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Letter

Biological safety of water-soluble fullerenes evaluated using tests for genotoxicity, phototoxicity, and pro-oxidant activity

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ABSTRACT — We evaluated the safety of water-soluble polymer-enwrapped fullerenes (PVP/fullerenes) as antioxidants in cosmetic and pharmaceutical preparations by studying the genotoxicity, phototoxicity, and pro-oxidant effects of these fullerenes. These materials were not mutagenic to any of the tested bacterial strains and did not induce chromosomal aberrations in cultured mammalian cells. The PVP/fullerenes did not exhibit cytotoxicity under ultraviolet or sham irradiation in the alternative phototoxicity test. Moreover, they did not show any pro-oxidant effect in the presence of Fe^{2+} or Cu^{2+}. Thus, we concluded that PVP/fullerenes are safe for use in cosmetic and pharmaceutical applications. This is the first study in which toxicity tests were performed on PVP/fullerenes.

Key words: Fullerene, Safety evaluation, Genotoxicity, Phototoxicity, Pro-oxidant

INTRODUCTION

The fullerene C_{60} has a unique truncated icosahedral structure that resembles a soccer ball—it is a polygon composed entirely of carbon atoms with 60 vertices and 32 faces, 12 of which are pentagonal and 20 are hexagonal (Kroto *et al*., 1985). In the 1990s, it was revealed that fullerenes (C_{60}, C_{70}, and higher), such as Radical Sponge®, are remarkably reactive to various free radicals (McEwen *et al*., 1992). Over the last few decades, radicals have been found to be associated with a variety of disorders and with skin aging. Therefore, fullerenes have attracted considerable attention as novel anti-aging treatments in the cosmetic and pharmaceutical industries. Fullerenes prevent the degeneration of articular cartilage in osteoarthritis (Yudoh *et al*., 2007) and may be useful in the treatment of secondary progressive multiple sclerosis (Basso *et al*., 2008). Furthermore, several fullerene derivatives such as fullerenol ($C_{60}OH_X$) inhibit allergic responses (Ryan *et al*., 2007) and prevent mitochondrial dysfunction in cellular models of Parkinson disease (Cai *et al*., 2008).

Significant advances in fullerene synthesis have enabled the large-scale production and inexpensive manufacture of these compounds in recent years. Thus, fullerenes are expected to be used for preventive purposes in different fields. However, the safety of fullerenes, which are typical nanomaterials, is a matter of growing concern. In recent years, toxicity studies have been conducted on fullerenes and their derivatives by using laboratory animals, aquatic organisms, and *in vivo* and *in vitro* microorganismal systems. Shinohara *et al*. (2009) showed that C_{60} in 0.1% carboxymethylcellulose sodium (CMC-Na) or 0.1% Tween-80 was not genotoxic by performing a bacterial reverse mutation assay, an *in vitro* chromosome aberration assay, and an *in vivo* micronucleus assay. Furthermore, the inhalation of the latter fullerene solution does not induce significant inflammation and tissue injury in rats (Fujita *et al*., 2009), and its intraperitoneal application does not result in peritoneal adhesions, fibrous thickening, or tumor formation in mice (Takagi *et al*., 2008). These findings suggest that fullerenes are nontoxic. Governments and individual researchers in several countries are currently attempting to formulate guidelines for the industrial application of nanomaterials, including fullerenes. Therefore, it is important to conduct further stud-

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ies and accumulate data on the toxicity and/or safety of fullerenes under various clinical and experimental conditions.

We have previously performed toxicity tests on a fullerene material to determine whether it could be used an additive in external-use quasi-drugs (Aoshima *et al*., 2009; Mori *et al*., 2006). Our findings indicated that highly purified fullerenes (HPFs) can be safely used in cosmetic skin applications prepared for human use. Fullerenes used for cosmetic and pharmaceutical purposes should form stable solutions or dispersions in water or other solvents. Therefore, in the present study, we aimed to confirm the solubility of polyvinylpyrrolidone (PVP)-enwrapped HPFs in water and their suitability as stable antioxidants in cosmetic ingredients. We investigated the potential toxicity of PVP-enwrapped fullerene solutions by determining their mutagenic potential, ability to induce chromosomal aberrations, alternative phototoxicity, and pro-oxidant effects.

MATERIALS AND METHODS

Test materials

HPFs (mixture of C_{60} and C_{70}, fullerite; sublimed and technical grade, 99.5% purity; Aoshima *et al*., 2009) obtained from Vitamin C60 BioResearch Corp. (Tokyo, Japan) was dissolved in water after enwrapping it in PVP (ISP Japan Ltd., Tokyo, Japan). The fullerene solutions thus obtained are hereafter referred to as PVP/fullerenes.

The test strains *Salmonella typhimurium* TA100, TA1535, TA98, and TA1537 were obtained from Prof. B. N. Ames of the University Of California at Berkeley, and *Escherichia coli* WP2*uvrA*/pKM101 were purchased from Japan Bioassay Research Center (Kanagawa, Japan), respectively. S9 mix was purchased from Kikkoman Corp. (Tokyo, Japan). The media used in the Ames test were as follows: Oxoid nutrient broth (Oxoid Ltd., Cambridge, UK), Bacto agar (Becton Dickinson and Co., NJ, USA), and minimal glucose agar plate (Oriental Yeast Co. Ltd., Tokyo, Japan). Soft agar was prepared from d-biotin, l-histidine hydrochloride monohydrate, and l-tryptophan (Wako Pure Chemical Industries Ltd., Osaka, Japan). 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) and sodium azide (NaN_3) from Wako and 2-aminoanthracene (2-AA) and 9-aminoacridine hydrochloride (9-AA) from Sigma-Aldrich Japan Inc. (Tokyo, Japan) were used as positive controls in the Ames test. Dimethyl sulfoxide (DMSO) and sodium chloride were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan).

CHL/IU cells, derived from the lungs of a female Chinese hamster, were purchased from Dainippon Pharmaceutical Co. Ltd. Eagle minimum essential medium (MEM) and Ca^{2+} and Mg^{2+}-free Dulbecco phosphate-buffered saline (PBS(-)) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan) were used to test for chromosomal aberrations. Mitomycin C (MMC; Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) and benzo[a]pyrene (BP; Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) were used as positive controls in the test for chromosomal aberrations.

The media and reagents used in the phototoxicity test were as follows: Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich); fetal bovine serum (FBS; Invitrogen, CA, USA); all the ingredients in Hanks buffered salt solution (HBS; Wako Pure Chemical Industries Ltd., and Sigma-Aldrich); chloromazine (CPZ; Sigma-Aldrich); sodium dodecyl sulfate (SDS), ethanol, and HCl (Wako); and neutral red (NR; Sigma-Aldrich).

Ames test

The Ames test was conducted according to the guidelines on genotoxicity tests for pharmaceuticals (notification no. 1604, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare (MHW), dated November 1, 1999) and the Organization for Economic Cooperation and Development (OECD) guideline for the testing of chemicals (no. 471, 1997). Mutagenicity of PVP/fullerenes was assessed using a bacterial reverse mutation assay with *S. typhimurium* TA100, TA1535, TA98, and TA1537 and *E. coli* WP2*uvrA*/pKM101 (Maron and Ames, 1983; Green and Muriel, 1976). The test was performed using the pre-incubation method in the presence or absence of S9 mix. The optimal dose of PVP/fullerene was determined by performing the test with the following doses: 156, 313, 625, 1,250, 2,500, and 5,000 μg/plate. The highest dose was determined on the basis of the required dose mentioned in the applied guideline. Distilled water (DW) was used as a negative control. The doses of the positive controls, AF-2, NaN_3, 9-AA, and 2-AA, were fixed at specific values that are known to induce positive responses. For each treatment, 0.1 ml of the test substance solution, negative control, or positive control solution was added to a sterilized test tube. Assays were performed by mixing 0.5 ml of a 0.1 mol/l sodium phosphate buffer (pH 7.4) or S9 mix with 0.1 ml of a bacterial suspension. The mixture was pre-incubated with gentle shaking (90 times/min) for 20 min at 37°C. After pre-incubation, 2 ml of the molten top agar was added to this mixture, which was then poured onto a minimal glucose agar plate. After the overlaid agar had solidified, the plates were incubated for 48 hr at 37°C. Revertant colonies in each plate were counted with an automatic colony analyzer (System Science

Co. Ltd., CA-11, Tokyo, Japan). The test substances were judged as mutagenic when they produced a dose-dependent and reproducible increase in the number of revertant colonies that was equal to or greater than twice the number of colonies in the case of the negative control, regardless of the presence or absence of S9 mix. The test substance was considered non-mutagenic when a reproducible increase in the number of revertant colonies was less than twice the number in the case of the negative control.

Chromosomal aberrations

The ability of the test substances to induce chromosomal aberrations was examined according to the guidelines used for the Ames test and the OECD guideline for the testing of chemicals (no. 473, 1997). CHL/IU cells were seeded in a culture medium (5 ml, 4.0E+03 cells/ml) and incubated at 37°C under 5% CO_2 for 3 days. The culture medium was removed, and the test substance solution (1/100 volume), S9 mix (1/6 volume), and MEM were added to the plate. The cells were incubated for 6 hr (short-term treatment) or 24 hr (continuous treatment). In the short-term treatment assay, the medium was removed from the plate after 6 hr, and the cells were washed thrice with MEM and further incubated in a fresh culture medium (5 ml) for 18 hr. In both types of assays, the cells were washed with PBS(-) and then treated with 0.25 w/v% trypsin for 5 min. The cells were then counted with a hemocytometer, and the cell growth index was calculated by considering the average value for the negative control as 100%.

Two hours before the end of culture, colcemid (final concentration, 0.1 µg/ml) was added to the medium in order to arrest the cells in metaphase. The cells were treated with 0.25 w/v% trypsin at 37°C for 5 min and dissociated after washing with PBS(-). The cell suspension was centrifuged (1,000 rpm, 5 min), and the cell pellet was subjected to hypotonic treatment with 75 mmol/l potassium chloride solution (4 ml) at 37°C for 15 min. An aliquot of the cell suspension was mixed with 0.5 ml ice-cold methanol-acetic acid mixture (3:1, v/v, fixative mixture) and centrifuged; the supernatant was discarded. The above procedure was conducted twice, and the cells were then suspended in a small amount of fixative mixture. A drop of the cell suspension was placed at 2 sites on a glass slide and allowed to dry. The cells were stained with 3 v/v% Giemsa stain for 20 min, and the slides were then washed with water and dried. The number of cells in metaphase among 500 cells/plate was determined. The mitotic and relative mitotic indexes were calculated for various concentrations of the test substances by using the following formulae:

Mitotic index (%) = (Number of cells in metaphase)/(Number of cells examined) * 100

Relative mitotic index (%) = (Mitotic index of cells treated with test substance)/(Mitotic index of cells treated with negative control) * 100

We microscopically examined 100 cells/plate for the presence of structural and numerical chromosomal aberrations. The incidence of both types of aberrations was < 5% under all treatment conditions for the substances judged as negative. This incidence was 5-10% for the substances judged as inconclusive and > 10% for those judged as positive.

Alternative phototoxicity

An *in vitro* 3T3 NR uptake phototoxicity test was performed by modifying the OECD guideline for the testing of chemicals (no. 432, 2004). The phototoxicity of each test substance was estimated on the basis of the differences in its cytotoxic concentrations for Balb/c 3T3 cells that had or had not been subjected to ultraviolet A (UVA) irradiation. Balb/c 3T3 cells were seeded in two 96-well microplates containing DMEM supplemented with 10% FBS and cultured overnight. After cultivation for 1 hr in Ca^{2+} and Mg^{2+}-containing HBS supplemented with various concentrations of test substances, the cells were irradiated with 5 J/cm^2 by using a illuminant (SOL500, honle uv technology, Munich, Germany) and a illuminometer (UV-Meter HightEnd, honle uv technology). UVA or sham-irradiated. CPZ and SDS were used as the positive and negative control respectively. After 24 hr, the concentrations of the substances that exhibited cytotoxicity were measured using the NR assay as follows. The cells were cultivated for 2 hr with NR (final concentration, 33 mg/l) in DMEM supplemented with 10% FBS. NR incorporated into living cells was quantified on the basis of the difference in the absorbance (Abs.) of cell lysates solubilized in 30% ethanol in 0.1 mol/l HCl at 550 and 650 nm. Cytotoxicity is expressed as a percentage of the difference in the Abs. (Abs. at 550 nm - Abs. at 650 nm) of the sample-treated/UVA-irradiated or sham-irradiated cells against that of untreated control cells. The photoirradiation factor (PIF) was estimated from the 50% toxic concentration (TC_{50}) of UVA- and sham-irradiated cells.

$PIF = TC_{50}(-UV)/TC_{50}(+UV)$

Pro-oxidant activity

The reaction mixture—ascorbic acid or PVP/fullerenes as an antioxidant (final 4.5-22.5 µmol/l), $FeCl_3$ or $CuCl_2$ as a metal ion (final 1.0 mmol/l), and DMSO (final 1.4 mol/l) in sodium phosphate buffer (pH 7.4), was incubat-

ed at 37°C for 60 min. Next, 100 µl 2,4-dinitrophenylhydrazine (DNPH) solution (500 mg DNPH, 2.5 ml conc H_2SO_4, 12.5 ml ethanol, 3.5 ml H_2O) was added to the reaction mixture, and was incubated at r.t. for 10 min. An authentic 2,4-dinitrophenylhydrazine (DNPH) derivative was synthesized DNPH and formaldehyde (HCHO). The DNPH derivative was quantified by high-performance liquid chromatography (HPLC) analysis. LC-6A HPLC system (Shimadzu, Kyoto, Japan) equipped with an ODS column (150 × 4.6 mm i.d.) was used as the HPLC apparatus. The elution and detection conditions were as follows: flow rate, 1 ml/min; column temperature, 30°C; elution solvent, acetonitrile:water = 4:6 (v/v); detection, 360 nm at 15 min.

RESULTS AND DISCUSSION

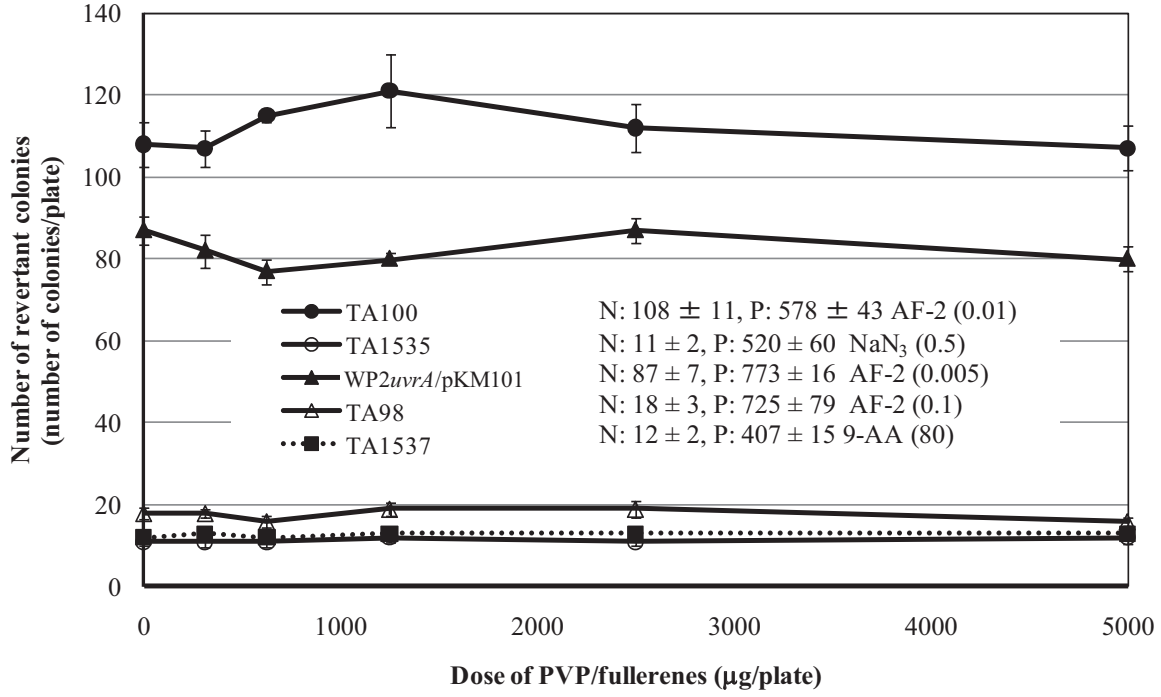
Ames test

The mutagenicity of PVP/fullerenes was examined using bacterial reverse mutation assays with various bacteria (Fig. 1). DW was selected as negative control for the test substance because PVP/fullerenes was dissolved in water. AF-2, NaN_3, 9-AA, and 2-AA were selected as positive control substances because these substances were widely used in a reverse mutation assay using bacteria and were recommended in the Guidelines on Genotoxicity Tests of Pharmaceuticals. The positive controls used in the assays on the presence or absence of S9 mix showed clear positive responses by the respective test strains, as evidenced by the number of revertant colonies being greater than 2-fold of the respective negative control value. Consequently, the validity of the test was confirmed. The number of revertant colonies in the PVP/fullerene-treated groups was less than twice that in the corresponding negative control-treated groups for all test strains, both in the presence and absence of S9 mix. Microbial toxicity was not observed for any of the test strains, regardless of the presence or absence of S9 mix. This result shows that PVP/fullerenes are not mutagenic under the conditions used in the present study. In our previous study, HPFs at a dose of 5,000 µg/plate were not mutagenic to any of the strains tested, regardless of metabolic activation (Mori *et al*., 2006). We also confirmed that 5,000 µg/plate of HPFs dissolved in squalane (200 mg/l C_{60}) and 1,000 µg/plate of HPFs enwrapped in hydrogenated lecithin, glycine soja (soybean) sterols (6 mg/l C_{60}) were not mutagenic to test strains (Kato *et al*., 2009a, 2009b). In addition, stable nano-sized C_{60} suspensions in 0.1% CMC-Na aqueous solution did not induce significant positive mutagenic responses in any test strain in the bacterial reverse mutation test, when used at concentrations of up to 1,000 µg/plate (Shinohara *et al*., 2009). These results indicate that regardless of the solution or dispersion used, fullerenes are not mutagenic.

Chromosomal aberrations

The chromosomal aberration of PVP/fullerenes was examined using cultured mammalian cells. DW was selected as negative control for the test substance because PVP/fullerenes was dissolved in water. MMC for the test without S9 mix and BP for the test with S9 mix were selected as positive control substances because these substances were widely used in a chromosomal aberration tests using cultured mammalian cells and were recommended in the applied guideline. The positive controls used in the assays on the presence or absence of S9 mix showed clear positive responses by the respective test strains, as evidenced by the number of structural or numerical aberrant cells being higher than 10% in all treatment conditions of the test substances (data not shown). Consequently, the validity of the test was confirmed. Cell growth was not inhibited by > 50% under any treatment condition (data not shown). None of the test substances precipitated in the medium at the beginning or end of the experiment under any treatment condition. The mitotic indexes of 1,000 cells are shown in Fig. 2A. The mitotic index of the cells treated with positive controls (MMC and BP) was not measured. The relative mitotic index was ≥ 71% for all test substance concentrations under all conditions. Furthermore, < 5% of cells showed structural or numerical chromosomal aberrations upon treatment with the test substances in all conditions. In the case of the positive controls, > 10% of cells had structural chromosomal aberrations, whereas in the case of the negative control, < 5% of cells had structural or numerical chromosomal aberrations (Fig. 2B). The mitotic index was not significantly lower than that in the negative control group for any of the test substances under any condition. Thus, PVP/fullerenes did not induce chromosomal aberrations under the conditions used in the present study. Therefore, we concluded that PVP/fullerenes do not significantly affect the cell cycle. Mori *et al*. (2006) indicated that HPFs at dose of 5,000 µg/ml were considered not to have the ability in induce chromosomal aberration. In addition, Shinohara *et al*. (2009) reported that stable nano-sized C_{60} suspensions in 0.1% CMC-Na aqueous solutions (100 µg/ml and 200 µg/ml as the highest dose in the test without irradiation and with irradiation) were judged to be negative in the presence and absence of S9 mix with and without irradiation. These results suggest that regardless of the solution or dispersion used, fullerenes do not have the ability to induce chromosom-

(A) Without S9 mix



(B) With S9 mix

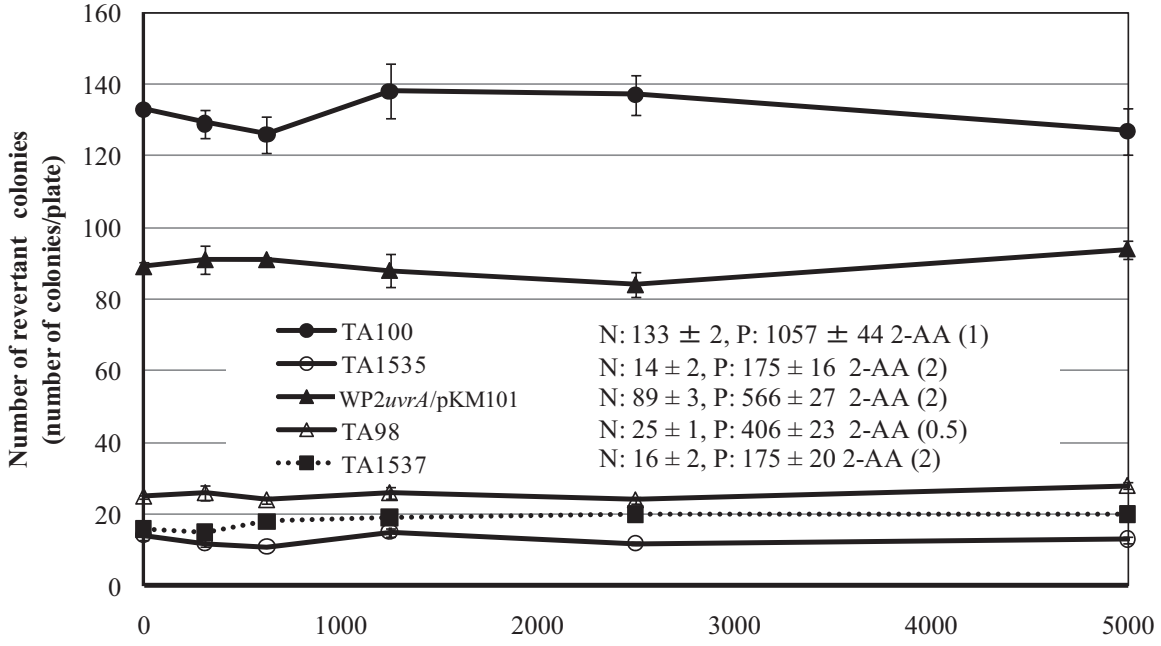


Fig. 1. Ames test of PVP/fullerenes: (A) without S9 mix, (B) with S9 mix. N: Negative control, DW; positive control: mean ± S.D. of various compounds (μg/plate), n = 3.

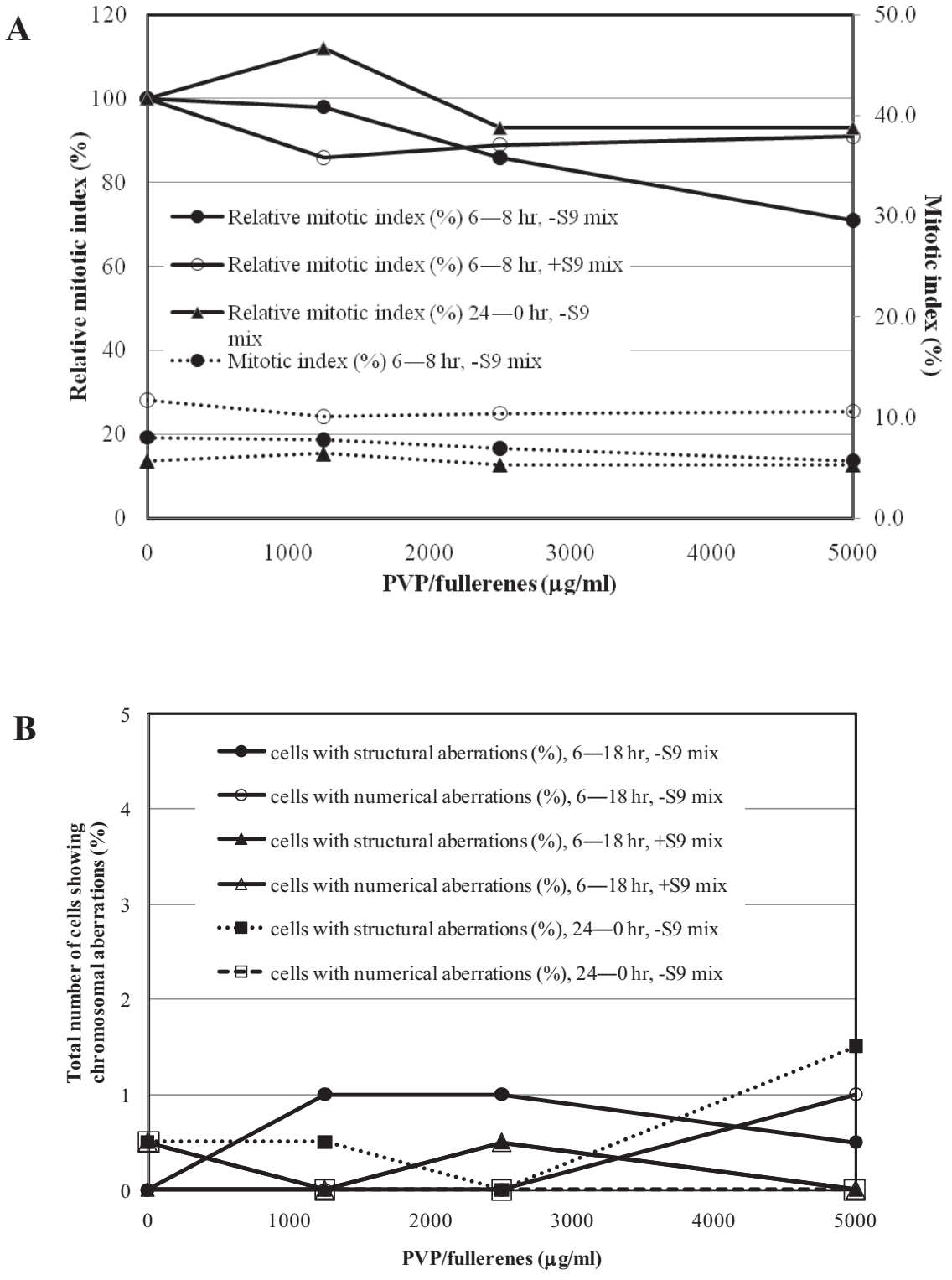


Fig. 2. Test for the ability of PVP/fullerenes to induce chromosomal aberrations: (A) Mitotic index and relative mitotic index, (B) number of cells showing structural and numerical chromosomal aberrations.

al aberration.

Alternative phototoxicity

The alternative phototoxicity of PVP/fullerenes was examined using cell viability assays with Balb/c 3T3 cells. The PIF values of all the tested substances are shown in Fig. 3. A median inhibitory concentration (IC_{50}) value of CPZ was 2.57 μg/ml under UVA-irradiation and 20.09 μg/ml under sham-irradiation. A median IC_{50} value of SDS was 26.12 μg/ml under UVA-irradiation and 30.16 μg/ml under sham-irradiation. Thus, this test system was considered correctly responsible. PVP/fullerenes at the maximum concentration used (1,000 μg/ml containing 73.0 mg C_{60}) did not exhibit any cytotoxicity toward the UV- or sham-irradiated cells. This result indicates that PVP/fullerenes are not phototoxic under the conditions employed in this study. We also confirmed that 1,000 μg/plate of HPFs dissolved in squalane and 1,000 μg/plate of HPFs enwrapped in hydrogenated lecithin, glycine soja (soybean) sterols did not also have alternative phototoxicity to the cells (Kato *et al*., 2009a, 2009b). We also demonstrated that HPFs in Propylene glycol had no contact phototoxicity potential in animal test (Aoshima *et al*., 2009). Therefore, these findings suggested that regardless *in vitro* or *in vivo*, fullerenes did not have phototoxicity.

Pro-oxidant activity

Ascorbic acid reduces divalent metal ions to monovalent ions, and the latter react with O_2 to produce active oxygen species. The pro-oxidant activity of PVP/fullerenes was evaluated on the basis of the hydroxyl radical (·OH)-dependent production of HCHO from DMSO. Since the HCHO concentration is low and therefore difficult to accurately evaluate, we converted HCHO to a DNPH derivative. Using HPLC, we measured the concentration of the derivative at various concentrations of PVP/fullerenes in the presence or absence of Cu^{2+} (Fig. 4A) or Fe^{3+} (Fig. 4B). Unlike ascorbic acid, PVP/fullerenes did not show any pro-oxidant activity under the present study conditions. The maximum applicable amount of PVP/fullerenes was used for all toxicity studies, except for the test for pro-oxidant activity. In the latter test, PVP/fuller-

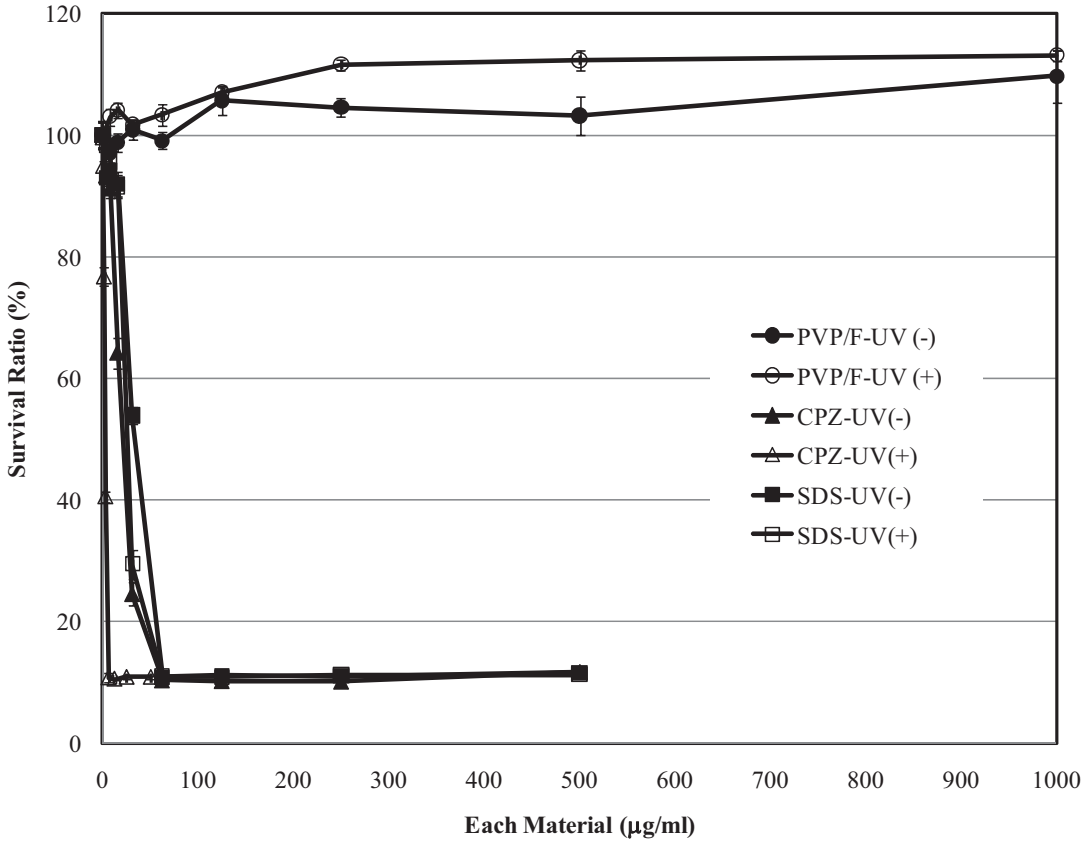


Fig. 3. Cell survival ratio after treatment with PVP/fullerenes, CPZ, and SDS; mean ± S.D. ; n = 3.

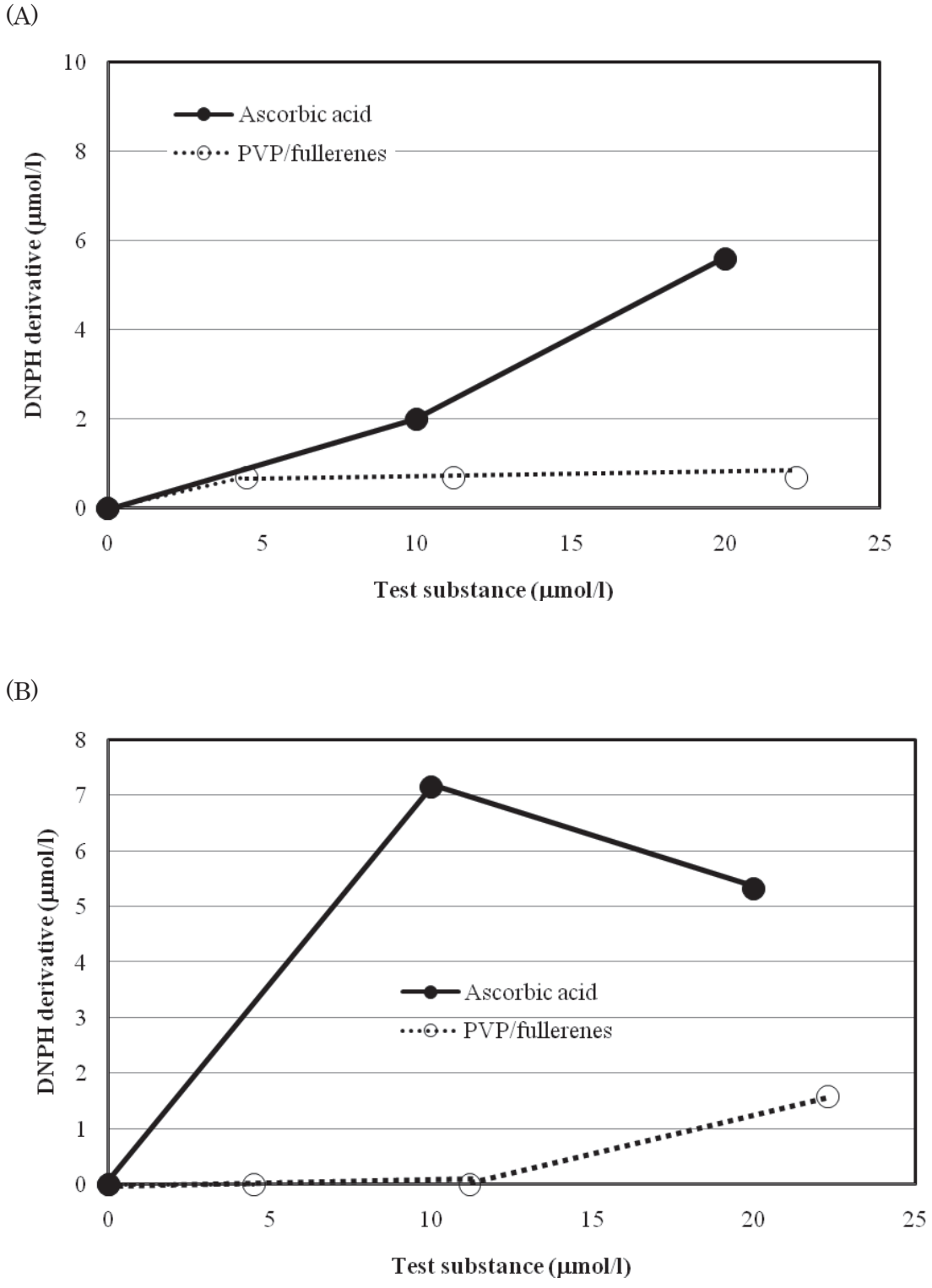


Fig. 4. HCHO generated by the pro-oxidant activity of PVP/fullerenes or ascorbic acid: (A) in the presence of Cu^{2+}, (B) in the presence of Fe^{3+}.

enes were used at a concentration equivalent to 23 μmol/l C_{60}, as determined in previous studies (Yudoh *et al*., 2007; Xiao *et al*., 2007).

In conclusion, this is the first study in which toxicity tests were performed on PVP/fullerenes. We found that PVP/fullerenes are not mutagenic, do not induce chromosomal aberrations, and are not cytotoxic under UV or sham irradiation in the alternative phototoxicity test. In addition, PVP/fullerenes have no pro-oxidant activity in the presence of metal ions. Water-soluble fullerenes have been shown to protect human skin keratinocytes from reactive oxygen species generated by UV irradiation or peroxylipids (Xiao *et al*., 2005, 2006). Fullerenes also inhibit UVA-induced melanogenesis in human melanocytes (Xiao *et al*., 2007). Yudoh *et al*. (2007) reported that water-soluble C_{60} is a potential therapeutic agent for the protection of articular cartilage in osteoarthritis. On the basis of the above findings, we conclude that PVP/fullerenes can be safely used for cosmetic and pharmaceutical purposes.

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