表 7 食品・食餌関係の特許

特開番号	発明名称	出願人	概要
特開 2011-229455	ヒスタミン吸着剤およびヒスタミン除去方法	独立行政法人　水産総合研究センター	液体上の食品に含まれるヒスタミンを除去する方法としてベントナイトを使用する。
特開 2007-306902	ベントナイト（主にナトリウム型モンモリロナイト）ダイエット食品	㈱エコトープ	水分を吸着し膨潤することによる満腹感を狙ったダイエット食品に関する特許
特表 2005-523714	動物の食餌サプリメント	アディセオ・アイルランド・リミテッド	動物の食餌サプリメントにキャリヤー物質（担体）としてベントナイト等の粘土鉱物が含まれる
特開 2005-295854	健康食品および栄養物質の吸収改善を目的とする組成物	㈱バイオセレンタック	健康食品・栄養食品をベントナイト等の微粒子体が小腸まで保持する経口デリバリーシステム

表 8 各用途におけるナノクレイの使用状況

用途 1	用途 2	製品名	使用部位	使用量	配合率	今後の需要・出荷予測
食品	包装容器材・PET ボトル・軟包装フィルム	軟包装フィルム A社一製品	不詳（コート層に含まれているため、表層であると推定される）	不詳	特許の実施例からコート剤の基材に対し 5wt%程度と推定される。（,特開 2004-18649）	積極的な展開を行っているため、出荷が増える可能性がある。
		軟包装フィルム B社一製品	不詳（コート層に含まれているため、表層であると推定される）	不詳	特許の実施例から約 2wt%または 5wt%程度と推定される。（,特許 4434908）	既存ユーザーへの供給のみで、出荷は横ばいと推定される。
		PET ボトル C社一製品	PET 層に挟まれたポリマー/ナノクレイナノコンポジット 層に使用されている。	1 本当りのバリア材（ポリマー/ナノクレイ ナノコンポジット層）は 3wt%使用とされている。	不詳	不詳
	食品添加物・ワインの澱引き	ー	（製造工程）	D 社の場合、ワイン 1000L に対して 100～600g の範囲を目安に添加する。	ー	不詳
	栄養補助食品	各種製品	粘土粉末の直接摂取や錠剤中の成分として使用される。	製品により異なる。	製品により異なる。粘土粉末の場合には無機鉱物（ベントナイト等）が主成分である。	不詳
	動物用飼料の添加剤	ー	不詳	不詳	不詳	不詳
農薬	フロアブル製剤用添加剤（増粘剤）	ー	増粘剤として使用されている。	トップシェアであるクニミネ工業㈱「クニピア」は年間 50～60t 出荷している。	0.1 ～ 1.0 %程度使用される。	不詳
	農薬造粒助剤（粘結剤）	ー	粘結剤として使用されている。	不詳	不詳	不詳

表 8 用途別のガスバリア性軟包装フィルムの販売量(2011 年)

材料系	販売量		
	販売量合計	用途別販売量	
PVDC フィルム	45,600	食品用途	1,700
		その他(家庭用ラップフィルム)	43,900
OPP/EVOH 複合フィルム	1,325	食品用途	1,275
		その他	50
ONY 系複合フィルム	8,800	食品用途	8,650
		その他	150
PVDC コート	9,600	食品用途	7,100
		その他	2,500
PVA コート	5,300	食品用途	4,800
		その他	500
ナノコンポジット系樹脂コート	**730**	**食品用途**	**730**
ハイブリッド材料コート	700	食品用途	700
アルミ蒸着	25,400	食品用途	23,600
		その他	1,800
透明蒸着	15,400	食品用途	9,400
		その他	6,000
合計	112,855	食品用途	57,955
		その他	54,900

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Matsumoto, M., Serizawa, H., Sunaga, M., Kato, H., Takahashi, M., Hirata-Koizumi, M., Ono, A., Kamata, E., Hirose, A.	No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats.	J. Toxicol. Sci.	37	463-474	2012
Takahashi, M., Kato, H., Doi, Y., Hagiwara, A., Hirata-Koizumi, M., Ono, A., Kubota, R., Nishimura, T., Hirose, A.	Sub-acute oral toxicity study with fullerene C60 in rats.	J. Toxicol. Sci.	37	353-361	2012
Takagi A, Hirose A, Futakuchi M, Tsuda H, Kanno J.	Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice.	Cancer Sci.	103	1440-1444	2012
Xu J, Futakuchi M, Shimizu H, Alexander DB, Yanagihara K, Fukamachi K, Suzui M, Kanno J, Hirose A, Ogata A, Sakamoto Y,	Multi-walled carbon nanotubes translocate into the pleural cavity and induce visceral mesothelial proliferation in rats.	Cancer Sci.	103	2045-2050	2012

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.37, No.3, 463-474, 2012

Original Article

No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats

Mariko Matsumoto[1], Hideki Serizawa[2], Masao Sunaga[3], Hina Kato[1], Mika Takahashi[1], Mutsuko Hirata-Koizumi[1], Atsushi Ono[1], Eiichi Kamata[1] and Akihiko Hirose[1]

[1]Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 185-8501, Japan
[2]Bozo Research Center Inc., 1284, Kamado, Gotenba-shi, Shizuoka 412-0039, Japan
[3]Safety Research Institute for Chemical Compounds Co., Ltd., 363-24 Shinei, Kiyota-ku, Sapporo-shi, Hokkaido 004-0839, Japan

(Received November 29, 2011; Accepted January 25, 2012)

ABSTRACT — Three female Crl:CD(SD) rats/group were dosed with single wall carbon nanotube (SWCNT) or multi wall carbon nanotube (MWCNT) four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour intervals). Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects, and thus the oral LD_{50} values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw, in rats respectively. Five or ten Crl:CD(SD) rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). Six or twelve Crl:CD(SD) rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 50 mg/kg bw/day groups). Based on no toxicological effects, the no observed adverse effect levels (NOAELs) of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day (the highest dose tested), respectively. It was suggested that SWCNT and MWCNT dosed by gavage reached the gastro-intestinal tract as agglomerates and were mostly excreted via feces.

Key words: Single wall carbon nanotube, Multi wall carbon nanotube, Acute oral toxicity, Repeated oral dose toxicity, Rat

INTRODUCTION

Nanomaterials possess different physico-chemical properties from bulk materials. Therefore, it is necessary to develop specialized approaches to testing their effects on human health and on the environment. Our study group has worked on the establishment of a human health risk assessment methodology of nanomaterials since 2005. As part of efforts, our co-researchers reported carcinogenic potential of intraperitoneal administration of multi wall carbon nanotube (MWCNT) in p53 heterozygous mice (Takagi *et al.*, 2008) and intrascrotal injection of MWCNT in intact Fischer 344 rats (Sakamoto *et al.*, 2009) and also suggested that nano-sized particles can be transferred to other organs. Subsequently, Sakamoto *et al.* (2010) showed that expression of renal carcinoma/mesothelin can be used as a biomarker of mesothelial proliferative lesions induced by intrascrotal administration of MWCNT.

In parallel of the establishment of our study group, the OECD Working Party on Manufactured Nanomaterials (WPMN) was established to assess the safety of the use of nanomaterials for human health and the environment in 2006, and the Sponsorship Programme on the Testing on Manufactured Nanomaterials was launched in 2007 (OECD, 2011). The aim of the Sponsorship Programme is to fill data gaps between existing data and a desired data set for 13 nanomaterials including single wall carbon nanotubes (SWCNTs) and MWCNTs. Japan has volunteered to act as the Joint Lead Sponsors with the US for the evaluation of mammalian toxicology for fullerenes (C60), SWCNTs and MWCNTs in the Sponsorship Programme. We recently reported no toxicological effects of gavage doses of fullerene C60 up to 1,000 mg/kg bw/day in rats (Takahashi *et al.*, 2012). After the 29-day

Correspondence: Akihiko Hirose (E-mail: hirose@nihs.go.jp)

administration period, blackish feces and black contents of the stomach and large intestine were observed. Fullerene C60 was not detected in the liver, spleen or kidney at the end of administration period. This study indicated that fullerene C60 dosed by gavage was excreted via feces and not distributed in major organs.

We also conducted acute and repeated dose toxicity studies of SWCNT and MWCNT by gavage, target endpoints of the Sponsorship Programme. In some past studies, the physical-chemical properties of tested materials are not clear due to the diversity of carbon nanotubes (CNTs) with respect to purity, production methods, purification methods or surface treatments/coatings (Kobayashi *et al.*, 2009; OECD, 2011). The clear characterization and good control of the size and shape of nano-sized particulate materials are essential for ensuring the reproducibility and reliability of tests. Therefore, well defined Nikkiso SWCNT and MWCNT were set as principal samples in this Sponsorship Programme to examine mammalian toxicity.

Major current uses of MWCNTs are electronics applications such as super-capacitors and batteries and structural composite applications such as sporting equipments and conductive sheets (OECD, 2010a) and the future applications of MWCNTs include medical care and fabrics (Kobayashi *et al.*, 2009; MHLW, 2010). Major expecting uses of SWCNTs in future are super-capacitors, high speed transistor, fuel cells, super high strength wires (OECD, 2010b). A total volume of production and import of MWCNTs was 500 tons in 2008 in Japan, and it is expected to increase in future (MHLW 2010). Oral exposure to CNTs may occur through the migration from food contact products or agricultural foods that uptake CNTs from environment (Magnuson *et al.*, 2011).

Many toxicity studies have been available for SWCNTs or MWCNTs dosed by pharyngeal aspiration (Erdely *et al.*, 2009; Shvedova *et al.*, 2008), intravenous injection (Yang *et al.*, 2008), intratracheal instillation (Inoue *et al.*, 2009; Elgrabli *et al.*, 2008; Inoue *et al.*, 2008) and inhalation (Shvedova *et al.*, 2008) in rats and mice, but this will be the first report to show the results of acute and repeated dose toxicity of SWCNT and MWCNT by gavage in rats according to the OECD guidelines (TG 423 and TG 407). Although a gavage dose is not likely to be representative of the anticipated exposure scenario, the findings of our studies will be useful to characterize the feature of CNTs toxicity and for risk assessment in humans.

MATERIALS AND METHODS

Acute and repeated dose toxicity studies for SWCNT or MWCNT were performed in the Gotemba Laboratory, Bozo Research Center Inc. or the Safety Research Institute for Chemical Compounds Co., Ltd., respectively. These studies were conducted in compliance with the OECD Guideline 423; Acute Oral Toxicity, the OECD Guideline 407; Repeated Dose 28-Day Oral Toxicity Study in Rodents and the Guideline for 28-Day Repeated Dose Toxicity Test in Mammalian Species (Chemical Substances Control Law of Japan) under GLP. The SWCNTs studies were conducted in compliance with the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of Environment, dated April 28, 2006) and the Guidelines for Proper Conduct of Animal Experiments (June 1, 2006). The MWCNT studies were conducted in compliance with the Guidelines for Animal Experimentation (May 22, 1987), along with the above described Acts and Standards.

Animals

Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Female rats (SWCNT: 8 weeks old; MWCNT: 9 weeks old) were used for acute toxicity studies, and male and female rats (SWCNT: 6 weeks old; MWCNT: 5 weeks old) were used for repeated dose toxicity studies. Rats were individually housed in metallic cages with wire mesh bottoms and reared on a basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. Rats were maintained at room temperature, a humidity of 50 ± 20%, 10-15 air changes per hour and a 12 hr dark/12 hr light cycle.

Chemicals and dosing

Principal single-wall carbon nanotubes (SWCNT: purity > 95%; Lot No.: SW1859/SW1860/SW1865) and principal multi-wall carbon nanotubes (MWCNT: purity: > 98%; Lot No.: 04-12/10#1-(4)) supplied by Nikkiso Co., Ltd. (Shizuoka, Japan) were used. These chemicals are principal samples in the OECD Sponsorship Programme on the Testing on Manufactured Nanomaterials. The structure of SWCNTs is a honeycomb carbon lattice rolled into a cylinder, and the basic morphology is in sheet form consisting of entangle SWCNTs (with a diameter of around 2 nm) bundles with diameters of several decade nanometers. The structure of MWCNTs is honeycomb carbon lattices rolled into a multi-layer tubular shape,

and the basic morphology is particles consisting of entangled MWCNTs with a diameter of around 30 nm. Both test materials were not coated or modified. The test materials were stored in a polycarbonate bottle with an airtight stopper to prevent dissemination at room temperature, and were accurately weighed and added to gum acacia (vehicle). This vehicle was chosen based on the results of the preliminary investigation with commonly used vehicles, in which CNTs showed the best dispersion state in 5% gum acacia of aqueous solution. The mixture of test materials was homogenized using an ultrasonic homogenizer (SWCNT: UR-200P, TOMY Seiko Co., Ltd., Tokyo, Japan; MWCNT: VC-130, Sonics & Materials Inc., Newtown, CT, USA) and a compact ultrasonic cleaning bath (SWCNT: US-1, As One Co., Ltd., Tokyo, Japan; MWCNT: USC-6, Iwaki Glass Co., Ltd., Chiba, Japan). The homogeneity of test suspensions was confirmed microscopically (Figs. 1 and 2).

Acute toxicity studies for SWCNT and MWCNT were conducted in a stepwise procedure. As a first step, three female rats/group were dosed with SWCNT or MWCNT four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour intervals), respectively. Because oral toxicity of the test materials was expected to be low, dosage levels were determined based on the maximum doses which could be prepared and administered. The concentrations of 0.625 mg/ml SWCNT and 2.5 mg/ml MWCNT were confirmed to be the limit to prepare. In addition, ethically, a dosing volume of 20 ml/kg was limit for gavage dosing and four times seemed to be the limit for the number of dosing times. In the first step, no deaths or adverse effects were found for either SWCNT or MWCNT dosing. Therefore, a second step was carried out with the same regimen to confirm the acute toxicity of the test materials.

Five or ten rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days. Five animals/sex at 0 and 12.5 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period. Six or twelve rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days. Six animals/sex at 0 and 50 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period. A high dose was set with the maximum doses which could be prepared, as described above, and the middle and low

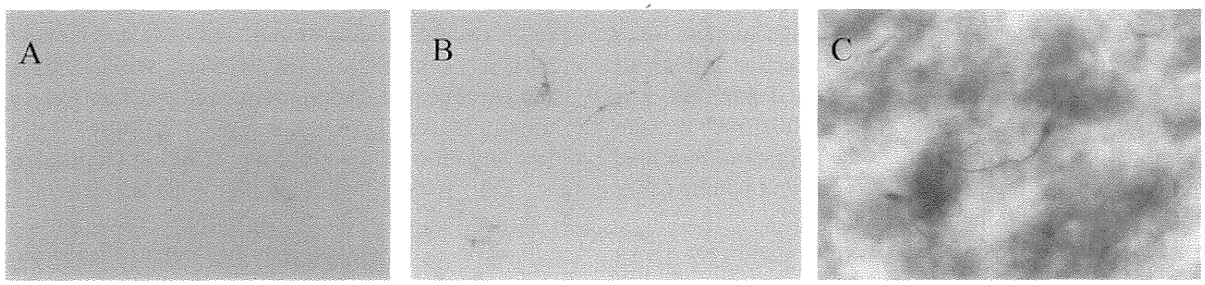

Fig. 1. Microscopic views of SWCNT suspensions (× 400). (A) 0.125 mg/kg bw/day (0.00625 mg/ml); (B) 1.25 mg/kg bw/day (0.0625 mg/ml); (C) 12.5 mg/kg bw/day (0.625 mg/ml).

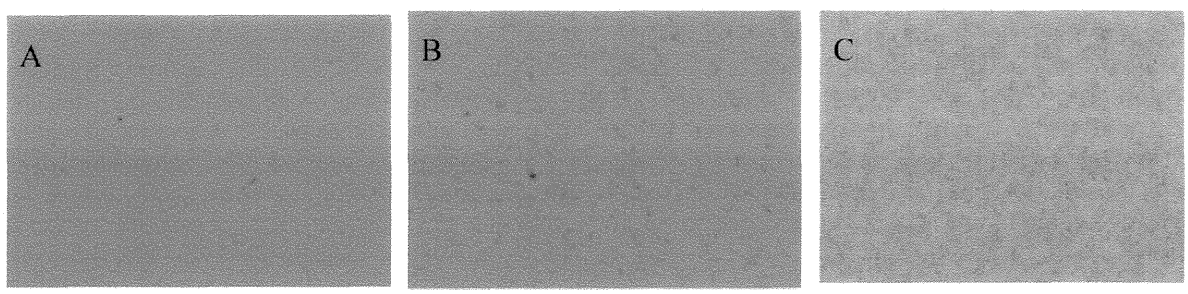

Fig. 2. Microscopic views of MWCNT suspensions (× 400). (A) 0.5 mg/kg bw/day (0.025 mg/ml); (B) 5.0 mg/kg bw/day (0.25 mg/ml); (C) 50 mg/kg bw/day (2.5 mg/ml).

doses were set with a common ratio of 10.

Observations

As for the acute toxicity studies, rats were observed for 14 days. Clinical observation was performed consecutively for several hours after administration and once or twice a day from the next day of administration. Animals were weighed just prior to administration, and 1, 3, 5, 7, 10 and 14 days (MWCNT) or 1, 3, 7 and 14 days (SWCNT) after administration. Necropsy was performed 14 days after administration.

As for the repeated dose studies, all males and females in the MWCNT study were observed twice per day, every day during administration and recovery periods, and in the SWCNT were observed three times per day, every day during the administration and once a day during the recovery period. A detailed clinical observation was carried out one day before administration, on days 7, 14, 21 and 28 days of the administration period and days 7 and 14 of the recovery period in the MWCNT study, and once a week in the SWCNT study. A functional examination was carried out in the fourth week of the administration period and in the second week of the recovery period. Body weight was measured on days 1, 4, 7, 14, 21 and 28 of the administration period and on days 7 and 14 of the recovery period in the MWCNT study, and on days 1, 4, 7, 10, 14, 17, 21, 24 and 28 of the administration period and on days 1, 3, 7, 10 and 14 of the recovery period. Food consumption was measured on days 1, 7, 14, 21 and 28 of the administration period, and on days 7 and 14 of the recovery period. Hematological examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Clinical chemistry examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Necropsy was performed under anesthesia on the day following the end of the administration or recovery period. The external surfaces of rats were examined and a gross internal examination was performed. Organ weights were measured and histopathological evaluations were performed on the organs. Urinary samples were collected for 3 or 4, and 20 hr in the fourth week of the administration period, and in the second week of the recovery period. Urine volume was calculated and a urinary examination was conducted.

Data analysis

For the SWCNT study, continuous data from the administration period were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, data were analyzed by the Dunnett test, whereas heterogeneous data were analyzed by the Dunnett-type mean rank test between the control group and individual treatment groups. For the recovery group data, homogeneity of variance was tested by the F-test. When homogeneity was recognized, the difference in mean values between the control group and treatment groups was analyzed by a Student's t-test, whereas heterogeneous data were analyzed by an Aspin-Welch's t-test.

For the MWCNT study, continuous data were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, the Dunnett test was conducted for comparison between the control group and individual treatment groups after a one-way layout analysis of variance. If not homogenous, the data were analyzed using the Kruskal-Wallis test followed by a Mann-Whitney's U-test. Qualitative data were analyzed by the Kruskal-Wallis test followed by Mann-Whitney's U-test.

RESULTS AND DISCUSSION

Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects on the clinical observation and body weight. Thus, the oral LD_{50} values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw in rats, respectively (data not shown).

A 28-day dose of SWCNT caused no death in both sexes. There were no differences in the clinical observation, detailed clinical observation, body weight and food consumption (Table 1) or histopathological examination (Table 2). In the functional examination, significantly low values of landing foot splay were observed in all the treatment groups in males at the end of the administration period (90 ± 11, 61 ± 15, 64 ± 18 and 75 ± 10 mm at 0, 0.125, 1.25 and 12.5 mg/kg bw/day, respectively). However, it was due to the high value in the control group and was not considered to be toxicological effects. In urinalysis, a significantly low urine volume was observed during the administration period in females at 1.25 mg/kg bw/day and above (Table 3). However, these values were within the historical background data of the test facility (Mean ± S.D.: 8.3 ± 4.0 ml/24 hr), and these were not dose dependent. In the hematological examination, significantly high erythrocyte counts in females and lymphocyte counts and basophil counts in males were observed at 12.5 mg/kg bw/day at the end of the administration period (Table 4). However, these changes were considered to be incidental because there were no changes in related parameters. In the serum biochemistry examination, significantly high alanine aminotransferase and triglyceride levels were observed in females in the 0.125 mg/kg bw/day group but not in the high dose groups at

Table 1. Body weight and food consumption in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight (g)								
Day 1	216 ± 7	212 ± 6	214 ± 8	214 ± 8	162 ± 5	165 ± 6	160 ± 7	161 ± 7
Day 4	243 ± 8	238 ± 8	241 ± 11	241 ± 10	174 ± 8	175 ± 5	172 ± 8	172 ± 7
Day 7	274 ± 12	267 ± 9	273 ± 16	270 ± 14	187 ± 9	186 ± 6	181 ± 9	184 ± 8
Day 14	335 ± 18	322 ± 16	337 ± 29	331 ± 20	211 ± 14	210 ± 10	198 ± 12	209 ± 10
Day 21	390 ± 26	368 ± 27	388 ± 34	378 ± 24	231 ± 18	234 ± 11	213 ± 17	227 ± 15
Day 28	426 ± 31	399 ± 34	426 ± 39	413 ± 30	250 ± 22	246 ± 15	227 ± 18	245 ± 17
Food consumption (g/rat/day)								
Day 0 - 1	25 ± 2	24 ± 1	25 ± 2	25 ± 1	18 ± 2	20 ± 1	18 ± 2	19 ± 2
Day 1 - 7	27 ± 2	26 ± 1	26 ± 2	26 ± 1	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 7 - 14	29 ± 3	28 ± 2	29 ± 3	29 ± 2	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 14 - 21	29 ± 3	28 ± 3	29 ± 3	29 ± 2	20 ± 2	20 ± 2	18 ± 1	19 ± 2
Day 21 - 28	29 ± 4	27 ± 3	29 ± 2	28 ± 2	20 ± 3	20 ± 2	18 ± 1	20 ± 2

Table 2. Histopathological findings of rats dosed with SWCNT by gavage for 28 days

Sex		Male				Female			
Dose (mg/kg bw/day)		0	0.125	1.25	12.5	0	0.125	1.25	12.5
	Grade								
Number of animals examined [a]		5	0	0	5	5	5	5	5
Kidney									
Tubular regeneration	+	2	-	-	1	0	1	0	0
Liver									
Vacuolation of midzonal hepatocyte	+	0	-	-	1	0	-	-	0
Vacuolation of periportal hepatocyte	+	0	-	-	0	2	-	-	3
	++	0	-	-	0	1	-	-	0
Focal necrosis	+	1	-	-	0	0	-	-	1
Microgranuloma	+	5	-	-	3	5	-	-	5
Lung (bronchus)									
Focal hemorrhage	+	1	-	-	1	0	-	-	0
Cell infiltration	+	0	-	-	1	0	-	-	0
Accumulation of foamy cell	+	0	-	-	1	0	-	-	0
Spleen									
Lymphoid hyperplasia	++	1	-	-	0	0	-	-	0

Grade; +: slight change; ++: mild change, -: Not applicable

[a] :The histopathological examination was carried out in 5 animals at 0 and 12.5 mg/kg bw/day and the kidney was examined in 5 females at 0.125 and 12.5 mg/kg bw/day.

Table 3. Water intake and urinalysis in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Water intake (ml/24 hr)	35 ± 6	30 ± 5	36 ± 4	35 ± 6	30 ± 8	31 ± 13	25 ± 5	26 ± 4
Urine volume (ml/24 hr)	17.7 ± 4.7	14.2 ± 2.7	16.7 ± 5	15.3 ± 3.6	12.8 ± 4.5	8.1 ± 1.5	5.5 ± 2.3**	8.1 ± 3.1*
Osmolality (mOsm/kg)	1696 ± 407	1710 ± 455	1459 ± 208	1758 ± 352	1874 ± 507	2046 ± 239	2296 ± 463	2104 ± 394

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

**: Significantly different from the control group ($p < 0.01$).

Table 4. Hematology in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
RBC ($\times 10^4$/μl)	841 ± 32	845 ± 30	819 ± 26	816 ± 83	810 ± 43	841 ± 13	858 ± 18	859 ± 34*
Hemoglobin (g/dl)	15.5 ± 0.6	15.6 ± 0.4	15.7 ± 0.6	15.2 ± 1.6	15.4 ± 0.7	15.9 ± 0.4	15.9 ± 0.4	16.2 ± 0.7
Hematocrit (%)	45.7 ± 1.4	45.5 ± 1.3	46 ± 1.8	44.8 ± 4.7	43.6 ± 2.3	45.5 ± 1	45.4 ± 1.1	46.3 ± 1.9
MCV (fl)	54.3 ± 1.3	53.8 ± 0.9	56.1 ± 1	54.9 ± 2	53.9 ± 2.5	54.1 ± 1.2	52.9 ± 0.9	53.9 ± 1.3
MCH (pg)	18.5 ± 0.5	18.5 ± 0.4	19.2 ± 0.3	18.6 ± 0.8	19 ± 0.8	19 ± 0.5	18.5 ± 0.4	18.8 ± 0.4
MCHC (g/dl)	34 ± 0.3	34.4 ± 0.1	34.2 ± 0.2	34 ± 0.2	35.3 ± 0.3	35 ± 0.3	35 ± 0.4	34.9 ± 0.1
Reticulocyte (%)	2.4 ± 0.4	1.9 ± 0.5	2.6 ± 0.3	2.8 ± 1.5	1.8 ± 0.3	1.5 ± 0.2	1.5 ± 0.1	1.7 ± 0.4
WBC ($\times 10^2$/μl)	89.1 ± 14.0	110.5 ± 24.2	105.5 ± 23.7	123.8 ± 34.8	70 ± 22.4	67.8 ± 11.4	81.0 ± 30.8	64.1 ± 10.9
Differential leukocyte counts ($\times 10^2$/μl)								
Lymphocyte	70.6 ± 9.7	86 ± 14.8	76.7 ± 13.6	99.9 ± 25.2*	52.4 ± 18	53.3 ± 13.7	64.1 ± 31.2	48.9 ± 11.3
Neutrophil	14.8 ± 3.2	20.1 ± 8.9	24.6 ± 16.3	19.2 ± 8.2	14.7 ± 3.4	11.8 ± 2.8	14.1 ± 5.4	12.7 ± 4.7
Eosinophil	1.0 ± 0.8	1.1 ± 0.7	0.9 ± 0.1	0.9 ± 0.4	1.0 ± 0.7	0.9 ± 0.2	0.9 ± 0.4	1.0 ± 0.3
Basophil	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.3*	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1
Monocyte	1.9 ± 0.8	2.3 ± 1.5	2.4 ± 1	2.6 ± 1.8	1.3 ± 0.7	1.3 ± 0.4	1.2 ± 0.5	1.2 ± 0.3

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.
*: Significantly different from the control group ($p < 0.05$).

Table 5. Serum biochemistry in rats dosed with SWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
AST (IU/l)	68 ± 4	68 ± 5	62 ± 4	64 ± 6	60 ± 7	86 ± 23	79 ± 29	72 ± 11
ALT (IU/l)	28 ± 5	30 ± 4	25 ± 3	28 ± 7	19 ± 2	34 ± 10**	27 ± 10	22 ± 1
LDH (IU/l)	54 ± 8	53 ± 7	52 ± 5	54 ± 6	52 ± 3	63 ± 16	57 ± 12	56 ± 9
γ-GTP (IU/l)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 0
ALP (IU/l)	647 ± 198	686 ± 82	655 ± 73	663 ± 84	415 ± 127	359 ± 102	378 ± 34	470 ± 102
T. bile acid (μmol/l)	15.1 ± 12.7	12.5 ± 4.9	10.5 ± 7.5	7.6 ± 2.9	12.2 ± 2.3	15.4 ± 4.9	11.3 ± 3.1	11.6 ± 6.3
T. cholesterol (mg/dl)	50 ± 5	48 ± 14	52 ± 7	50 ± 6	55 ± 7	57 ± 14	66 ± 16	50 ± 17
Triglyceride (mg/dl)	56 ± 19	55 ± 22	50 ± 18	59 ± 24	14 ± 3	29 ± 12*	21 ± 11	14 ± 6
Phospholipid (mg/dl)	93 ± 8	92 ± 14	94 ± 10	93 ± 10	100 ± 11	108 ± 18	123 ± 20	95 ± 26
T. bilirubin (mg/dl)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
Glucose (mg/dl)	126 ± 13	121 ± 5	135 ± 18	141 ± 12	113 ± 11	106 ± 11	122 ± 8	123 ± 23
BUN (mg/dl)	13 ± 2	14 ± 3	12 ± 1	14 ± 2	16 ± 2	16 ± 2	15 ± 2	16 ± 2
Creatinine (mg/dl)	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.03	0.27 ± 0.02	0.26 ± 0.04	0.27 ± 0.02	0.28 ± 0.01
Sodium (mmol/l)	144 ± 1	144 ± 1	144 ± 1	143 ± 1	144 ± 1	143 ± 1	143 ± 1	143 ± 2
Potassium (mmol/l)	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.7 ± 0.2	4.3 ± 0.2	4.4 ± 0.3	4.2 ± 0.2	4.2 ± 0.2
Chloride (mmol/l)	105 ± 1	106 ± 1	105 ± 1	106 ± 1	107 ± 1	107 ± 1	107 ± 2	107 ± 1
Calcium (mg/dl)	10 ± 0.2	9.8 ± 0.2	10.2 ± 0.3	9.8 ± 0.2	10 ± 0.3	10 ± 0.1	9.9 ± 0.1	10 ± 0.1
I. phosphorus (mg/dl)	7.8 ± 0.3	7.5 ± 0.4	7.9 ± 0.5	7.5 ± 0.6	7 ± 0.3	7 ± 0.6	6.9 ± 0.9	6.9 ± 0.8
T. protein (g/dl)	5.8 ± 0.1	6 ± 0.1	6 ± 0.1	5.9 ± 0.2	6.4 ± 0.3	6.2 ± 0	6.2 ± 0.2	6.1 ± 0.1
Albumin (g/dl)	3.2 ± 0	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.6 ± 0.2	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
Albumin/Globlin	1.21 ± 0.09	1.14 ± 0.07	1.15 ± 0.08	1.23 ± 0.06	1.31 ± 0.12	1.31 ± 0.09	1.43 ± 0.08	1.35 ± 0.07

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: lactate dehydrogenase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen

Table 6. Absolute and relative organ weight in rats dosed with SWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight [a]	406 ± 22	371 ± 33	390 ± 34	379 ± 21	232 ± 10	227 ± 13	212 ± 18	225 ± 16
Pituitary (mg) [b]	12.3 ± 1.5	11.3 ± 2	11.7 ± 1.2	10.6 ± 0.6	14.9 ± 1.1	12.5 ± 2	12.6 ± 1.9	13.2 ± 2.4
(mg%) [c]	3 ± 0.3	3.1 ± 0.5	3 ± 0.4	2.8 ± 0.2	6.4 ± 0.4	5.5 ± 0.7	6 ± 0.9	5.8 ± 0.7
Thyroid (mg)	21.9 ± 4.8	18 ± 2.4	20.2 ± 2.8	17.6 ± 2.1	14.2 ± 0.8	15.2 ± 2.5	13.2 ± 2.5	14.5 ± 1.7
(mg%)	5.4 ± 0.9	4.9 ± 0.6	5.2 ± 0.7	4.7 ± 0.4	6.1 ± 0.5	6.7 ± 1	6.3 ± 1.3	6.4 ± 0.6
Thymus (mg)	619 ± 131	538 ± 123	630 ± 96	541 ± 79	484 ± 45	480 ± 62	439 ± 112	450 ± 85
(mg%)	152 ± 25	144 ± 24	161 ± 12	143 ± 18	208 ± 13	212 ± 32	206 ± 36	199 ± 28
Heart (g)	1.34 ± 0.08	1.22 ± 0.11	1.32 ± 0.16	1.28 ± 0.11	0.82 ± 0.07	0.88 ± 0.06	0.78 ± 0.08	0.83 ± 0.08
(g%)	0.33 ± 0.01	0.33 ± 0.02	0.34 ± 0.03	0.34 ± 0.01	0.35 ± 0.02	0.39 ± 0.02*	0.37 ± 0.02	0.37 ± 0.02
Liver (g)	12.04 ± 1.17	11 ± 1.22	12.03 ± 1.54	11.52 ± 1.16	6.57 ± 0.43	6.59 ± 0.54	6.06 ± 0.66	6.42 ± 0.89
(g%)	2.97 ± 0.16	2.96 ± 0.16	3.08 ± 0.14	3.03 ± 0.15	2.83 ± 0.12	2.9 ± 0.13	2.86 ± 0.18	2.84 ± 0.19
Spleen (g)	0.81 ± 0.2	0.71 ± 0.12	0.71 ± 0.1	0.64 ± 0.01*	0.5 ± 0.03	0.48 ± 0.07	0.42 ± 0.07	0.44 ± 0.06
(g%)	0.20 ± 0.04	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.01	0.22 ± 0.02	0.21 ± 0.03	0.2 ± 0.02	0.2 ± 0.02
Kidney (g)	2.9 ± 0.15	2.69 ± 0.18	2.84 ± 0.2	2.71 ± 0.1	1.81 ± 0.09	1.75 ± 0.16	1.67 ± 0.12	1.75 ± 0.14
(g%)	0.72 ± 0.03	0.73 ± 0.03	0.73 ± 0.03	0.72 ± 0.04	0.78 ± 0.03	0.77 ± 0.03	0.79 ± 0.03	0.78 ± 0.02
Adrenal (mg)	72 ± 12	59 ± 14	67 ± 9	62 ± 8	69 ± 11	61 ± 8	62 ± 7	71 ± 12
(mg%)	18 ± 3	16 ± 3	17 ± 3	16 ± 2	30 ± 4	27 ± 2	29 ± 2	32 ± 7
Testis (g)	3.35 ± 0.33	3.37 ± 0.33	3.29 ± 0.24	3.05 ± 0.42				
(g%)	0.82 ± 0.04	0.91 ± 0.08	0.85 ± 0.07	0.81 ± 0.11				
Epididymis (mg)	907 ± 66	911 ± 50	863 ± 67	809 ± 123				
(mg%)	224 ± 6	247 ± 22	223 ± 27	214 ± 32				
Ovary (mg)					87.4 ± 8.6	84.9 ± 4.5	81.3 ± 12.3	91.9 ± 11.6
(mg%)					37.7 ± 4.1	37.4 ± 1.6	38.5 ± 5.5	41.1 ± 6.7
Uterus (mg)					470 ± 143	334 ± 52	460 ± 107	407 ± 84
(mg%)					202 ± 56	148 ± 25	218 ± 45	180 ± 30

*: Significantly different from the control group ($p < 0.05$).
[a]: Values are given as the mean ± S.D.
[b]: Absolute organ weight.
[c]: Relative organ weight.

the end of the administration period (Table 5). In organ weight measurements, a significantly low absolute weight of the spleen was observed in males in the 12.5 mg/kg group at the end of the administration period (Table 6). However, there were no changes in the relative weight and histopathological examination of the spleen. A significantly high relative weight of the heart was observed in females in the 0.125 mg/kg group but not in the high dose groups at the end of the administration period. At necropsy, a dark red focus in the lung was observed sporadically in the control group and 12.5 mg/kg bw/day group at the end of the administration and recovery periods. At the histopathological examination, focal hemorrhage in the lung was observed in the animals showed a dark red focus. Blackish contents were observed in the cecum of animals in the treatment groups at the end of administration period. However, the histopathological examination revealed no effects in these organs. The contents in the intestines were not observed after the recovery period. Significant differences noted at the end of administration period all disappeared by the end of the recovery period.

A 28-day dose of MWCNT caused no death in both sexes. Black feces were observed in both sexes in all the treatment groups. No effects were found on detailed clinical observation, functional examination, body weight and food consumption (Table 7), organ weights, urinalysis and histopathology (Table 8). In the hematological examination, eosinophil counts were significantly low in females in the 5.0 and 50 mg/kg bw/day groups at the end of the administration period (Table 9) and in the 50 mg/ bw/day group at the end of recovery period ($1.13 \pm 0.38 \times 10^2/\mu l$ vs. $0.73 \pm 0.23 \times 10^2/\mu l$), but the changes were within the historical background data of the test facility ($0.9 \pm 0.86 \times 10^2/\mu l$). A few female animals incidentally showed high values for eosinophils in the control group, and a significant decrease in eosinophil counts was considered to be due to the high value of eosinophils in the control group. Therefore, it was not considered to be clinically

Table 7. Body weight and food consumption in rats dosed with MWCNT by gavage for 28 days

Dose	Male				Female			
(mg/kg bw/day)	0	0.5	5	50	0	0.5	5	50
Body weight (g)								
Day 1	183.7 ± 11.1	185.7 ± 9.7	186.7 ± 11.4	186.8 ± 8.4	149.7 ± 8.6	150.2 ± 10.8	150.5 ± 10.3	149.0 ± 10.3
Day 4	212.9 ± 13.3	213.2 ± 10.9	215.5 ± 14.0	215.0 ± 10.9	161.7 ± 9.9	161.8 ± 9.4	160.8 ± 10.7	159.1 ± 10.8
Day 7	242.0 ± 15.0	242.0 ± 14.4	244.5 ± 13.8	241.5 ± 13.0	172.0 ± 11.9	172.7 ± 15.4	172.0 ± 15.9	168.7 ± 13.3
Day 14	305.7 ± 18.5	304.5 ± 16.8	308.3 ± 17.5	305.3 ± 17.3	197.4 ± 12.3	193.0 ± 19.8	198.0 ± 19.9	194.0 ± 17.7
Day 21	362.4 ± 20.0	359.5 ± 17.2	367.2 ± 23.3	359.7 ± 24.1	219.7 ± 16.1	215.2 ± 22.1	218.8 ± 22.0	213.2 ± 19.9
Day 28	399.7 ± 24.1	392.2 ± 21.8	401.8 ± 30.0	394.5 ± 27.2	237.8 ± 18.7	227.7 ± 17.3	236.8 ± 23.2	230.4 ± 22.2
Food consumption (g/rat/day)								
Day 0 - 1	22.42 ± 2.02	22.00 ± 2.37	22.00 ± 1.67	22.58 ± 1.56	16.42 ± 2.19	16.83 ± 2.64	17.00 ± 1.26	17.50 ± 2.39
Day 1 - 4	23.33 ± 1.49	23.33 ± 1.45	23.90 ± 1.62	23.33 ± 1.12	16.76 ± 1.17	16.38 ± 0.98	16.17 ± 1.60	16.48 ± 1.68
Day 4 - 7	25.36 ± 1.75	24.95 ± 2.02	24.98 ± 1.55	24.65 ± 1.17	16.96 ± 1.39	16.77 ± 1.85	17.00 ± 2.31	17.10 ± 1.86
Day 7 - 14	26.94 ± 1.92	26.20 ± 1.62	26.53 ± 1.99	26.14 ± 1.18	18.27 ± 1.47	17.22 ± 1.02	17.92 ± 2.22	17.74 ± 1.82
Day 14 - 21	28.98 ± 1.42	28.25 ± 1.47	28.33 ± 3.34	27.69 ± 1.44	19.31 ± 1.96	18.50 ± 1.52	18.83 ± 2.33	18.62 ± 2.30
Day 21 - 28	29.09 ± 1.89	27.62 ± 2.14	28.18 ± 2.94	28.03 ± 2.26	19.66 ± 1.94	18.62 ± 1.86	19.20 ± 2.66	19.32 ± 2.51

Table 8. Histopathological findings of rats dosed with MWCNT by gavage for 28 days

Sex		Male				Female			
Dose (mg/kg bw/day)	Grade	0	0.5	5	50	0	0.5	5	50
Number of animals examined [a]		6	0	1	6	6	0	0	6
Lung	+								
Aggregation of alveolar macrophage	+	1	-	-	0	0	-	-	0
Mineralization of artery	+	0	-	-	0	1	-	-	0
Pancreas									
Atrophy of focal acinar cell	+	1	-	-	0	0	-	-	0
Ileum									
Diverticulum	+	0	-	1	0	1	-	-	0
Liver									
Microgranuloma	+	3	-	-	2	4	-	-	2
Kidney									
Tubular epithelium regeneration	+	2	-	-	1	0	-	-	0
Hyaline droplet in proximal tubular epithelium	+	0	-	-	1	0	-	-	0
Eosinophilic body in proximal tubular epithelium	+	0	-	-	1	0	-	-	0
Focal inflammatory cell infiltration in cortex	+	0	-	-	0	0	-	-	1

Grade; +: slight change. -: Not applicable.

[a] :The histopathological examination was carried out in 6 animals at 0 and 50 mg/kg bw/day and in animals which showed gross findings at 0.5 and 5 mg/kg bw/day.

or toxicologically important. Table 10 shows the results of serum biochemistry in rats dosed MWCNT. A significantly low γ-globulin fraction in females in the 50 mg/kg group was not accompanied by significant differences in other fractions, and no changes were noted in related parameters such as white blood cell counts. This was also considered to be due to a high value in the control group. Gamma-GTP in males in the 0.5 mg/kg group was significantly lower than that in the control group, but it was not dose-dependent. These changes in serum biochemistry were not observed at the end of the recovery period. Necropsy at the end of the administration period revealed swelling of the submandibular lymph node in one male in the 0.5 mg/kg group. This change was not dose-dependent and was not considered to be toxicologically important. Grayish green/dark green contents in the cecum, colon and/or rectum were observed in both sexes at 5.0 mg/kg bw/day and higher. These contents in the intes-

Table 9. Hematology in rats dosed with MWCNT by gavage for 28 days

Dose	Male				Female			
(mg/kg bw/day)	0	0.5	5	50	0	0.5	5	50
RBC ($\times 10^4/\mu l$)	878.8 ± 39.3	865.2 ± 27.7	862.8 ± 18.9	878.7 ± 35.5	879.3 ± 30.3	866.8 ± 48.2	855.8 ± 15.6	864.5 ± 24.0
Hemoglobin (g/dl)	17.20 ± 0.45	16.97 ± 0.56	17.12 ± 0.33	17.08 ± 0.28	16.77 ± 0.45	16.90 ± 0.82	16.50 ± 0.24	16.58 ± 0.43
Hematocrit (%)	48.57 ± 0.88	48.00 ± 1.56	48.72 ± 0.81	47.95 ± 1.09	46.13 ± 0.94	46.77 ± 1.94	45.98 ± 0.79	45.80 ± 1.45
MCV (fl)	55.33 ± 1.73	55.52 ± 1.89	56.47 ± 0.87	54.62 ± 1.86	52.48 ± 1.39	54.05 ± 2.16	53.73 ± 0.92	52.98 ± 1.12
MCH (pg)	19.58 ± 0.47	19.60 ± 0.41	19.85 ± 0.18	19.45 ± 0.68	19.07 ± 0.54	19.50 ± 0.68	19.30 ± 0.39	19.20 ± 0.37
MCHC (g/dl)	35.42 ± 0.40	35.35 ± 0.58	35.13 ± 0.30	35.63 ± 0.24	36.35 ± 0.38	36.12 ± 0.33	35.88 ± 0.40	36.22 ± 0.37
Reticulocyte (%)	3.683 ± 0.405	3.603 ± 0.448	3.545 ± 0.534	3.445 ± 0.434	2.907 ± 0.730	3.265 ± 0.431	3.013 ± 0.557	2.875 ± 0.644
WBC ($10^2/\mu l$)	128.57 ± 38.56	116.95 ± 15.35	116.48 ± 29.80	129.37 ± 27.96	91.07 ± 12.67	109.07 ± 27.58	88.83 ± 26.32	79.32 ± 18.52
Differential leukocyte counts ($\times 10^2/\mu l$)								
Lymphocyte	111.07 ± 35.91	100.13 ± 12.75	102.98 ± 27.80	111.30 ± 23.02	78.73 ± 11.18	95.97 ± 27.81	78.45 ± 26.20	68.18 ± 18.27
Neutrophil	12.63 ± 3.89	11.82 ± 2.65	9.95 ± 2.83	13.88 ± 5.17	9.32 ± 3.07	10.58 ± 4.12	8.48 ± 2.78	8.88 ± 4.68
Eosinophil	1.43 ± 1.02	1.67 ± 0.31	1.18 ± 0.64	1.07 ± 0.39	1.68 ± 0.89	1.08 ± 0.41	0.87 ± 0.33*	0.88 ± 0.34*
Basophil	0.07 ± 0.05	0.00 ± 0.00	0.03 ± 0.05	0.02 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.00 ± 0.00
Monocyte	3.37 ± 0.81	3.33 ± 0.76	2.33 ± 1.00	3.10 ± 0.88	1.33 ± 0.62	1.43 ± 0.48	1.02 ± 0.52	1.37 ± 1.18

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.
*: Significantly different from the control group ($p < 0.05$).

Table 10. Serum biochemistry in rats dosed with MWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.5	5	50	0	0.5	5	50
T. protein (g/dl)	5.67 ± 0.18	5.65 ± 0.12	5.57 ± 0.23	5.70 ± 0.28	5.80 ± 0.32	5.65 ± 0.23	5.87 ± 0.21	5.75 ± 0.16
Glucose (mg/dl)	164.7 ± 8.2	162.2 ± 18.9	168.3 ± 16.8	173.5 ± 13.9	137.3 ± 17.3	134.5 ± 20.1	136.5 ± 13.4	138.8 ± 18.6
Triglyceride (mg/dl)	50.7 ± 11.5	48.7 ± 18.3	39.7 ± 13.5	44.7 ± 9.5	10.0 ± 2.5	11.2 ± 3.7	10.7 ± 4.1	11.2 ± 5.0
T. cholesterol (mg/dl)	59.5 ± 12.1	51.7 ± 9.3	56.8 ± 11.4	56.0 ± 10.1	56.0 ± 5.8	56.7 ± 9.0	60.8 ± 7.7	62.8 ± 13.7
BUN (mg/dl)	14.42 ± 2.12	13.53 ± 2.22	14.32 ± 1.57	15.22 ± 2.61	16.02 ± 1.36	17.22 ± 2.80	16.08 ± 4.45	15.77 ± 4.60
Creatinine (mg/dl)	0.497 ± 0.027	0.483 ± 0.023	0.502 ± 0.035	0.502 ± 0.022	0.505 ± 0.037	0.513 ± 0.043	0.507 ± 0.058	0.508 ± 0.045
T. bilirubin (mg/dl)	0.047 ± 0.008	0.038 ± 0.008	0.043 ± 0.008	0.040 ± 0.009	0.055 ± 0.012	0.063 ± 0.012	0.060 ± 0.021	0.057 ± 0.008
AST (IU/l)	67.3 ± 7.4	64.2 ± 7.7	66.3 ± 4.2	67.7 ± 10.0	69.3 ± 5.2	65.0 ± 4.7	61.5 ± 5.8	69.7 ± 11.6
ALT (IU/l)	26.2 ± 3.2	26.2 ± 2.6	25.7 ± 2.6	25.2 ± 2.6	20.3 ± 2.9	22.7 ± 2.4	21.2 ± 3.4	22.2 ± 4.4
ALP (IU/l)	688.8 ± 46.3	693.3 ± 122.5	614.2 ± 112.8	700.7 ± 171.2	423.8 ± 44.8	412.8 ± 91.3	333.2 ± 65.3	398.7 ± 138.4
γ-GTP (IU/l)	0.85 ± 0.21	0.62 ± 0.12*	0.68 ± 0.12	0.70 ± 0.14	1.22 ± 0.19	1.18 ± 0.12	0.95 ± 0.20	1.20 ± 0.27
Calcium (mg/dl)	10.18 ± 0.30	9.90 ± 0.14	9.93 ± 0.21	10.07 ± 0.27	9.88 ± 0.08	9.87 ± 0.46	10.00 ± 0.39	9.78 ± 0.43
I. phosphorus (mg/dl)	8.78 ± 0.49	8.67 ± 0.22	8.53 ± 0.48	8.77 ± 0.36	8.03 ± 0.42	8.02 ± 0.67	7.93 ± 0.38	7.70 ± 0.91
Sodium (mEq/l)	143.2 ± 1.2	143.2 ± 0.8	143.3 ± 0.8	143.8 ± 1.0	144.0 ± 1.4	143.5 ± 1.9	143.8 ± 1.5	144.3 ± 0.8
Potassium (mEq/l)	4.873 ± 0.268	4.923 ± 0.265	4.858 ± 0.117	4.762 ± 0.331	4.593 ± 0.372	5.108 ± 0.420	4.862 ± 0.351	4.755 ± 0.500
Chloride (mEq/l)	104.3 ± 1.9	105.0 ± 0.6	105.2 ± 1.2	105.0 ± 0.9	107.3 ± 2.1	107.2 ± 1.9	106.8 ± 1.9	106.7 ± 1.6
Albumin (%)	51.03 ± 2.11	51.93 ± 1.67	52.93 ± 1.25	53.50 ± 2.68	54.83 ± 2.31	55.53 ± 1.87	55.65 ± 1.94	55.88 ± 1.40
Alpha-1 globlin (%)	22.93 ± 3.10	23.35 ± 1.51	21.20 ± 2.81	21.47 ± 3.08	17.60 ± 2.54	17.93 ± 2.17	19.28 ± 2.38	19.32 ± 0.65
Alpha-2 globlin (%)	7.03 ± 0.34	6.65 ± 0.45	7.10 ± 0.51	6.98 ± 0.55	6.95 ± 0.77	7.45 ± 0.29	6.40 ± 0.75	6.57 ± 0.45
Beta globlin (%)	15.48 ± 0.98	14.72 ± 1.09	15.15 ± 1.1	14.48 ± 0.44	15.27 ± 0.85	14.57 ± 0.99	14.40 ± 1.11	14.35 ± 0.9
Gamma globlin (%)	3.52 ± 0.60	3.35 ± 0.63	3.62 ± 0.95	3.57 ± 0.43	5.35 ± 1.29	4.52 ± 0.76	4.27 ± 1.04	3.88 ± 0.67*
Albumin/Globlin	1.045 ± 0.089	1.083 ± 0.074	1.123 ± 0.057	1.153 ± 0.128	1.218 ± 0.115	1.252 ± 0.091	1.257 ± 0.101	1.268 ± 0.070

BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

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

tines were not observed after the recovery period. Based on the absence of toxicological effects, the no observed adverse effect levels (NOAELs) of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day in rats (the highest dose tested), respectively.

Our findings showed no toxicological effects of orally administered CNTs, but available data show CNTs, under some conditions, may induce harmful effects. Both SWCNTs and MWCNTs showed the capacity to induce toxicity such as inflammation and fibrosis in the lung when administered by pharyngeal aspiration, intratracheal instillation or inhalation (Shvedova *et al.*, 2008; Erdely *et al.*, 2009; Inoue *et al.*, 2008; Pauluhn, 2010; Warheit *et al.*, 2004; Lam *et al.*, 2004; Han *et al.*, 2008). The carcinogenic potential of intraperitoneally administered MWCNT was observed in p53 heterozygous mice (Takagi *et al.*, 2008) and intrascrotal injection of MWCNT in intact Fischer 344 rats (Sakamoto *et al.*, 2009). The cytotoxic potential of CNTs was also observed in skin and lung cells *in vitro* (Jia *et al.*, 2005; Monteiro-Riviere *et al.*, 2005) although cytotoxicity can be influenced by various factors such as material impurities, length and size distribution, surface area, and so on (Hussain *et al.*, 2009). Intravenously injected CNTs increased oxidative stress markers in the lung and liver in mice (Yang *et al.*, 2008) or induced slight hepatotoxicity by inflammation and oxidative damage in mice (Ji *et al.*, 2009).

Deng *et al.* (2007) showed that intratracheally dosed taurine-functionalized MWCNT accumulated mainly in the lung and remained there until 28 days following administration whereas an oral gavage dose led to distribution in the small/large intestines and stomach, and about 74% was excreted in the feces after 12 hr. Intravenously injected taurine-functionalized MWCNT accumulated and remained until 28 days following administration in the liver, lung and spleen. The authors of this study suggested that taurine-functionalized-MWCNT cannot enter the blood circulation or be absorbed by the intestine tracts, and that routes of exposure influence the distribution of CNTs although the distribution pattern was for functionalized MWCNT. The findings of a study by Deng *et al.* (2007) together with our study suggested that SWCNTs and MWCNTs dosed by gavage reached the gastro-intestinal tract as agglomerates and were rapidly excreted via feces in rats.

However, there is a study in which rats were dosed with SWCNT by gavage once at 0.64 mg/kg bw, and the levels of oxidatively damaged DNA increased in the liver and lung tissue (Folkmann *et al.*, 2009). Furthermore, significant increases in the number of resorptions, and fetal morphological and skeletal abnormalities, were observed in fetuses of CD-1 mouse dams that were administered functionalized CNTs by gavage at 10 mg/kg bw/day on day 9 of gestation (Philbrook *et al.*, 2011). Surface functionalization increases solubility of CNTs, and therefore absorbability of CNTs could have been increased in this study. However, these two studies suggested that CNTs could be absorbed from the gastro-intestinal tract into the blood circulation.

The findings of a study by Philbrook *et al.* (2011) are in discord from a study by Lim *et al.* (2011), in which pregnant SD rats were given MWCNTs by gavage at 0-1,000 mg/kg bw/day on days 6-19 of gestation, and no adverse effects were found in fetuses. Although controversial findings were observed in oral dose studies, an intravenous study obviously showed the teratogenic potential of CNTs (Pietroiusti *et al.*, 2011). Pregnant CD-1 mice were intravenously injected with SWCNT, oxidized-SWCNT and ultra oxidized-SWCNT at 0-30 μg/animal on day 5.5 of gestation. Increases in incidence of early miscarriages and fetal malformations were observed in all treatment groups at 0.1 μg/animal and higher. This study suggested that low-dose SWCNTs may induce fetal malformations. Our study group will undertake further teratogenic studies to confirm whether CNTs cause developmental toxicity.

In conclusion, rats were dosed with SWCNT or MWCNT once daily by gavage for 28 days with a 14-day recovery period, and no toxicological effects were found up to the highest dose tested. However, the possibility that CNTs dosed by oral administration are absorbed from the gastro-intestinal tract and persistent in the body for the long term cannot be ruled out, and a further study for chronic oral toxicity will be helpful to confirm the safety of CNTs. Because of the small size of CNTs, particles may spread over the entire body, and causing adverse effects like observed in intravenous studies, once absorbed.

ACKNOWLEDGMENTS

This study was undertaken under the safety programmes for existing chemicals funded by the Ministry of Health, Labour and Welfare (MHLW), Japan, and was supported by a Health and Labour Sciences Research Grant (Research on Risk of Chemical Substances: H21-Chemistry-Ippan-008) from the MHLW, Japan. The test materials were kindly provided from the National Institute of Advanced Industrial Science and Technology (AIST), Japan.

REFERENCES

Deng, X., Jia, G., Wang, H., Sun, H., Wang, X., Yang, S., Wang, T. and Liu, Y. (2007): Translocation and fate of multi-walled carbon nanotubes in vivo. Carbon, **45**, 1419-1424.

Elgrabli, D., Abella-Gallart, S., Robidel, F., Rogerieux, F., Boczkowski, J. and Lacroix, G. (2008): Induction of apoptosis and absence of inflammation in rat lung after intratracheal instillation of multiwalled carbon nanotubes. Toxicology, **253**, 131-136.

Erdely, A., Hulderman, T., Salmen, R., Liston, A., Zeidler-Erdely, P.C., Schwegler-Berry, D., Castranova, V., Koyama, S., Kim, Y.A., Endo, M. and Simeonova, P.P. (2009): Cross-talk between lung and systemic circulation during carbon nanotube respiratory exposure. Potential biomarkers. Nano Lett., **9**, 36-43.

Folkmann, J.K., Risom, L., Jacobsen, N.R., Wallin, H., Loft, S. and Moller, P. (2009): Oxidatively damaged DNA in rats exposed by oral gavage to C60 fullerenes and single-walled carbon nanotubes. Environ. Health. Perspect., **117**, 703-708.

Han, S.G., Andrews, R., Gairola, C.G. and Bhalla, D.K. (2008): Acute pulmonary effects of combined exposure to carbon nanotubes and ozone in mice. Inhal. Toxicol., **20**, 391-398.

Hussain, M.A., Kabir, M.A. and Soood, A.K. (2009): On the cytotoxicity of carbon nanotubes. Current Science, **96**, 664-673.

Inoue, K., Koike, E., Yanagisawa, R., Hirano, S., Nishikawa, M. and Takano, H. (2009): Effects of multi-walled carbon nanotubes on a murine allergic airway inflammation model. Toxicol. Appl. Pharmacol., **237**, 306-316.

Inoue, K., Takano, H., Koike, E., Yanagisawa, R., Sakurai, M., Tasaka, S., Ishizaka, A. and Shimada, A. (2008): Effects of pulmonary exposure to carbon nanotubes on lung and systemic inflammation with coagulatory disturbance induced by lipopolysaccharide in mice. Exp. Biol. Med. (Maywood), **233**, 1583-1590.

Ji, Z., Zhang, D., Li, L., Shen, X., Deng, X., Dong, L., Wu, M. and Liu, Y. (2009): The hepatotoxicity of multi-walled carbon nanotubes in mice. Nanotechnology, **20**, 445101.

Jia, G., Wang, H., Yan, L., Wang, X., Pei, R., Yan, T., Zhao, Y. and Guo, X. (2005): Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene. Environ. Sci. Technol., **39**, 1378-1383.

Kobayashi, N., Ogura, I., Gamo, M., Kishimoto, A. and Nakanishi, J. (2009): Risk Assessment of Manufactured Nanomaterials -Carbon Nanotubes (CNTs)- Interim Report issued on October 16, 2009 Exective Summary. http://goodnanoguide.org/tiki-download_wiki_attachment.php?attId=31

Lam, C.W., James, J.T., McCluskey, R. and Hunter, R.L. (2004): Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol. Sci., **77**, 126-134.

Lim, J.H., Kim, S.H., Shin, I.S., Park, N.H., Moon, C., Kang, S.S., Kim, S.H., Park, S.C. and Kim, J.C. (2011): Maternal exposure to multi-wall carbon nanotubes does not induce embryo–fetal developmental toxicity in rats. Birth. Defects. Res. B Dev. Reprod. Toxicol., **92**, 69-76.

Magnuson, B.A., Jonaitis, T.S. and Card, J.W. (2011): A Brief Review of the occurrence, use, and safety of food-related nanomaterials. J. Food Sci., **76**, R126-R133.

MHLW, J. (2010): The results of "An Investigative Research on Consumer's Safety in Utilizing Nanomaterials (2009)". www.nihs.go.jp/mhlw/chemical/nano/nanopdf/H21houkoku/honbun/all.pdf

Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y. and Riviere, J.E. (2005): Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicol. Lett., **155**, 377-384.

OECD (2010a): DRAFT DOSSIER DEVELOPMENT PLAN: Multi- walled Carbon Nanotubes (MWCNTs), April 28, 2010.

OECD (2010b): DRAFT DOSSIER DEVELOPMENT PLAN: Single- walled Carbon Nanotubes (SWCNTs), April 15, 2010.

OECD (2011): Sponsorship Programme for the Testing of Manufactured Nanomaterials. http://www.oecd.org/document/47/0,3746,en_2649_37015404_41197295_1_1_1_1,00.html

Pauluhn, J. (2010): Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures. Toxicol. Sci., **113**, 226-242.

Philbrook, N.A., Walker, V.K., Afrooz, A.R., Saleh, N.B. and Winn, L.M. (2011): Investigating the effects of functionalized carbon nanotubes on reproduction and development in Drosophila melanogaster and CD-1 mice. Reprod. Toxicol., **32**, 442-448

Pietroiusti, A., Massimiani, M., Fenoglio, I., Colonna, M., Valentini, F., Palleschi, G., Camaioni, A., Magrini, A., Siracusa, G., Bergamaschi, A., Sgambato, A. and Campagnolo, L. (2011): Low doses of pristine and oxidized single-wall carbon nanotubes affect mammalian embryonic development. ACS Nano, **5**, 4624-4633.

Sakamoto, Y., Dai, N., Hagiwara, Y., Satoh, K., Ohashi, N., Fukamachi, K., Tsuda, H., Hirose, A., Nishimura, T., Hino, O. and Ogata, A. (2010): Serum level of expressed in renal carcinoma (ERC)/ mesothelin in rats with mesothelial proliferative lesions induced by multi-wall carbon nanotube (MWCNT). J. Toxicol. Sci., **35**, 265-270.

Sakamoto, Y., Nakae, D., Fukumori, N., Tayama, K., Maekawa, A., Imai, K., Hirose, A., Nishimura, T., Ohashi, N. and Ogata, A. (2009): Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J. Toxicol. Sci., **34**, 65-76.

Shvedova, A.A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., Hubbs, A.F., Mercer, R.R., Keohavong, P., Sussman, N., Jin, J., Yin, J., Stone, S., Chen, B.T., Deye, G., Maynard, A., Castranova, V., Baron, P.A. and Kagan, V.E. (2008): Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. Am. J. Physiol. Lung Cell. Mol. Physiol., **295**, L552-565.

Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., Kitajima, S. and Kanno, J. (2008): Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotubes. J. Toxicol. Sci., **33**, 105-116.

Takahashi, M., Kato, H., Doi, Y., Hagiwara, A., Hirata-Koizumi, M., Ono, A., Kubota, R., Nishimura, T. and Hirose, A. (2012) Sub-acute oral toxicity study with fullerene C60 in rats. J. Toxicol. Sci., **37**, 353-61.

Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A. and Webb, T.R. (2004): Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol. Sci., **77**, 117-125.

Yang, S.T., Wang, X., Jia, G., Gu, Y., Wang, T., Nie, H., Ge, C., Wang, H. and Liu, Y. (2008): Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice. Toxicol. Let., **181**, 182-189.

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.37, No.2, 353-361, 2012

Original Article

Sub-acute oral toxicity study with fullerene C60 in rats

Mika Takahashi[1], Hina Kato[1], Yuko Doi[2], Akihiro Hagiwara[2], Mutsuko Hirata-Koizumi[1], Atsushi Ono[1], Reiji Kubota[3], Tetsuji Nishimura[3] and Akihiko Hirose[1]

[1]Division of Risk Assessment, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya, Tokyo 158-8501, Japan
[2]DIMS Institute of Medical Science, Inc., 64 Goura, Nishiazai, Azai-cho, Ichinomiya, Aichi 491-0113, Japan
[3]Division of Environmental Chemistry, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya, Tokyo 158-8501, Japan

(Received October 20, 2011; Accepted January 5, 2012)

ABSTRACT — To obtain initial information on the possible repeated-dose oral toxicity of fullerene C60, Crl:CD(SD) rats were administered fullerene C60 by gavage once daily at 0 (vehicle: corn oil), 1, 10, 100, or 1,000 mg/kg/day for 29 days, followed by a 14-day recovery period. No deaths occurred in any groups, and there were no changes from controls in detailed clinical observations, body weights, and food consumption in any treatment groups. Moreover, no treatment-related histopathological changes were found in any organs examined at the end of the administration period and at the end of the recovery period. Blackish feces and black contents of the stomach and large intestine were observed in males and females at 1,000 mg/kg/day in the treatment group. There were no changes from controls in the liver and spleen weights at the end of the administration period, but those weights in males in the 1,000 mg/kg/day group increased at the end of the recovery period. Using liquid chromatography-tandem mass spectrometry, fullerene C60 were not detected in the liver, spleen or kidney at the end of the administration period and also at the end of the recovery period. In conclusion, the present study revealed no toxicological effects of fullerene C60; however, the slight increases in liver and spleen weights after the 14-day recovery period may be because of the influence of fullerene C60 oral administration. In the future, it will be necessary to conduct a long-term examination because the effects of fullerene C60 cannot be ruled out.

Key words: Fullerene C60, Gavage, Rat, Repeated dose toxicity

INTRODUCTION

Since the publication of a paper on fullerenes in 1985 (Kroto *et al.*, 1985), the application of fullerenes has been considered due to their fascinating properties, such as substituent modification, endohedrality, and superconductivity. The production and use of fullerenes in the market is limited at present, but is expected to grow significantly (Aschberger *et al.*, 2010), and the potential of general public exposure as well as occupational exposure at manufacturing sites to pristine fullerene (fullerene C60) will increase in the future.

The main exposure routes of fullerene C60 in the occupational setting are considered to be inhalation and dermal contact. Aschberger *et al.* (2010) summarized as follows; fullerenes have low acute and sub-chronic inhalation toxicity, and as for dermal toxicity, fullerenes did not induce acute toxic effects to the skin, and no long term dermal studies were available.

In the general population, the possible exposure routes of concern include oral exposure; there is a possibility of oral intake by contamination of food and drinking water with fullerene C60 and from fullerene C60-containing products that the consumer touches directly. Moreover, in workers who inhale fullerene C60, it could also be taken up via the gastrointestinal tract because nanosized particles cleared from the respiratory tract via the mucociliary escalator can subsequently be ingested into the GI tract (Oberdörster *et al.*, 2005).

There are three acute oral dose toxicity tests for fullerenes available. In an acute oral toxicity test of fullerene C60 using an *in vivo* micronucleus test carried out with male and female mice at doses of 20-78 mg/kg, no mice died and no abnormalities were detected (Shinohara *et*

Correspondence: Akihiko Hirose (E-mail: hirose@nihs.go.jp)

al., 2009); in an acute oral toxicity test of the mixture of fullerenes C60 and C70 with male and female rats at a dose of 2,000 mg/kg, no deaths or abnormalities were observed in any rats and the body weights of both sexes in the treated group increased in a similar pattern to the control group (Mori *et al.*, 2006); and in an acute oral toxicity test of water-soluble polyalkylsulfonated C60 with female rats at a dose of 2,500 mg/kg, no deaths occurred (Chen *et al.*, 1998). From these outcomes of acute oral studies, it can be concluded that the acute oral toxicity of fullerenes is very low; however, no information on repeated oral dosing tests of fullerenes is available.

In the present study, an oral repeated dose toxicity study of pristine fullerene C60 was conducted according to the test guidelines. In addition, we measured the amount of fullerene C60 in the liver, spleen, and kidney using liquid chromatography-tandem mass spectrometry (LC-MS/MS) after administration of fullerene C60. We report and discuss the results of the study.

MATERIALS AND METHODS

The present study was conducted in 2010-2011 at DIMS Institute of Medical Science, Inc. (Aichi, Japan). The study design complied with the Test Guideline of the Japanese Chemical Control Act (law concerning examination and regulation of manufacture, etc., of chemical substances), "Twenty-eight-day Repeated Dose Toxicity Test in Mammalian Species" (EA *et al.*, 1986). All procedures involving the use and care of animals were performed in accordance with the principles for Good Laboratory Practice (MOE *et al.*, 2003) and "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain" (MOE, 2006). This experiment was approved by the institutional animal care and use committee of DIMS Institute of Medical Science.

Chemicals and reagents

Fullerene C60 (Nanom Purple SU, 0.71 nm in diameter, black powder. CAS No. 99685-96-8) was obtained from Frontier Carbon Corp. (Fukuoka, Japan). The fullerene C60 (lot no. 10B0098-A) used in the present study was 99.9% pure and was kept at room temperature (17-22°C) in a dark place. Corn oil, as a vehicle, was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents used in present study were specific purity grade.

Animals

Crl:CD(SD) male and female rats (4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). All animals were maintained in an air-conditioned room at 20.0-22.5°C, with a relative humidity of 48-62%, a 12-hr light/dark cycle, and ventilation with at least 10 air changes per hour. They were housed one or two of the same sex per cage in plastic cages with stainless steel covers.

A basal diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The initial numbers of rats were 10/sex in the control and the highest dose group, and 5/sex in other dose groups. After 7-day acclimation, they were subjected to treatment at 5 weeks of age.

Administration

The dosage levels were determined based on the guideline and the maximum dose was 1,000 mg/kg/day. The lowest dose was set at 1 mg/kg/day (concentration of fluid: 0.1 mg/ml) based on the solubility of fullerene C60 in olive oil being approximately 0.1 mg/ml (Yamakoshi, 1999). The intermediate doses were selected as 100 and 10 mg/kg/day with a proportional factor of 10.

Fullerene C60 was weighed for each dosing level and the vehicle (corn oil) was added. Each dosing fluid including that for the vehicle control was sonicated 3 times (for 5 min each) in a beaker cooled with ice. Sonication was performed at 5- to 10-min intervals after it was confirmed that the fluid was sufficiently cool. The dosing fluids were prepared from 1 p.m. to 5 p.m. on the day before each administration day, and mixed using a stirrer at room temperature (17-22°C) in a dark place until just before administration.

For each fluid dose, samples collected from the upper, central, and lower parts of the glass container were observed and photographed under an optical microscope on the first and last day of the administration period. All doses, even the lowest dose of 0.1 mg/ml, did not completely dissolve in corn oil, and included visible and invisible residues which could be seen at 40 x magnification, although we assumed the lowest dose as completely soluble. Photographs of samples of each dosage looked similar between the first and last day. Typical microscopic photographs of samples on the last day are shown in Fig. 1. Black particles shown in Fig. 1 are aggregated fullerene C60. Administration was by oral dosage at 10 ml/kg using a disposable syringe and a disposable gastric tube. The dosing volume was adjusted by the latest body weight of each rat.

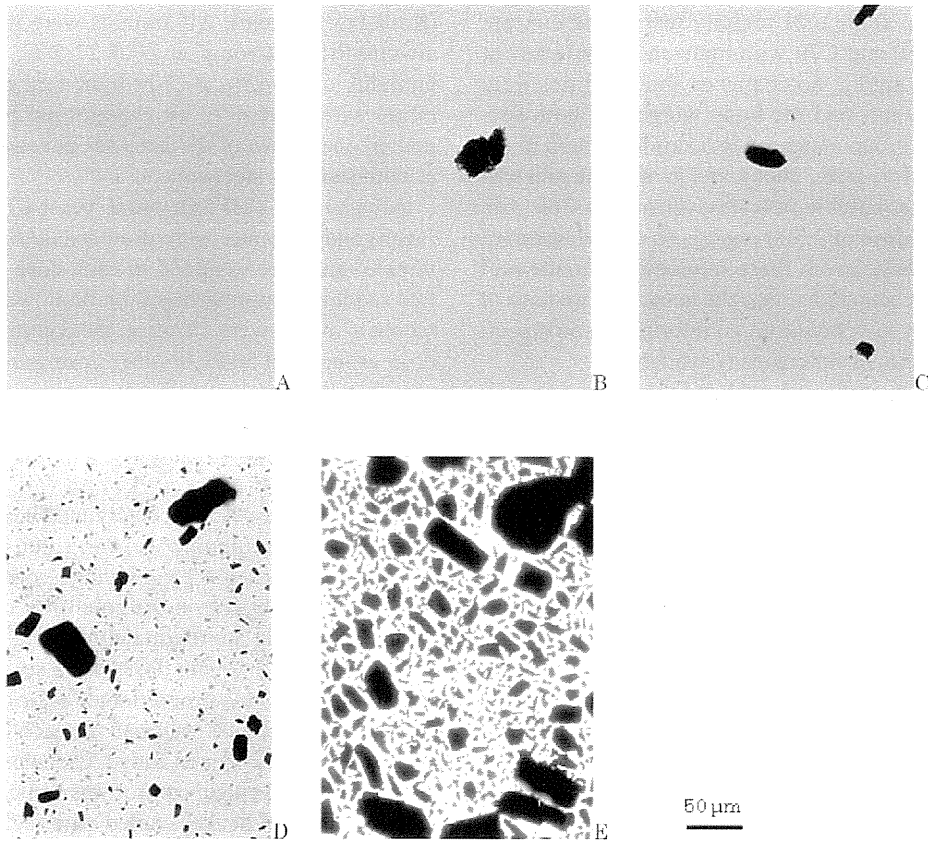

Fig. 1. Typical microscopic photographs of each dosage fluid on the last day of the administration period, with a single scale bar for all microscopic photographs. (A) 0 mg/kg/day, vehicle, (B) 1 mg/kg/day, (C) 10 mg/kg/day, (D) 100 mg/kg/day, and (E) 1,000 mg/kg/day. Black particles are aggregated fullerene C60. No staining, original magnification × 40.

Experimental design

Rats were given fullerene C60 by gavage once daily at 0 (vehicle control), 1, 10, 100, or 1,000 mg/kg/day for 29 days. On the day after the last dosing, five males and five females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The respective remaining five rats/sex at 0 and 1,000 mg/kg/day were kept without treatment for 14 days as a recovery period and then fully examined.

Daily observation and a functional observation battery

All animals were observed at least twice daily for clinical signs of toxicity in their cage. A functional observation battery (FOB), including observations in hands: ease of removal, respiration, salivation, nose secretion, lacrimation, exophthalmos, ptosis, eyeball opacity, skin, soiled perineal region, handling reactivity, and open field observations: exploration, gait, behavior, posture, fur, twitch, convulsion, tremor, rearing, defecation, urination, was conducted once a week during treatment, and sensory reactivity to stimuli of different types (reactivity to sensory stimulation: visual, auditory, tactile, and nociceptive; cranial nerve reflexes: palpebral reflex, pinna reflex, and papillary reflex; spinal reflexes: flexor reflex and extensor thrust reflex; postural reaction: proprioceptive positioning reaction; righting reactions: surface righting reaction and aerial righting reaction; and landing foot splay), grip strength (fore/hind limb), and motor activity (DAS system, model DAS-008; Neuroscience, Inc., Tokyo, Japan), once during the fourth week of treatment. Body weight was recorded on days 0, 7, 14, 21, and 28 of the dosing period and days 6 and 13 of the recovery period. Food

consumption was measured once a week during the dosing and recovery periods.

Urinalysis, hematology and blood biochemistry

One day in the fourth week of the dosing period, urine was collected for 4 hr and analyzed for dipstick parameters, such as the volume of the urine, color, occult blood, ketone bodies, glucose, protein, urobilinogen, bilirubin, specific gravity, and pH. Prior to necropsy at the end of the administration period and at the end of the recovery period, blood was collected from the abdominal aorta under deep ether anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematologic parameters such as red blood cell count, hemoglobin, hematocrit, white blood cell count, platelet count, and differential leukocyte count. Another blood sample was treated with sodium citrate, and blood clotting parameters, such as prothrombin time and activated partial thromboplastin time, were examined. Serum from one remaining portion of blood was analyzed for blood biochemistry [total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, lactate dehydrogenase, phospholipid, calcium, inorganic phosphorus, sodium, potassium, chlorine]. Serum from the remaining portion of blood was analyzed for levels of triiodothyronine, thyroxine, and thyroid stimulating hormone at Bozo Research Center Inc. (Shizuoka, Japan).

Organ weights and histopathological analysis

After blood collection, all animals were sacrificed by exsanguination, and the surface and cavity of the body and the organs and tissues of the entire body were observed macroscopically. The pituitary, thymus, thyroids (including parathyroids), heart, liver, spleen, kidneys, adrenals, testes, epididymides, uterus, and ovaries were then removed and weighed (after formalin fixation of the pituitary and thyroids). The trachea, lungs (including bronchus), lymph nodes (mandibular, mesenteric, and axillary), stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, mammary gland (male), brain, spinal cord (cervical, pectoral, and lumbar part), sciatic nerve, prostates, bone marrow (femur) as well as the above organs were fixed in 10% neutral-buffered formalin phosphate (after Bouin fixation for testes and epididymides). Histopathological examination was conducted for all of these organs of the control and the highest dose groups at the end of the administration period. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin. If any pathological effects at the end of the administration period or any toxicological effects after the recovery period were found, histopathological examination of related organs was also conducted at the end of the recovery period.

Measurement of fullerene C60 in organs

For the determination of concentrations of fullerene C60 in liver (median lobe), right and left kidneys, and spleen samples, samples of all males in the control and the highest groups were obtained at the end of the administration period and at the end of the recovery period, weighed, frozen with liquid nitrogen, and stored in a deep freezer (-80 to -74°C) until used. The mean values of the wet weight of organs were 0.1 g (spleen) to 0.4 g (liver). The analytical method of LC-MS/MS and the extraction procedure from tissues of experimental animals were as reported previously (Kubota *et al.*, 2009, 2011), and C70 was used as an internal standard for quantification. The detection limits for each organ were 0.102 μg/g wet wt. (liver), 0.146 μg/g wet wt. (kidneys), and 0.587 μg/g wet wt. (spleen).

Data analysis

Parametric data, such as FOB findings, body weight, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological and blood biochemical findings, serum hormone level, and organ weights, were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. If homogenous, Dunnett's test (Dunnett, 1964) was conducted and, if not homogenous, Steel's multiple comparison test (Steel, 1959) was conducted to compare control and individual treatment groups. For two groups, parametric data were analyzed by the F-test (Snedecor and Cochran, 1967) for homogeneity of distribution. If homogenous, Student's t-test (Steel and Torrie, 1980) was conducted and, if not homogenous, Aspin-Welch's t-test (Snedecor and Cochran, 1967) was conducted for comparison. For significant differences in the incidences of FOB, urinalysis, and histopathological findings, Fisher's exact test (Fisher, 1973) was performed, and the grade of lesions was compared using the Mann-Whitney U-test (Mann and Whitney, 1947). A 5% level of probability was used as the criterion for significance.

RESULTS

No deaths or clinical signs of toxicity occurred in any groups. In general appearance, blackish feces were observed in males and females at 1,000 mg/kg/day from

dosing day 4 to the end of the administration period, and from day 0 to day 1 of the recovery period. In the detailed clinical observation, the number of urinations was significantly increased at 1 mg/kg/day in females on one day in week 2, and the number of defecations was significantly decreased at 1,000 mg/kg/day in males on one day in week 3, but these were not persistent changes. There were no changes from controls in the manipulation test, grip strength, motor activity, body weight, and food consumption.

In urinalysis at the end of the administration period, only an increase in the number of positive incidences of ketone bodies was observed at 10 and 1,000 mg/kg/day in males (data not shown). In the hematological examination, a decrease in the differential lymphocyte ratio and an increase in the differential eosinophil ratio were observed at 10 mg/kg/day in males at the end of the administration period, but not at the end of the recovery period (data not shown). Blood chemistry results are shown in Table 1. An increase in creatinine at 100 mg/kg/day in males, and a decrease in albumin at 1,000 mg/kg/day in males were observed only at the end of the administration period, and an increase in total protein was observed in females only at the end of the recovery period. No changes from controls were found in serum levels of triiodothyronine, thyroxine, and thyroid stimulating hormone.

At necropsy, black contents of the stomach and large intestine were observed in all animals at 1,000 mg/kg/day at the end of the administration period, but not at the end of the recovery period. No other macroscopic changes were observed in all treated animals at the end of the administration period and at the end of the recovery period. Body and organ weights at the end of the administration period or the recovery period are shown in Table 2. An increase in relative thymus weight at 100 mg/kg/day in females and a decrease in relative kidney weight at 1,000 mg/kg/day in males were observed at the end of the administration period, but not at the end of the recovery period. Increases in absolute and relative liver weights and absolute spleen weight were observed in males in the treatment group only at the end of the recovery period. Histopathological findings are shown in Table 3. There were no changes from controls in all organs examined at the end of the administration period and also no changes in the liver and spleen of males examined in the recovery period. In the analysis using LC-MS/MS, the contents of fullerene C60 were under the detection limit in all samples of the liver, kidneys, and spleen at the end of the administration period and at the end of the recovery period (data not shown).

DISCUSSION

The present study was conducted to obtain initial information on the possible repeated-dose toxicity of fullerene C60 in rats. There were no treatment-related effects during and at the end of the administration period. Although there were statistically significant differences in the following findings at the end of the administration period, they were not considered to be toxicological because there was no dose-dependency; an increase in the number of positive incidences of urine ketone bodies in males, a decrease in the differential lymphocyte ratio and an increase in the differential eosinophil ratio in males in hematology, an increase in serum creatinine in males, and an increase in relative thymus weight in females. As for decreases in serum albumin and the relative kidney weight at 1,000 mg/kg/day in males at the end of the administration period, and also an increase in total protein at 1,000 mg/kg/day in females at the end of the recovery period, their toxicological significance remains to be elucidated in the present study.

Blackish feces and black contents of the stomach and large intestine observed in the present study were considered to result from the administered fullerene C60 itself. Blood clots cannot be included in these blackish feces and black contents because there were no necropsy and histopathological findings including bleeding and erosion in the gastrointestinal tract. It would appear that a large amount of fullerene C60 passed through the gastrointestinal tract without significant absorption. Fullerene C60 which had not dissolved in vehicle was considered to mix with food in the gut, and to have been excreted outside of the body.

In a recent solubility study of fullerenes in natural oils and animal fats (Semenov *et al.*, 2009), the solubility of fullerene C60 in corn oil was 0.6 mg/ml at 20°C. In the present study, however, 0.1 mg/ml fullerene C60 did not completely dissolve in corn oil. This difference in the dissolution amount of fullerene C60 may be due to differences in the materials and methods used.

Regarding the absorption of water-soluble fullerene synthesized using dipolar trimethylenemethane administered orally to male rats, virtually all radioactivity (97%) was excreted in the feces, and trace amounts of fullerene derivatives were identified in the urine (less than 3%) (Yamago *et al.*, 1995). This result shows at least that dissolved fullerene can be absorbed from the intestines. Moreover, absorption of pristine fullerene C60 administered orally to female rats was suggested because levels of 8-oxo-2'-deoxyguanosine, one of the products of DNA oxidation, in the liver and lung are higher than those of