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Original Article

Lung toxicity of a vapor-grown carbon fiber in comparison with a multi-walled carbon nanotube in F344 rats

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Abstract: Carbon fibers have excellent physicochemical and electrical properties. Vapor-grown carbon fibers are a type of carbon fibers that have a multi-walled carbon tube structure with a high aspect ratio. The representative vapor-grown carbon fiber, VGCF™-H, is extremely strong and stable and has superior thermal and electrical conductivity. Because some high-aspect-ratio multi-walled carbon nanotubes (MWCNTs) have been reported to have toxic and carcinogenic effects in the lungs of rodents, we performed a 13-week lung toxicity study using VGCF™-H in comparison with one of MWCNTs, MWNT-7, in rats. Male and female F344 rats were intratracheally administered VGCF™-H at doses of 0.2, 0.4, and 0.8 mg/kg bw or MWNT-7 at doses of 0.4 and 0.8 mg/kg bw once a week for 8 weeks and then up to week 13 without treatment. The lung burden was equivalent in the VGCF™-H and MWNT-7 groups; however, the lung weight had increased and the inflammatory and biochemical parameters in the broncho-alveolar lavage fluid and histopathological parameters, including inflammatory cell infiltration, alveolar type II cells proliferation, alveolar fibrosis, pleural fibrosis, lung mesothelium proliferation, and diaphragm fibrosis, were milder in the VGCF™-H group than in the MWNT-7 group. In addition, the proliferating cell nuclear antigen (PCNA)-positive index in the visceral and pleural mesothelium was significantly higher in the MWNT-7 group than in the controls, but not in the VGCF™-H group. Thus, the results of this study indicate that the lung and pleural toxicities of VGCF™-H were less than those of MWNT-7. (DOI: 10.1293/tox.2020-0064; J Toxicol Pathol 2021; 34: 57–71)

Key words: vapor-grown carbon fiber, multi-walled carbon nanotubes, intratracheal instillation, lung toxicity

Introduction

Carbon fibers with nanoscale to submicron-scale diameters and high aspect ratios have excellent physicochemical and electrical properties. Multi-walled carbon nanotubes (MWCNTs) have a number of industrial applications, and their production is increasing with an expected market of over 3 billion USD by 2022. MWCNTs produced under different manufacturing conditions have different diameters, lengths, and shapes and hence different physicochemical and electrical properties. MWCNTs with needle-like/fibrous structures and high aspect ratios, such as MWNT-7, are considered potentially biopersistent in the lung when inhaled[1, 2] and can result in pulmonary toxicity. Indeed, MWNT-7 showed persistent lung toxicity in two- and thirteen-week whole-body inhalation studies with rats[3, 4] and carcinogenicity in the rat lung in a two-year whole-body inhalation study[5]. In addition, the inhalation of MWNT-7 promoted lung carcinogenesis initiated by methylcholanthrene[6].

Vapor grown carbon fibers have a multi-walled carbon tube structure and are formulated to enhance the electrical and thermal properties of high-performance materials. VGCF™-H is a vapor grown carbon fiber with a thick fiber diameter and is used in lithium-ion batteries and fuel cells because of its excellent thermal and electrical conductivity. VGCF™-H has a larger diameter (VGCF™-H: 148 nm; MWNT-7: 75 nm) but a shorter average fiber length than MWNT-7 (VGCF™-H: 5.2 µm; MWNT-7: 9 µm). However, like MWNT-7, it has a fibrous structure with a high aspect ratio. These facts raise the possibility that the inhalation of VGCF™-H may also induce lung toxicity and carcinogenicity.

A nose-only 13-week inhalation study exposed rats to 0.54, 2.5, and 25 mg/m^3 VGCF™-H for 13 weeks, followed by a 3-month recovery period. This study reported a no-adverse-effect level of 0.54 mg/m^3 for VGCF™-H[7]. However, the effects of a long-term exposure to VGCF™-H remain unknown. Whole-body or nasal inhalation exposure

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is the standard method for evaluating lung toxicity using respirable chemicals and particulate matter. However, inhalation testing requires specialized facilities, equipment, techniques, and a large quantity of test material. Consequently, the number of materials that can be tested is limited, which can result in an extended delay in the identification of toxic materials. For example, at the time of this manuscript writing, while a large number of different types of MWCNTs were being produced, only a single MWCNT, i.e., MWNT-7, had undergone long-term inhalation testing. In contrast to inhalation testing, the administration of respirable test materials by intratracheal instillation requires no special facilities, is inexpensive, and is useful for screening and hazard identification[8–12]. Furthermore, since the amount of test material delivered into the lung by intratracheal instillation is known, it is possible to determine a precise dose-response relationship between the amount of test material in the lung and toxicological parameters[13, 14]. Therefore, in the present study, we performed a 13-week toxicity study comparing VGCF™-H with MWNT-7, a known carcinogen in the lung and pleural cavity of the rat[5, 15], using intratracheal instillation to administer the test materials. The purpose of this study was to determine and compare the subchronic lung toxicities of VGCF™-H and MWNT-7 when administered by instillation, using a combination of bronchoalveolar lavage fluid (BALF) analysis and histopathological examination.

Materials and Methods

Test materials and their preparation

The VGCF™-H used in this study (Showa Denko K.K., Tokyo, Japan) had a diameter of 148 ± 51.5 nm, a length of 5.2 ± 2.7 µm, and a surface area of 15 m^2/g, and MWNT-7 (Bussan Nanotech Research Institute Inc., Tokyo, Japan) had a diameter of 75 ± 20.4 nm, a length of 9.0 ± 6.1 µm, and a surface area of 25 m^2/g. Both VGCF™-H and MWNT-7 were dispersed in saline containing 0.3% w/v Kolliphor™ P188 (KP188) (Sigma-Aldrich Japan Ltd., Tokyo, Japan) using a desktop-type ultrasonic bus. MWNT-7 was dispersed during deaeration. These suspensions were sonicated using a probe-type ultrasonic generator and then dispersed by a wet dispersion device to prepare their administration solutions. Subsequently, the average hydrodynamic diameter of fibers in a 0.4 mg/mL solution in saline containing 0.3% w/v KP188 was measured by dynamic light scanning (DLS) (ELSZ-2000S, Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C. The average hydrodynamic diameters of VGCF™-H and MWNT-7 were 668 ± 37 and 611 ± 54 nm, respectively, and these values were the average of eight measurements. The average hydrodynamic diameter did not change before and after passing through the microsprayer aerosolizer.

Our unpublished preliminary toxicity study using F344 rats showed that at a dose of 0.8 mg/kg bw, a single intratracheal instillation of MWNT-7, but not of VGCF™-H, induced a persistent pulmonary inflammation at 2 weeks after instillation. Therefore, the highest dose of both VGCF™-H and MWNT-7 were 0.8 mg/kg bw in the present experiment. The dose volume for instillation was set at 2.0 mL/kg bw, and the concentrations of the VGCF™-H solution was adjusted to 0.1, 0.2, and 0.4 mg/mL, and those of the MWNT-7 solution was adjusted to 0.2 and 0.4 mg/mL. Therefore, the final concentrations were 1.6, 3.2, and 6.4 mg/kg bw in the VGCF™-H treated group and 3.2 and 6.4 mg/kg bw in the MWNT-7 treated group. The dosing volume was calculated for each individual animal based on body weight at the time of instillation. The prepared solutions were stored in the refrigerator before instillation. Prior to intratracheal instillation, the vehicle and VGCF™-H solutions were redispersed using a tabletop ultrasonic processor (Model: M1800-J, Emerson Japan, Ltd., Tokyo, Japan) for 10 min, and then vortexed for several seconds. As of instilling the MWNT-7 dosing solution, a degassing/redispersing treatment was executed for 1 min using a vacuum pump (Vacuum Pump V-100, Nihon Buchi Co., Ltd., Tokyo, Japan) and the tabletop ultrasonic processor, and then subjected to the redispersion treatment for further 9 min without degassing, and furthermore, the container was gently stirred by shaking. All dosing solutions were used within 1 h after redispersion and were gently mixed to produce a homogeneous solution immediately prior to instillation.

Animals and husbandry

Eight-week-old pathogen-free F344/DuCrlCrlj rats of both sexes were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were housed in a barriered-system animal room maintained under controlled conditions (temperature, 22 ± 3°C; humidity, 55 ± 15%; 12-h light-dark cycle) and were given the pellet diet CRF-1 sterilized by 30 kGy gamma irradiation (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. After a two-week quarantine and acclimation period, the 10 week-old rats of each sex were randomized by body weight and assigned to seven groups (six rats in the untreated group, 11 in the vehicle group, and 16 in each of the VGCF™-H and MWNT-7 groups) on the day prior to the initial instillation. No significant differences in the average body weights were observed between groups at the commencement of the study, Bartlett test ($p<0.05$), and Tukey test ($p<0.05$, two-sided). In addition, no abnormalities were observed in the general condition of the animals during the quarantine period.

The study was approved by the Animal Experimental Committee at the DIMS Institute of Medical Science, Inc. and conducted in accordance with the "Law for the Humane Treatment and Management of Animals" (Law No. 46, May 2014), "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 84 of the Ministry of the Environment, September 2013), "Basic policies for the conduct of animal experiment in academic research institutions under the jurisdiction of the Ministry of Health, Labour, and Welfare" (Notice No. 0220-1 of the Ministry of Health, Labour and Welfare, February 2015), "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 2006), and "Standards for

Care and Use of Laboratory Animals of DIMS Institute of Medical Science, Inc." (June 1, 2016).

Experimental design and treatment of intratracheal instillation

Animal handling during and after intratracheal instillation was performed as described previously[15, 16]. Briefly, rats were placed under isoflurane anesthesia using the NARCOBIT-E for small laboratory animals (Natsume Seisakusho Co., Ltd., Tokyo, Japan), and the instillation of the test material solution was performed intratracheally with a DIMS-type microsprayer aerosolizer (for rats) connected to a 1-mL disposable syringe (Osaka Chemical Co., Ltd., Osaka, Japan). Instillation was performed once a week for 8 weeks (8 times in total). The single doses of VGCF™-H were set at 0 (vehicle control), 0.2, 0.4, and 0.8 mg/kg bw, and those of MWNT-7 were set at 0.4 and 0.8 mg/kg bw. The animals in the untreated control group did not undergo either isoflurane anesthesia or insertion of the microsprayer aerosolizer. Animals were then observed for 6 weeks without further treatment and sacrificed after the observation period.

General observation and examination of animals

The general physical condition of the rats was checked three times on the day of instillation: immediately before and after instillation and once in the afternoon. After the 8-week dosing period, all animals were observed for clinical signs twice per day until the end of the experimental period.

Body weights and food consumption

All animals were individually weighted on the day of instillation and then weekly until the end of the experimental period. The weight at the end of the study was also measured. Food intake was measured weekly until the end of the experimental period, and the average daily food consumption was calculated.

Collection and analysis of bronchoalveolar lavage fluid (BALF)

At the end of the experimental period, all surviving animals were placed under deep isoflurane anesthesia and exsanguinated from the abdominal aorta. After blood collection, BALF was collected from three animals in each group and subjected to cannulation through the trachea, which was ligated tightly with a cotton band. The cannula was attached to a three-way stopcock. Subsequently, 8 mL of sterilized buffered physiological saline (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) at room temperature was allowed to flow into both lungs through a three-way stopcock by gravity feed from a height of 30 cm. After the influx stopped, the inlet cock of the three-way stopcock was closed, and the outlet cock was opened to collect the discharge from the lungs by gravity feed. The influx/discharge operation was repeated twice. The collected BALF was centrifuged at 4°C and 1,000 rpm for 10 min, and the supernatant was collected and stored at −80°C until use. The residual cell pellet was resuspended in 1 mL of sterilized buffered physiological saline and subjected to cytological analysis.

Analysis of inflammatory cells and clinical chemistry in BALF

Cytological analysis of the resuspended cell pellet and clinical chemistry of the BALF were performed as described previously[16]. Briefly, the resuspended cell pellets, described above, were processed for differential leukocyte count using an automatic multi-item blood cell analyzer (XT-2000i, Sysmex Corp., Hyogo, Japan). The supernatants were analyzed for alkaline phosphate (ALP), lactate dehydrogenase (LDH), protein concentration (total protein), and albumin (ALB) using an automatic analyzer (Hitachi 7070, Hitachi, Ltd., Tokyo, Japan). Interleukin 8 (IL-8), a marker of neutrophil migration factor[17, 18], was measured using an ELISA microplate reader (Model: Sunrise Rainbow RC, Tecan Japan Co., Ltd., Kanagawa, Japan).

Macroscopic pathological examination and organ weight

The animals that were not subjected to BALF analysis were exsanguinated from the abdominal aorta under deep isoflurane anesthesia. After blood collection, a gross pathological examination of all organs and tissues was performed, and the organs were weighed and then preserved in 10% buffered formalin solution. Among these animals, five rats in each of the VGCF™-H and MWNT-7 groups were used for performing a lung burden analysis. To measure the amount of VGCF™-H and MWNT-7 in the lung, fixed lung tissues were sent to the Japan Bioassay Research Center, Japan Organization of Occupational Health and Safety (Kanagawa, Japan).

Histopathological examination

For histopathological examination, the lung and diaphragm were sliced into 5-mm-thick sections, embedded in paraffin, and then processed for hematoxylin and eosin (H&E) staining and histopathological examination. The lungs subjected to BALF analysis were not used for histopathological examination. The terminology used in this study conforms to the INHAND Project[19]. The degree of change was classified into five levels (minimal, slight, moderate, marked, and severe).

Immunohistochemical staining

Sections prepared for histopathological examination, as described above, were immunostained with anti-PCNA antibody (Merck KGaA, Germany, Monoclonal Mouse Anti-PCNA, Clone: PC 10) using the polymer method, and the PCNA-positive cells in the lung and pleural specimens were counted. The PCNA index was calculated as the percentage of total cells that stained positive for PCNA.

Statistical analysis

For comparisons of the vehicle and treated groups, the homogeneity of variance was analyzed by Bartlett's test ($p<0.05$). If homogeneous, the data were analyzed using the parametric Dunnett's test (2-sided); if not homogeneous, the data were analyzed by the non-parametric Steel's test (2-sided). For comparisons of the untreated group vs. the vehicle group and for comparisons between the two groups supplied with the same doses of VGCF™-H and MWNT-7, the means were analyzed using the F-test. If the differences in means were non-significant in the F-test, a Student's *t*-test (2-sided) was used; however, if the differences in means were significant in the F-test, a Welch's *t*-test (2-sided) was used. For histopathological alterations with an assigned value for the degree of change, a two-sided Wilcoxon's test was employed. The p-values <0.05 were considered statistically significant.

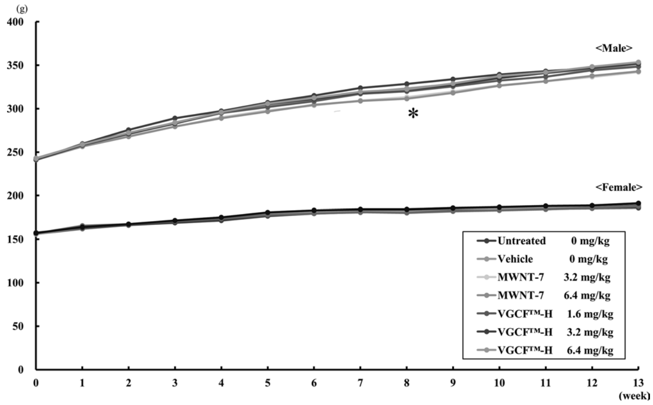

Fig. 1. Body weight curves of male and female rats in the untreated, vehicle-treated, and VGCF™-H- (1.6, 3.2 and 6.4 mg/kg) and MWNT-7-treated (3.2 or 6.4 mg/kg) groups.

Results

General condition of animals

No deaths were observed in any of the groups. After the intratracheal instillation procedure, a moist rale was observed in the treated animals. However, no difference in the incidence or severity of the moist rale was observed among the treated groups, and the moist rale disappeared by the day after intratracheal instillation. No other obvious changes were observed in the general condition of the animals in any of the groups.

Body weight and food consumption

The body weights are shown in Fig. 1. The body weight gain was significantly suppressed in the male 6.4 mg/kg MWNT-7 group compared to the vehicle control group at week 8. However, the suppression of body weight gain was temporary, and no significant differences in the body weights were observed between the male 6.4 mg/kg MWNT-7 group and the control group after week 8. Therefore, the suppression of body weight gain in the male 6.4 mg/kg MWNT-7 group compared to the control group was assumed to be a minor effect of the intratracheal instillation of MWNT-7. No statistically significant differences in body weights were observed between untreated and other treated groups at any time during the experimental period. Furthermore, no significant differences in food consumption were observed among the groups during the experimental period (data not shown).

Macroscopic pathological examination, lung burden, and organ weight

Representative macroscopic findings of the lung are shown in Fig. 2. Macroscopically, no abnormalities were observed in the lungs of the untreated and vehicle-treated groups (Fig. 2A). A discoloration (black or gray) of the lung lobe was observed in all of the VGCF™-H- and MWNT-7-treated groups; Fig. 2B and C show lungs from a male rat administered with 6.4 mg/kg of VGCF™-H and MWNT-7, respectively. There was no difference in the incidence of lung discoloration between the VGCF™-H- and MWNT-7-treated groups. Lung discoloration was due to the color of the test materials instilled into the lung. The test material was distributed to all lung lobes but with somewhat less deposition in the cranial and peripheral areas.

The average total amount of the test material instilled into the lung (µg/rat), lung burden (µg/rat) at autopsy, body weight at autopsy, and lung weight (absolute and relative weight) are shown in Table 1. The total lung burden increased in a dose-dependent manner. A significantly lower lung burden was observed in the male 3.2 mg/kg VGCF™-H group and the female 6.4 mg/kg VGCF™-H group compared to the corresponding MWNT-7-treated groups. There were no significant differences in lung burden between the other VGCF™-H- and MWNT-7-treated groups.

Significant increases in absolute and relative lung weights were observed in both the male and female vehicle-treated groups compared to the untreated group. KP188, which is known to cause weak toxic effects in the lungs, has been used in several intratracheal instillation studies for dispersing test materials[15, 16, 20–24]. The increase in the absolute and relative lung weights of the rats in the vehicle-treated groups was accompanied by a slight toxic effect due to repeated intratracheal instillation of the vehicle solution, KP188, as shown previously[16].

The 3.2 and 6.4 mg/kg VGCF™-H- and MWNT-7-treated groups had significantly higher absolute and relative lung weights compared to the vehicle-treated groups. There were no significant differences in absolute or relative lung weights in either male or female 1.6 mg/kg VGCF™-H-treated groups and their respective vehicle controls. The absolute and relative lung weights were significantly lower in both the male and female 3.2 and 6.4 mg/kg VGCF™-H-treated groups compared to the 3.2 and 6.4 mg/kg MWNT-7-treated groups.

The absolute and relative liver, kidney, and spleen weights are shown in Table 2. Significantly higher absolute

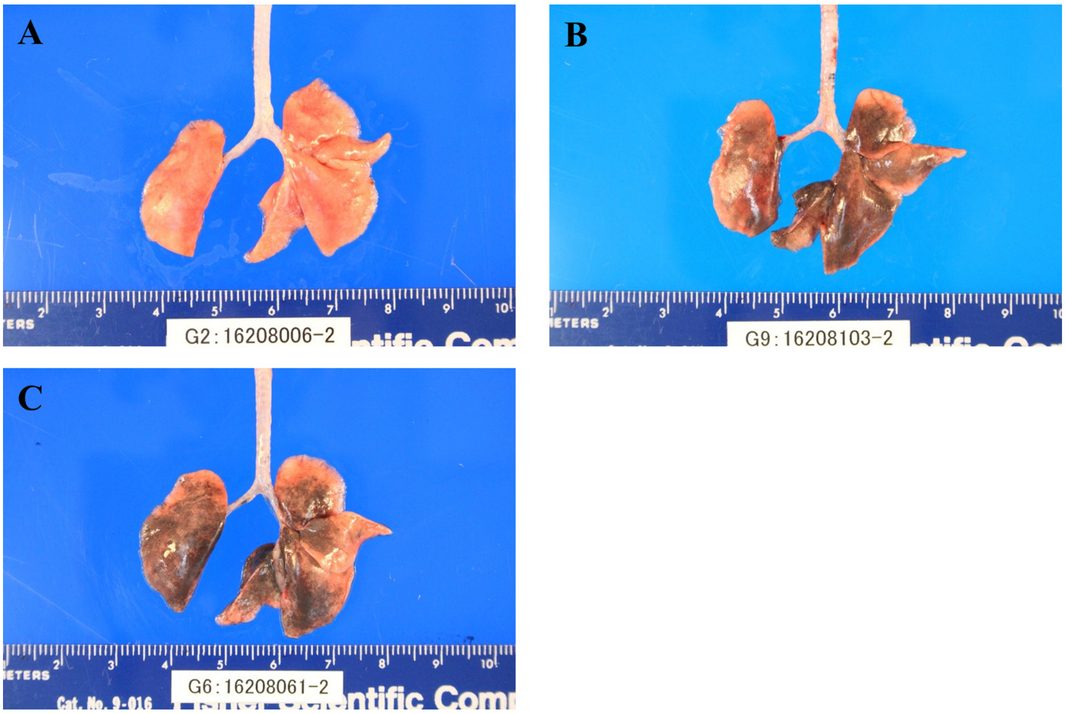

Fig. 2. Macroscopic observation of the lung. Representative photographs of the lung in the males from the vehicle-treated group (A), VGCF™-H-treated group (B: 6.4 mg/kg) and MWNT-7-treated group (C: 6.4 mg/kg).

Table 1. Total Amount of Test Material in the Lung, Lung Burden, Body Weight, and Lung Weight in Rats

Sex	Test material	Dose (mg/kg)	No. of rats examined	Total amount instilled[a] (μg/rat)	Lung burden[b] (μg/rat)	Body weight (g)[c]	Lung weight	
							Absolute (g)	Relative (%)
Male	Untreated	0	5	-	NE[d]	355 ± 10 [e]	0.96 ± 0.04	0.27 ± 0.01
	Vehicle	0	8	-	NE	355 ± 14	1.15 ± 0.07 ##	0.32 ± 0.01 ##
	VGCF™-H	1.6	8	460	305 ± 35	350 ± 19	1.17 ± 0.05	0.33 ± 0.01
	VGCF™-H	3.2	8	910	550 ± 73 $	360 ± 8	1.22 ± 0.05 *, ††	0.34 ± 0.01 **, ††
	VGCF™-H	6.4	8	1830	1337 ± 238	355 ± 14	1.25 ± 0.05 **, ††	0.35 ± 0.01 **, ††
	MWNT-7	3.2	8	900	646 ± 32	353 ± 9	1.51 ± 0.04 **	0.43 ± 0.01 **
	MWNT-7	6.4	8	1790	1157 ± 163	342 ± 14	1.79 ± 0.06 **	0.52 ± 0.01 **
Female	Untreated	0	5	-	NE	188 ± 10	0.70 ± 0.04	0.37 ± 0.01
	Vehicle	0	8	-	NE	189 ± 5	0.79 ± 0.04 ##	0.42 ± 0.02 ##
	VGCF™-H	1.6	8	280	159 ± 22	190 ± 6	0.81 ± 0.03	0.42 ± 0.02
	VGCF™-H	3.2	8	550	293 ± 37	189 ± 4	0.84 ± 0.02 **, ††	0.45 ± 0.02 **, ††
	VGCF™-H	6.4	8	1110	551 ± 74 $$	190 ± 5	0.86 ± 0.02 **, ††	0.46 ± 0.01 **, ††
	MWNT-7	3.2	8	550	318 ± 66	188 ± 6	1.05 ± 0.05 **	0.56 ± 0.02 **
	MWNT-7	6.4	8	1090	723 ± 31	188 ± 5	1.24 ± 0.04 **	0.66 ± 0.01 **

a: Calculated by each body weight; b: Measured at week 13(N=5); c: The day of autopsy; d: Not examined, e: Mean ± S.D. ##: significantly different from the untreated group at $p<0.01$. * and **: significantly different from the vehicle-treated group at $p<0.05$ and $p<0.01$, respectively. $ and $$: significantly different from the same dose of each sex in the MWNT-7-treated group at $p<0.05$ and $p<0.01$, respectively. ††: significantly different from the same dose of the MWNT-7-treated group at $p<0.01$.

liver and spleen weights were observed in the female 6.4 mg/kg VGCF™- H-treated group compared to the vehicle control group. Compared to the vehicle controls, significantly higher relative liver weights in the male 6.4 mg/kg MWNT-7-treated group, significantly higher absolute liver weights in the female 6.4 mg/kg MWNT-7-treated group, and significantly higher relative spleen weights in the female 3.2 mg/,kg MWNT-7-treated group were also observed. However, all of these weight changes were slight and, therefore, not considered to be of toxicological significance.

Cytological analysis in the BALF

Figure 3 shows total cells (A), neutrophils (B), macrophages (C), lymphocytes (D), and eosinophils (E) in the

Table 2. Weights of Liver, Kidney and Spleen in Rats

Sex	Test material	Dose (mg/kg)	No. of rats examined	Liver weight		Kidney weight		Spleen weight	
				Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Male	Untreated	0	5	10.57 ± 0.59[a]	2.98 ± 0.09	2.22 ± 0.07	0.63 ± 0.01	0.62 ± 0.03	0.18 ± 0.01
	Vehicle	0	8	10.46 ± 0.44	2.95 ± 0.07	2.17 ± 0.07	0.61 ± 0.02	0.64 ± 0.03	0.18 ± 0.01
	VGCF™-H	1.6	8	10.31 ± 0.71	2.94 ± 0.10	2.13 ± 0.11	0.61 ± 0.02	0.64 ± 0.04	0.18 ± 0.01
	VGCF™-H	3.2	8	10.67 ± 0.36	2.96 ± 0.04	2.17 ± 0.12	0.60 ± 0.03	0.66 ± 0.02	0.18 ± 0.00
	VGCF™-H	6.4	8	10.56 ± 0.83	2.97 ± 0.13	2.18 ± 0.12	0.61 ± 0.03	0.64 ± 0.03	0.18 ± 0.01
	MWNT-7	3.2	8	10.61 ± 0.39	3.01 ± 0.08	2.15 ± 0.07	0.61 ± 0.01	0.63 ± 0.02	0.18 ± 0.01
	MWNT-7	6.4	8	10.56 ± 0.55	3.09 ± 0.07**	2.10 ± 0.10	0.61 ± 0.02	0.63 ± 0.02	0.18 ± 0.01
Female	Untreated	0	5	5.39 ± 0.23	2.87 ± 0.12	1.29 ± 0.08	0.69 ± 0.05	0.40 ± 0.02	0.21 ± 0.00
	Vehicle	0	8	5.29 ± 0.19	2.80 ± 0.12	1.28 ± 0.06	0.68 ± 0.03	0.39 ± 0.01	0.20 ± 0.01
	VGCF™-H	1.6	8	5.21 ± 0.14	2.74 ± 0.13	1.26 ± 0.05	0.66 ± 0.03	0.40 ± 0.02	0.21 ± 0.01
	VGCF™-H	3.2	8	5.46 ± 0.22	2.89 ± 0.11	1.28 ± 0.06	0.68 ± 0.03	0.40 ± 0.02	0.21 ± 0.01
	VGCF™-H	6.4	8	5.56 ± 0.13*	2.93 ± 0.09	1.30 ± 0.01	0.69 ± 0.02	0.41 ± 0.02 *	0.22 ± 0.01
	MWNT-7	3.2	8	5.49 ± 0.28	2.92 ± 0.13	1.30 ± 0.05	0.69 ± 0.02	0.41 ± 0.03	0.22 ± 0.01*
	MWNT-7	6.4	8	5.54 ± 0.41*	2.96 ± 0.18	1.29 ± 0.05	0.69 ± 0.02	0.40 ± 0.02	0.21 ± 0.01

a: Mean ± S.D. * and **: significantly different from vehicle treated group at $p<0.05$ and $p<0.01$, respctively.

BALF. In both the male and female VGCF™-H-treated and MWNT-7-treated groups, there was a general increase in all of these parameters compared to the vehicle control groups. None of the increases were significant in the male or female 1.6 mg/kg VGCF™-H-treated groups. In the VGCF™-H-treated groups, an increasing trend of total cells in both sexes of 1.6, 3.2, and 6.4 mg/kg groups, significantly higher values or an increasing trend of neutrophils in both sexes of 1.6, 3.2, and 6.4 mg/kg groups, an increasing trend of macrophages in the males of 6.4 mg/kg and in the females of 1.6, 3.2, and 6.4 mg/kg groups, an increasing trend of lymphocytes in the males of 1.6, 3.2, and 6.4 mg/kg groups, significantly higher values or an increasing trend of eosinophils in the males of 3.2 and 6.4 mg/kg and in the females of 1.6, 3.2, and 6.4 mg/kg groups were observed as compared to those of the vehicle-treated group. In the MWNT-7-treated groups, significantly higher values or an increasing trend of total cells, neutrophils, lymphocytes in both sexes of 3.2 and 6.4 mg/kg groups, and significantly higher values or an increasing trend of macrophages in the males of 6.4 mg/kg and the females of 3.2 and 6.4 mg/kg groups, and an increasing trend of eosinophils in both sexes of 3.2 and 6.4 mg/kg groups were observed. When comparing the same dose groups of VGCF™-H and MWNT-7 in each sex, significantly lower values of total cells, neutrophils, and lymphocytes in the females of 3.2 and 6.4 mg/kg VGCF™-H-treated groups and significantly lower values of macrophages in the females of the 6.4 mg/kg VGCF™-H-treated group were observed as compared to those of the MWNT-7-treated groups.

Overall, both VGCF™-H and MWNT-7 fibers induced inflammatory responses in the lung; however, the inflammatory response was milder in the VGCF™- H-treated rats compared to that in the MWNT-7-treated rats.

Clinical chemistry in BALF

Figure 4 shows total protein (A), ALB (B), ALP (C), LDH (D), and IL-8 (E) levels in the BALF. In the VGCF™-H-treated groups, an increasing trend or significantly higher values of ALB in the males of the 1.6 and 6.4 mg/kg groups and in the females of the 1.6 and 6.4 mg/kg groups, significantly higher value of ALP in the males of the 6.4 mg/kg group, an increasing trend of LDH in the males of the 1.6, 3.2, and 6.4 mg/kg groups and in the females of the 1.6 and 6.4 mg/kg groups, and an increasing trend or significantly higher levels of IL-8 in the males and females of the 1.6, 3.2, and 6.4 mg/kg groups were observed as compared to those of the vehicle-treated group. In the MWNT-7-treated groups, an increasing trend or significantly higher values of total protein, ALB, ALP, LDH, and IL-8 in the males and females of 3.2 and 6.4 mg/kg groups were observed. When comparing the same doses of VGCF™-H and MWNT-7, the levels of total protein, ALB, ALP, LDH, and IL-8 were significantly lower in the 3.2 and 6.4 mg/kg VGCF™-H-treated group as compared to that of MWNT-7-treated group, and the toxic effect of VGCF™-H on the lung was thought to be milder than that of MWNT-7.

Histopathological examination

The results of the histopathological findings of the lung are summarized in Table 3 for males and in Table 4 for females. The lungs of the untreated rats showed normal histology (Fig. 5A). In the vehicle-treated group, grade 1 (minimal) alveolar macrophage aggregation was significantly increased in the alveolar area (Fig. 5B). The severity of alveolar macrophage aggregation was significantly higher in all male and female VGCF™-H- and MWNT-7-treated groups compared to their respective vehicle control groups. The severity of alveolar macrophage aggregation was not different between the male VGCF™-H- and MWNT-7-treated groups. The severity of alveolar macrophage aggregation was milder in the female 3.2 and 6.4 mg/kg VGCF™-H-treated groups compared to the female 3.2 and 6.4 mg/kg MWNT-7-treated groups.

Deposition of the test material in alveolar macrophages

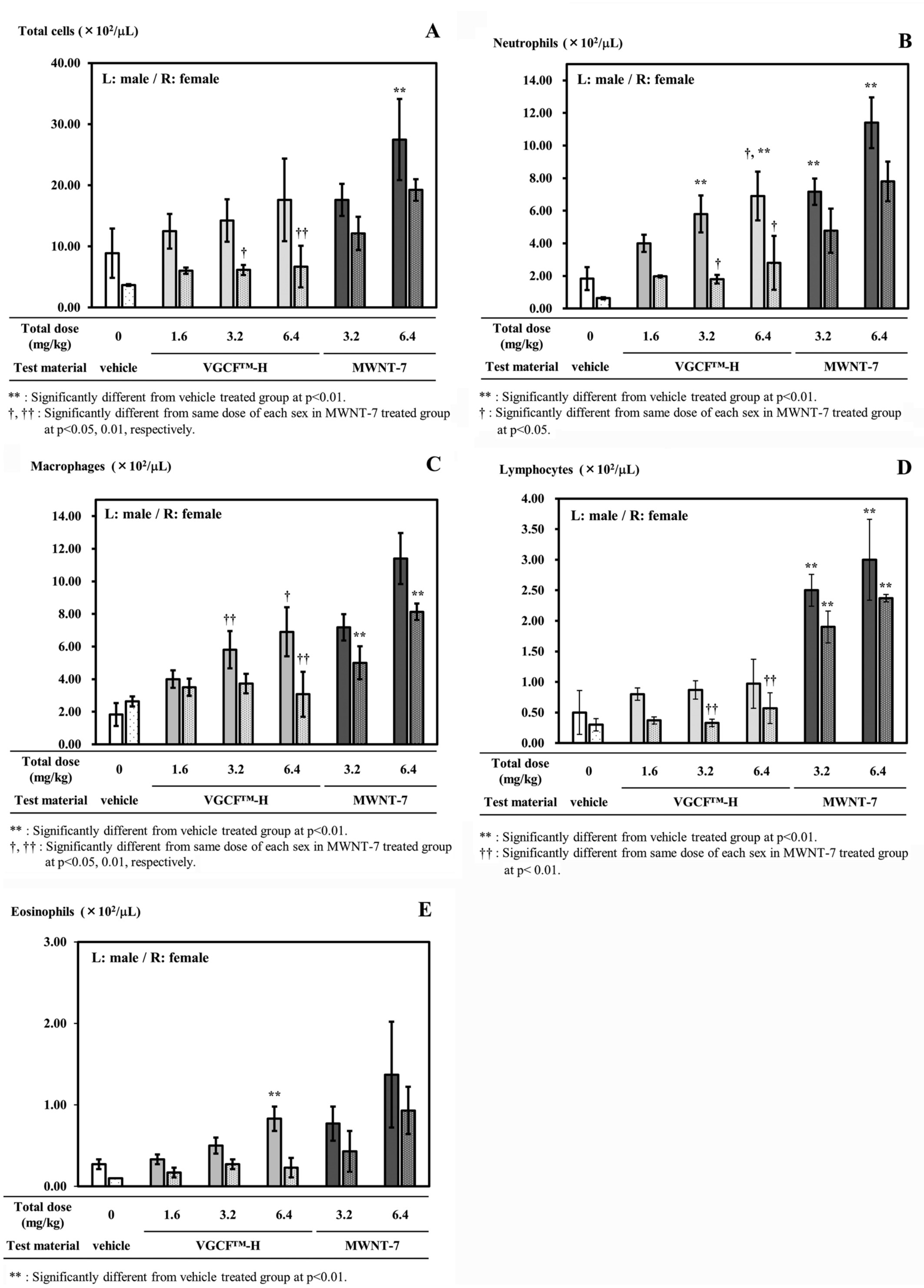

Fig. 3. Leukocyte counts in broncho-alveolar lavage fluid (BALF). Total numbers of leukocytes (A), neutrophils (B), macrophages (C), lymphocytes (D) and eosinophils (E) in the BALF. Values are presented as mean ± SD. The left bar indicates male rats, and the right bar indicates female rats. **: p<0.01 vs. vehicle-treated group. † and ††: p<0.05 and p<0.01 vs. the same dose and gender in the MWNT-7-treated group.

was observed in all male and female VGCF™-H- and MWNT-7-treated groups. In males, the severity was milder in the 3.2 and 6.4 mg/kg VGCF™-H-treated groups compared to the 3.2 and 6.4 mg/kg MWNT-7-treated groups.

Grade 1 (minimal) granulomatous change was observed in one male rat of the 1.6 mg/kg VGCF™-H-treated group and in two female rats of the 3.2 mg/kg MWNT-7-treated group.

Inflammatory cell infiltration in the alveoli was observed in all male and female VGCF™-H- and MWNT-

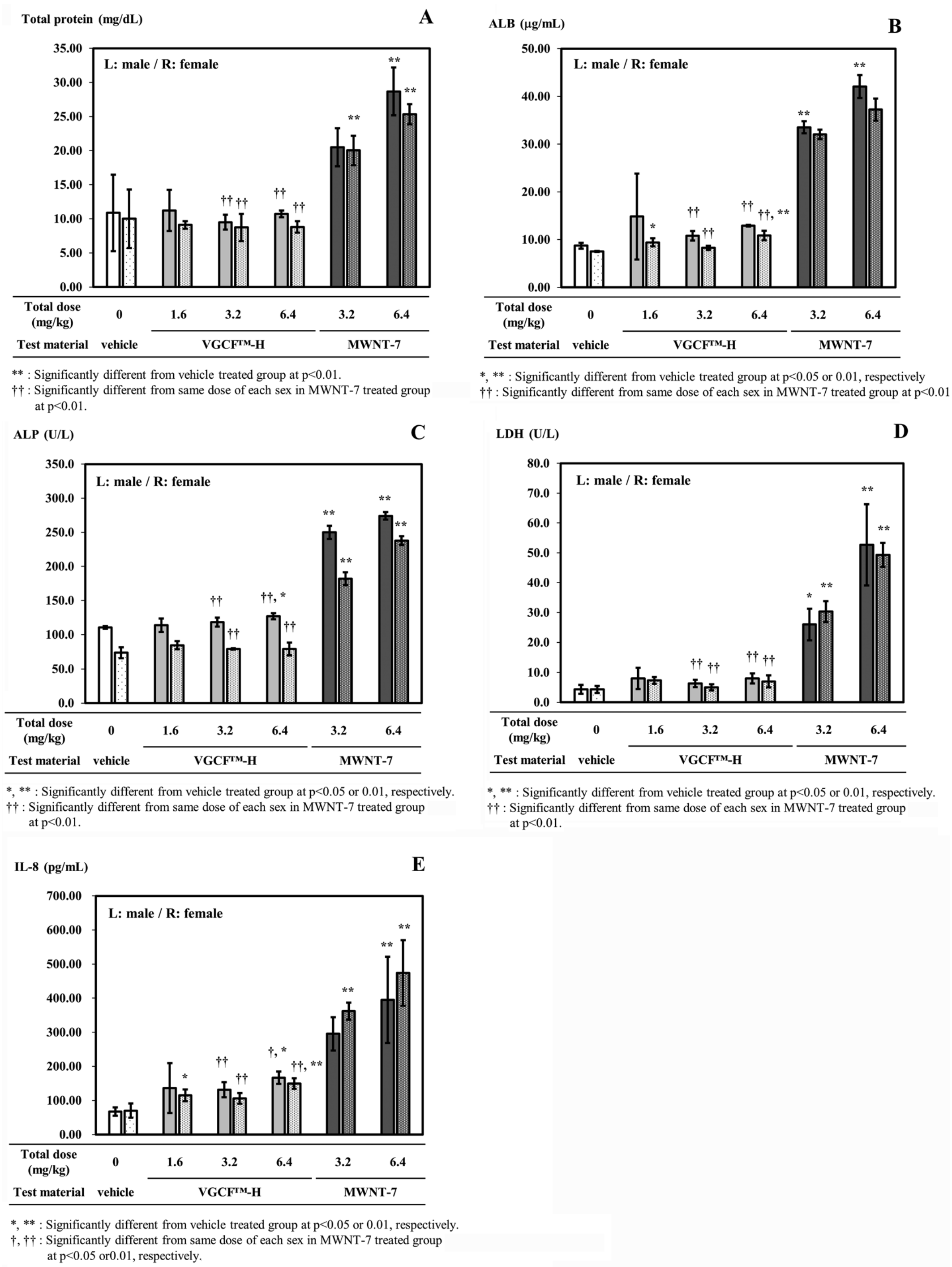

Fig. 4. Total protein, albumin (ALB), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and interleukin-8 (IL-8) levels in the bronchoalveolar lavage fluid (BALF). Values are presented as mean ± SD. The left bar indicates male rats, and the right bar indicates female rats. * and **: p<0.05 and p<0.01 vs. the vehicle-treated group. † and ††: p<0.05 and p<0.01 vs. the same dose and gender in the MWNT-7-treated group.

7-treated groups, and both the incidence and severity were significantly higher in the VGCF™-H- and MWNT-7-treated groups than in the respective vehicle control groups. The severity of inflammatory cell infiltration was milder in the VGCF™-H-treated groups compared to the MWNT-7-treated groups.

Proliferation of alveolar type II cells (Fig. 5C) was observed in the male 6.4 mg/kg VGCF™-H- and 3.2 and 6.4 mg/kg MWNT-7-treated groups and in all female VGCF™-H- and MWNT-7-treated groups. The incidence and severity of the proliferation of alveolar type II cells were significantly higher in these groups compared to their respective

Table 3. Histopathological Findings of the Lung in Male Rats

Organ and findings	Untreated	Vehicle	VGCF™-H			MWNT-7	
Sex	Male						
Test material	Untreated	Vehicle	VGCF™-H			MWNT-7	
Dose (mg/kg)	0	0	1.6	3.2	6.4	3.2	6.4
No. of rats examined	5	8	8	8	8	8	8
Lung/bronchial							
Normal	5	0	0	0	0	0	0
Alveolar macrophage aggregation/(1)a	0	8 ##	0 **	0 **	0 **	0 **	0 **
/(2)	0	0	8	0	0	0	0
/(3)	0	0	0	8	0	8	0
/(4)	0	0	0	0	8	0	8
Deposition of test material, alveolar macrophage/(1)	0	0	8 **	0 **	0 **	0 **	0 **
/(2)	0	0	0	8	0	0	0
/(3)	0	0	0	0	8	8	0
/(4)	0	0	0	0	0	0	8
Granulomatous change/(1)	0	0	1	0	0	0	0
Infiltration, inflammatory cell/(1)	0	1	7 **	4 **	3 **	1 **	0 **
/(2)	0	0	1	4	5	6	0
/(3)	0	0	0	0	0	1	7
/(4)	0	0	0	0	0	0	1
Proliferation, alveolar type II cell/(1)	0	0	0	0	2	5 *	5 **
/(2)	0	0	0	0	0	0	3
Pulmonary fibrosis, alveoli/(1)	0	1	8 **	8 **	0 **	0 **	0 **
/(2)	0	0	0	0	8	0	0
/(3)	0	0	0	0	0	8	1
/(4)	0	0	0	0	0	0	7
Pulmonary fibrosis, pleura/(1)	0	0	0	0	1	0 **	0 **
/(2)	0	0	0	0	0	7	6
/(3)	0	0	0	0	0	1	2
Proliferation, mesothelium/(1)	0	0	0	1	4 *	6 **	2 **
/(2)	0	0	0	0	0	2	6

a: Numbers in parentheses indicate lesion grades (1) Minimal, (2) Slight, (3) Moderate, and (4) Marked. ##: Significantly different from the untreated group at p<0.01. * and **: Significantly different from the vehicle-treated group at p<0.05 and p<0.01, respectively.

vehicle controls. The severity of the proliferation of alveolar type II cells in the VGCF™-H-treated groups was milder than in the MWNT-7-treated groups.

Pulmonary fibrosis (Fig. 5D) was observed in all male and female VGCF™-H- and MWNT-7-treated groups. The incidence and severity of pulmonary fibrosis was significantly higher in these groups compared to their respective vehicle controls. The severity of pulmonary fibrosis in the VGCF™-H-treated groups was milder than that in the MWNT-7-treated groups.

Grade 1 (minimal) fibrosis of the visceral pleura was observed in one male of the 6.4 mg/kg VGCF™-H-treated group (Fig. 5E), one female of the 3.2 mg/kg VGCF™-H-treated group, and one female of the 6.4 mg/kg VGCF™-H-treated group. Fibrosis of the visceral pleura occurred in all MWNT-7-treated rats. Notably, the MWNT fibers were seen near the visceral pleura (Fig. 5F). The incidences and severity of visceral pleural fibrosis were significantly higher in the MWNT-7-treated groups than in their respective vehicle controls. The incidences and severity of visceral pleural fibrosis were markedly milder in the 3.2 and 6.4 mg/kg VGCF™- H-treated groups than in the 3.2 and 6.4 mg/kg MWNT-7-treated groups.

Proliferation of lung mesothelium was observed in one male of the 3.2 mg/kg VGCF™-H-treated group and in four males of the 6.4 mg/kg VGCF™-H-treated group but not in any of the females of the VGCF™-H-treated groups. However, it was observed in all MWNT-7-treated males, in 7 of 8 females of the 3.2 mg/kg MWNT-7-treated group, and in 7 of 8 females of the 6.4 mg/kg MWNT-7-treated group. The incidence and severity were significantly higher in the male 6.4 mg/kg VGCF™-H-treated group and all MWNT-7-treated groups compared to their respective vehicle control groups. The incidences and the severity of proliferation of the lung mesothelium were markedly milder in the 3.2 and 6.4 mg/kg VGCF™-H-treated groups compared to the 3.2 and 6.4 mg/kg MWNT-7-treated groups.

The results of the histopathological findings of the pleura are summarized in Table 5 for males and Table 6 for females. The parietal pleura did not show any lesion development in the untreated group. Inflammatory cell infiltration in the subpleural tissue was observed in both sexes of the vehicle-, VGCF™-H-, and MWNT-7-treated groups. However, no significant differences were observed in inflammatory cell infiltration in the VGCF™-H-treated groups compared to the vehicle control groups. It was significantly higher in the male 3.2 mg/kg MWNT-7-treated group, but not in the male 6.4 mg/kg MWNT-7-treated group or in the female

Table 4. Histopathological Findings of the Lung in Female Rats

Sex	Female						
Organ and findings / Test material	Untreated	Vehicle	VGCF™-H			MWNT-7	
Dose (mg/kg)	0	0	1.6	3.2	6.4	3.2	6.4
No. of rats examined	5	8	8	8	8	8	8
Lung/bronchial							
Normal	5	0	0	0	0	0	0
Alveolar macrophage aggregation/(1)a	0	8 ##	0 **	0 **	0 **	0 **	0 **
/(2)	0	0	8	8	0	0	0
/(3)	0	0	0	0	8	8	0
/(4)	0	0	0	0	0	0	8
Deposition of test material, alveolar macrophage/(1)	0	0	8 **	0 **	0 **	0 **	0 **
/(2)	0	0	0	8	0	8	0
/(3)	0	0	0	0	8	0	8
Granulomatous change/(1)	0	0	0	0	0	2	0
Infiltration, inflammatory cell/(1)	0	0	3	5 *	8 **	2 **	0 **
/(2)	0	0	0	0	0	6	0
/(3)	0	0	0	0	0	0	8
Proliferation, alveolar type II cell/(1)	0	0	1	1	4 *	4 *	7 **
/(2)	0	0	0	0	0	1	0
Pulmonary fibrosis, alveoli/(1)	0	1	7 **	8 **	4 **	0 **	0 **
/(2)	0	0	0	0	4	1	0
/(3)	0	0	0	0	0	7	0
/(4)	0	0	0	0	0	0	8
Pulmonary fibrosis, pleura/(1)	0	0	0	1	1	1 **	0 **
/(2)	0	0	0	0	0	7	7
/(3)	0	0	0	0	0	0	1
Proliferation, mesothelium/(1)	0	0	0	0	0	4 **	4 **
/(2)	0	0	0	0	0	3	3

a: Numbers in parentheses indicate lesion grades (1) Minimal, (2) Slight, (3) Moderate, and (4) Marked. ##: Significantly different from the untreated group at $p<0.01$. * and **: Significantly different from the vehicle-treated group at $p<0.05$ and $p<0.01$, respectively.

3.2 and 6.4 mg/kg MWNT-7-treated groups compared to the vehicle control groups. No fibrosis was detected in the pleural cavity of any of the rats in the VGCF™-H-treated groups (Fig. 5G). In contrast, the fibrosis of the parietal pleura (Fig. 5H) was significantly increased in the male 3.2 6.4 mg/kg MWNT-7-treated and female 6.4 mg/kg MWNT-7-treated groups compared with their respective controls. There were no significant differences in the proliferation of the diaphragm mesothelium in any of the VGCF™-H- and MWNT-7-treated groups compared to the vehicle control groups.

PCNA-positive index

The PCNA-positive index (%) of the mesothelium of the visceral and parietal pleura are shown in Table 7. There were no differences between any of the VGCF™-H-treated groups and the vehicle control groups. In contrast, all MWNT-7-treated groups had significantly higher PCNA indices in the visceral pleura compared to the vehicle controls, and the male 6.4 mg/kg MWNT-7-treated group and the female 3.2 and 6.4 mg/kg MWNT-7-treated group had significantly higher PCNA indices in the parietal pleura than the vehicle controls. Notably, all VGCF™- H-treated groups had significantly lower PCNA indices compared to their respective MWNT-7-treated counterparts.

Discussion

In the present study, the lung toxicity of VGCF™-H was evaluated by a 13-week intratracheal instillation study. Rats were instilled with a vehicle or the test substance, VGCF™-H at doses of 0.2, 0.4, or 0.8 mg/kg bw (total amounts of 1.6, 3.2, or 6.4 mg/kg bw) or MWNT-7 at doses of 0.2, 0.4, or 0.8 mg/kg bw (total amounts of 3.2 or 6.4 mg/kg bw), once a week from the beginning of week 1 to the beginning of week 8. Following the 8th instillation, the rats were observed for 6 weeks and sacrificed after the observation period. Both VGCF™-H and MWNT-7 induced an increase in lung weight, changes in BALF consistent with fiber-induced lung toxicity, and histopathological changes associated with an inflammatory response in the lung. Notably, all of the changes associated with fiber-induced toxicity in the lung were clearly milder in the VGCF™-H-treated groups compared to the MWNT-7-treated groups. In addition, the MWNT-7 treatment caused the fibrosis of the parietal pleura and a significant increase in the PCNA-positive indices in the visceral and parietal pleura, whereas VGCF™-H did not.

Inhalation of airborne material may induce adverse effects in the human lung[25]. However, the toxicity assessment of respirable materials, almost all of which are used for rodents, is not widespread because the systemic inhala-

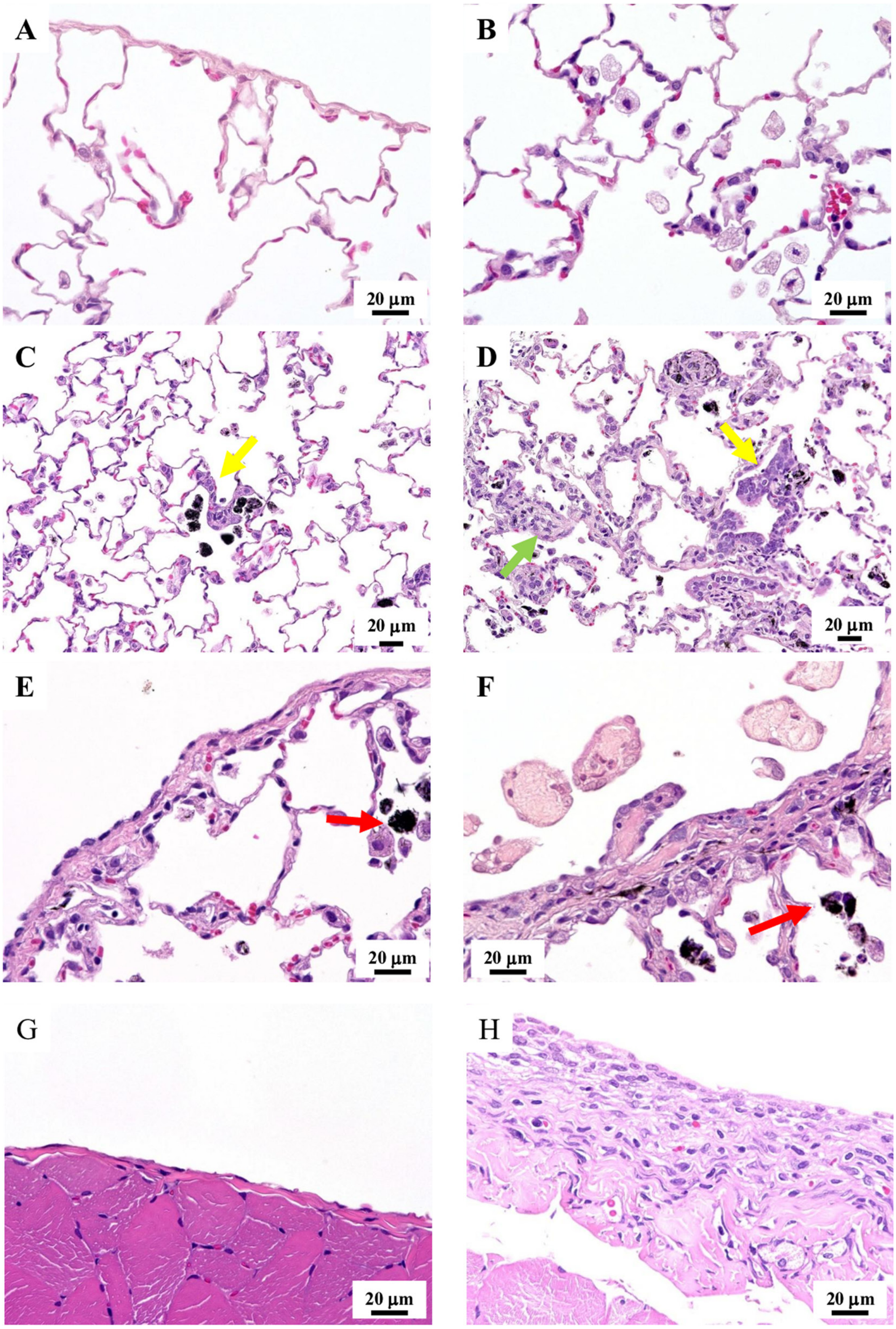

Fig. 5. Histopathological observations. A; Normal histology in the alveolar and pleural areas of the lung of a male rat in the untreated group. B; Alveolar macrophage aggregation in a male rat from the vehicle-treated group. C; Proliferation of alveolar type II cells in a male rat from the VGCF™-H-treated group. The yellow arrow indicates the proliferation of alveolar type II cells. D; Pulmonary fibrosis and proliferation of alveolar type II cells in the alveoli of a male rat from the MWNT-7-treated group. The green arrow indicates pulmonary fibrosis involving inflammatory cell infiltration, and the yellow arrow indicates alveolar type II cell proliferation. E; Minimal fibrosis of the visceral pleura in a male rat from the VGCF™-H-treated group. The red arrow indicates the deposition of test material in alveolar macrophages. F; Proliferation of pulmonary mesothelium in a male rat from the MWNT-7-treated group. The red arrow indicates the deposition of test material in alveolar macrophages. G; Normal histology of the parietal pleura in a male rat from the VGCF™-H-treated group. F; Fibrosis on the parietal pleura in a male rat from the MWNT-7-treated group.

Table 5. Histopathological Findings of the Pleura in Male Rats

Organ and findings	Sex	Male						
	Test material	Untreated	Vehicle	VGCF™-H			MWNT-7	
	Dose (mg/kg)	0	0	1.6	3.2	6.4	3.2	6.4
Diaphragm								
Normal		5	6	8	5	7	0	0
Infiltration, inflammatory cell/(1)[a]		0	2	0	3	0	5*	4
/(2)		0	0	0	0	0	2	1
Fibrosis/(1)		0	0	0	0	0	3**	3**
/(2)		0	0	0	0	0	1	5
/(3)		0	0	0	0	0	2	0
Proliferation, mesothelium/(1)		0	0	0	0	1	0	0

a: Numbers in parentheses indicate lesion grades (1) Minimal, (2) Slight, and (3) Moderate. * and **: Significantly different from the vehicle-treated group at $p<0.05$ and $p<0.01$, respectively.

Table 6. Histopathological Findings of the Pleura in Female Rats

Organ and findings	Sex	Female						
	Test material	Untreated	Vehicle	VGCF™-H			MWNT-7	
	Dose (mg/kg)	0	0	1.6	3.2	6.4	3.2	6.4
Diaphragm								
Normal		5	7	8	8	5	5	0
Infiltration, inflammatory cell/(1)a		0	1	0	0	3	3	3
Fibrosis/(1)		0	0	0	0	0	0	4**
Fibrosis/(2)		0	0	0	0	0	1	3
Proliferation, mesothelium/(1)		0	0	0	0	0	1	1

a: Numbers in parentheses indicate lesion grades (1) Minimal and (2) Slight. **: Significantly different from the vehicle-treated group at $p<0.01$.

tion studies require specialized facilities, large amounts of test materials, and technical expertise for aerosol generation. In contrast, the intratracheal instillation method does not require specialized facilities, uses small amounts of test materials, and is relatively simple to perform. These factors allow the widespread use of intratracheal instillation for testing the toxicity of respirable materials; for example, some types of MWCNTs induce pulmonary toxicity and pleural toxicity[26]. In addition, the precise dose administered into the lung by intratracheal instillation is known, allowing the dose-response relationship between the amount of test material in the lung and toxicological parameters to be determined[13, 14]: Rodents are obligate nasal breathers, and the nasal barrier has a considerable effect on the penetration of respirable fibers into the lung. Any fibers that are deposited on the nasal mucosa are removed by mucociliary clearance. Seven-week-old rats and mice have nasal surface areas of 798.6 and 277.7 mm^2, respectively[27]; the nasal cavity surface area per body weight is clearly much higher in rodents than in humans[28]. Using non-fibrous chemicals, only 10% of the aerosolized test chemical reached the lung when administered by inhalation to rats[29, 30]; thus, the penetration of fibers into the lung was dramatically reduced. Therefore, when administered by inhalation to rodents, the amount of test material that reaches the lung is unknown. In addition, given the same concentration of aerosolized fibers, the amount and size of fibers inhaled by rodents, with their highly effective nasal filtering, will be substantially different from what is inhaled by humans. Intratracheal instillation, however, directly injects the test material into the trachea, allowing the materials that may not be inhaled by a rodent to reach the lung, and the amount of material administered into the lung is known. Consequently, the use of intratracheal instillation for hazard identification and characterization is becoming increasingly recognized[8–12, 16, 20, 31], making intratracheal instillation an important method of toxicity evaluation of micro and nanomaterials that have already been developed and marketed.

Carbon fibers with submicron-scale diameters, which include the MWCNTs and the vapor grown carbon fiber tested in the present study, are attracting market attention because of their excellent physicochemical properties. However, with progress in the development and production of these materials, concerns about the toxic effects of these respirable fibers are increasing. VGCF™-H is already being used for lithium-ion batteries and fuel cells, but its needle-like fibrous structure highlights the issue of insufficient information on the toxicity and carcinogenicity of VGCF™-H when inhaled[15, 24]. Therefore, we conducted this initial study comparing the subchronic toxicities of VGCF™-H and MWNT-7, a known carcinogen in the rat lung and pleura[5, 15].

No significant differences in lung weights were observed in either male or female rats administered 1.6 mg/kg VGCF™-H compared to the vehicle controls. In rats ad-

Table 7. Proliferation Cell Nuclear Antigen (PCNA) labeling Index (%) of the Mesothelium of the Visceral and Parietal Pleura of Rats

Sex	Test Material	Dose (mg/kg)	No. of rats examined	PCNA labeling index (%)	
				Visceral pleura	Parietal pleura
Male	Untreated	0	5	3.24 ± 2.23[a]	13.68 ± 5.39
	Vehicle	0	8	1.91 ± 2.15	22.40 ± 17.14
	VGCF™-H	1.6	8	1.91 ± 1.00	17.00 ± 7.74
	VGCF™-H	3.2	8	2.88 ± 2.18 ††	20.19 ± 13.84 ††
	VGCF™-H	6.4	8	3.05 ± 2.25 ††	19.64 ± 10.30 ††
	MWNT-7	3.2	8	10.39 ± 4.25 **	40.24 ± 15.40
	MWNT-7	6.4	8	12.88 ± 5.28 **	44.48 ± 16.70 *
Female	Untreated	0	5	2.40 ± 0.85	13.80 ± 10.51
	Vehicle	0	8	3.05 ± 1.85	17.29 ± 9.48
	VGCF™-H	1.6	8	2.35 ± 1.52	14.35 ± 9.10
	VGCF™-H	3.2	8	5.31 ± 3.02 ††	22.81 ± 14.66 †
	VGCF™-H	6.4	8	3.64 ± 1.74 ††	18.19 ± 12.38 ††
	MWNT-7	3.2	8	14.83 ± 5.60 **	40.84 ± 12.63 **
	MWNT-7	6.4	8	20.26 ± 6.82 **	43.31 ± 12.22 **

a: Mean ± S.D. * and **: Significantly different from the vehicle-treated group at p<0.05 and p<0.01, respectively. † and ††: Significantly different from the same dose of each sex in the MWNT-7-treated group at p<0.05 and p<0.01, respectively.

ministered 3.2 6.4 mg/kg VGCF™-H, the lung weight increased, respectively, by 6% and 9% in males and by 7% and 10% in females. The increase in lung weight in the rats administered MWNT-7 was dramatically higher. In rats administered 3.2 and 6.4 mg/kg MWNT-7, the lung weight increased, respectively, by 34% and 63% in males and by 33% and 58% in females. These results indicate that VGCF™-H exerts a much milder effect than MWNT-7 in rat lungs.

Histopathological changes related to inflammation, such as inflammatory cell infiltration, alveolar type II cell proliferation, and pulmonary fibrosis in the lung were observed in both VGCF™-H- and MWNT-7-treated groups. However, these changes were minimal in the male and female 1.6 mg/kg VGCF™-H-treated groups, and the severity of the changes was markedly milder in the VGCF™-H-treated groups compared to the MWNT-7-treated groups. These results support the lung weight results, that is, VGCF™-H exerts a much milder effect in the lung compared to MWNT-7.

BALF analysis showed that both VGCF™-H and MWNT-7 induced inflammatory responses in the lung. In general, the 1.6 mg/kg VGCF™- H-treated groups did not have a significant elevation in any of the BALF parameters. While females in the 1.6 mg/kg VGCF™-H-treated group had elevated ALB and IL-8 levels, the females in the 3.2 mg/kg VGCF™-H-treated group did not have elevated ALB or IL-8 levels, and the lungs of the female rats in the 1.6 mg/kg VGCF™-H-treated group showed very minor changes, approximately within the normal range. Therefore, the increased levels of ALB and IL-8 in the females of the 1.6 mg/kg VGCF™-H-treated group were not considered to be of toxicological significance. Inflammatory cell infiltration was lower in the VGCF™-H-treated groups than in the MWNT-7-treated groups, and the difference was often significant. The levels of total protein and ALB (indicators of vascular permeability), LDH (an indicator of general cytotoxicity), and ALP (an indicator of type II cell toxicity) were all generally similar in the vehicle control and VGCF™-H-treated groups, but were generally markedly elevated in the MWNT-7-treated groups. These results support the results discussed above, that is, VGCF™-H exerts a much milder effect than MWNT-7 in the lung.

The lung burdens at autopsy in the rats of the VGCF™-H- and MWNT-7-treated groups was higher in males than in females. The clearance rates from the lung in the 1.6, 3.2, and 6.4 mg/kg VGCF™-H-treated groups, respectively, were 66%, 60%, and 73% in males and 57%, 53%, and 50% in females, and the clearance rates from the lung in the 3.2 and 6.4 mg/kg MWNT-7-treated groups, respectively, were 72% and 65% in males and 58% and 66% in females. These data indicate that the clearance rates of VGCF™-H and MWNT-7 from the lung were similar, suggesting that the difference in the lung toxicities of VGCF™-H and MWNT-7 was due to the factors other than lung burden. For example, the surface areas of the VGCF™-H and MWNT-7 fibers used in this study were 15 and 25 m^2/g, respectively. In the present study, we did not characterize the VGCF™-H or MWNT-7 fibers in the lung or pleural cavity; the results of the lung burden analysis indicate that the characterization of fibers retained in the tissues will be required in future studies.

MWCNTs are biopersistent and remain in the lung after their inhalation[4], and a small fraction of these fibers can translocate into the thoracic cavity where they induce proliferation of mesothelial cells in the parietal pleura[5]. Histopathological examination showed no changes in any of the histopathological parameters in the pleura of any of the VGCF™-H-treated groups compared to the vehicle controls. The administration of VGCF™-H also did not cause pleural mesothelial cell proliferation. In contrast, MWNT-7 caused fibrosis in the parietal pleura and mesothelial cell proliferation in both visceral and parietal pleura. These results are in agreement with a previous intraperitoneal injection study of carbon fibers that reported thinner straight fibers to be potentially more carcinogenic to the mesothelium than thicker straight fibers[32], and with our previous long-term study in which the rats instilled with 1.5 mg/rat MWNT-7 developed mesothelioma[15].

In conclusion, to evaluate the lung toxicity of VGCF™-H, a 13-week intratracheal instillation study comparing VGCF™-H to MWNT-7 was carried out using male and female F344 rats. Although the lung burden analysis at the end of the study showed similar results for the VGCF™-H- and MWNT-7-treated groups, the lung toxicity of VGCF™-H was obviously less than that of MWNT-7. In addition, the histopathological examination of the pleura indicated that VGCF™-H, unlike MWNT-7, did not induce toxicity in either visceral or parietal pleura.

Disclosure of Potential Conflicts of Interest: The authors declare no conflicts of interest associated with this manuscript.

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