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Biochemical and hematologic effects of polyvinylpyrrolidone-wrapped fullerene C_{60} after oral administration

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The fullerene C_{60} is used in consumer products such as cosmetics owing to its antioxidative effects and is being developed for nanomedical applications. However, knowledge regarding the safety of fullerene C_{60}, especially after oral administration, is sparse. Here, we examined the safety of fullerene C_{60} in mice after 7 d of exposure to orally administered polyvinylpyrrolidone (PVP)-wrapped fullerene C_{60} (PVP-fullerene C_{60}). Mice treated with PVP-fullerene C_{60} showed few changes in the plasma levels of various markers of kidney and liver injury and experienced no significant hematologic effects. Furthermore, the histology of the colon of PVP-fullerene C_{60}-treated mice was indistinguishable from that of control mice. These results suggest that PVP-fullerene C_{60} lacks toxicity after high-dose oral administration and indicate that PVP-fullerene C_{60} can be considered safe for oral medication. These data provide basic information that likely will facilitate the production of safe and effective forms of fullerene C_{60}.

1. Introduction

Advances in nanotechnology have led to the recent development of many nanomaterials, including nanoscale silica particles, titanium dioxide nanoparticles, and carbon nanomaterials (Augustin and Sanguansri 2009; Bowman et al. 2010; Konstantatos and Sargent 2010; Petros and DeSimone 2010). Nanomaterials typically are defined as materials that are 1 to 100 nm in length or diameter. Compared with micro-sized particles, nanomaterials have a high surface area, with increased structural integrity and unique mechanical, chemical, electrical, and magnetic properties. These properties have led to the use of nanomaterials in electronics, foods, and cosmetics and as drug delivery vehicles (Augustin and Sanguansri 2009; Bowman et al. 2010; Konstantatos and Sargent 2010; Petros and DeSimone 2010).

The fullerene C_{60} is one of the most promising nanomaterials because of its unique chemical and physical properties (Chen et al. 2012). Fullerene C_{60} is a remarkably stable compound consisting of 60 carbon atoms, with a diameter of approximately 0.7 nm. Thirty carbon double bonds are present in the structure, to which free radicals easily bond, leading to fullerene C_{60}'s characterization as a "radical sponge" (Krusic et al. 1991). Because of this strong antioxidative feature, fullerene C_{60} is used in cosmetics to reduce oxidative stress in the skin (Benn et al. 2011; Kato et al. 2010). In addition, various water-soluble fullerene C_{60} derivatives have been synthesized for use in a wide range of biologic applications (Aoshima et al. 2009; Kokubo et al. 2008; Lin and Lu 2012; Yin et al. 2009). For example, water-soluble fullerene C_{60} has stronger antimelanogenic potential than do naturally occurring whitening agents (Kato et al. 2009; Xiao et al. 2007). Furthermore, water-soluble fullerene C_{60} derivatives show promise for the treatment of various inflammatory diseases including rheumatoid arthritis (Hu et al. 2007; Yudoh et al. 2009a,b). Because of these potential biologic applications, several studies have assessed the safety of fullerene C_{60} and its water-soluble derivatives (Aoshima et al. 2010; Kato et al; 2009).

One water-soluble derivative, polyvinylpyrrolidone (PVP)-wrapped fullerene C_{60} (PVP-fullerene C_{60}), is used as a very stable, strongly antioxidative ingredient of cosmetics (Aoshima et al. 2010; Xiao et al. 2007). When applied to the skin, PVP-fullerene C_{60} exhibits protective activity against the apoptosis of keratinocytes that is caused by reactive oxygen species (Xiao et al. 2007). Furthermore, *in vitro* chromosomal aberration assays were conducted using mammalian cells and negative results were reported for PVP-fullerene C_{60} (Aoshima et al. 2010). However, only a few studies have addressed the safety of orally administered PVP-fullerene C_{60} *in vivo*. Therefore assessment of the safety of PVP-fullerene C_{60} after oral administration is a key area in the development of nanomedicines using PVP-fullerene C_{60}.

Here, we examined the safety of PVP-fullerene C_{60} after oral administration to mice. Our data show that oral administration of PVP-fullerene C_{60} induced negligible changes in various biochemical and hematologic parameters. These data provide useful basic safety information that likely will facilitate the development of safe and effective forms of fullerene C_{60}.

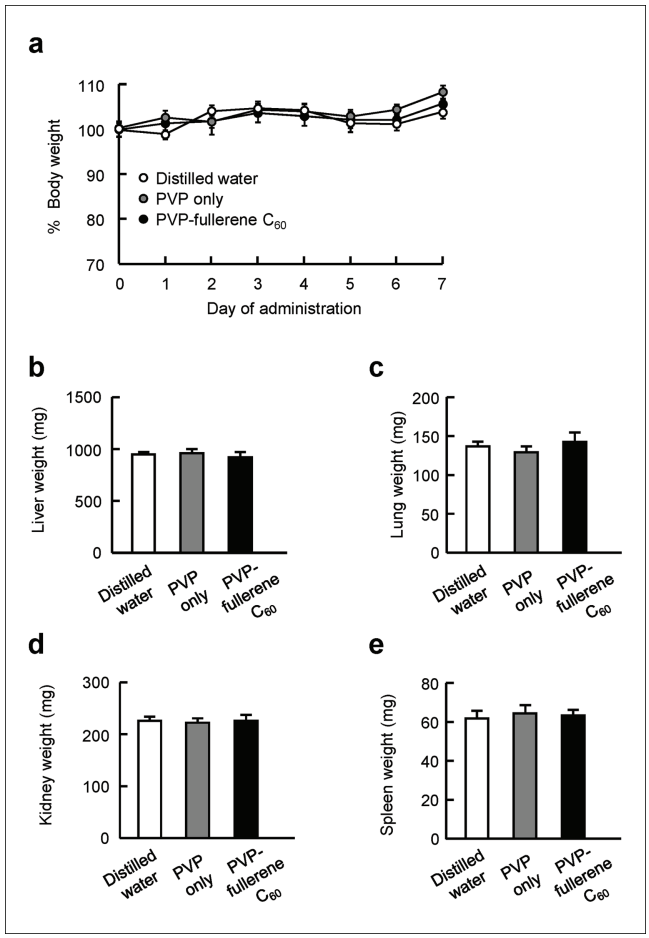

Fig. 1: Effect of oral administration of PVP-fullerene C_{60} on body weight and wet organ weights of mice. PVP-fullerene C_{60} solution in distilled water (50 mg/500 μL/mouse) was administrated orally. Control mice received distilled water or PVP only; all mice were treated by oral gavage daily for 7 d. (a) Body weight during oral administration of PVP-fullerene C_{60}, PVP only, or distilled water. Wet weight of (b) liver, (c) lung, (d) kidney, and (e) spleen after 7 d of treatment. Data are given as mean ± SEM (n = 8)

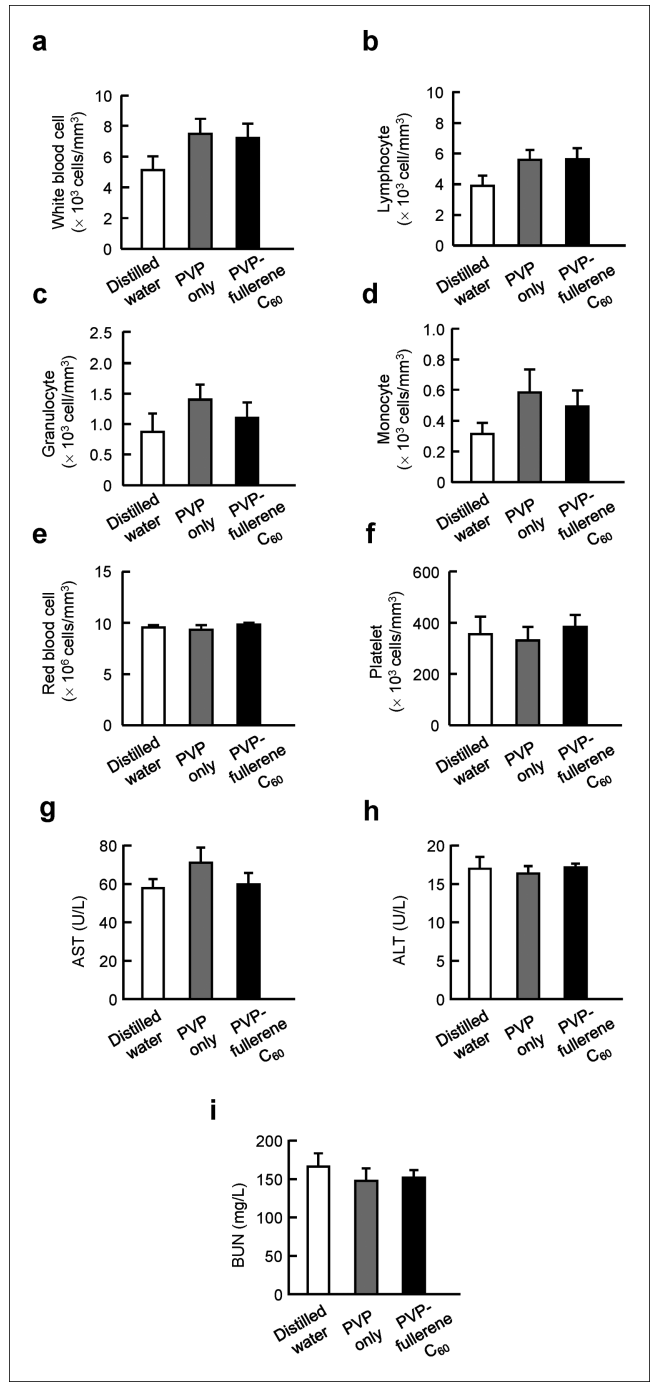

Fig. 2: Effect of oral administration of PVP-fullerene C_{60} on hematologic and biochemical parameters of mice. (a–f) Hematologic parameters were measured after oral administration of PVP-fullerene C_{60} for 7 d. (g–i) Biochemical parameters in the plasma were measured after oral administration of PVP-fullerene C_{60} for 7 d. Data are given as mean ± SEM (n = 6 or 7)

2. Investigations, results and discussion

We first used dynamic light scattering to measure the hydrodynamic diameters of PVP-fullerene C_{60}. The particle size of PVP-fullerene C_{60} in the distilled water was 127 nm, and its zeta potential was –2.2 mV.

To examine the safety of PVP-fullerene C_{60} after oral administration to mice, each mouse received 0.5 ml of distilled water, PVP only, or PVP-fullerene C_{60} solution by oral gavage once daily for 7 d. Daily behavior including eating, drinking, and activity did not differ between groups; no mice died; and there were no overt differences in body weight gain between groups (Fig. 1a). In addition, wet organ weight after 7 d of oral treatment did not differ significantly between groups (Fig. 1b–e). Hematologic parameters including numbers of red blood cells, platelets, white blood cells, lymphocytes, granulocytes, and monocytes in mice did not show significant differences between groups (Fig. 2a–f). Similarly, plasma biochemical parameters including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as indicators of hepatic injury and blood urea nitrogen (BUN) as a marker of renal damage did not differ significantly between groups (Fig. 2g–i).

Disease symptom scores and colon length are well-known indicators of colonic inflammation, which is the most common adverse effect after oral administration of test compounds. We scored fecal occult blood as a disease symptom in mice. Similar to those for the distilled water group (1.6 ± 0.1) or PVP only group (1.5 ± 0.1), the score for the PVP-fullerene C_{60}-treated group (1.5 ± 0.1) did not indicate any occult or gross rectal bleeding (Fig. 3a). Furthermore neither colon length (Fig. 3b) nor histology (Fig. 3c–e) differed between groups. Taking together all of our results, we consider that oral administration of 50 mg PVP-fullerene C_{60} daily for 7 d has negligible effects on the health of the colon in mice (Fig. 3).

Various *in vitro* and *in vivo* safety assessments of fullerene C_{60} and its derivatives have been reported previously (Metanawin et al. 2011; Nielsen et al. 2008; Zhang et al. 2009). Most studies have shown that fullerene C_{60} and its derivatives are not genotoxic under *in vitro* conditions (Aoshima et al. 2010; Ema

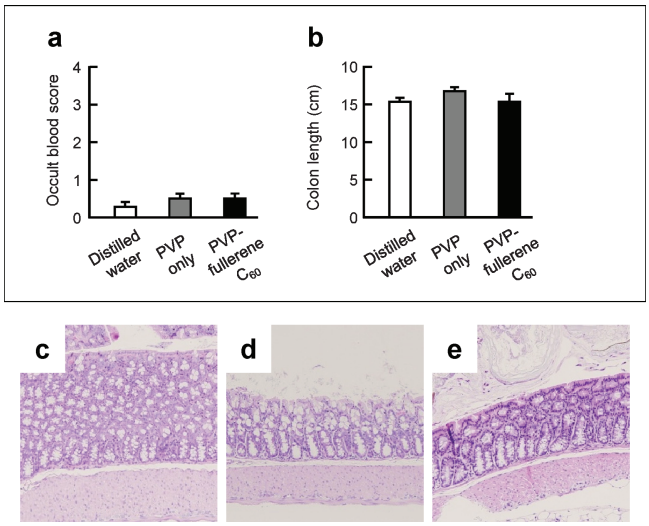

Fig. 3: Effect of oral administration of PVP-fullerene C_{60} on the histology of the colon in mice. (a) Occult blood scores were determined after 7 d of treatment by assessing the consistency, overt blood, and occult blood of feces. (b) Effect of PVP-fullerene C_{60} on colon length. All data are expressed as mean ± SEM (n = 8). Histopathology of the distal colon in C57/BL6 mice after oral administration of distilled water (c), PVP only (d) or PVP-fullerene C_{60} (e) for 7 d. Representative sections were stained with hematoxylin and eosin and examined by using light microscopy

et al. 2012; Shinohara et al. 2009). In addition, water-soluble fullerene C_{60} derivatives can safely be used for dermal and intraperitoneal injection (Aoshima et al. 2010; Gharbi et al. 2005). However, insufficient information is available regarding the safety of water-soluble fullerene C_{60} derivatives after oral administration. In this study, we evaluated the safety and toxicity of oral PVP-fullerene C_{60} by monitoring the body weight, hematologic and biochemical parameters, and colonic health of treated mice. Our results indicate that oral PVP-fullerene C_{60} has no adverse effects on the evaluated parameters in mice.

Guidelines from the Organization for Economic Co-operation and Development (OECD) recommend 28- and 90-d repeated-dose oral toxicity studies in rodents for the safety assessment of chemicals used as nanomaterials. As a first step in the safety assessment of PVP-fullerene C_{60}, we here performed a 7-d oral toxicity study. Now we are trying to perform safety evaluations after long-term exposure.

In conclusion, we showed that oral administration of PVP-fullerene C_{60} induced negligible change in various hematologic, biochemical, and histologic parameters in mice. Although additional studies are needed to further examine the safety of PVP-fullerene C_{60}, we consider that our data provide the basic information that likely will facilitate the development of safe and effective forms of fullerene C_{60}.

3. Experimental

3.1. Particles

PVP-fullerene C_{60} was provided by Vitamin C60 BioResearch (Tokyo, Japan) and is composed of purified fullerene C_{60} and PVP of 60 to 80 kDa. The C_{60} content in PVP-fullerene C_{60} was determined by HPLC analysis on a 5PBB column (Nacalai Tesque, Kyoto, Japan) and found to be approximately 3000 ppm. PVP-fullerene C_{60} was used after 5 min of sonication (280 W output; Ultrasonic Cleaner, AS One, Tokyo, Japan) and 1 min of vortexing. Particle size and zeta potential were measured by using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). The mean size and size distribution of particles were measured by using dynamic light scattering; zeta potential was measured by using laser doppler electrophoresis.

3.2. Mice

Female C57BL/6 mice were purchased from Nippon SLC (Kyoto, Japan) and used at 6 weeks of age. Mice were housed in a ventilated animal room maintained at 20 ± 2 °C with a 12:12-h light:dark cycle. Distilled water and sterilized mouse chow were available *ad libitum*. All procedures were performed in accordance with institutional ethical guidelines for animal experiments. During the treatment period, each mouse received 0.5 ml distilled water, PVP only, or PVP-fullerene C_{60} in distilled water (total dose, 50 mg) by oral gavage once daily for 7 d. Mice were euthanized 24 h after administration of the final dose, and liver, lung, kidney, and spleen tissues were harvested and weighed. Blood samples were collected in tubes containing 5 IU/ml heparin sodium, and plasma was harvested. Colons were resected for the determination of colon length (from cecum to anus) and histopathologic examination. Feces were collected and evaluated for occult blood.

3.3. Hematologic analysis

The numbers of white blood cells, granulocytes, lymphocytes, monocytes, red blood cells, and platelets in whole blood were measured by using an auto analyzer (VetScan HMII Hematology System, Abaxis, Union City, CA). Liver function was assessed by measuring plasma levels of AST and ALT. Nephrotoxicity was evaluated by measuring plasma levels of BUN. AST, ALT, and BUN were assayed by using a biochemical autoanalyzer (Fuji Dri-Chem 7000, Fujifilm, Tokyo, Japan).

3.4. Histopathologic examination

For histology of paraffin-fixed tissue, colons were excised and fixed overnight in 10% neutral buffered formalin, embedded in paraffin blocks, sliced, and placed on glass slides. Sections were deparaffinized, rehydrated through a graded series of ethanol, and stained with hematoxylin and eosin. Stained sections were dehydrated through a graded ethanol series and mounted using permount (OCT Compound, Sakura Finetek, Tokyo, Japan). Representative histologic images were recorded by a CCD digital camera that was affixed to a microscope. Fecal occult blood was scored by using the Coloscreen Occult Blood Card Test (Shionogi, Osaka, Japan), with the scale ranging from 0 for negative to 4 for strongly positive.

3.5. Statistical analysis

All results are presented as mean ± standard error of the mean (SEM). Statistical significance in differences was evaluated by analysis of variance (ANOVA) followed by Bonferroni correction. The P value used to define significance ($P < 0.05$).

Competing interests: The authors declare that there are no conflicts of interest.

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