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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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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Original Article

Effects of preparation methods for multi-wall carbon nanotube (MWCNT) suspensions on MWCNT induced rat pulmonary toxicity

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ABSTRACT — Since there is a possibility of inhaling the fibers of multi-wall carbon nanotube (MWCNT) without any agglomeration, it is important that the pulmonary toxicity is evaluated by intratracheal instillation without agglomeration. MWCNT suspended in an artificial lung surfactant (ALS) with or without grinding in an agate mortar was instilled once intratracheally to rats to determine whether differences of the effects to pulmonary toxicity by different amounts of agglomerated MWCNT particle. The MWCNT suspension preparation method with grinding was effective at reducing agglomerates and in increasing uniform dispersion of the fibers. The ground MWCNT induced higher LDH levels and neutrophil ratios in the bronchoalveolar lavage fluid (BALF). There were no remarkable responses in rats in the non-ground MWCNT group, with the exception of inflammatory responses in the early phase. Some histopathological findings varied between rats given the ground MWCNT and non-ground MWCNT. A major difference was an MWCNT-laden macrophage infiltration site in the lung, which were in the alveolus in the ground MWCNT group, and in the interstitium in non-ground MWCNT group. Accordingly, the preparation method with grinding is considered to be effective at reducing agglomerates and ensuring uniform dispersion of the fibers. These findings lead us to conclude that the amount of agglomerates in the suspension is an important factor affecting the pulmonary toxicity of MWCNT.

Key words: Multi-wall carbon nanotube (MWCNT), Rats, Lung toxicity, Inflammation

INTRODUCTION

The production of multi-wall carbon nanotube (MWCNT), a representative industrial nanomaterial, is increasing worldwide due to their high potentials in industrial usage. Recent reports (Lam *et al.*, 2006; Donaldson *et al.*, 2006) suggested that the health effects of such materials must be evaluated properly. It is estimated that a possible exposure route of MWCNT is inhalation, especially in occupational environments. The shape of MWCNT is similar to asbestos and has been reported to induce mesothelioma like lesions (Takagi *et al.*, 2008; Poland *et al.*, 2008). Therefore, assessment of pulmonary toxicity of MWCNT with experimental animals is important to ascertain the possible effects on human health. The first evaluation of MWCNT inducing pulmonary toxicity was made by an intratracheal instillation with rodents. However, the information regarding MWCNT induced pulmonary toxicity in rodents is limited (Muller *et al.*, 2005).

It is well known that MWCNT tends to agglomerate into large particles, such as in the micrometer-order scale (Lam *et al.*, 2006). Studies in which large particulate of MWCNT are intratracheally administered and evaluated may lead to a misunderstanding of its pulmonary toxicity. Therefore, the preparation method of finely dispersing MWCNT is essential to assess its pulmonary toxicity.

The present study examined the preparation methods of MWCNT suspensions for intratracheally instilling finely dispersed fibers as well as the effects of different dispersion conditions of MWCNT fibers in suspensions on pulmonary toxicity in rats.

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MATERIALS AND METHODS

Experimental animals

Female Crl:CD(SD) rats with a body weight range of 170 to 200 g were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were kept in an animal room of the Kashima Laboratory, Mitsubishi Chemical Medience Corporation (Ibaraki, Japan), which was maintained at temperature of 22 ± 2°C, relative humidity of 55 ± 20%, with a 12 hr light-dark cycle. They were given tap water and a diet for experimental animals (MF: Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum*. The rats were 8 weeks old at dosing. The present study was carried out in accordance with the Guidelines for Animal Studies (Toxicological Science Division, Mitsubishi Chemical Medience Corporation). Moreover, the protocol of this study was approved by the Committee for Ethics in Animal Studies of the Kashima Laboratory prior to commencing the study.

Materials

MWCNT (MITSUI MWCNT-7, Lot No. 060125-01k) and crystalline silica (Min-U-Sil #5: provided by Hayashi-Kasei Co., Ltd., Osaka, Japan) were used in this study. The property of the MWCNT is the same as those formerly reported (Takagi *et al.*, 2008). Briefly, MWCNT has a density of 3.55×10^{11} particles/g. The length and width were examined with a scanning electron microscope and are indicated in Fig. 1. MWCNT contained; Fe (approximately 3,500 ppm), sulfur (470 ppm), and chlorine (20 ppm) as well as fluorine (5 ppm) and bromide (40 ppm) that were below the detection levels.

Preparation of particle suspension

There were large numbers of agglomerates in suspensions prepared using media such as, 0.5% CMC-Na solution, 0.1% Tween 80 solution, or 0.5% CMC-Na solution containing 0.1% Tween 80. In contrast, the numbers of agglomerates decreased when an artificial lung surfactant (ALS, Surfacten®: Mitsubishi Tanabe Pharma Corp., Osaka, Japan) was used as the suspension media. Therefore, the ALS was selected as the suspension media for this study. Surfacten® is an extraction from bovine lung, which include a constant ratio of phospholipids, free fatty acids, and triglyceride. ALS was prepared by dissolving Surfacten® at 120 mg into sterilized saline (Otsuka Normal Saline: Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at 4 ml. MWCNT was suspended in ALS at a concentration of 10 mg/ml with or without grinding in an agate mortar, the suspension was subjected to sonication for 3 min with an ultrasonic disruptor (UD-201: Tomy Seiko Co., Ltd., Tokyo, Japan). The grinding was conducted under wet conditions: ground after addition of a small amount of ALS to an adequate amount of MWCNT, and filled up to the proper volume with ALS to make target concentrations. The dispersion conditions were evaluated with a light microscope, transmission electron microscope (H-7600: Hitachi, Tokyo, Japan), and scanning electron microscope (JSM-5200: JEOL, Tokyo, Japan).

Min-U-Sil #5 was also suspended in ALS at a concentration of 10 mg/ml, and sonicated for 3 min.

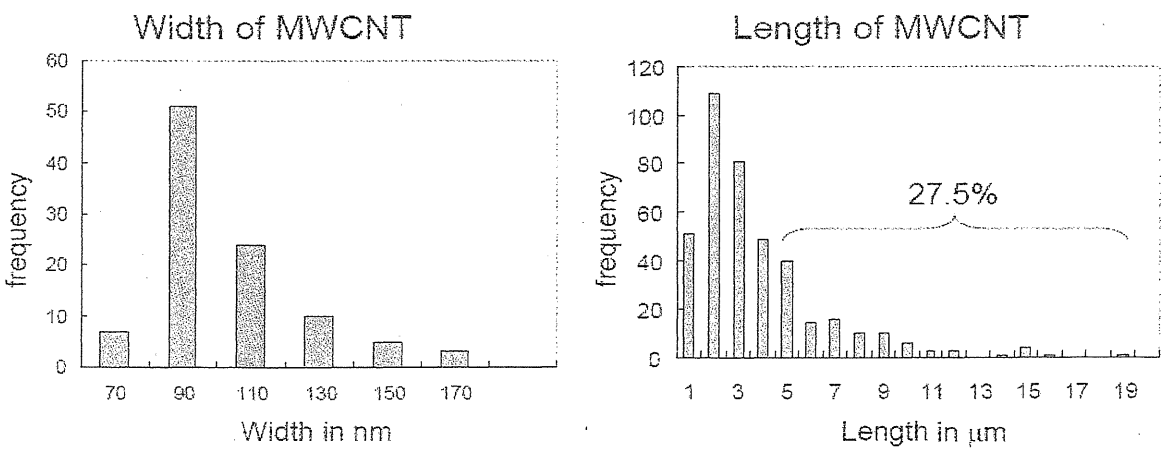

Fig. 1. Width and length distribution of MWCNT: The average width was about 100 nm, and 27.5% of the particles were longer than 5 μm.

Particle instillation

The light anesthetization was achieved by ether inhalation. An 18-G intravenous catheter (Terumo Corp., Tokyo, Japan) was inserted to the larynx as a guide to insert a narrow catheter. The suspensions (volume: 0.5 ml/animal) were instilled to the trachea via the narrow catheter (outside diameter: 0.80 mm).

General experimental design

The suspensions of the ground MWCNT, non-ground MWCNT, Min-U-Sil, and ALS alone (as a negative control) were administered intratracheally at a dose of 5 mg/animal with a dosage volume of 0.5 ml/animal to 12 rats/group. Since it was reported that the dose at 5 mg/head of MWCNT had toxicological effects on the lung according to Muller *et al.* (2005), this dose was selected for this study. Furthermore, this dosage volume is widely used in similar studies. The day of administration was designated as Day 1. Three rats from each group were subjected to necropsy on Days 2, 8, 29, and 92 after peritoneal injection of pentobarbital sodium (Nembutal®: Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Bronchoalveolar lavage fluid (BALF) was obtained from all rats on each day of necropsy. The difference in the effects on pulmonary toxicity by the amount of agglomerated particles in the instilled suspension was assessed by BALF analysis and histological examination.

BALF analysis

Firstly, the right lung lobes after ligating of the left bronchus were lavaged three times with 3 ml of saline (Otsuka Normal Saline: Otsuka Pharmaceutical Factory Inc.) heated up to 37°C in advance for the cell-counting procedure. A cell pellet was obtained from the BALF after centrifugation (300 × g, 10 min) at about 4°C. The cell pellet was re-suspended in 0.1% BSA containing phosphate buffered saline (PBS^-) + 0.05 mM EDTA-2K and cell count and deferential ratio were examined with a hemocytometer (XT-2000iV: Sysmex Corp., Hyogo, Japan). Secondary, the right lung lobes were lavaged once with 2 ml of saline. The obtained BALF was mixed with the previously obtained supernatant and centrifuged (300 × g, 10 min) at about 4°C. The supernatant thus obtained was subjected to the quantification of protein (Biuret method) and LDH (UV-rate method, JSCC modified method) with an automatic analyzer (TBA-200FR: Toshiba Corp., Tokyo, Japan).

Histological examination

The left lung lobes and bronchiolar lymph nodes were removed and fixed in 10% phosphate-buffered formalin. After conventional processing, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and examined histopathologically under a light microscope. Moreover, a part of the lung was collected from one animal of each group and fixed in 2.5% glutaraldehyde with negative pressure. The lungs were embedded in epoxy resin and stained with uranyl acetate and lead citrate for examination with a transmission electron microscope (H-7600: Hitachi).

Statistical analysis

The cell differential ratio as well as protein and LDH concentrations in BALF were statistically compared with the control values for each time point. Initially, the variance was assessed with Bartlett's test. When the variance was equal, one-way analysis of variance was used; otherwise, the Kruskal-Wallis test was used. When significant differences were seen between the groups, they were evaluated with Dunnett's method (homogeneous variance) or a Dunnett's type (Steel method; non-homogeneous variance) multiple comparison test. The significance was judged at the 0.01 and 0.05 probability levels. Additionally, the above values were examined between the non-ground MWCNT and ground MWCNT groups. Initially, the variance was assessed with the F test with a significant level of 5%. Significant differences between the groups were then analyzed using the Student's t test when the variance was equal; otherwise, the Aspin-Welch's test was used.

RESULTS

Grinding effects

The effects of grinding with an agate mortar were evaluated by observation of the suspensions with a light microscope and electron microscope (Figs. 2, 3, and 4). The agglomerates in the ground MWCNT were smaller than those in the non-ground MWCNT. The number of fibers finely dispersed was observed in the ground MWCNT. The length of the fiber in the ground MWCNT was smaller than those in the non-ground MWCNT.

BALF analysis

The following results were obtained as compared to the control values. The total cell count in BALF elevated in rats given the ground MWCNT on Day 8 and Min-U-Sil on Day 91. Higher neutrophil ratios in BALF were observed in rats given the non-ground MWCNT and ground MWCNT on Day 2, ground MWCNT and Min-U-Sil on Day 29, and Min-U-Sil on Day 91 (Fig. 5). Higher LDH concentrations in BALF were observed in rats given

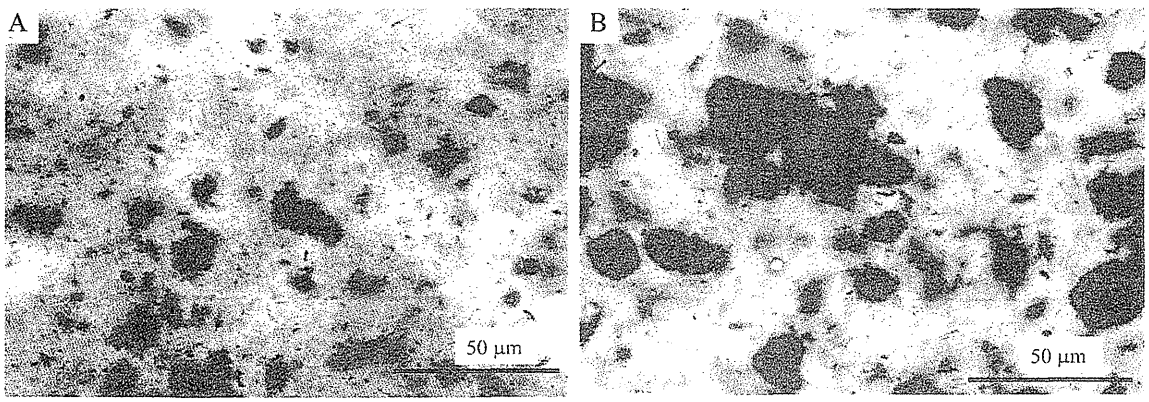

Fig. 2. Light microscopic view of administered MWCNT suspension: A) Ground MWCNT suspension, sizes of agglomerates were smaller than non-ground MWCNT suspension and finely dispersed fibers were seen. B) Non-ground MWCNT suspension, large agglomerates and small number of dispersed fibers were seen. Original magnifications were x 400.

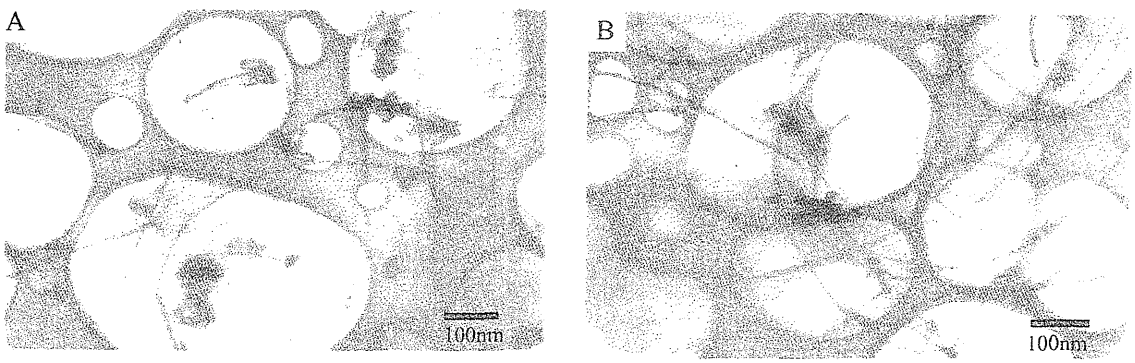

Fig. 3. Transmission electron microscopic view of administered MWCNT suspension: A) Ground MWCNT suspension, short fibers were seen. B) Non-ground MWCNT suspension, long fibers were seen.

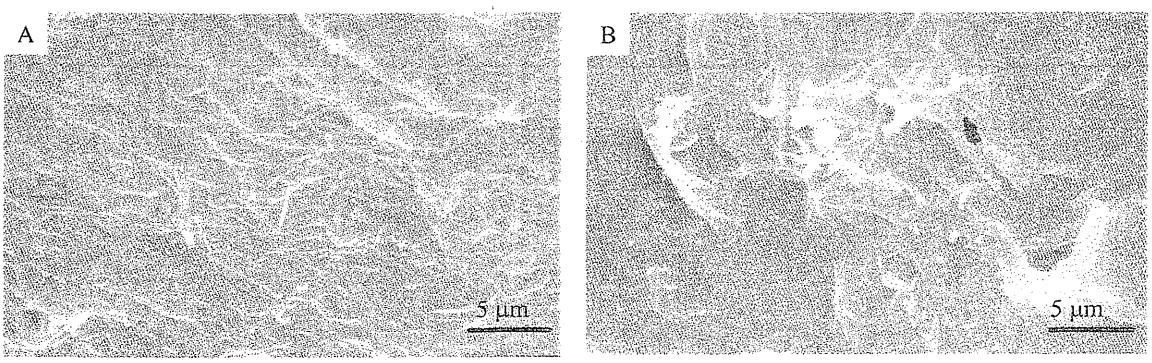

Fig. 4. Scanning electron microscopic view of administered MWCNT suspension: A) Ground MWCNT suspension, uniform suspended condition was seen with small size of fibers. B) Non-ground MWCNT suspension, non-uniform suspended condition and agglomerates were seen.

the non-ground MWCNT and ground MWCNT on Day 8, ground MWCNT and Min-U-Sil on Day 29, as well as Min-U-Sil on Day 91. Higher protein levels in BALF were observed in all treated rats on Day 91 (Fig. 6). There were no remarkable differences in these parameters between rats given the non-ground and ground MWCNT.

Histological examination

Tables 1 and 2 summarized histopathological findings. Black patches in the lung and blackish change of the bronchiolar lymph nodes were macroscopically observed in rats given the non-ground and ground MWCNT. MWCNT, which was "blackish" in color, and was microscopically observed in the alveolar region of rats given the ground MWCNT on Day 2. In rats given the non-ground MWCNT, MWCNT was slightly observed in the bronchial region. On Day 8 or later, focal infiltration of MWCNT- laden macrophages were observed in the interstitium of the lung of rats given the non-ground MCNT, compared to rats given the ground MWCNT, where macrophages were observed mainly in the alveolus. Ultrastructurally, the macrophages in the alveolus of rats given the ground MWCNT had well-developed lysosomes (Figs. 7 and 9). In the bronchiolar lymph nodes of rats given the non-ground and ground MWCNT, there were black colored macrophage infiltrations, which were thought to be phagocytosed MWCNT. The severity of the black colored macrophage infiltrations in the bronchiolar lymph nodes progressively increased after Day 29 in rats given the ground MWCNT (Fig. 8). Focal inflammatory cell infiltration in the lungs was observed in rats given the non-ground and ground MWCNT on Day 2 only, however, it was observed in rats given Min-U-Sil not only on Day 2 but also on Day 92. In rats given Min-U-Sil, granuloma in the bronchiolar lymph nodes was observed on Day 92.

DISCUSSION

This study led to a conclusion that the ALS is one of the most suitable vehicle media to suspend MWCNT for intratracheal instillation. Buford *et al.* (2007) reported that the vehicle containing some protein, lipid or protein/

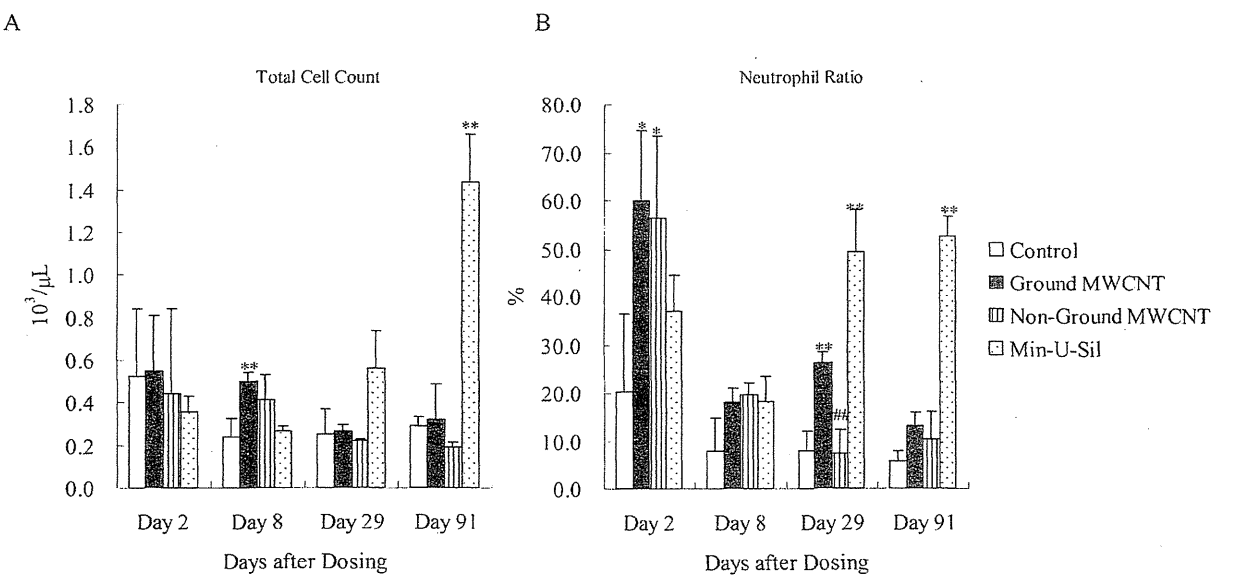

Fig. 5. A) Total cell count in broncho-alveolar lavage fluids (BALF) from rats exposed to non-ground and ground MWCNT, and corresponding controls on days 2, 8, 29, and 91 (day of dosing designated as day 1). Values are mean ± S.D. Ground MWCNT induced significantly increasing of total cell count in BALF on day 8, $^{**}p < 0.01$. Min-U-Sil induced significantly increasing of total cell count in BALF on day 91, $^{**}p < 0.01$.
B) Neutrophil ratio in BALF from rats exposed to ground, non-ground MWCNT and corresponding controls on days 2, 8, 29, and 91. Values are mean ± S.D. Ground and non-ground MWCNT induced higher neutrophil ratio in BALF on days 2, $^{*}p < 0.05$, and ground MWCNT induced the higher neutrophil ratio on day 29, $^{**}p < 0.01$. Neutrophil ratio in non-ground MWCNT on day 29 significantly lower than ground MWCNT, $^{\#\#}p < 0.05$. Min-U-Sil induced higher neutrophil ratio on days 29 and 91, $^{*}p < 0.01$.

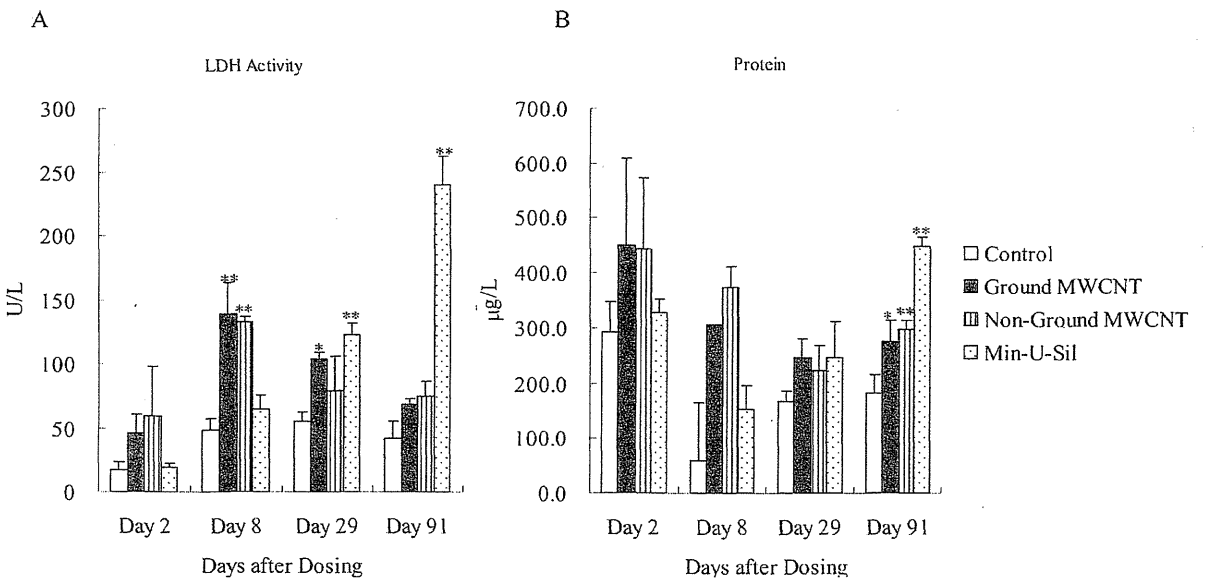

Fig. 6. A) LDH activity in BALF from rats exposed to non-ground and ground MWCNT, and corresponding controls on days 2, 8, 29, and 91 (day of dosing designated as day 1). Values are mean ± S.D. Ground MWCNT induced significantly increasing of LDH activity on days 8 and 29, non-ground MWCNT induced higher LDH activity on day 8 only, $*p < 0.05$ and $**p < 0.01$. Min-U-Sil induced higher LDH activity on days 29 and 91, $**p < 0.01$.
B) Total protein concentration in BALF from rats exposed to ground, non-ground MWCNT and corresponding controls on days 2, 8, 29, and 91. Values are mean ± S.D. Ground and non-ground MWCNT, and Min-U-Sil induced higher protein level in BALF on day 91, $*p < 0.05$ and $**p < 0.01$.

lipid component could disperse carbon nanotubes appropriately. Since the ALS used in this study is an extraction from bovine lung, which includes phospholipids, free fatty acids, and triglyceride, the results of this study did not contradict their report (Buford *et al.*, 2007). Additionally, although no remarkable adverse effects were noted by dosing of the xenogeneic media, the possible pulmonary toxicity of MWCNT may have to be evaluated by removing the influence of the xenogeneic agent. It is considered that the suspension preparation method recently reported (Buford *et al.*, 2007; Sager *et al.*, 2007) is suitable for this purpose.

The results of this study suggested that the wet-grinding in an agate mortar was useful to prepare uniform dispersed suspension, because it was confirmed that the grinding reduced the number and the size of the agglomerates in the suspensions. Since grinding with a mortar is a simple procedure, it was concluded that this method is appropriate for preparation of any other CNT suspensions for intratracheal instillation. Furthermore, this method reduced generation of MWCNT aerosol when conducted under wet conditions. This suggested that the possibility of inhalation of aerosolized MWCNT to human could be reduced with this method.

There were remarkable differences between the ground MWCNT and non-ground MWCNT in the BALF chemistry analysis on Day 29. Higher neutrophil ratios and LDH levels in the BALF were observed in rats given the ground MWCNT on Day 29. In contrast, such changes were not noted in rats given the non-ground MWCNT. There were remarkable differences in histological changes of the lungs between rats given the ground and non-ground MWCNT. In rats given the ground MWCNT, remarkable macrophage infiltration in the alveolus was observed, but in the interstitium it was relatively weak. In contrast, there was predominant macrophage infiltration in the interstitium of rats given the non-ground MWCNT.

Muller *et al.* (2005) reported that ground MWCNT led the pulmonary lesion characterized by the interstitial fibrosis in the lungs. MWCNT-laden macrophage infiltration in the alveolus was considered to be attributable to the relatively short fiber, which was generated by grinding. The acceleration of phagocytic activity, which was demonstrated as an increase of developed lysosomes supported our speculation.

There were no inflammatory responses in the bronchi-

Table 1. Numbers of rats indicating the histopathological lesions in the bronchiolar lymph node and trachea of rats given ground or non-ground multiwall carbon nanotube, Min-U-Sil, or artificial lung surfactant

Organ			Group name															
Findings			Control				Ground MWCNT				Non-Ground MWCNT				Min-U-Sil			
	Grade	Day:	2	8	29	92	2	8	29	92	2	8	29	92	2	8	29	92
Lymph node, bronchial																		
Cell infiltration, macrophage	1		0	0	0	0	0	3	0	0	0	0	3	1	0	0	0	0
	2		0	0	0	0	0	0	3	3	0	0	0	1	0	0	0	0
Granuloma	1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
	2		0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
	3		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Trachea																		
Accumulation, administered substance	1		0	0	0	0	3	0	0	0	2	0	0	0	0	0	0	0
Cell infiltration, inflammatory, mucosa	1		0	0	0	0	1	0	0	0	0	0	0	0	2	0	0	0
Regeneration, mucosa, focal	1		1	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0
Ulcer	1		0	0	0	0	3	0	0	0	1	0	0	0	0	0	0	0
	2		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	3		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Hypertrophy, mucosal epithelium	1		0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
Cell infiltration, macrophage, lamina propria, focal	1		0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0

Note: values indicate number of animals indicating lesion.
Grade: 1 = minimal, 2 = mild, 3 = moderate. Day: day of intratracheal instillation designated as Day 1.

Table 2. Numbers of rats indicating the histopathological lesions in the lung of rats given ground or non-ground multiwall carbon nanotube, Min-U-Sil, or artificial lung surfactant

Organ			Group name															
Findings			Control				Ground MWCNT				Non-Ground MWCNT				Min-U-Sil			
	Grade	Day:	2	8	29	92	2	8	29	92	2	8	29	92	2	8	29	92
Lung (and bronchus)																		
Accumulation, administered substance, alveolus	1		0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
	2		0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
	3		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Accumulation, administered substance, bronchus	1		0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
	2		0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0
	3		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Atelectasis, focal	1		0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0
	2		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Cell infiltration, inflammatory, focal	1		0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	3
	2		0	0	0	0	3	0	0	0	2	0	0	0	0	0	0	0
	3		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Cell infiltration, macrophage, alveolus, focal	1		0	0	0	0	3	1	1	0	0	3	1	1	0	1	1	0
	2		0	0	0	0	0	2	2	3	0	0	0	0	0	0	0	3
Cell infiltration, macrophage, interstitium, focal	1		0	0	0	0	0	3	3	3	0	0	1	0	0	0	1	2
	2		0	0	0	0	0	0	0	0	0	3	1	3	0	0	0	0
Erosion, bronchus	1		0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Hyperplasia, lymphoid tissue	1		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Hypertrophy, alveolar epithelium, focal	1		0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Hypertrophy, bronchial epithelium	1		0	0	0	0	3	1	0	0	2	1	0	0	0	0	0	0

Note: values indicate number of animals indicating lesion.
Grade: 1 = minimal, 2 = mild, 3 = moderate. Day: day of intratracheal instillation designated as Day 1.

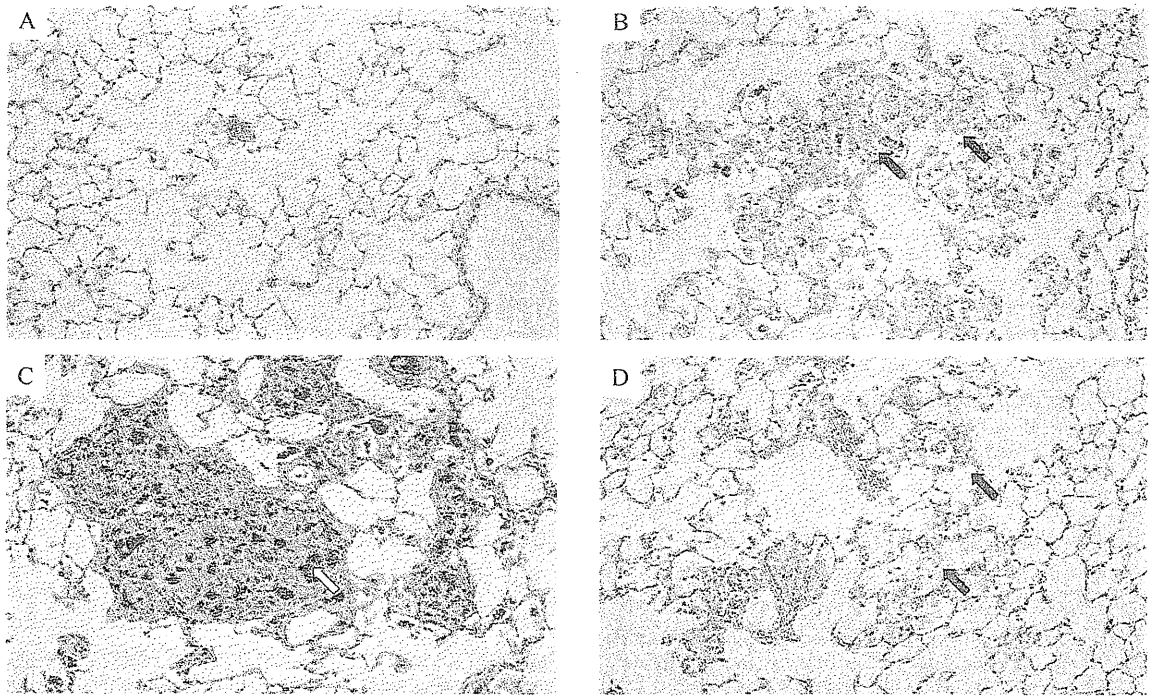

Fig. 7. Light microscopy of the lungs of rats exposed to non-ground and ground MWCNT, and corresponding controls on day 29. A) Control, no remarkable change. B) Ground MWCNT, foamy macrophage infiltration in alveolus. C) non-ground MWCNT, macrophage infiltration in interstitium. D) Min-U-Sil, foamy macrophage infiltration in alveolus.

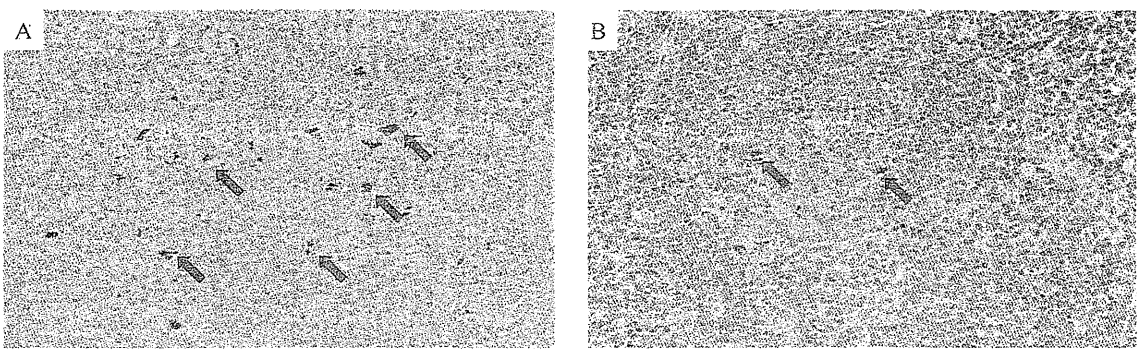

Fig. 8. Light microscopic view of bronchiolar lymph node of rats exposed to non-ground or ground MWCNT on day 29. A) Ground MWCNT: remarkable infiltration of macrophage including MWCNT without any inflammatory reactions than non-ground MWCNT. B) Non-ground MWCNT: infiltration of macrophage including MWCNT without any inflammatory reactions.

olar lymph nodes of rats given MWCNT. Since inflammatory change in the bronchiolar lymph nodes was observed in rats given Min-U-Sil, the biological activity on the discharge process of foreign body from the respiratory tract is considered to be remarkably different between MWCNT and Min-U-Sil.

It is estimated that the aerodynamic sizes of MWCNT become smaller than their original size, because MWCNT is widely used after the grinding process (Liu *et al.*, 2004). In fact, it is expected that MWCNT, which has

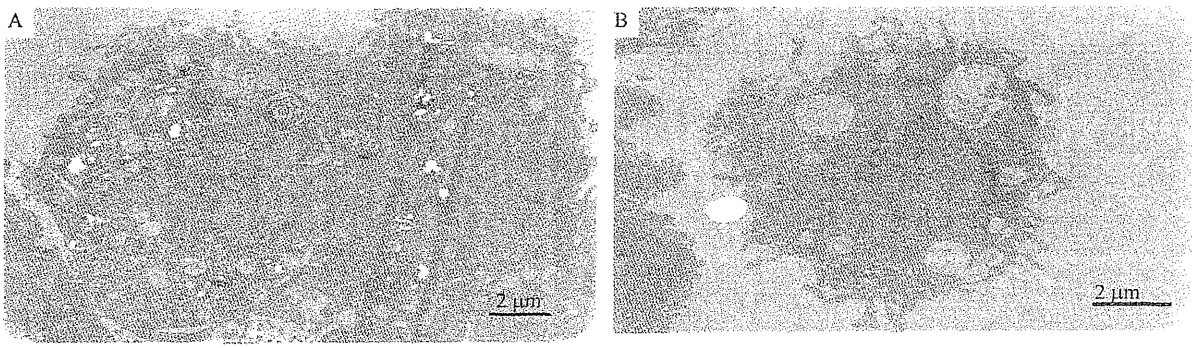

Fig. 9. Transmission electron microscopic view of macrophage in alveolus of rat exposed to non-ground or ground MWCNT. A) Ground MWCNT: macrophage in alveolus had lysosomes which developed remarkably. B) Non-ground MWCNT: macrophage in alveolus on day 29, there were no remarkable lysosomes.

small aerodynamic diameter due to the grinding, reaches deep into the lung in an occupational environment. The pulmonary toxicity of MWCNT obtained in this study was different from the recent report (Muller *et al.*, 2005). Consequently, it is considered that the available information on MWCNT induced pulmonary toxicity is not sufficient to evaluate the effects on human health. Since both of the above-mentioned studies did not have characterization data of the instilled suspensions, the pulmonary toxicity could not be compared between the studies. Therefore, the fibers in the suspensions must be characterized, at least for their length, diameter, and dispersion conditions in order to make a comparison with recent reports. Furthermore, the present study indicated that the different pulmonary toxicity occurred depending on the size of the particles in the suspension. These results suggested that the proper evaluation of the pulmonary toxicity of nanomaterial must include characterization of the instilled suspensions, even if it was only a single material.

There were no inflammatory reactions in the lung or bronchiolar lymph nodes in the rats given MWCNT 3 months after instillation. However, higher protein concentrations in BALF were observed in rats given MWCNT. Moreover, the long term effect of MWCNT on the lungs caused by grinding or non-grinding remained unclear in this study. These findings lead us to conclude that additional studies, such as examination of the fate of MWCNT in the lung and its long term effects on the lung are necessary.

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【特集】

OECD 化学物質対策の動向（第 16 報）

－第 27 回 OECD 高生産量化学物質初期評価会議（2008 年オタワ）

Progress on OECD Chemicals Programme (16) — SIAM 27 in Ottawa, 2008

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要旨：第 27 回 OECD 高生産量化学物質初期評価会議（SIAM 27）が 2008 年 10 月にオタワ（カナダ）で開催され、日本が担当した 3 物質（*p*-トルエンスルホン酸ナトリウム：CAS 番号 657-84-1、レゾルシノール：CAS 番号 108-46-3、N-シクロヘキシル-2-ベンゾチアゾールスルフェンアミド：CAS 番号 95-33-0）の初期評価プロファイル（SIAP）について合意が得られた。本稿では本会議で合意の得られたこれら 3 物質の初期評価文書について紹介する。
キーワード：OECD、HPV プログラム、SIDS 初期評価会議

Abstract: The 27th Screening Information Data Set (SIDS) Initial Assessment Meeting (SIAM 27) was held in Ottawa, hosted by Canada. The initial assessment documents of three substances, sodium *p*-toluenesulfonate (CAS number: 657-84-1), 1,3-benzenediol (CAS number: 108-46-3), and N-cyclohexyl-2-benzothiazolsulfenamide (CAS number: 95-33-0) were submitted by the Japanese Government with or without the collaboration with International Council of Chemical Associations (ICCA). These SIDS Initial Assessment Profiles (SIAPs) of the substances were agreed at the meetings. In this report, the documents of these substances are introduced.
Keywords: OECD, HPV programme, SIDS Initial Assessment Meeting

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