.51 ± 2.12	18.83 ± 2.39	17.72 ± 1.60
On PND 14	35.79 ± 3.52	36.18 ± 3.63	37.32 ± 4.15	35.44 ± 2.73
On PND 21	59.61 ± 5.45	59.44 ± 5.67	60.12 ± 7.12	56.36 ± 4.47
Female pup weight during lactation (g)[a]				
On PND 0	6.66 ± 0.69	6.38 ± 0.78	6.41 ± 0.65	6.50 ± 0.49
On PND 4	10.22 ± 1.63	9.70 ± 1.23	10.36 ± 1.54	9.98 ± 0.91
On PND 7	17.03 ± 1.99	16.36 ± 2.35	17.40 ± 2.18	16.89 ± 1.23
On PND 14	34.82 ± 3.52	34.17 ± 3.58	34.96 ± 4.24	34.01 ± 2.09
On PND 21	57.33 ± 4.90	56.11 ± 5.54	56.41 ± 6.04	54.16 ± 2.82*

[a] Values are given as the mean ± S.D.
[b] Sex ratio = total no. of male pups/total no. of pups.
[c] Viability index on PND 0 (%) = (no. of live pups on PND 0/no. of pups delivered) × 100.
[d] Viability index on PND 4 (%) = (no. of live pups on PND 4/no. of live pups on PND 0) × 100.
[e] Viability index on PND 21 (%) = (no. of live pups on PND 21/no. of live pups on PND 4 after cull) × 100.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

male reproductive systems, causing decreases in testicular and epididymal sperm counts, necrosis of spermatocytes/spermatids in the testes, and reduction of fertility, etc., in rats and mice [19,20,47]. Although aluminium has extremely low oral bioavailability (less than 1%) [48,49], male reproductive toxicity was also observed in oral gavage studies [22,23,50]. In the 6-month oral gavage study in rats, changes in the number of spermatozoa and motility, and the substantial proliferation of interstitial cells in the testes were observed at doses as low as 2.5 mg Al/kg/day (as aluminium chloride) [50]. The primary reason why such effects were not detected in the present study might be a difference in the administration method because the toxicokinetic behavior of chemicals given as a bolus dose by gavage must differ significantly from those after continuous administration via drinking water. Another possible factor is aluminium content in the laboratory animal feed, for which substantial brand-to-brand and lot-to-lot variations, ranging from 60 to 8300 ppm, have been reported [51]. Since the dietary intake of aluminium was not considered in these oral gavage studies, toxic effects of aluminium could be overestimated. As for continuous exposure studies, taking into account the aluminium content in the basal diet, Hicks et al. [52] demonstrated that 28-day continuous dietary exposure to basic sodium aluminium phosphate or aluminium hydroxide did not affect the testicular histopathology up to 302 mg Al/kg/day in Sprague–Dawley rats. In the 26-week feeding study of basic sodium aluminium phosphate in beagle dogs, germinal epithelial cell degeneration and atrophy in the seminiferous tubules were observed at 75 mg Al/kg bw/day [21], but such effects on male reproductive organs were not detected up to 88 mg Al/kg/day in the similar subchronic dietary study for acidic sodium aluminium phosphate in beagle dogs [53]. These dietary studies used water-insoluble or sparingly soluble forms of aluminium [8,51]. Since it is widely assumed that insoluble aluminium compounds are less bioavailable than soluble compounds, such as AS, aluminium chloride and aluminium lactate [8], there is a possibility that the male reproductive toxicity of aluminium was underestimated in these previous dietary exposure studies. The present two-generation study provided useful information that the male reproductive system is not affected even after continuous exposure to a water-soluble aluminium compound, at least up to around 50 mg Al/kg bw/day.

In the present study, some developmental effects were observed. Male and female F1 pups and female F2 pups in the

Table 3
Absolute and relative organ weight of F1 male and female weanlings.

AS (ppm)		0 (control)	120	600	3000
Males					
No. of animals		22	20	22	22
Body weight	(g)	90.8 ± 6.9	93.4 ± 10.5	89.7 ± 6.1	79.4 ± 7.5**
Brain	(g)	1.69 ± 0.06	1.73 ± 0.08	1.72 ± 0.07	1.68 ± 0.05
	(g/100 g bw)	1.88 ± 0.13	1.87 ± 0.19	1.92 ± 0.09	2.14 ± 0.17**
Thymus	(mg)	375 ± 55	384 ± 86	357 ± 58	305 ± 51**
	(mg/100 g bw)	414 ± 56	409 ± 64	398 ± 59	383 ± 36
Liver	(g)	4.33 ± 0.43	4.40 ± 0.60	4.22 ± 0.45	3.49 ± 0.53**
	(g/100 g bw)	4.77 ± 0.30	4.71 ± 0.33	4.70 ± 0.27	4.37 ± 0.30**
Kidney[a]	(g)	1.06 ± 0.09	1.09 ± 0.14	1.03 ± 0.11	0.95 ± 0.13**
	(g/100 g bw)	1.17 ± 0.06	1.16 ± 0.07	1.15 ± 0.08	1.20 ± 0.07
Spleen	(mg)	394 ± 49	410 ± 68	388 ± 74	301 ± 43**
	(mg/100 g bw)	436 ± 63	437 ± 40	432 ± 73	379 ± 37**
Testis[a]	(mg)	596 ± 65	583 ± 67	569 ± 65	539 ± 51*
	(mg/100 g bw)	657 ± 64	626 ± 49	635 ± 64	682 ± 58
Epididymis[a]	(mg)	81.8 ± 8.6	76.8 ± 10.9	76.5 ± 8.4	72.0 ± 9.9**
	(mg/100 g bw)	90.4 ± 10.3	82.0 ± 6.1	85.4 ± 8.4	91.5 ± 14.6
Females					
No. of animals		22	20	22	21
Body weight	(g)	84.3 ± 6.3	85.9 ± 9.2	80.5 ± 7.0	75.8 ± 6.4**
Brain	(g)	1.64 ± 0.06	1.66 ± 0.06	1.63 ± 0.05	1.63 ± 0.07
	(g/100 g bw)	1.96 ± 0.12	1.95 ± 0.18	2.04 ± 0.17	2.16 ± 0.14**
Thymus	(mg)	383 ± 66	373 ± 74	345 ± 46	313 ± 33**
	(mg/100 g bw)	453 ± 63	433 ± 64	429 ± 57	415 ± 41
Liver	(g)	3.83 ± 0.47	3.92 ± 0.48	3.61 ± 0.35	3.24 ± 0.34**
	(g/100 g bw)	4.53 ± 0.30	4.57 ± 0.31	4.48 ± 0.30	4.27 ± 0.25*
Kidney[a]	(g)	0.99 ± 0.11	0.99 ± 0.09	0.93 ± 0.10	0.93 ± 0.10
	(g/100 g bw)	1.17 ± 0.08	1.15 ± 0.07	1.15 ± 0.09	1.23 ± 0.09
Spleen	(mg)	337 ± 62	356 ± 55	341 ± 64	292 ± 43*
	(mg/100 g bw)	400 ± 67	415 ± 44	422 ± 53	386 ± 47
Ovary[a]	(mg)	25.3 ± 4.8	25.3 ± 3.8	22.5 ± 4.6	24.7 ± 3.2
	(mg/100 g bw)	30.1 ± 5.1	29.7 ± 5.0	27.9 ± 5.0	32.5 ± 4.2
Uterus	(mg)	70.6 ± 16.6	74.2 ± 32.0	59.2 ± 11.9*	55.4 ± 13.4**
	(mg/100 g bw)	83.8 ± 19.2	85.5 ± 32.4	73.3 ± 11.9	73.3 ± 18.0

Values are given as the mean ± S.D.
[a] Values represent the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

3000 ppm group had a lower body weight on PND 21 while no difference was found in the birth weight. Such inhibition of the preweaning body weight gain may be simply attributable to the decreased palatability of the drinking water, which would decrease the water intake of pups themselves or might decrease the amount of maternal breast milk; however, similar developmental effects of aluminium were reported in the previous three dietary exposure studies, in which aluminium lactate was mixed in the diet and fed to mice from day 0 of gestation throughout the lactation period [28,30,32], and the lowest effect level was 500 ppm, which was estimated to be equal to 94–273 mg Al/kg bw/day based on the body weight and food consumption during the lactation period [28]. In these dietary exposure studies, food consumption was decreased, but Golub et al. [28] indicated that the mean body weight of pups on PNDs 15 and 20 in the 1000 ppm group was lower than that of the pair-fed control. In contrast, the effects on body weight of preweaning mice were not found in the other two dietary exposure studies [29,31], which were conducted using a similar study design by the same research group. Donald et al. [29] discussed differences in the constituents of the diet as a possible cause of the inconsistent results, but this speculation appears to be contradicted by a subsequent study [30]. Although it is still unclear why different results were obtained in these dietary exposure studies, these results suggest that the fall in body weight around weaning in the present study might not be explained only by decreased water intake. Aluminium ingested by pups themselves and/or taken via maternal milk may affect preweaning growth, or impairment of maternal nursing behavior or the lactation status could be considered possible factors.

In F1 and F2 weanlings, various organ weight changes were found in the 3000 ppm group. Among them, an increase in the relative brain weight is considered to be a secondary change that occurs with the fall in body weight because the absolute weight did not change. Similarly, decreased absolute weights of the kidneys, testes, epididymides, uterus, etc., without changes in the relative weight, were thought to be associated with decreased body weight. On the other hand, the effects on the liver and spleen, the absolute and relative weights of which were decreased in both generations, could not be explained only by the fall of body weight. Since similar effects were observed in the above-mentioned dietary exposure study of aluminium lactate [28], the effects on the liver and spleen weight might be a direct effect of aluminium ingested by pups themselves and/or taken via maternal milk; however, in the present study, no histopathological changes were detected in the liver and spleen. Furthermore, the changes in the liver or spleen weight were not detected in adults, except for F0 males in the 3000 ppm group; therefore, organ weight changes observed in F1 and F2 weanlings in the 3000 ppm group were not deemed to be adverse effects. Organ weight changes in the 120 and 600 ppm groups were not considered to have toxicological significance because these changes were not dose-dependent or were inconsistent across generations.

As for effects on the developmental landmarks, vaginal opening was slightly delayed in F1 females in the 3000 ppm group while no compound-related changes were found in the other developmental landmarks, including male preputial separation. Vaginal opening generally occurs around the time of first ovulation in response to an increase in serum estradiol levels as females enter puberty [54], and therefore, it is widely used as a marker of female puberty.

Table 4
Absolute and relative organ weight of F2 male and female weanlings.

AS (ppm)		0 (control)	120	600	3000
Males					
No. of animals		21	18	22	21
Body weight	(g)	87.7 ± 5.8	89.0 ± 8.7	87.0 ± 9.6	79.2 ± 6.8**
Brain	(g)	1.66 ± 0.05	1.69 ± 0.06	1.70 ± 0.06	1.67 ± 0.06
	(g/100 g bw)	1.90 ± 0.13	1.91 ± 0.17	1.97 ± 0.16	2.13 ± 0.17**
Thymus	(mg)	382 ± 50	348 ± 49	357 ± 66	305 ± 36**
	(mg/100 g bw)	439 ± 70	392 ± 52*	411 ± 57	386 ± 40**
Liver	(g)	3.93 ± 0.37	4.04 ± 0.64	3.91 ± 0.39	3.45 ± 0.41**
	(g/100 g bw)	4.49 ± 0.34	4.52 ± 0.44	4.50 ± 0.24	4.36 ± 0.23
Kidney[a]	(g)	1.02 ± 0.09	1.01 ± 0.13	0.99 ± 0.13	0.94 ± 0.10
	(g/100 g bw)	1.16 ± 0.08	1.14 ± 0.06	1.14 ± 0.07	1.19 ± 0.06
Spleen	(mg)	368 ± 54	381 ± 62	361 ± 49	296 ± 48**
	(mg/100 g bw)	421 ± 64	427 ± 50	416 ± 48	372 ± 42**
Testis[a]	(mg)	559 ± 67	549 ± 98	543 ± 77	534 ± 54
	(mg/100 g bw)	637 ± 60	615 ± 81	624 ± 47	680 ± 92
Epididymis[a]	(mg)	75.3 ± 6.9	78.3 ± 8.8	75.1 ± 10.7	70.5 ± 5.7*
	(mg/100 g bw)	86.1 ± 8.3	88.4 ± 9.0	86.5 ± 9.0	89.4 ± 8.2
Females					
No. of animals		22	18	21	21
Body weight	(g)	80.8 ± 6.0	80.0 ± 7.2	80.8 ± 9.1	73.8 ± 4.4**
Brain	(g)	1.60 ± 0.06	1.61 ± 0.05	1.64 ± 0.05*	1.61 ± 0.04
	(g/100 g bw)	1.99 ± 0.14	2.03 ± 0.16	2.05 ± 0.20	2.19 ± 0.15**
Thymus	(mg)	337 ± 45	364 ± 36	347 ± 49	312 ± 37
	(mg/100 g bw)	419 ± 61	457 ± 50	431 ± 47	424 ± 54
Liver	(g)	3.56 ± 0.35	3.61 ± 0.39	3.61 ± 0.48	3.07 ± 0.26**
	(g/100 g bw)	4.41 ± 0.21	4.51 ± 0.26	4.47 ± 0.26	4.17 ± 0.29**
Kidney[a]	(g)	0.95 ± 0.07	0.93 ± 0.10	0.92 ± 0.10	0.88 ± 0.08
	(g/100 g bw)	1.18 ± 0.08	1.16 ± 0.09	1.14 ± 0.06	1.20 ± 0.07
Spleen	(mg)	320.9 ± 46.7	331.8 ± 59.3	331.3 ± 57.1	269.9 ± 55.2**
	(mg/100 g bw)	398.4 ± 59.0	414.8 ± 64.3	409.0 ± 42.2	365.0 ± 67.4
Ovary[a]	(mg)	23.9 ± 3.7	22.8 ± 3.6	23.2 ± 3.5	20.2 ± 2.3**
	(mg/100 g bw)	29.7 ± 4.9	28.8 ± 5.6	29.0 ± 4.7	27.5 ± 3.5
Uterus	(mg)	60.5 ± 17.0	63.8 ± 18.4	65.0 ± 41.7	49.3 ± 11.6*
	(mg/100 g bw)	74.6 ± 19.2	79.3 ± 19.3	78.7 ± 40.4	67.0 ± 16.2

Values are given as the mean ± S.D.
[a] Values represent the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

On the other hand, vaginal opening is closely related to body weight, and growth retardation can delay the day of acquisition [55]. In the present study, body weight at the time of vaginal opening was slightly heavier in F1 females of the highest dose group, compared with the control. However, other hormone-dependent events, including estrous cyclicity and AGD, were not changed in AS-treated groups. It seems unlikely that aluminium have a clear impact on the hormonal event. Further studies are required to draw a definitive conclusion on the effects of aluminium on the sexual maturation.

Aluminium has been reported to affect the developing nervous system [56,57]. In continuous exposure studies using rodents, the neurobehavioral effects were reported as follows: dietary exposure of pregnant rats to aluminium chloride or lactate at more than 96 mg Al/kg bw/day impaired the righting reflex, gasping reflex, negative geotaxis and/or locomotor coordination of preweaning pups [25,26]. Impaired performance of operant conditioning tasks, accompanied by a decrease in locomotor activity, was observed on PND 65 in the offspring of rats receiving a diet containing aluminium lactate at 400 mg Al/kg bw/day during gestation [58], suggesting that the effects continue after maturation. Unfortunately, aluminium concentration in the basal diet was not reported in these feeding studies using rats. Similar neurobehavioral effects were found in the preweaning, juvenile and adult periods of the offspring after dietary exposure of mice to aluminium lactate throughout the gestation and lactation periods [28–32]. These mouse studies took the basal diet content of the aluminium diet into consideration, and the lowest observed effect level was 500 ppm, corresponding to 100 mg Al/kg bw/day at the beginning of pregnancy and 210 mg Al/kg/day near the end of lactation [29]. In the present study, no changes were found in the reflex ontogeny of F1 and F2 pups and in spontaneous locomotor activity tested at 4 weeks of age for F1 animals. As for the performance in a water-filled multiple *T*-maze, a transient decrease in the elapsed time and the number of errors were found in F1 females in the 600 ppm group, but this change was not considered to be treatment-related because of the lack of dose-dependency. These findings indicate that previous findings of developmental neurotoxic effects were possibly related to the toxic effects of aluminium given at higher doses than those given in this study.

In summary, AS administered via drinking water at 120, 600 or 3000 ppm resulted in decreased water consumption. This change was associated with decreased food consumption in the 600 and 3000 ppm groups and decreased body weight in the 3000 ppm group. In the 3000 ppm group, male and female pups had a lower body weight on PND 21. At this dose, vaginal opening was slightly delayed. No definitive effects were found in the other reproductive/developmental parameters, including developmental neurobehavioral toxicity. Although there is a possibility that observed developmental effects are results of decreased water consumption, more definitive conclusions could not be reached since paired-comparison data are not available to assess the effects of decreased water intake in the absence of AS exposure. Based on these considerations, a conservative evaluation of the data led to the conclusion that the no observed adverse effect level of AS in this two-generation study is 600 ppm (41.0 mg/kg bw/day) for parental systemic toxicity and reproductive/developmental toxicity. The total ingested dose of aluminium from food and drinking

water combined in this 600 ppm group was calculated to be 8.06 mg Al/kg bw/day.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Original Article

Influence of coefficient of variation in determining significant difference of quantitative values obtained from 28-day repeated-dose toxicity studies in rats

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ABSTRACT — In order to understand the influence of coefficient of variation (CV) in determining significant difference of quantitative values of 28-day repeated-dose toxicity studies, we examined 59 parameters of 153 studies conducted in accordance with Chemical Substance Control Law in 12 test facilities. Sex difference was observed in 12 parameters and 10 parameters showed large CV in females. The minimum CV was 0.74% for sodium. CV of electrolytes was comparatively small, whereas enzymes had large CV. Large differences in CV were observed for major parameters among 7-8 test facilities. The changes in CV were grossly classified into 11. Our study revealed that a statistical significant difference is usually detected if there is a difference of 7% in mean values between the groups and the groups have a CV of about 7%. A parameter with a CV as high as 30% may be significantly different, if the difference of the mean between the groups is 30%. It would be ideal to use median value to assess the treatment-related effect, rather than mean, when the CV is very high. We recommend using CV of the body weight as a standard to judge the adverse effect level.

Key words: Coefficients of variation, Repeated-dose study, Quantitative value, Standard deviation, Chemical substance control law

INTRODUCTION

Repeated dose toxicity studies with rodents are usually conducted with a minimum of three treatment doses and a control (OECD, 1995). The quantitative data obtained from these studies are subjected to statistical analysis, using parametric or nonparametric statistical tools. If the data do not show heterogeneity and show a normal distribution, a parametric statistical tool is used, otherwise a nonparametric statistical tool. When the individual values of a parameter distribute in a wider range, it is most likely that the data show heterogeneity in variance. Distribution of data around mean can be estimated in terms of standard deviation and coefficient of variation (CV). CV is a numerical value where the proportion of the standard deviation in the mean value is shown as a percentage. Generally, the distribution of the quantitative values is broad for serum enzymes and narrow for electrolytes. Statistical significant difference of a parameter between groups is influenced by the difference of means between the groups, variance of the data and number of animals of the groups. A large difference observed between the mean values of control and dosage groups may not be statistically significant, if the variance of one or more groups explodes in a wider range.

Reports on the influence of CV in determining significant difference of quantitative values obtained from toxicity studies are rare. Matsuzawa *et al.* (1993) analyzed historical control data of clinical pathology testing provided by 67 member companies of the Japan Pharmaceutical Manufacturers Association covering study populations of approximately 14,000 rats, 10,000 dogs and 1,400 mon-

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keys. The authors assessed the potential factors contributing to variation in reference data based on weighted means and standard deviations. The authors described that the serum assay values showed greater variation than the plasma values.

In the present investigation, we examined the data of control groups of 28-day repeated-dose toxicity studies in rats performed according to the Chemical Substance Control Law (CSCL) in 12 test facilities. We examined 59 parameters of 153 studies. The number of animals per group, administration period and other factors were standardized according to the guidelines of CSCL. The distribution for each quantitative item (males and females separately) was converted into CV, and the influence of it in determining the significant difference between the groups was studied.

MATERIALS AND METHODS

The data investigated (Table 1) were from male and female SD rats of the control groups in the repeated-dose 28-day toxicity studies (Opening 153; MHLW, 2009) conducted by CSCL (NITE, 2007). CAS (Chemical Abstracts Service) numbers of the test substance administered in these studies are shown in Table 2. In these animals the vehicle was administered by oral gavage using a stomach tube. The number of animals in the group was 5-7 in most of the clinical examinations and organ weight determination and 10-12 in body weight and the feed consumption measurements. Most of the data were obtained on day 28 of the experimental period. However, few data pertaining to body weight, feed consumption, urinalyses and water consumption were obtained on days 29, 22-28, 26 and 24-26, respectively, during the experiment. The organ weight/body weight ratio was not included in the present investigation. CV (%) was calculated for each parameter using the standard formula.

The cluster analysis was conducted using the SAS JMP software (ver. 5.0; SAS Institute, Cary, NC, USA). The analysis of variance (ANOVA) and *t*-test were performed using software by Aoki (Aoki, 2010). Sex differences in CV were analyzed by the *F*-test for homogeneity of variance. When the variances were homogeneous at a significance level of 5%, Student's *t*-test was performed. When the variances were not homogeneous, Welch's *t*-test was performed. These *t*-tests were two-sided.

Items examined were (1) sex differences in CV of each quantitative value, (2) rank order of the parameters based on CV, (3) classification of CV of parameters by cluster analysis, (4) changes in CV of parameters in different test facilities, and (5) significant difference detection pattern when the difference between two groups was set constant and CV was changed.

RESULTS

CV for each quantitative item

Sex differences in CV of the 59 quantitative values are shown in Table 3. Statistically significant differences were observed in 12 items. Among them, large CVs were observed for prothrombin time (PT) and adrenal weights in the males compared with females (Table 4).

Rank order of parameters with regard to their CVs

CVs of the parameters of males and females were

Table 1. Parameters investigated

Item	Parameter
Animal care	Body weight (BW), Feed consumption (FC), Water consumption (WC)
Urinalyses	Urine volume (UV), Specific gravity (SG/urine), Osmotic pressure (OP/urine), *etc.*
Hematology	White Blood cell (WBC), Differential lymphocyte ratio (Lymph), Differential neutrophil ratio (Neut-seg), Red blood cell (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Platelet (PLT), Reticulocyte (RET), Fibrinogen (Fib), Prothrombin time (PT), Activated partial thromboplastin time (APTT), Methemoglobin, *etc.*
Blood chemistry	Total protein (TP), Albumin (Alb), Albumin/Globulin ratio (A/G), Total cholesterol (Cho), Total bilirubin (Bili), Triglyceride (TG), Glucose (Glu), Blood urea nitrogen (BUN), Creatinine (CRN), Triglyceride (TG), Phospholipid (PL), Alkaline phosphate (ALP), Lactic dehydrogenase (LDH), Cholinesterase (ChE), Creatine phosphokinase (CPK), Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT), γ Glutamyl transpeptidase (γ-GTP), Calcium (Ca), Inorganic phosphorus (IP), Sodium (Na), Potassium (K), Chloride (Cl), *etc.*
Absolute organ weights	Brain, Thymus, Thyroids, Heart, Lungs, Liver, Spleen, Kidneys, Adrenals, Testes, Epididymis, Ovaries, *etc.* The right and left attached table record adopts the right.

Table 2. CAS number of test substance administered in the 28-day repeated-dose toxicity studies

95-64-7	99-71-8	96-29-7	83-32-9	842-18-2	84-51-5	657-84-1	109-59-1	111-17-1
100-61-8	106-37-6	96-69-5	87-59-2	1066-40-6	88-18-6	824-78-2	109-64-8	112-26-5
103-69-5	121-47-1	111-41-1	95-33-0	16219-75-3	88-89-1	1328-53-6	1552-42-7	1025-15-6
105-99-7	526-78-3	119-47-1	97-52-9	25321-09-9	95-50-1	1333-16-0	3846-71-7	27676-62-6
88-53-9	1806-54-8	126-33-0	100-54-9	79-39-0	95-57-8	5039-78-1	4286-23-1	102-81-8
140-66-9	7756-94-7	526-73-8	100-69-6	80-09-1	96-76-4	6505-28-8	7803-57-8	620-92-8
538-75-0	26967-76-0	585-07-9	103-83-3	88-19-7	102-06-7	6731-36-8	12033-89-5	96-45-7
544-76-3	583-39-1	626-17-5	108-69-0	121-45-9	106-48-9	26471-62-5	26630-87-5	77-90-7
629-62-9	623-26-7	1570-64-5	123-30-8	793-24-8	108-39-4	76-83-5	56539-66-3	96-49-1
4390-04-9	1477-55-0	1843-05-6	1241-94-7	2416-94-6	109-70-6	98-51-1	80-51-3	100-47-0
5460-09-3	56-93-9	2216-69-5	3586-14-9	5707-44-8	110-30-5	108-73-6	95-32-9	107-95-9
86-87-3	87-02-5	3319-31-1	101-83-7	38640-62-9	123-07-9	5124-25-4	97-39-2	108-87-2
95-64-7	87-84-3	3648-21-3	127-68-4	51-28-5	591-27-5	6099-57-6	97-99-4	118-75-2
100-61-8	88-44-8	25154-52-3	130-13-2	75-59-2	599-64-4	79-27-6	99-94-5	121-60-8
103-69-5	95-63-6	78-51-3	135-51-3	80-43-3	620-17-7	101-72-4	100-74-3	134-62-3
141-02-6	141-17-3	461-72-3	517-23-7	1314-98-3	2580-78-1	3710-84-7	4435-53-4	5468-75-7
9014-90-8	9016-45-9	20679-58-7	25791-96-2	40766-31-2	112-18-5	29836-26-8	118-91-2	95-68-1

combined, arranged in order and are given in Table 5. The smallest CV was 0.74% for Na and next in order were Na, Cl, SG-urine, MCHC, Ca, MCV, MCH, HGB, brain weight, HCT, Alb, TP and RBC (up to 4% CV). The CV was between 4 and 10% for PT, lymph, K, lungs weight, Fib, testes weight, A/G ratio, IP, APTT, submaxillary gland weight, heart weight, kidneys weight, epididymis weight, PLT, liver weight and CRN. For Glu, BW, FC, adrenals weight, pituitary weight, BUN, Bili, spleen weight, ASAT, PL, ovaries weight, ALAT, thyroid weight, Cho, thymus weight and RET the CV was between 10-20%. For ALP, prostate gland weight, WC, ChE, WBC, OP-urine, LDH, uterus weight, methemoglobin and â-GTP the CV varied between 20-30%, whereas for TG, UV, CPK and Neut-seg it varied between 30-40%.

The parameters with CV similar to body weight (7.09%) were PT, Fib, lungs weight, testes weight and submaxillary glands weight. Electrolytes showed smaller CVs. On the other hand, enzymes, urine volume, Neut-seg and methemoglobin showed larger CVs.

Classification of CV of quantitative values by cluster analysis

Mean, S.D., 95% confidence limits to mean (upper and lower), median, and maximum and minimum values for the 59 parameters of males and females were combined and cluster analysis was carried out using these parameters. The results of cluster analysis are shown in Table 6 and Fig. 1. There were 11 clusters. In cluster 1, 14 parameters were classified, whereas the number of parameters classified in clusters 2, 3, 4 and 5 were 12, 14, 6 and 7, respectively. In clusters 6-11, one parameter each having larger CV was classified.

Changes in CV of parameters with regard to the test facility

Several factors, like methodology employed, equipment used, expertise of personnel, laboratory environment, etc. to quantitatively determine a parameter in laboratory animals may differ from one test facility to the other. An analysis was carried out to understand the influence of the above factors on CVs of few selected parameters among the test facilities which carried out the 28 day repeated dose toxicity studies examined in the present investigation. The parameters selected and rationale for their selection is given in Table 7. Eight test facilities, viz., A1, B2, C3, D4, E5, F6, G7 and H8, which conducted 10 or more than 10 studies, were selected for the analysis. Differences among the facilities were analyzed by ANOVA. The parameter with minimum CV was considered as control. Comparison with control was done by a one-sided Student's or Welch's *t*-test. Test for homogeneity of variance (*F*-test) was conducted before the *t*-tests. Changes in CV of quantitative values according to the test facility are shown in Table 8.

Body weight: ANOVA did not indicate significant difference among the eight test facilities. However, the minimum CV value of F6 was significantly different from H8, E5, A1 and C3.

Feed consumption, Urine volume and Lymphocyte: ANOVA revealed a significant difference among the groups. The value of these parameters with the minimum

Table 3. Sex difference in CV (%) for each parameter

Parameter	Male					Female					Sex diff., *P*
	Mean ± S.D. (*N*)	95% confidence limit of mean	Median	Min.	Max.	Mean ± S.D. (*N*)	95% confidence limit of mean	Median	Min.	Max.	
BW	7.03 ± 1.77 (151)	6.75–7.32	6.91	3.22	12.8	7.15 ± 1.71 (151)	6.87–7.42	8.05	2.5	13.1	NS
FC	10.1 ± 2.90 (150)	9.66–10.5	10.2	1.56	20.0	12.3 ± 4.54 (150)	11.5–13.0	12.4	2.53	27.7	$P < 0.05$
WC	21.9 ± 9.18 (42)	15.0–24.7	20.1	8.33	39.6	24.6 ± 13.5 (42)	20.4–28.9	21.6	4.81	83.5	NS
UV	35.8 ± 13.7 (107)	33.2–38.4	34.6	10.8	81.8	39.5 ± 14.6 (109)	36.7–42.2	37.5	9.52	83.5	NS
SG-urine	1.45 ± 1.43 (82)	1.13–1.76	1.23	0.39	13.5	1.54 ± 1.12 (84)	1.29–1.78	1.43	0.45	10.4	NS
OP-urine	26.6 ± 9.01 (24)	22.8–30.4	25.8	8.87	46.3	26.5 ± 8.35 (24)	23.0–30.1	25.4	9.49	41.8	NS
HCT	3.48 ± 1.50 (150)	3.24–3.72	3.30	1.06	9.52	3.68 ± 1.51 (150)	3.44–3.93	3.42	0.64	9.35	NS
HGB	3.57 ± 2.37 (150)	3.19–3.96	3.24	0.64	20.8	3.46 ± 1.40 (150)	2.24–3.69	3.34	0.71	8.85	NS
RBC	4.16 ± 1.69 (150)	3.89–4.43	3.89	1.52	12.3	4.16 ± 1.48 (150)	3.92–4.40	4.05	1.1	8.98	NS
MCV	2.77 ± 1.13 (149)	2.59–2.95	2.73	0.44	6.66	2.55 ± 0.88 (149)	2.41–2.69	2.37	0.80	7.76	NS
MCH	2.89 ± 1.47 (149)	2.74–3.21	2.90	0.93	15.2	2.72 ± 0.94 (149)	2.57–2.87	2.61	0.50	5.39	NS
MCHC	1.62 ± 1.37 (149)	1.40–1.83	1.46	0.29	15.3	1.64 ± 1.13 (149)	1.46–1.82	1.44	0.29	10.2	NS
RET	17.7 ± 9.07 (119)	16.1–19.4	15.3	4.30	65.3	22.7 ± 11.4 (119)	20.6–24.7	20.8	1.22	72.2	$P < 0.05$
PLT	8.95 ± 3.58 (150)	8.38–9.53	8.31	1.94	28.3	9.64 ± 4.74 (150)	8.88–10.3	9.04	2.04	34.5	NS
WBC	24.6 ± 8.78 (150)	23.2–26.1	23.6	6.92	50.0	26.8 ± 8.04 (150)	25.5–28.1	26.4	6.38	50.0	$P < 0.05$
Neut-seg	39.8 ± 17.2 (150)	37.1–42.6	38.2	7.69	116	42.7 ± 17.1 (150)	39.9–45.4	41.5	0.00	95.0	NS
Lymph	4.88 ± 2.46 (150)	4.48–5.27	4.44	1.11	15.7	5.38 ± 3.17 (150)	4.87–5.89	4.76	1.07	22.5	NS
PT	8.63 ± 6.95 (150)	7.52–9.75	6.15	1.43	6.15	4.00 ± 2.20 (150)	3.65–4.36	3.55	0.57	16.7	$P < 0.05$
APTT	7.87 ± 3.07 (150)	7.38–8.36	7.40	2.51	17.6	7.51 ± 3.68 (150)	6.92–8.10	7.33	1.31	24.6	NS
Fib	6.40 ± 2.31 (37)	5.63–7.17	6.19	1.86	12.0	7.56 ± 2.94 (37)	6.58–8.55	7.32	2.27	13.6	NS
Methemoglobin	33.6 ± 20.2 (6)	12.3–54.9	34.3	12.7	56.0	34.9 ± 21.8 (6)	12.0–57.9	29.0	11.1	6.33	NS
BUN	13.3 ± 5.30 (150)	12.4–14.1	12.4	0.00	37.5	14.4 ± 6.25 (150)	13.4–15.4	13.3	4.54	13.7	NS
CRN	10.8 ± 6.26 (150)	9.83–11.8	10.6	0.00	10.6	10.9 ± 6.66 (150)	9.90–12.0	9.81	0.00	9.81	NS
Cho	16.8 ± 7.27 (151)	15.6–17.9	15.7	2.56	45.0	18.6 ± 7.55 (151)	17.4–19.8	18.0	6.67	53.3	$P < 0.05$
Bili	16.9 ± 20.8 (118)	13.3–20.6	11.7	0.00	100	16.0 ± 15.1 (118)	13.2–18.7	14.2	0.00	100	NS
TP	3.80 ± 1.98 (151)	3.48–4.12	3.69	0.00	20.7	4.26 ± 1.60 (151)	4.00–4.51	3.92	0.90	11.1	$P < 0.05$
Alb	3.70 ± 1.66 (151)	3.43–3.96	3.32	0.00	9.37	5.11 ± 2.40 (151)	4.72–5.49	5.06	0.00	13.5	$P < 0.05$
A/G	7.32 ± 3.19 (149)	6.81–7.83	7.29	1.06	19.1	8.26 ± 4.71 (149)	7.50–9.02	7.20	2.16	7.20	$P < 0.05$
Glu	10.2 ± 4.00 (150)	9.62–10.9	10.2	2.93	24.6	9.97 ± 3.62 (150)	9.39–10.5	10.1	2.58	18.2	NS
TG	34.8 ± 13.4 (151)	32.6–36.9	33.4	9.75	75.9	34.0 ± 17.1 (151)	31.3–36.7	32.3	7.40	96.4	NS
PL	12.7 ± 4.57 (25)	10.9–14.6	13.0	4.39	25.6	15.1 ± 5.95 (25)	12.6–17.6	15.2	6.61	26.3	NS
AST	13.4 ± 6.38 (151)	12.4–14.4	12.7	2.08	34.3	14.0 ± 6.52 (151)	12.9–15.0	13.1	4.34	42.1	NS
ALT	15.7 ± 6.65 (151)	14.6–16.7	15.3	3.57	40.7	16.8 ± 8.63 (151)	15.4–18.2	15.1	1.50	61.2	NS
ALP	18.8 ± 7.12 (150)	17.7–19.9	18.3	6.46	46.8	22.5 ± 7.31 (150)	21.3–23.6	22.2	6.68	45.1	$P < 0.05$
LDH	31.3 ± 15.8 (52)	26.9–35.7	26.1	10.3	78.4	27.7 ± 12.9 (52)	24.1–31.3	24.8	5.55	70.8	NS
γ-GTP	41.1 ± 48.2 (138)	33.1–49.2	27.9	0.00	210	51.4 ± 60.8 (142)	41.4–61.4	33.1	0.00	318	NS
CPK	48.3 (2)					36.3 (2)					
ChE	21.9 ± 11.3 (27)	17.4–26.3	22.2	5.50	51.8	26.4 ± 11.7 (27)	21.8–31.1	24.6	9.16	57.5	NS
Na	0.73 ± 0.30 (149)	0.68–0.77	0.69	0.06	1.99	0.76 ± 0.32 (149)	0.71–0.81	0.70	0.13	1.70	NS
K	6.06 ± 4.99 (149)	5.25–6.86	5.02	1.03	41.0	6.47 ± 5.74 (149)	5.55–7.39	5.57	0.00	50.6	NS
Cl	1.34 ± 0.60 (149)	1.24–1.44	1.21	0.37	4.68	1.30 ± 0.65 (149)	1.20–1.40	1.19	0.37	6.03	NS
Ca	2.70 ± 1.14 (149)	2.51–2.88	2.38	0.61	8.79	2.73 ± 1.12 (149)	2.55–2.91	2.47	0.86	7.77	NS
IP	6.33 ± 3.08 (149)	5.83–6.82	5.66	1.16	22.7	9.24 ± 3.56 (149)	8.67–9.81	8.82	1.36	23.7	$P < 0.05$
Brain weight	3.41 ± 1.40 (150)	3.24–3.69	3.33	0.47	9.26	3.54 ± 1.25 (150)	3.34–3.74	3.35	0.82	7.21	NS
Submaxillary gland weight	9.31 ± 2.39 (3)	3.36–15.2	10.7	6.55	10.7	5.83 ± 2.71 (3)	0.00–12.5	7.31	2.7	7.5	NS
Pituitary weight	12.0 ± 3.92 (42)	10.8–13.2	11.9	4.2	20.6	13.8 ± 4.27 (42)	12.5–15.1	12.4	6.20	24.9	$P < 0.05$
Thyroid weight	16.6 ± 5.14 (51)	15.2–18.1	16.1	5.00	28.4	15.8 ± 5.08 (51)	14.4–17.2	15.9	3.37	15.9	NS
Thymus weight	18.2 ± 5.95 (124)	17.2–19.3	17.2	5.79	46.4	18.1 ± 6.68 (124)	16.9–19.2	17.7	4.72	49.3	NS
Heart weight	8.25 ± 3.43 (108)	7.60–8.90	7.62	2.83	20.6	8.85 ± 4.41 (108)	9.02–9.68	8.17	3.03	8.17	NS
Lungs weight	7.16 ± 4.28 (49)	5.92–8.39	6.65	1.45	31.6	7.16 ± 3.43 (49)	6.17–8.14	6.66	0.99	23.2	NS
Liver weight	9.95 ± 3.53 (150)	9.39–10.5	9.63	2.46	19.4	9.19 ± 3.26 (150)	8.67–9.71	8.75	2.63	18.4	NS
Kidneys weight	8.51 ± 3.69 (150)	7.92–9.10	8.11	1.55	32.4	7.92 ± 2.59 (150)	7.50–8.33	7.73	1.91	15.6	NS
Spleen weight	13.9 ± 5.80 (133)	12.9–14.1	13.0	2.73	33.3	13.3 ± 4.89 (150)	12.4–14.1	12.7	0.16	28.9	NS
Adrenals weight	13.1 ± 4.75 (150)	12.3–13.8	12.9	3.12	24.7	11.8 ± 4.81 (150)	11.0–12.6	11.5	2.66	27.2	$P < 0.05$
Testes v	7.44 ± 4.16 (150)	6.77–8.10	6.93	1.43	35.4						
Epididymis weight	8.88 ± 4.33 (88)	7.96–9.80	8.17	1.88	28.2						
Prostate weight	8.88 ± 4.33 (88)	7.96–9.80	8.17	1.88	28.2						
Ovaries weight						14.7 ± 4.93 (145)	13.9–15.5	14.4	3.40	25.7	
Uterus weight						27.6 ± 13.7 (16)	20.3–34.9	26.3	6.21	55.5	

NS, not significant difference. Vide Table 1 for abbreviations.

Table 4. Parameters that showed changes in CV with regard to sex

Sex	Parameter that increased compared to the other sex
Male	PT, Adrenals
Female	FC, RET, WBC, Cho, TP, Alb, A/G, ALP, IP, Pituitary

CVs were different from those respective parameters of 7 test facilities.

GOT and Sodium: Differences were observed among the test facilities. Compared to the value of G7, GOT was different in the other test facilities, whereas sodium was different in 5 facilities compared to the value of C3.

Brain, liver and spleen weights: These parameters were not statistically different among the facilities as per ANOVA. However, the value with smallest CV was significantly different in 6 facilities in the case of brain weight, 4 facilities in the case of liver weight and 3 facilities in the case of spleen weight as per t test.

Rank order of test facilities with regard to the parameters evaluated is given in Table 9. The mean CV ranks in the increasing order were D4, F6, A1, H8, G7, C3, E5, and B2.

Next, a cluster analysis was performed using the data given in Table 9 to understand the features of each test facility. Test facility G7 was excluded because data on urine volume were not available in this test facility.

The clusters were grossly divided into two bunches (Fig. 2). One bunch consisted of D4 and F6, with several parameters having small CV. The second bunch included five test facilities that had two sub-bunches, A1, H8, and E5 in the first bunch and C3 and B2 in the second bunch. The distance (difference) between D4 and B2 was the largest.

Significant difference detection pattern when the difference of mean between two groups is set constant and the CV changed

Changes in body weight are considered as the most important index in toxicity studies (MHLW, 2009). In the present study, the power of detection pattern of statistically significant difference as result of change in CV was assessed using the body weight values. In a study (CAS No. 26471-62-5), the value of 10 males in the control group was 336 g (100%) at 4 weeks after the test substance administration. A normal distribution was assumed for these rats with a CV of 7.7%. The mean value of the dosage group was set as constant (312 g, 92.8%). The CV of both the groups was changed in the range of 2 to 45%. These data were analyzed by a two-sided Student's *t*-test.

A significant difference ($P < 0.05$) was observed if the

Table 5. Rank order of parameters with regard to CV (%)

Rank order	Parameter	Mean ± S.D. (Number of study*)	95% confidence limit in mean	Median
1	Na	0.74 ± 0.31 (298)	0.71–0.78	0.69
2	Cl	1.32 ± 0.63 (298)	1.25–1.39	1.25
3	SG/urine	1.49 ± 1.28 (166)	1.30–1.69	1.33
4	MCHC	1.63 ± 1.26 (298)	1.49–1.77	1.44
5	MCV	2.66 ± 1.02 (298)	2.55–2.78	2.54
6	Ca	2.72 ± 1.13 (298)	2.59–2.84	2.46
7	MCH	2.85 ± 1.24 (298)	2.71–2.99	2.87
8	Brain	3.51 ± 1.32 (300)	3.36–3.66	3.35
9	HGB	3.52 ± 1.94 (300)	3.30–3.74	3.27
10	HCT	3.58 ± 1.51 (300)	3.41–3.75	3.37
11	TP	4.03 ± 1.82 (302)	3.82–4.23	3.85
12	RBC	4.16 ± 1.59 (300)	3.98–4.34	3.97
13	Alb	4.40 ± 2.18 (302)	4.16–4.65	3.73
14	Lymph	5.13 ± 2.84 (300)	4.81–5.45	4.55
15	K	6.26 ± 5.38 (298)	5.65–6.87	5.48
16	PT	6.32 ± 5.64 (300)	5.68–6.96	4.27
17	Fib	6.98 ± 2.69 (74)	6.36–7.61	6.88
18	Lungs	7.16 ± 3.86 (98)	6.38–7.93	6.65
19	BW	7.09 ± 1.74 (302)	10.7–11.6	10.7
20	Testes	7.44 ± 4.16 (150)	6.77–8.10	6.93
21	Submaxillary gland	7.57 ± 2.98 (6)	4.48–10.7	7.40
22	APTT	7.69 ± 3.39 (300)	7.31–8.07	7.33
23	IP	7.78 ± 3.63 (298)	7.37–8.20	7.31
24	A/G	7.79 ± 4.05 (298)	7.33–8.25	7.24
25	Kidneys	8.22 ± 3.20 (300)	7.85–8.58	7.89
26	Heart	8.55 ± 3.95 (216)	8.02–9.08	7.87
27	Epididymis	8.88 ± 4.33 (88)	7.96–9.80	8.17
28	PLT	9.28 ± 4.21 (300)	8.82–9.77	8.70
29	Liver	9.57 ± 3.41 (300)	9.18–9.96	9.19
30	Glu	10.1 ± 3.81 (300)	9.69–10.5	10.2
31	CRN	10.9 ± 6.45 (300)	10.1–11.6	10.0
32	FC	11.2 ± 3.96 (300)	10.7–11.6	10.7
33	Adrenals	12.4 ± 4.81 (300)	11.9–13.0	12.1
34	Pituitary	12.9 ± 4.17 (84)	12.0–13.8	12.2
35	Spleen	13.6 ± 5.36 (266)	12.9–14.2	12.8
36	ASAT	13.7 ± 6.44 (302)	13.0–14.4	12.8
37	BUN	13.8 ± 5.81 (300)	13.2–14.5	12.6
38	PL	13.9 ± 5.38 (50)	12.4–15.4	13.3
39	Ovaries	14.7 ± 4.90 (145)	13.9–15.5	14.4
40	Thyroid	16.2 ± 5.10 (102)	15.2–17.2	16.1
41	ALAT	16.2 ± 7.71 (302)	15.4–17.1	15.2
42	Bili	16.4 ± 17.8 (236)	14.2–18.7	12.6
43	Cho	17.7 ± 7.46 (302)	16.8–18.5	16.8
44	Thymus	18.1 ± 6.31 (248)	17.4–18.9	17.6
45	Prostate	18.9 ± 9.74 (7)	9.92–27.9	20.5
46	RET	20.2 ± 10.5 (238)	18.9–21.5	18.4
47	ALP	20.6 ± 7.43 (300)	19.8–21.5	20.2
48	WC	23.3 ± 11.5 (84)	20.7–25.8	21.5
49	ChE	24.2 ± 11.6 (54)	21.0–27.3	23.0
50	WBC	25.7 ± 8.47 (300)	21.8–26.7	25.1
51	OP/urine	26.6 ± 8.59 (48)	24.1–29.1	25.5
52	Uterus	27.6 ± 13.7 (16)	20.3–34.9	26.3
53	LDH	29.5 ± 14.5 (104)	36.7–32.3	25.8
54	Methemoglobin	34.3 ± 20.1 (12)	21.5–47.1	29.0
55	TG	34.4 ± 15.4 (302)	32.7–36.1	33.0
56	UV	37.9 ± 14.2 (216)	35.7–39.6	35.3
57	Neut-seg	41.3 ± 17.1 (300)	39.3–43.2	40.0
58	CPK	42.3 ± 16.3 (4)	16.3–68.2	36.3
59	γ-GTP	46.3 ± 55.1 (280)	39.9–52.8	30.0

* Values given in parentheses are number of observations from both males and females.

difference in body weight mean was about 7% between the groups and the CV was 7-8% (Table 10). A significant difference was observed for the γ-GTP, CPK, neutrophil cell, urine volume, and triglyceride levels which showed a CV of 30% or more and a 30% difference of mean between groups.

Table 6. Classification of CVs of 59 parameters by cluster analysis

Custer No.	Parameter
1	BW, Fib, Submaxillary gland weight, PLT, Epididymis weight, Kidneys weight, Heart weight, APTT, IP, A/G ratio, Lungs weight, Testes weight, PT, K
2	FC, Adrenals weight, Gul, Liver weight, BUN, Spleen weight, ASAT, CRA, PL, Pituitary weight, Thyroid weight, Ovaries weight
3	SG-urine, MCHC, Na, Cl, HCT, Brain weight, RBC, MCV, Ca, MCH, HGB, TP, Alb, Lymph
4	WC, ChE, LDH, Uterus weight, OP-urine, WBC
5	RET, Cho, ALAT, ALP, Thymus weight, Prostate gland weight
6	Bili
7	UV, TG
8	Methemoglobin
9	Neut-seg
10	CPK
11	γ-GTP

DISCUSSION

We calculated CV for 59 quantitative values obtained from the 153 numbers of 28-day repeated-dose toxicity studies carried out in accordance with the CSCL in 12 test facilities. We conclude the following from our findings:

1. Sex difference in CV was observed in 12 out of 59 items including body weight. Larger CV was observed in 10 items of male.

2. The quantitative value with the smallest CV was 0.74% for sodium. Electrolytes and calculated hematology values showed smaller CVs, whereas, enzymes showed larger CVs.

3. The values based on their CVs were classified into 11 clusters, which were grossly divided into 4 to 5 bunches.

4. CVs for liver, brain and spleen weights were similar in all the test facilities studied. However, large differences in body weight and some clinical laboratory tests were observed in few test facilities. Therefore it is most likely that statistically significant differences could be detected for the data with small CVs in some test facilities. Similarly, no statistical significant difference will be shown by the data with large CVs in some test facilities.

5. A significant difference is seen at 5% probability level, if the difference of the mean value between the groups is about 7% and CV 7%-8%.

6. A statistically significant difference can be detect-

Table 7. Influence of laboratory factors on CVs of parameters analysed. List of parameters selected and the rationale for their selection

Parameter	Rationale for selection
Body weight	Toxicity of a chemical is primarily reflected on body weight. Several factors like poor animal husbandry, animal house condition and improper handling of the animals can affect body weight.
Feed consumption	The factors that may affect body weight can affect feed consumption of the animals. Accurate measurement of feed consumption is not an easy task because of spillage. Feed hoppers vary from one laboratory to the other.
Urine volume	Metabolic cages are used for urine collection. These cages differ from one testing facility to the other.
Lymphocyte	Lymphocyte count usually done under a microscope, varies among the technicians who perform the count.
GOT	Variation in GOT estimation may occur with regard to the analytical reagents, methodology, instrument calibration and the nature of the plasma/serum sample (hemolysed samples may show erratic GOT values).
Sodium	Fluctuation in sodium is the least compared to other clinical chemistry parameters.
Brain weight	Absolute weight of the brain can be determined accurately in all the test facilities
Liver weight	Onslaught of toxicity effect of a chemical is reflected on liver and variation in absolute weight of this organ is usually seen.
Spleen weight	Most of the test facilities determine absolute weight of the spleen accurately.
Other parameters	Instruments used to determine other hematology and blood chemistry parameters are not different from those used to determine above parameters. Enzymes that show very larger variations are not also not included. Erythrocyte indices *etc.* have a lot of calculation values. However, these parameters were seemed no large changes.

Table 8. Changes in CV of quantitative values according to test facilities

Parameter	Test facility	Mean ± S.D. (%)	No. of study (male + female)	*P* ANOVA	*P* *t*-test
Body weight	F6	6.36 ± 1.81 (100)	20	0.485	vs. F6
	D4	6.81 ± 1.54 (107)	40		0.156
	G7	6.98 ± 1.45 (109)	36		0.084
	B2	7.08 ± 1.83 (111)	28		0.091
	H8	7.17 ± 1.50 (112)	30		0.045
	E5	7.18 ± 1.27 (112)	26		0.038
	A1	7.31 ± 1.87 (114)	48		0.028
	C3	7.32 ± 2.14 (115)	50		0.040
Feed consumption	D4	7.00 ± 2.11 (100)	40	0.000	vs. D4
	B2	10.6 ± 4.03 (152)	28		< 0.01
	A1	10.7 ± 3.04 (153)	48		< 0.01
	G7	11.4 ± 3.25 (163)	34		< 0.01
	C3	12.2 ± 3.55 (174)	49		< 0.01
	H8	12.6 ± 3.74 (180)	30		< 0.01
	E5	12.8 ± 4.92 (183)	26		< 0.01
	F6	13.2 ± 4.16 (189)	20		< 0.01
Urine volume	F6	1.43 ± 0.40 (100)	20	0.000	vs. F6
	D4	19.3 ± 17.2 (1349)	14		< 0.01
	C3	29.0 ± 10.8 (2027)	36		< 0.01
	H8	35.5 ± 12.0 (2482)	28		< 0.01
	A1	35.9 ± 15.6 (2510)	46		< 0.01
	E5	42.4 ± 16.5 (2965)	26		< 0.01
	B2	44.4 ± 13.8 (3104)	28		< 0.01
Lymphocyte	E5	3.10 ± 0.99 (100)	26	0.000	vs. E5
	H8	4.32 ± 1.97 (139)	28		< 0.01
	A1	4.35 ± 2.65 (140)	48		< 0.01
	D4	4.44 ± 1.72 (143)	40		< 0.01
	H8	5.49 ± 1.94 (177)	36		< 0.01
	C3	6.16 ± 3.52 (198)	50		< 0.01
	B2	6.49 ± 3.05 (209)	28		< 0.01
	F6	7.38 ± 4.04 (238)	20		< 0.01
GOT	G7	10.1 ± 4.36 (100)	36	0.001	vs. G7
	H8	12.8 ± 5.78 (126)	30		0.022
	C3	12.9 ± 8.29 (127)	50		0.026
	A1	12.9 ± 6.39 (127)	48		0.012
	F6	14.9 ± 4.95 (147)	20		< 0.01
(Continued)					
	D4	15.3 ± 4.25 (151)	40		< 0.01
	E5	15.8 ± 7.08 (156)	26		< 0.01
	B2	16.0 ± 6.41 (158)	28		< 0.01
Sodium	C3	0.61 ± 0.26 (100)	50	0.000	vs. C3
	F6	0.62 ± 0.21 (101)	20		0.452
	D4	0.65 ± 0.19 (106)	40		0.184
	A1	0.72 ± 0.29 (118)	48		0.023
	B2	0.79 ± 0.25 (129)	28		< 0.01
	H8	0.81 ± 0.28 (132)	30		< 0.01
	E5	0.82 ± 0.43 (134)	26		0.015
	G7	0.89 ± 0.29 (145)	34		< 0.01
Brain weight	F6	2.79 ± 0.87 (100)	20	0.081	vs. F6
	D4	3.22 ± 1.12 (115)	38		0.074
	A1	3.40 ± 1.20 (121)	48		0.022
	H8	3.45 ± 1.09 (123)	30		0.014
	G7	3.56 ± 1.49 (127)	36		< 0.01
	E5	3.58 ± 1.17 (128)	26		< 0.01
	C3	3.79 ± 1.33 (135)	50		< 0.01
	B2	3.83 ± 1.72 (137)	28		< 0.01
Liver weight	F6	8.17 ± 2.41 (100)	20	0.726	vs. F6
	D4	9.35 ± 3.67 (115)	38		0.102
	A1	9.46 ± 3.88 (121)	48		0.052
	H8	9.53 ± 2.41 (123)	30		0.028
	E5	9.61 ± 3.31 (127)	26		0.054
	B2	9.69 ± 3.19 (128)	28		0.040
	G7	9.70 ± 2.74 (135)	36		0.021
	C3	10.0 ± 4.05 (137)	50		0.010
Spleen weight	F6	11.4 ± 4.49 (100)	20	0.549	vs. F6
	D4	12.6 ± 3.60 (110)	30		0.212
	A1	13.1 ± 5.63 (114)	48		0.129
	G7	13.6 ± 5.12 (119)	28		0.072
	E5	13.9 ± 6.06 (121)	20		0.081
	H8	13.9 ± 4.47 (121)	30		0.032
	B2	14.1 ± 5.44 (123)	28		0.042
	C3	14.3 ± 6.40 (125)	38		0.042

Table 9. Rank order of test facilities with regard to the parameters evaluated. Ranking is done from smallest to largest CV

Parameter	Test facility							
	D4	F6	A1	H8	G7	C3	E5	B2
Body weight	2	1	7	5	3	8	6	4
Feed consumption	1	8	3	6	4	5	7	2
Urine volume	2	1	5	4		3	6	7
Lymphocyte	4	8	3	2	5	6	1	7
GOT	6	5	4	2	1	3	7	8
Sodium	3	2	4	6	8	1	7	5
Brain weight	2	1	3	4	5	7	6	8
Liver weight	2	1	3	4	7	8	5	6
Spleen weight	2	1	3	6	4	8	5	7
Mean rank	2.6	3.1	3.8	4.3	4.6	5.4	6.6	7.1

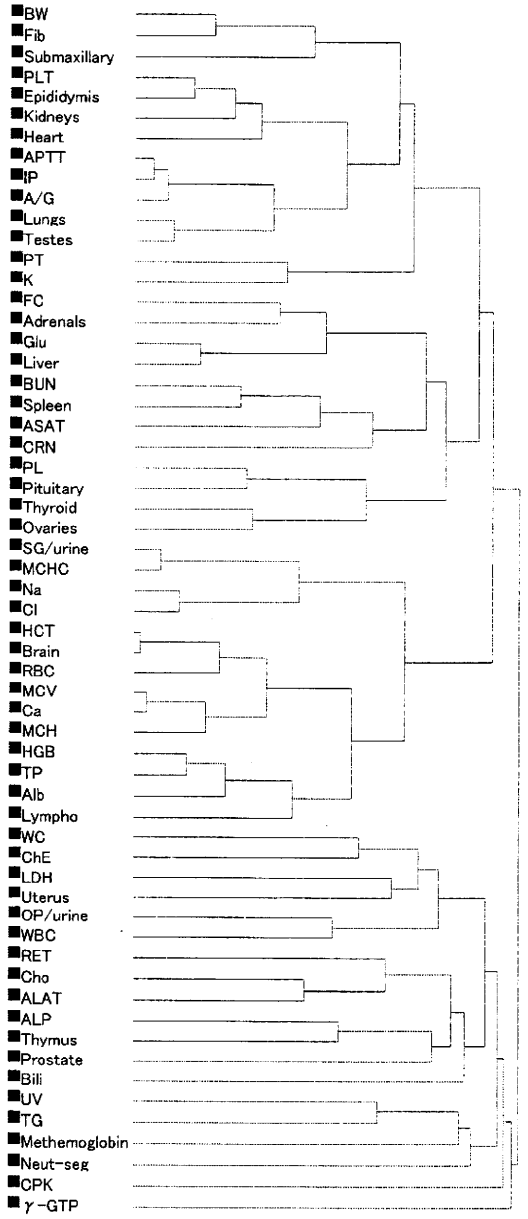

Fig. 1. Classification of CV of quantitative value by cluster analysis.

ed for parameters with extremely small CVs even for a smaller difference in mean values.

Information on the influence of CV in determining significant difference of quantitative parameters obtained from animal toxicity studies is scarce. Aoyama (2005) suggested that when the number of animals is adjust-

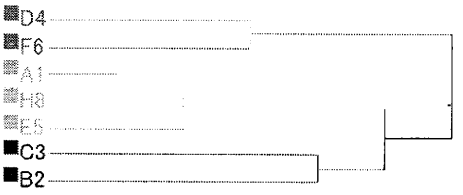

Fig. 2. Classification of CV of test facilities by cluster analysis.

Table 10. Significant difference power pattern when the body weight difference between two groups is set to a constant and CV is changed

Control group: 336 g vs. dosed group: 312 g	
Coefficient of variation (%) of two groups	*P* value
2	0.000
3	0.000
4	0.000
5	0.003
6	0.012
7	0.029
8	0.053
9	0.082
10	0.115
12	0.184
14	0.252
16	0.314
18	0.369
20	0.418
25	0.516
30	0.587
35	0.641
40	0.683
45	0.717

ed, the decentralization of data, like body weight and the organ weight, becomes comparatively smaller, and a CV of about 10% is obtained. CV for blood levels of various hormones, even data in the control group are large. Often, the standard deviation exceeds the mean value by more than 50% for these parameters. Present study also reveals similar findings. CVs have greater influence in determining the significant difference of a parameter in repeated dose toxicity studies and the CVs vary considerably for certain parameters in different test facilities. Thus priority should be given to a judgment of toxicological effects, not to a statistically significant difference when such a dif-

ference is smaller than that based on CV of body weight measurements.

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Prenatal developmental toxicity of gavage or feeding doses of 2-*sec*-butyl-4,6-dinitrophenol in rats

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ABSTRACT

This study evaluated the prenatal developmental toxicity of the pesticide 2-*sec*-butyl-4,6-dinitrophenol (dinoseb). Pregnant rats were given dinoseb by gavage at 0, 8.0 or 10 mg/kg bw/day on days 6–15 of gestation, or in the diet at 0, 120 or 200 ppm (0, 6.52 or 8.50 mg/kg bw/day) on days 6–16 of gestation, and litters were evaluated on day 20 of gestation. Maternal toxicity was observed as evidenced by significantly decreased body weight gain and reduced food consumption during the administration period in all the dinoseb-treated groups, and two dams died at 10 mg/kg bw/day. Significantly lower fetal weights and delayed skeletal ossification was observed in the dinoseb-treated groups except for the group fed dinoseb at 120 ppm. The teratogenic potential of the gavage dose of dinoseb was confirmed as evidenced by increased incidences of fetuses with external and skeletal malformations at 10 mg/kg bw/day. The incidence of fetuses with microphthalmia was significantly increased at this dose. On the other hand, feeding doses of dinoseb up to 200 ppm did not induce teratogenicity in this study. These data indicate that dinoseb is teratogenic at maternally toxic doses, but the exposure range of dinoseb at which malformations occur seems to be narrow.

1. Introduction

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7) was approved for sale in the US in 1948 as a nitrophenolic herbicide in soybeans, vegetables, fruits, nuts, citrus and other field crops for the selective control of grass and broadleaf weeds [1,2]. Dinoseb is also used as an insecticide for grapes and as a seed crop-drying agent [2]. Dinoseb is one of the chemicals permitted on the market on the basis of safety tests conducted by Industrial Bio-Test Laboratory, a concern later found to have submitted many flawed and even fraudulent reports on its procedures and results [3]. Subsequently, several studies showed that dinoseb has the potential to produce developmental toxicity including teratogenicity in rats, mice and rabbits [4–7].

Dinoseb as a pesticide was banned in the US in 1986 and the EU in 1991 owing to the potential risk of adverse health effects in humans [2,8], but dinoseb and its salts are still widely used as other agricultural products [9]. Dinoseb is a high volume chemical with production or importation exceeding 1000 tons/year in Organisation for Economic Co-operation and Development (OECD) member countries [10]. Dinoseb as a pesticide is also banned in Japan but its import is permitted [9], and the volumes of dinoseb imported into Japan were estimated to be 827 tons in fiscal year 2007 and 615 tons in fiscal year 2008 [11].

Exposure to dinoseb may occur by direct contact, ingestion or inhalation by users and producers. Indirect exposure to dinoseb via the environment is also anticipated. The microbial breakdown of dinoseb has been demonstrated in soils, but dinoseb persists for about 2–4 weeks after application [12]. A soil persistence of 24–42 months was also observed in potato fields in Canada [13]. It has been reported that dinoseb was detected in water supplies in Canada and the US, and dinoseb residues were found in a cotton meal sample [12].

In previous review papers, we showed that dinoseb possesses testicular toxicity [14] and developmental toxicity [15] in experimental animals. We reported the results of a combined repeated dose toxicity study with a reproduction/developmental toxicity screening test, in which Crj:CD(SD)IGS rats were administered dinoseb by gavage at 0, 0.78, 2.33 or 7.0 mg/kg bw/day. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. The numbers of dams that delivered their pups and dams with live pups at delivery were significantly reduced at this dose. Because only two females in the highest dose group delivered their pups, the developmental toxicity of dinoseb was not fully assessed in this study [16], but gross internal and external examinations revealed no significant differences in the incidence of pups with malformations. In a previous review

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[15], we concluded that teratogenic susceptibility to dinoseb was greater in rabbits than in rats and mice. Several studies failed to demonstrate the teratogenicity of dinoseb in rats [16–18], but we consider that the teratogenic potential of dinoseb in rats is unclear for various reasons. The feeding dose of dinoseb to rats on days 5–14 of gestation increased the incidence of fetuses with microphthalmia at 200 ppm (15 mg/kg bw/day), but this was not observed by gavage dosing at 15 mg/kg bw/day [4]. The incidence of fetuses with microphthalmia also increased when dinoseb was given in a certain composition of diet (protein 21%, fat 4.8%, fiber 4.2%, ash 8.5% and N-free extractives 61.5%) at 200 ppm or by gavage with the same diet at 15 mg/kg bw/day on days 5–13 of gestation, but this effect was not observed when a different diet (protein 21%, fat 3.5%, fiber 6.5%, ash 7.5% and N-free extractives 61.5%) was fed to pregnant rats [19]. As described above, adequate experimental conditions for the production of fetal malformations by the administration of dinoseb to pregnant rats remain unknown. Therefore, the present study was conducted to clarify the experimental conditions that produce fetal malformations when dinoseb is given to pregnant rats.

2. Materials and methods

2.1. Animals

This study was performed in 2008 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). The study was conducted in accordance with "Act on Welfare and Management of Animals" [Act No. 105, October 1, 1973, revised December 22, 1999, Revised Act No. 221; revised June 22, 2005, Revised Act No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006] and "Basic Policies for the Conduct of Animal Experiments in Research Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006].

Male and female SPF Crl:CD (SD) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, and historical control data are available. Rats at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and were quarantined and acclimated to the laboratory for 3 weeks prior to the start of the experiment. Male and female rats found to be in good health were selected for use. The animals were reared on a sterilized basal diet (CRF-1; protein 22%, fat 5.7%, fiber 2.9%, ash 6.3% and N-free extractives 55.3%; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. After the quarantine and acclimation, male and female rats were housed individually except during the mating period. Daily vaginal lavage samples of each female were evaluated for estrous cyclicity. Females showing proestrous vaginal smears were paired overnight with males on a 1:1 basis. The females were examined next morning for vaginal plugs and sperm in vaginal smears. The day on which the presence of sperm in the vaginal smear and/or a vaginal plug was detected was designated day 0 of gestation. The mated females were separated into three groups to equalize the female body weights in the gavage dose groups or the feeding dose groups. The animals were maintained in an air-conditioned room at a room temperature of 22 ± 3 °C, a relative humidity of 50 ± 20%, a 12-hour light/dark cycle and 10–15 air changes per hour.

2.2. Chemicals and dosing

Dinoseb was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The dinoseb (Lot No. 010608LB-AC) used in this study was 100% pure, and was stored under refrigeration prior to use. The purity and stability of the chemical were verified by analysis before the study. Dose levels were determined on the basis of the results of studies by Giavini et al. [4,19]. At these doses, maternal and/or developmental toxicity was/were expected to be observed in the dinoseb-treated groups. For the gavage dose groups, 12 females per group were given dinoseb once daily by gastric intubation at 0 (control), 8.0 and 10 mg/kg bw from day 6 to day 15 of gestation. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. The volume of each dose was adjusted to 5 ml/kg body weight on the basis of the latest body weight. The dosing suspensions were prepared once per 7 days, and were stored in the dark and cold conditions before use. For the feeding dose groups, 12 females per group were given dinoseb in the diet from day 6 to day 16 of gestation at 0 (control), 120 and 200 ppm, and were thus expected to consume similar amounts of dinoseb to those in the gavage groups. The control rats were given the basal diet. The diet for the dose groups was prepared more than once every 4 days and was stored at room temperature before use.

2.3. Observations

All female rats were observed for clinical signs of toxicity once a day before and after the administration period, twice a day during the administration period and once on the day of sacrifice. Body weight was recorded once a day during the administration period and on days 0, 18 and 20 of gestation, and body weight gain was calculated. Food consumption was recorded on days 0, 6, 9, 12, 16, 18 and 20 of pregnancy. Rats that died during the administration period were autopsied and grossly examined. The pregnant rats were killed by exsanguination under ether anesthesia on day 20 of gestation. The organs and tissues were grossly examined. The ovary and uterus were removed from the maternal body, and gravid uterine weight was recorded. The numbers of corpora lutea, implantation sites, live and dead fetuses and resorptions were recorded. The placenta was removed and weighed. The live fetuses were removed from the uterus, sexed, weighed and inspected for external malformations and malformations within the oral cavity. The live fetuses were put down using an intraperitoneal injection of a sodium pentobarbital solution, and the eyes of the fetuses were examined after the removal of the skin of the head. Then, approximately one-half of the live fetuses in each litter were fixed in Bouin's solution for the examination of internal anomalies. Their heads were subjected to free-hand razor-blade sectioning [20], and the thoracic areas were subjected to microdissection [21]. The remaining live fetuses in each litter were fixed in 70% ethanol, stained with Alizarin red S and alician blue, and examined for skeletal anomalies.

2.4. Data analysis

Maternal body weight gain, gravid uterine weight, food consumption, number of corpora lutea, number of implantations, number of live fetuses, number of dead or resorbed embryos/fetuses, fetal weight, placental weight and degree of ossification were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the group variances were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. Fetal weight, placental weight and degree of ossification were analyzed using the litter as a unit. Implantation index, viability index of fetuses, total incidence of dead or resorbed embryos/fetuses, incidence of fetuses with malformations or variations and sex ratio of live fetuses were analyzed by Wilcoxon's rank sum test using the litter as a unit. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the maternal findings in rats given dinoseb by gavage or in the diet. At 10 mg/kg bw/day, death occurred on days 10 and 13 of gestation in one female each. No changes in clinical findings were observed in the feeding dose and the other gavage dose groups. Maternal body weight gain was significantly decreased on days 6–16 and 0–20 of gestation in all the dinoseb-treated groups and significantly increased on days 16–20 of gestation at 200 ppm. Food consumption was significantly decreased in the gavage dose groups on days 6–9 and 9–12 of gestation at both 8.0 and 10 mg/kg bw/day. After the administration period, food consumption was increased at 8.0 and 10 mg/kg bw/day, and a significant increase was observed on days 16–18 of gestation at 8.0 mg/kg bw/day. Similarly, food consumption was significantly decreased during the administration period in the feeding dose groups at 120 and 200 ppm, and it was significantly increased at 200 ppm after the administration period. The average intakes of dinoseb at 120 and 200 ppm were 6.52 and 8.50 mg/kg bw/day, respectively. At autopsy, dilatation of renal pelvis was observed in only one rat at 8.0 mg/kg bw/day, which was suggested to be spontaneous occurrence. Two animals that died during the administration period at 10 mg/kg bw/day showed abnormal findings such as discoloration of the lung and spleen, atrophy of the thymus, thickening limiting ridge of the stomach and/or dark red patch in the glandular stomach. No changes were observed in the feeding dose groups at autopsy (data not shown).

Table 2 presents the reproductive findings in rats given dinoseb by gavage or in the diet. Body weights of live fetuses were decreased in the dinoseb-treated groups, and significantly decreased body weights were noted in male fetuses at 10 mg/kg bw/day, in

Table 1
Maternal findings in rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
No. of pregnant rats	12	12	12	12	12	12
Initial body weight	263.3 ± 10.4[a]	263.7 ± 10.0	262.8 ± 11.0	299.1 ± 21.8	298.9 ± 22.9	298.9 ± 25.9
No. of females showing clinical signs of toxicity						
Death	0	0	2	0	0	0
Body weight gain during pregnancy (g)						
Days 0–6	42.3 ± 7.9	36.8 ± 5.7	39.6 ± 5.4	27.1 ± 5.8	26.3 ± 7.4	24.2 ± 6.4
Days 6–16	59.3 ± 9.5	31.3 ± 7.4**	25.6 ± 8.2** (10)	48.7 ± 12.9	25.3 ± 5.2**	−11.4 ± 5.8**
Days 16–20	67.1 ± 8.4	70.8 ± 9.8	68.8 ± 9.9 (10)	64.1 ± 9.9	64.3 ± 9.8	81.4 ± 15.1**
Days 0–20	168.8 ± 18.4	138.9 ± 12.4**	133.3 ± 14.7** (10)	139.8 ± 20.1	115.9 ± 14.8**	94.2 ± 19.9**
Food consumption during pregnancy (g/day)						
Days 0–6	23.4 ± 1.8	22.9 ± 1.6	23.3 ± 1.4	21.5 ± 2.1	22.2 ± 2.5	20.4 ± 1.9
Days 6–9	21.0 ± 1.9	17.1 ± 1.4**	16.2 ± 2.4**	21.1 ± 2.3	16.8 ± 0.9**	12.0 ± 1.1**
Days 9–12	22.3 ± 2.2	19.7 ± 1.7*	19.5 ± 2.8** (11)	21.8 ± 4.2	17.2 ± 1.4**	11.7 ± 1.5**
Days 12–16	21.5 ± 2.1	20.5 ± 1.1	22.1 ± 1.9 (10)	22.4 ± 2.4	20.6 ± 3.2	15.6 ± 2.0**
Days 16–18	25.5 ± 2.2	28.2 ± 2.1**	27.6 ± 1.9 (10)	24.0 ± 2.5	25.2 ± 2.6	28.2 ± 2.9**
Days 18–20	26.3 ± 1.5	27.9 ± 2.6	28.0 ± 1.6 (10)	23.1 ± 2.6	24.2 ± 2.6	27.3 ± 2.8**

Values in parentheses are the number of animals examined.
[a] Values are given as the mean ± SD.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

female fetuses at 8.0 and 10 mg/kg bw/day and in both sexes at 200 ppm. Weight of the placenta was significantly decreased at 10 mg/kg bw/day, but it was not affected by the feeding dose of dinoseb. Gravid uterine weight was decreased dose-dependently. No effects were observed in other reproductive parameters.

The summarized results of external and internal examinations of fetuses are shown in Table 3. External malformations were found in 1 out of the 171 fetuses (1 out of 12 litters) at 8.0 mg/kg and 18 out of the 147 fetuses (4 out of the 10 litters) at 10 mg/kg bw/day, and the incidence of fetuses with external malformations was significantly increased at 10 mg/kg bw/day. Among the fetuses at 10 mg/kg bw/day, there were 1 each with cleft palate or filamentous tail, 2 each with runt, anotia, brachymelia or ectrodactyly and 17 fetuses with microphthalmia. The incidence of fetuses with microphthalmia was significantly increased at this dose. No significant differences were found upon external examinations of the feeding dose groups. Runt was observed in one fetus at 8 mg/kg bw/day and each one fetus in two different litters at 10 mg/kg bw/day. In the internal examinations, no significant differences were observed in the gavage and feeding dose groups.

The summarized results of skeletal examinations of the fetuses are presented in Table 4. There were no significant differences between the dinoseb-treated and control groups in the incidence

Table 2
Reproductive findings in rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
No. of litters	12	12	10	12	12	12
No. of corpora lutea per litter	16.3 ± 2.3[a]	16.0 ± 2.2	15.9 ± 1.7	15.5 ± 1.6	15.4 ± 1.0	13.9 ± 2.9
No. of implantations per litter	14.9 ± 3.4	14.8 ± 2.5	15.2 ± 2.2	15.2 ± 1.9	14.4 ± 1.1	13.6 ± 3.0
Implantation index (%)[b]	90.5 ± 14.8	92.6 ± 12.5	95.4 ± 6.7	97.8 ± 4.4	93.6 ± 5.8	97.5 ± 3.7
Dead or resorbed embryos and fetuses						
Early stage[c]	0.4 ± 0.5	0.5 ± 0.5	0.4 ± 0.5	0.8 ± 0.7	0.7 ± 0.8	0.8 ± 1.3
Late stage[d]	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total incidence (%)[e]	2.7 ± 3.4	3.1 ± 3.2	3.4 ± 3.7	5.4 ± 4.4	4.7 ± 5.5	6.6 ± 9.0
No. of live fetuses	14.5 ± 3.4	14.3 ± 2.1	14.7 ± 2.3	14.3 ± 1.7	13.8 ± 1.4	12.8 ± 3.2
Viability index of fetuses (%)[f]	97.3 ± 3.4	96.9 ± 3.2	96.6 ± 3.7	94.6 ± 4.4	95.4 ± 5.5	93.4 ± 9.0
Sex ratio of live fetuses[g]	0.472 ± 0.152	0.472 ± 0.136	0.447 ± 0.163	0.503 ± 0.133	0.506 ± 0.141	0.427 ± 0.152
Body weight of live fetuses (g)						
Male	4.043 ± 0.283	3.792 ± 0.285	3.425 ± 0.279**	4.033 ± 0.293	3.858 ± 0.281	3.620 ± 0.217**
Female	3.873 ± 0.228	3.587 ± 0.221*	3.240 ± 0.315**	3.780 ± 0.288	3.641 ± 0.253	3.399 ± 0.261**
Gravid uterine weight (g)	84.3 ± 19.1	78.9 ± 11.2	74.7 ± 11.5	84.1 ± 12.7	77.5 ± 8.5	70.1 ± 18.4*
Placental weight (g)	0.483 ± 0.047	0.467 ± 0.030	0.435 ± 0.046*	0.502 ± 0.045	0.477 ± 0.037	0.518 ± 0.096

[a] Values are given as the mean ± SD.
[b] (Number of implantations/number of corpora lutea) × 100.
[c] Includes implantation sites and placental remnants.
[d] Includes macerated fetuses and dead term fetuses.
[e] (Number of dead or resorbed embryos and fetuses/number of implantations) × 100.
[f] (Number of live fetuses/number of implantations) × 100.
[g] Number of live male fetuses/number of live fetuses.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

Table 3
External and internal examinations of fetuses of rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
External examination						
Total no. of fetuses (litters) examined	174 (12)	171 (12)	147 (10)	172 (12)	165 (12)	153 (12)
No. of fetuses (litters) with external malformations	0	1 (1)	18 (4)*	0	0	0
Microphthalmia	0	0	17 (4)*	0	0	0
Cleft palate	0	0	1 (1)	0	0	0
Anotia	0	0	2 (1)	0	0	0
Brachygnathia	0	1 (1)	0	0	0	0
Brachymelia	0	0	2 (1)	0	0	0
Ectrodactyly	0	0	2 (1)	0	0	0
Filamentous tail	0	0	1 (1)	0	0	0
No. of runt fetuses (litters)	0	1 (1)	2 (2)	0	0	0
Internal examination						
Total no. of fetuses (litters) examined	83 (12)	84 (12)	72 (10)	83 (12)	80 (12)	75 (12)
No. of fetuses (litters) with malformations	1 (1)	1 (1)	2 (1)	0	0	0
Small cerebrum/small inner ear	0	0	2 (1)	0	0	0
Dilatation of lateral ventricle	0	1 (1)	0	0	0	0
Situs inversus totalis	1 (1)	0	0	0	0	0
Small intermediate lobe of lung	1 (1)	0	0	0	0	0
No. of fetuses (litters) with variations	7 (5)	6 (3)	7 (6)	7 (5)	3 (3)	9 (7)
Thymic remnant in neck (partially undescended horn of thymus)	5 (4)	5 (2)	5 (4)	5 (3)	0	8 (6)
Dilatation of renal pelvis	1 (1)	1 (1)	2 (2)	2 (2)	1 (1)	1 (1)
Left-sided umbilical artery	1 (1)	0	0	1 (1)	2 (2)	0

* Significantly different from the control ($p < 0.05$).

of fetuses with skeletal malformations. At 10 mg/kg bw/day, there were between one and five fetuses with split thoracic centrum, thoracic hemivertebra, fusion of cervical/thoracic vertebral arches, absence or fusion of ribs, fusion of clavicle and scapula, short humerus and absence of radius, absence of forelimb phalanges or short/absent metacarpals. These anomalies were not observed in the control data of 12 studies in the laboratory that performed this study for past 7 years. The incidences of fetuses with skeletal variations were significantly increased in all dinoseb-treated groups. A significantly increased incidence of fetuses with supernumerary ribs was noted in all dinoseb-treated groups. The incidences of fetuses with unossified thoracic centrum, 27 presacral vertebrae and lumbarization of sacral vertebra were also significantly higher at 10 mg/kg bw/day. Significantly delayed ossification was noted as evidenced by the numbers of cervical centrum and metacarpi at 8.0 and 10 mg/kg bw/day and of cervical centrum at 200 ppm.

Table 4
Skeletal examinations of fetuses of rats given dinoseb by gavage or in the diet.

Dose	Gavage dose			Feeding dose		
	Control	8.0 mg/kg	10 mg/kg	Control	120 ppm	200 ppm
Total no. of fetuses (litters) examined	91 (12)	87 (12)	75 (10)	89 (12)	85 (12)	78 (12)
No. of fetuses (litters) with malformations	3 (3)	1 (1)	6 (2)	3 (3)	0	1 (1)
Splitting of cervical centrum	1 (1)	1 (1)	0	0	0	1 (1)
Splittting of thoracic centrum	2 (2)	0	5 (1)	2 (2)	0	0
Fusion of cervical centrum	0	0	0	1 (1)	0	0
Thoracic hemivertebra	0	0	4 (2)	0	0	0
Fusion of cervical/thoracic vertebral arches	0	0	2 (1)	0	0	0
Absence of ribs	0	0	4 (2)	0	0	0
Fusion of ribs	0	0	1 (1)	0	0	0
Fusion of clavicle and scapula	0	0	1 (1)	0	0	0
Short humerus and absence of radius	0	0	1 (1)	0	0	0
Absence of forelimb phalanges	0	0	3 (1)	0	0	0
Short/absent metacarpals	0	0	2 (1)	0	0	0
No. of fetuses (litters) with variations	12 (6)	38 (10)**	69 (10)**	14 (6)	30 (10)*	29 (10)*
Bipartite ossification of thoracic centrum	0	1 (1)	3 (2)	2 (1)	1 (1)	3 (2)
Dumbbell ossification of thoracic centrum	0	0	1 (1)	5 (2)	1 (1)	1 (1)
Unossified thoracic centrum	0	3 (2)	10 (5)**	0	0	1 (1)
25 presacral vertebrae	0	0	0	1 (1)	0	0
27 presacral vertebrae	0	3 (2)	19 (5)**	0	1 (1)	1 (1)
Short supernumerary ribs	12 (6)	37 (10)**	66 (10)**	9 (6)	29 (10)*	24 (10)*
Lumbarization of sacral vertebra	0	2 (2)	9 (5)**	0	0	0
Bipartite ossification of sternebra	0	0	0	0	0	1 (1)
Misaligned ossification of sternebra	0	0	0	0	0	1 (1)
Degree of ossification						
Number of cervical centrum	0.55 ± 0.51[a]	0.26 ± 0.54*	0.04 ± 0.05**	0.88 ± 0.62	0.40 ± 0.58	0.23 ± 0.22*
Number of metacarpi	6.80 ± 0.52	6.33 ± 0.49*	6.02 ± 0.08**	7.18 ± 0.64	6.90 ± 0.55	6.64 ± 0.76

[a] Values are given as the mean ± SD (the litter is the unit evaluated).
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

Lower number of cervical centrum was also observed at 120 ppm, but it was within the historical control range (0.35–0.87) of the laboratory that performed this study.

4. Discussion

In this study, the effect of dinoseb on the morphological development of embryos was determined by administering relatively high doses of dinoseb by gavage or in the diet to pregnant rats during organogenesis. As expected, maternal toxicity was observed in all the dinoseb-treated groups. Dinoseb induced dose-dependent decreases in body weight gain and food consumption during pregnancy in the dinoseb-treated groups. The decrease in food consumption was greater in the feeding dose groups than the gavage dose groups; therefore, the decreased food consumption may be related to a reduced palatability of the diet in the feeding groups.

Although there was no increased incidence of intrauterine deaths in any dinoseb-treated groups, significantly decreased weights of fetuses were observed in all the dinoseb-treated groups, except for the group fed dinoseb at 120 ppm. A decrease in the gravid uterine weight, reflecting the decreases in the fetal weights, was also found in the treatment groups, and a significant decrease at 200 ppm seemed partly related to the incidentally low number of corpora lutea. Skeletal examinations of fetuses revealed an increased incidence of fetuses with skeletal variations in all dinoseb-treated groups and delayed ossification at 8.0 and 10 mg/kg bw/day and at 200 ppm. These findings indicate that dinoseb is developmentally toxic at 8.0 and 10 mg/kg bw/day by gavage and 120 and 200 ppm by feeding when administered during organogenesis.

An increased incidence of fetuses with external malformations was observed at 10 mg/kg bw/day, but there was no increased incidence of fetuses with external, internal or skeletal malformations in the groups given dinoseb at 8.0 mg/kg bw/day by gavage or 120 or 200 ppm by feeding. The results of morphological examinations of fetuses revealed that dinoseb is teratogenic at the maternally toxic dose of 10 mg/kg bw/day when administered by gavage during organogenesis.

A recent study analyzing 125 developmental toxicity bioassays indicated that reduced maternal body weight gain was associated with fetal development [22]. To further evaluate dinoseb-induced developmental toxicity, maternal toxicity in the 10 mg/kg bw/day group was compared between litters with malformations and litters without malformations. A remarkable reduction in maternal body weight gain over days 6–16 was observed in the litters with malformations (19.0 ± 6.7 g vs. 30.0 ± 6.1 g; with vs. without malformations). In addition, placental weight was reduced in the litters with malformations (0.415 ± 0.024 g) compared to the litters without malformations (0.448 ± 0.054 g). These findings indicated that dinoseb was teratogenic at maternally toxic doses, but seems unrelated to maternal dietary deficiency.

Although the feeding dose of dinoseb at 200 ppm (15 mg/kg bw/day) was previously reported to be teratogenic in rats [4], the feeding dose of dinoseb up to 200 ppm (8.5 mg/kg bw/day) did not induce teratogenicity in the present study. Dose levels of dinoseb in the current study might not have been sufficiently high to induce teratogenicity; however, pregnant rats did not consume sufficiently high amounts of dinoseb to produce fetal malformations because food consumption was reduced in the feeding groups. It seems unlikely that a feeding study is appropriate to evaluate the toxicity of dinoseb.

Microphthalmia, which was found in rats after exposure to dinoseb by gavage or feeding [4,19] and in rabbits by gavage [23] or dermal application [7], was predominantly observed after administration of dinoseb at 10 mg/kg bw/day by gavage. As a rule, the administration of a suitable dosage of a teratogen generally results in the production of some normal offspring, some malformed offspring, and some dead or resorbed offspring [24]. In the present study, the increased incidence of malformed fetuses was not accompanied by an increased incidence of intrauterine deaths of offspring after the administration of dinoseb. This phenomenon was also observed in the previous studies of Giavini et al. [4,19]. One possible explanation for this is that microphthalmia itself is not lethal *in utero* as well as probably postnatally.

Giavini et al. showed that teratogenic potential in rats was influenced by the mode of administration or even the dietary composition [4,19]; however, conditions under which malformations occurred were not clearly described in these papers. The diets used in these studies did not meet the nutrient requirement of rats for fat (more than 5%) [25,26] while the diet used in our study is a standard rat diet; however, fat concentration seems unrelated to dinoseb-induced teratogenicity. Teratogenic effects were not observed after the gavage dose of dinoseb at 8.0 mg/kg bw/day. Because maternal death was observed after the gavage dose of dinoseb at 10 mg/kg bw/day, the exposure range of dinoseb where malformations are observed seems to be narrow. The findings of the present study confirmed the experimental condition that could induce malformation in rats fed a standard diet.

Dinitro-o-cresol, a structural and mechanical analogue of dinoseb, also induced external or internal malformations in 29 out of 64 fetuses when pregnant rabbits were administered it by gavage from day 6 to day 18 of gestation at 25 mg/kg bw/day [27]. The most frequent malformations were microphthalmia/anophthalmia and hydrocephaly/microcephaly. These results were quite similar to the findings of a gavage dose study of dinoseb in rabbits [23]. Further teratology studies of other uncoupling agents may be needed to clarify that uncoupling agents can produce malformations with the same mode of action.

It is considered that the basic mechanism of toxicity of dinoseb is stimulation of oxidative metabolism in cell mitochondria by the uncoupling of oxidative phosphorylation, and the energy is released as heat [28,29]. However, there is no clear understanding of the fundamental mechanism of developmental toxicity of dinoseb, although an energy-deficient intrauterine environment due to uncoupling of cellular oxidative phosphorylation may explain dinoseb-induced developmental toxicity. A decreased placental weight was observed in the gavage dose group at 10 mg/kg bw/day, which may suggest intrauterine energy deficiency. A prenatal dose of thiabendazole, an ATP-synthesis inhibitor, induced a deformity involving reduced limb size in mice fetuses [30], and ATP levels in fore and hind limb buds of fetuses were related to the incidence of this deformity [31]. Dinoseb-induced teratogenicity may be related to the degree of reduction in ATP expression influenced by variable factors such as the mode of administration used in experiments. Recent studies have investigated the role that mitochondria play in mediating apoptotic signals [32–34]. Programmed cell death (PCD) is an essential component of normal physiological processes such as embryogenesis and normal tissue development [35]. Altering normal patterns of PCD could be teratogenic because areas of the body with a high incidence of malformations coincide with areas where PCD occurs [36,37]. Some studies showed a positive correlation between mitochondrial uncoupling activity and PCD [38,39], and 2,4-dinitrophenol, an uncoupling agent, enhanced the Fas apoptotic signal in Jurkat Bcl-2 cells [33]. These findings imply that the enhanced uncoupling of oxidative phosphorylation in mitochondria may alter normal patterns of PCD. However, the link between malformations and mitochondrial uncoupling activity are still poorly understood. Further mechanistic studies are necessary to clarify the teratogenicity of dinoseb.

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