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Original Article

Sub-acute oral toxicity study with fullerene C60 in rats

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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*

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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.

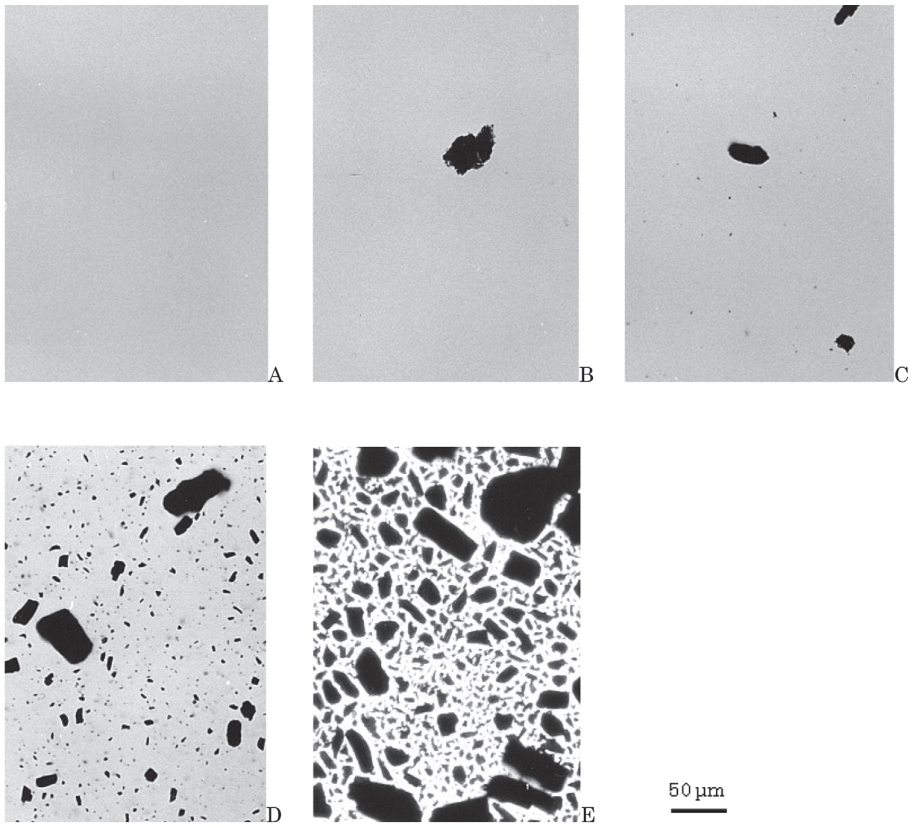

Fig. 1. Typical microscopic photographs of each dosage fluid on the last day of the administration period, with a single scale bar for all microscopic photographs. (A) 0 mg/kg/day, vehicle, (B) 1 mg/kg/day, (C) 10 mg/kg/day, (D) 100 mg/kg/day, and (E) 1,000 mg/kg/day. Black particles are aggregated fullerene 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

Table 1. Principal blood biochemical values in male and female rats given fullerene C60 by gavage

Dose (mg/kg/day)	At the end of the administration period					At the end of the recovery period	
	0	1	10	100	1000	0	1000
Male							
No. of animals	5	5	5	5	5	5	5
AST (U/l)	88.4 ± 20.2	92.8 ± 29.4	72.8 ± 13.8	80.6 ± 10.5	64.6 ± 11.6	127.8 ± 29.2	100.6 ± 34.9
ALT (U/l)	33.8 ± 4.1	33.4 ± 4.2	33.4 ± 6.1	33 ± 8.4	32.6 ± 7.4	33.6 ± 5.9	31 ± 4.9
ALP (U/l)	588.2 ± 114.1	634.8 ± 67	649.6 ± 100.3	564 ± 101.4	610.4 ± 134.2	423.4 ± 75.9	410 ± 43.7
γ-GTP (U/l)	0.58 ± 0.28	0.42 ± 0.13	0.5 ± 0.19	0.6 ± 0.07	0.66 ± 0.34	0.58 ± 0.22	0.4 ± 0.34
Lactate dehydrogenase (U/l)	127.6 ± 32.6	142.2 ± 34.4	121.8 ± 47.2	133.6 ± 50.2	113.2 ± 15.8	184.4 ± 40.4	161.4 ± 66.5
Urea nitrogen (mg/dl)	9.44 ± 1.21	10.22 ± 1.14	9.78 ± 1.67	10.8 ± 2.16	9.6 ± 1.44	13.52 ± 1.32	12.84 ± 0.67
Creatinine (mg/dl)	0.232 ± 0.044	0.282 ± 0.022	0.268 ± 0.035	0.32 ± 0.049**	0.27 ± 0.019	0.286 ± 0.029	0.27 ± 0.016
Glucose (mg/dl)	145.8 ± 21.4	150.6 ± 15.8	149.4 ± 15.9	155.8 ± 30.7	165.2 ± 10.7	138.6 ± 8	145 ± 27.3
Total cholesterol (mg/dl)	50.8 ± 10.5	48.8 ± 11	55.4 ± 6.2	59.8 ± 7.6	55 ± 12.1	55.4 ± 10.7	65.8 ± 17
Phospholipid (mg/dl)	93.2 ± 15.3	88 ± 13.8	101.2 ± 8.8	106 ± 7.6	101 ± 16.2	92 ± 13.4	106 ± 20.5
Triglycerides (mg/dl)	59.4 ± 22.5	47.6 ± 9.2	54.2 ± 5.2	36.6 ± 9.3	66.4 ± 28.2	38.6 ± 16.4	64.6 ± 34.2
Total protein (g/dl)	5.78 ± 0.08	5.72 ± 0.11	5.52 ± 0.13	5.76 ± 0.27	5.68 ± 0.13	5.94 ± 0.23	6.06 ± 0.17
Albumin (g/dl)	2.52 ± 0.13	2.46 ± 0.05	2.38 ± 0.11	2.42 ± 0.11	2.34 ± 0.11*	2.42 ± 0.08	2.44 ± 0.11
A/G	0.774 ± 0.054	0.758 ± 0.036	0.76 ± 0.06	0.73 ± 0.064	0.704 ± 0.057	0.69 ± 0.023	0.674 ± 0.027
Female							
No. of animals	5	5	5	5	5	5	5
AST (U/l)	115.6 ± 35.4	119.4 ± 26.5	122 ± 28.6	108.6 ± 30.2	99.4 ± 40.2	109.8 ± 28.9	130.4 ± 58.9
ALT (U/l)	31.6 ± 6.8	35.8 ± 6.3	34.8 ± 10.5	30.4 ± 9.4	33.4 ± 11	29 ± 4	35 ± 21.8
ALP (U/l)	533.2 ± 238.9	399.4 ± 99	432.4 ± 86.1	343.4 ± 48.6	379.4 ± 45.9	270.8 ± 14	301 ± 42.3
γ-GTP (U/l)	0.7 ± 0.32	0.78 ± 0.16	0.8 ± 0.14	0.64 ± 0.15	0.66 ± 0.25	0.74 ± 0.18	0.8 ± 0.48
Lactate dehydrogenase (U/l)	177.6 ± 73	143.2 ± 41.4	169.6 ± 47.5	150.6 ± 15.3	168.6 ± 11.7	144.6 ± 31.7	129.2 ± 44.9
Urea nitrogen (mg/dl)	10.88 ± 1.29	11.98 ± 2.08	11.32 ± 1.88	12.66 ± 1.27	12.84 ± 1.44	16.78 ± 2.47	17.9 ± 1.67
Creatinine (mg/dl)	0.31 ± 0.029	0.322 ± 0.041	0.316 ± 0.029	0.326 ± 0.032	0.318 ± 0.029	0.352 ± 0.022	0.352 ± 0.013
Glucose (mg/dl)	132.6 ± 13	124.6 ± 21.1	133.2 ± 16.1	139 ± 15.5	138.8 ± 18	124 ± 10.4	130.4 ± 5.9
Total cholesterol (mg/dl)	56.4 ± 15.8	61.4 ± 6	56 ± 8.2	65.8 ± 12.4	60.4 ± 8.8	78 ± 8.2	75.6 ± 13.6
Phospholipid (mg/dl)	107.8 ± 25.6	111.4 ± 11.1	104.6 ± 13.3	125.6 ± 18.6	116.2 ± 14.4	141.2 ± 15.1	142 ± 25.2
Triglycerides (mg/dl)	25.8 ± 14.9	21.2 ± 12.6	19 ± 7	20.8 ± 6.9	16.2 ± 11.2	26 ± 8.4	35.6 ± 14.8
Total protein (g/dl)	5.76 ± 0.21	5.82 ± 0.31	5.78 ± 0.24	5.94 ± 0.15	5.92 ± 0.11	6.06 ± 0.21	6.32 ± 0.11*
Albumin (g/dl)	2.56 ± 0.05	2.58 ± 0.18	2.54 ± 0.11	2.7 ± 0.19	2.72 ± 0.11	2.64 ± 0.09	2.72 ± 0.13
A/G	0.802 ± 0.037	0.8 ± 0.089	0.784 ± 0.03	0.836 ± 0.081	0.85 ± 0.05	0.776 ± 0.081	0.756 ± 0.062

Values are expressed as the mean ± S.D. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; A/G, albumin-globulin ratio. *: Significantly different from control group at $p < 0.05$. **: Significantly different from control group at $p < 0.01$.

Table 2. Principal organ weights of male and female rats given fullerene C60 by gavage

Dose (mg/kg/day)	At the end of the administration period					At the end of the recovery period	
	0	1	10	100	1000	0	1000
Male							
No. of animals	5	5	5	5	5	5	5
Body weight[a] (g)	415.0 ± 39.0	426.6 ± 36.8	408.6 ± 32.8	424.4 ± 50.8	422.2 ± 39.4	454.4 ± 49.4	485.6 ± 19.3
Thymus (g)	0.54 ± 0.16	0.45 ± 0.09	0.50 ± 0.10	0.51 ± 0.13	0.50 ± 0.13	0.46 ± 0.08	0.46 ± 0.11
	(0.130 ± 0.032)[b]	(0.104 ± 0.017)	(0.121 ± 0.024)	(0.119 ± 0.016)	(0.117 ± 0.025)	(0.100 ± 0.015)	(0.095 ± 0.026)
Liver (g)	12.80 ± 1.93	13.33 ± 1.64	11.93 ± 0.71	13.96 ± 2.95	12.98 ± 1.39	12.17 ± 1.27	13.99 ± 1.15*
	(3.076 ± 0.288)	(3.123 ± 0.253)	(2.926 ± 0.136)	(3.274 ± 0.385)	(3.073 ± 0.121)	(2.681 ± 0.113)	(2.878 ± 0.137*)
Kidneys (g)	2.84 ± 0.40	2.83 ± 0.26	2.71 ± 0.20	2.71 ± 0.17	2.59 ± 0.16	2.91 ± 0.20	3.15 ± 0.34
	(0.684 ± 0.046)	(0.663 ± 0.017)	(0.664 ± 0.035)	(0.643 ± 0.036)	(0.615 ± 0.031*)	(0.644 ± 0.045)	(0.646 ± 0.047)
Spleen (g)	0.56 ± 0.08	0.65 ± 0.08	0.61 ± 0.07	0.66 ± 0.15	0.61 ± 0.10	0.67 ± 0.09	0.82 ± 0.05*
	(0.134 ± 0.009)	(0.153 ± 0.017)	(0.149 ± 0.005)	(0.153 ± 0.021)	(0.144 ± 0.019)	(0.148 ± 0.017)	(0.169 ± 0.015)
Female							
No. of animals	5	5	5	5	5	5	5
Body weight[a] (g)	217.6 ± 20.5	222.4 ± 12.2	216.8 ± 12.8	211.6 ± 16.8	218.2 ± 7.4	235.0 ± 16.7	234.8 ± 22.8
Thymus (g)	0.36 ± 0.07	0.45 ± 0.11	0.39 ± 0.05	0.47 ± 0.09	0.40 ± 0.09	0.41 ± 0.08	0.41 ± 0.11
	(0.163 ± 0.021)	(0.200 ± 0.042)	(0.180 ± 0.032)	(0.222 ± 0.032*)	(0.185 ± 0.041)	(0.172 ± 0.026)	(0.176 ± 0.053)
Liver (g)	6.43 ± 0.91	6.89 ± 0.57	6.66 ± 0.34	6.48 ± 0.54	6.85 ± 0.56	6.28 ± 0.42	6.19 ± 0.98
	(2.950 ± 0.203)	(3.097 ± 0.129)	(3.080 ± 0.252)	(3.066 ± 0.131)	(3.142 ± 0.236)	(2.675 ± 0.109)	(2.626 ± 0.214)
Kidneys (g)	1.48 ± 0.12	1.41 ± 0.12	1.43 ± 0.07	1.51 ± 0.12	1.52 ± 0.17	1.67 ± 0.13	1.51 ± 0.28
	(0.682 ± 0.076)	(0.637 ± 0.045)	(0.662 ± 0.041)	(0.717 ± 0.050)	(0.697 ± 0.061)	(0.714 ± 0.048)	(0.640 ± 0.068)
Spleen (g)	0.40 ± 0.04	0.46 ± 0.06	0.41 ± 0.04	0.43 ± 0.06	0.42 ± 0.05	0.45 ± 0.07	0.44 ± 0.07
	(0.185 ± 0.012)	(0.206 ± 0.027)	(0.187 ± 0.011)	(0.200 ± 0.018)	(0.195 ± 0.028)	(0.190 ± 0.020)	(0.189 ± 0.020)

Values are expressed as the mean ± S.D. Values in parentheses are relative organ weights (organ weight per body weight, %). a: The values presented were obtained after the animals were fasted overnight. *: Significantly different from control group at $p < 0.05$.

Table 3. Number of animals with histopathological findings in male and female rats given fullerene C60 by gavage

	Male		Female	
Dose (mg/kg/day)	0	1000	0	1000
No. of animals	5	5	5	5
At the end of the administration period				
Liver				
Normal	2	1	1	0
Granuloma, minimal	1	3	3	1
Granuloma, slight	0	0	0	1
Granuloma, moderate	0	0	0	1
Tension lipidosis, slight	0	1	0	0
Vacuolation, cytoplasmic, minimal	3	3	3	4
Vacuolation, cytoplasmic, slight	0	0	1	1
Kidney				
Normal	4	5	4	2
Mineralization, minimal	0	0	1	3
Scar, mininal	1	0	0	0
Prostate				
Normal	4	5		
Cellular infiltration, lymphocyte, minimal	1	0		
Uterus				
Normal			3	5
Dilatation, lumen, slight			2	0
At the end of the recovery period				
Liver				
Normal	3	3		
Granuloma, minimal	2	1		
Vaculoation, cytoplasmic, minimal	0	1		
Spleen				
Normal	5	5		

controls (Folkmann *et al.*, 2009), although the presence of fullerenes in the liver and lungs was not demonstrated. In three acute oral dose toxicity tests (Shinohara *et al.*, 2009; Mori *et al.*, 2006; Chen *et al.*, 1998), there was no discussion about absorption of the fullerene used.

In the study, when intravenously injected into female rats, ^{14}C-labeled fullerene C60 was rapidly (within 1 min) cleared from the circulation and the majority accumulated in the liver (about 92%), followed by the spleen (about 4%), 2-hr post-injection, and the 14C-labeled fullerene C60 was not eliminated from the liver, but from the spleen, 120-hr post-injection (Bullard-Dillard *et al.*, 1996). In a study (Kubota *et al.*, 2011) of tail vein injection of fullerene C60 into rats using liposomes as a carrier, burdens of fullerene C60 were widely distributed in five tissues, the liver, lungs, spleen, kidneys, and brain (in descending order) although no fullerene C60 was detected in the blood on day 1 after completion of the injections. Fullerene C60 accumulated in the liver did not decrease until 14 days, and for up to 28 days after the completion of injections, and a time-dependent decrease in fullerene C60 concentration was not observed in the spleen until 28 days (Kubota *et al.*, 2011).

Because these above-mentioned studies suggested that fullerene C60 could be absorbed via the gastrointestinal tract (Folkmann *et al.*, 2009; Yamago *et al.*, 1995) and distribute in the spleen and liver (Bullard-Dillard *et al.*, 1996; Kubota *et al.*, 2011), increased liver and spleen weights after the recovery period in the present study may relate to the oral administration of fullerene C60. However, it was clear that there was no accumulation of fullerene C60 which could change the weights of the liver and spleen directly. The causal relationships between the possible absorption of fullerene C60 and those weight changes were unknown because the pathological findings as indirect influences, such as swelling and congestion, were not also observed in the liver and spleen.

In conclusion, the results of this study of no marked change after 29-day repeated dosing of fullerene C60 by gavage indicated that its toxicity by oral administration is relatively low; however, increased liver and spleen weights observed after the recovery period may be asso-

ciated with fullerene C60 administration although no histopathological changes were found and absorbed fullerene C60 was under the detection limits in these organs. Therefore, with the prospective exposure by increased uses in future because of low toxic substance, more long-term exposure study is necessary to clarify the effects of fullerene C60 via oral exposure.

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