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The polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ protects mice from ionizing-radiation-induced immune and mitochondrial dysfunction

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ABSTRACT

Although the protective effect of the polyhydroxylated fullerene derivative $C_{60}(OH)_n$ against ionizing radiation is an area of much interest, the mechanisms relating to how polyhydroxylated fullerene derivatives improve mitochondrial dysfunction remain unknown. In order to find new and effective radioprotective agents, we synthesized a new polyhydroxylated fullerene molecule with 24 hydroxyl groups of known positions on C_{60} and studied its protective effects in mice subjected to irradiation. Mice were pretreated with $C_{60}(OH)_{24}$ for 2 weeks (daily, 40 mg/kg i. p.), then subjected to a lethal dose of whole body γ-irradiation (from a ^{60}Co source). Survival was observed for 30 days after irradiation. Immune and mitochondrial dysfunction and oxidative damage were analyzed in mice with the same $C_{60}(OH)_{24}$ pretreatment and irradiation except that the animals were euthanized at day 5 after the irradiation. It was found that 2-week $C_{60}(OH)_{24}$ pretreatment effectively reduced whole body irradiation-induced mortality without apparent toxicity. $C_{60}(OH)_{24}$ pretreatment also showed significant protective effects against ionizing-radiation-induced decreases in immune and mitochondrial function and antioxidant defense in the liver and spleen. These results suggest that the polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ protects against ionizing-radiation-induced mortality, possibly by enhancing immune function, decreasing oxidative damage and improving mitochondrial function.

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Introduction

It has long been recognized that the damaging effects of ionizing radiation are brought about by both direct and indirect mechanisms (Shirazi et al., 2007). Direct action includes the disruption of sensitive molecules in cells, and indirect action is attributed to the interaction of ionizing radiation with water molecules in the cell, resulting in the production of highly reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$) and superoxides ($O_2^{\bullet -}$) (Ewing and Jones, 1987). ROS have the potential to damage cellular macromolecules such as nucleic acids, proteins and lipids, which eventually results in physical and chemical damage to tissues that may lead to cell death or neoplasia (Cerutti, 1985). The chemistry of free radical generation has led to the proposal of the "Free Radical Theory of Aging" by Harman in 1956 (Harman, 1956).

Radiotherapy is one of the most effective treatments for cancer. Eighty percent of cancer patients need radiotherapy at some time, either for curative or palliative purposes (Nair et al., 2001). Over the past 50 years, research has focused on screening chemical and biological compounds to find effective radioprotectors. Several synthetic compounds such as sulfhydryl radioprotectors have been developed, but they have limited use due to their inherent toxicity and short active periods (Maisin, 1998; Capizzi and Oster, 2000). Therefore, the urgent search for new protective agents against ionizing radiation is essential for treating individuals at risk for environmental exposure or undergoing cancer therapy.

With the recent rapid development of nanoscience and nanotechnology, interest in carbon nanomaterials has been gradually diverted to biological and medical fields. Fullerene (C_{60}), which was discovered by H. W. Kroto et al. in 1985 (Kroto et al., 1985), is an attractive agent for biological and medical applications (Bosi et al., 2003). It has been

Abbreviations: BSA, bovine serum albumin; $DCFH_2$-DA, dichlorofluorescein diacetate; DNPH, 2,4-dinitrophenylhydrazine; GSH, reduced glutathione; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MDA, malondialdehyde; MMP, mitochondrial membrane potential; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; SOD, superoxide dismutase; ROS, reactive oxygen species.

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shown to have anti-HIV activity (Friedman et al., 1993), antimicrobial activity (Tang et al., 2007), anti-apoptotic activity (Huang et al., 1998), and neuroprotection (Dugan et al., 1996, 1997). Polyhydroxylated fullerenes [$C_{60}(OH)_x$], also known as fullerenols, are particularly efficient antioxidants (Chiang et al., 1995). Both *in vitro* and *in vivo* studies have shown that polyhydroxylated fullerene derivatives and other fullerene derivatives are potential antioxidant agents in biological systems (Lin et al., 1999; Dugan et al., 2001; Lai et al., 2003; Gharbi et al., 2005; Chirico et al., 2007), and can reduce ROS-mediated neuronal death induced by heightened glutamate receptor activity (Jin et al., 2000).

Though polyhydroxylated and carboxy-C_{60} derivatives have been shown to protect stylonychia mytilus and zebrafish against ionizing radiation (Zhao et al., 2005; Daroczi et al., 2006). The protective effects of polyhydroxylated fullerene derivatives $C_{60}(OH)_x$ ($x = 22$–24) on rodents have not been well studied, except for one study in mice and rats (Trajkovic et al., 2007). Recently, Dugan LL demonstrated a positive effect of the carboxylated C60 on cognitive impairment and life span in aging mice (Quick et al., 2008). In addition, no studies have been conducted with structurally identified polyhydroxylated fullerene derivatives regarding the mechanisms of radioprotection against mitochondrial dysfunction. We have recently synthesized a new polyhydroxylated fullerene with the 24 hydroxyl groups on C_{60}. In the present study, we investigate the effects of $C_{60}(OH)_{24}$ on $^{60}C_O$ γ radiation-induced mortality and immune dysfunction in mice, and the underlying mechanisms of mitochondrial dysfunction and oxidative damage in the spleen and liver.

Materials and methods

Materials. C_{60} was purchased from Sigma (St. Louis, MO); carboxy-dichlorofluoresce in diacetate ($DCFH_2$-DA), JC-1 and propidium iodide (PI) from Molecular Probes (Eugene, OR); Oxyblot Protein Oxidation Detection kit from Chemicon International Inc. (Temecula, CA); BCA Protein Assay Reagent kit from PIERCE (Rockford, IL); and Luminol Western blotting reagent from Santa Cruz Biotechnology. All other chemicals were purchased from China National Medicines Group, Shanghai Chemical Reagents Company.

Synthesis and characterization of $C_{60}(OH)_{24}$. $C_{60}(OH)_{24}$ was synthesized in alkaline media by complete substitution of bromine atoms from $C_{60}Br_{24}$, which was synthesized by catalytic ($FeBr_3$) reaction of C_{60} in Br_2 (Tebbe et al., 1992). The $C_{60}(OH)_{24}$ nanostructure was investigated by atomic force microscopy (AFM) and dynamic light scattering (DLS). AFM observations were performed using a Nanoscope IIIa AFM system (Veeco Instruments, Santa Barbara, USA) in the tapping mode in air with a silicon cantilever (resonance frequency, Ca. 300 KHz; length, 125 μm). AFM was used to analyze the height of fullerenol aggregates, the height difference was determined via a cross-section analysis by AFM system software. DLS measurements were performed on a DLS-820(Otsuka Electronics, Co. Ltd., Osaka, Japan) light scattering system equipped with a He-Ne laser (22 mW output power) operating at 632.8 nm at 25 °C. Each measurement was repeated at least three times.

Animal model and experimental protocol. Anti-irradiation studies of $C_{60}(OH)_{24}$ were performed on male ICR mice (Lee and Park, 2003, 2004). The animals were purchased from Shanghai Laboratory Animal Co., LTD, Shanghai, China. Animals were housed five per cage in a climate-controlled, ventilated standardized sterile room according to institutional animal care and use (IACUC) protocols. The animals were, on average, 60–80 days old at the time of radiation and weighed 20–30 g. The animal model and experimental protocol were the same as described by Lee and Park (2003, 2004).

The animals were randomly divided into four groups with 20 animals in each group. Group 1, Control group: Mice were treated with saline (0.5 ml i.p.) for continuous 14 days without irradiation; Group 2, $C_{60}(OH)_{24}$ pretreated without irradiation group: Mice were treated with $C_{60}(OH)_{24}$ (40 mg/kg in 0.5 ml saline i.p.) for continuous 14 days without irradiation; Group 3, irradiation group: Mice were treated with saline (0.5 ml i.p.) for continuous 14 days with irradiation on day 15, and Group 4, $C_{60}(OH)_{24}$ pretreated with irradiation group: Mice were treated with $C_{60}(OH)_{24}$ (40 mg/kg in 0.5 ml saline i.p.) for continuous 14 days with irradiation on day 15.

The mice in groups 3 and 4 received a dose of 8 Gy at a dose rate of 1 Gy/min from a ^{60}Co source (Gamma Cell-40 Irradiator, Atomic Energy of Canada Ltd.) in a well-ventilated wooden box. After the irradiation, the mice were returned to climate-controlled cages with access to food and water. All animal protocols were approved by IACUC of the shanghai Institute of Applied Physics, Chinese Academy of Sciences.

Impact of $C_{60}(OH)_{24}$ on the animal survival rate. The animal survival rate was assessed 30 days after irradiation following the experimental protocol described by Lee and Park (2003, 2004). Eighty mice were used for this experiment. Mice were killed by euthanasia when appeared moribund or when demonstrating greater than 20% weight loss, according to IACUC protocols.

Impact of $C_{60}(OH)_{24}$ on the animal hemograms. Hemograms were evaluated in 32 mice (eight animals from each group) using the same animal model and experimental protocol. Blood was taken from the retro-orbital sinus/plexus using heparin-coated blood collection tubes 5 days after irradiation. Hematological parameters were analyzed using an autoanalyzer (XE, 2100, Sysmex, Tokyo, Japan).

Sample preparation for biochemical assays. Biochemical analysis was evaluated with 64 mice (16 animals from each group) using the same animal model and experimental protocol. All animals were euthanized by decapitation 5 days after irradiation. This time point was chosen based on the protocol of Lee and Park (2003, 2004) to assess the effects of radioprotectors prior to animal death.

The livers from half of the mice (eight animals from each group) were immediately dissected and frozen at −80 °C until used for determination of reduced glutathione (GSH) and malondialdehyde (MDA) levels, superoxide dismutase (SOD) enzymatic activities, or Western blotting. Livers from the other half (eight animals from each group) were immediately dissected and used to isolate liver mitochondria.

Spleens from eight mice in each group were immediately dissected to obtain suspensions of dissociated splenocytes to evaluate cell proliferation and mitochondrial membrane potential (MMP). Spleens of the other eight mice in each group were immediately dissected for flow cytometric evaluation of splenocyte apoptosis.

Preparation of liver tissue extracts. Liver tissue was homogenized with a solution of 0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.4, containing 8.5 μM leupeptin, and 100 μg/ml aprotinin, using a homogenizer at maximum speed for 15 s. Each sample was then centrifuged at 4000 × *g* for 15 min at 4 °C and the resulting supernatants were stored at −80 °C and used for the assays. The protein concentration in the supernatant was measured by the BCA™ Protein Assay kit (PIERCE, Rockford, IL) with bovine serum albumin (BSA) as the standard.

SOD activity assays and determination of MDA and GSH levels in liver tissue extract. SOD activity was determined using a variation of the classical NBT (nitroblue tetrazolium) method (Spitz and Oberley, 1989). The experimental method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliun chloride to form a red formazan dye. SOD activity is measured by the degree of inhibition of this reaction, expressed as U/mg protein. MDA was

determined as an measurement of lipid peroxidation using a spectrophotometric assay for TBAS (thiobarbuturic acid-reactive substances) (Ohkawa et al., 1979). The concentration of total glutathione was determined by the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4 M^{-1} cm^{-1}$) as described by Akerboom and Sies (1981).

Preparation of liver mitochondria. Mitochondria were isolated as described (Krahenbuhl et al., 1991) with a slight modification. Briefly, liver tissue was rinsed with saline, weighed, and put into ice-cold isolation buffer containing 0.25 M sucrose, 10 mM Tris base, 0.5 mM ethylenediaminetetraacetic acid, pH 7.4. Tissues were carefully sheared and rinsed to wash off residual blood, and then homogenized with isolation buffer. The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant fraction was decanted and saved. The pellet was washed with isolation buffer, and the supernatant was combined with the former saved aliquot, and centrifuged at 10,000 × *g* for 10 min. The mitochondrial pellet was washed twice with isolation buffer. All manipulations were carried out at 4 °C. The mitochondrial protein concentration was determined using a BCA (bicinchoninic acid) method with BSA as a standard. All aliquots of freshly prepared mitochondria were used to assay mitochondrial reactive oxygen species (ROS), and the remainder was frozen at −80 °C for other assays.

Detection of ROS and hydrogen peroxide in liver mitochondria. The production of liver mitochondrial ROS was measured by 2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA) oxidation (Cathcart et al., 1983). Freshly isolated mitochondria were incubated with 2 μM $DCFH_2$-DA for 30 min, then washed with isolation buffer and the fluorescence was measured at 502 nm excitation and 530 nm emission on a spectrophotometer (Flex Station 384, Molecular Devices, USA). Hydrogen peroxide levels were determined using horseradish peroxidase and 2,2′-azino-bis(3-ehylbenzthiazoline-6-sulfonic acid as described by Makinen and Tenovuo (1982).

Detection of protein carbonyls in liver mitochondria. Protein carbonyls were assayed with the Oxyblot protein oxidation detection kit with a slight modification (Chemicon International, CA). The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNPH-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting (Levine et al., 1990). Polyacrylamide resolving gels (12% w/v) were stained with Coomassie Brilliant Blue R250 and processed immediately for immunoblotting using the Western blotting technique.

Detection of cell proliferation of splenocytes. Mitogen concanavalin A (Con A)-stimulated proliferation of splenocytes, an index of immune function, was assayed by the method of Mosmann (1983). Isolated splenocytes were washed twice with PBS, adjusted to 10^6 cells/ml with RPMI-1640 medium, and seeded in a 96-well plate (100 μl/well) in the presence of the mitogen concanavalin A (10 μg/ml; Sigma), then cultured in a 5% CO_2 humidified incubator at 37 °C for 24 h. Cells were incubated with 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) for 3 h at 37 °C, 5% CO_2. The MTT solution was removed, and 200 μl dimethyl sulfoxide was added to each well to dissolve the formazan. Absorbance was measured at 550 nm (600 nm as a reference) on a microplate reader (SpectraMAX 190, Molecular Devices, USA).

Detection of mitochondrial membrane potential (MMP) in splenocytes. Determination of MMP was carried out using the ratiometric dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), which is a dual emission potential-sensitive probe (Tirosh et al.,

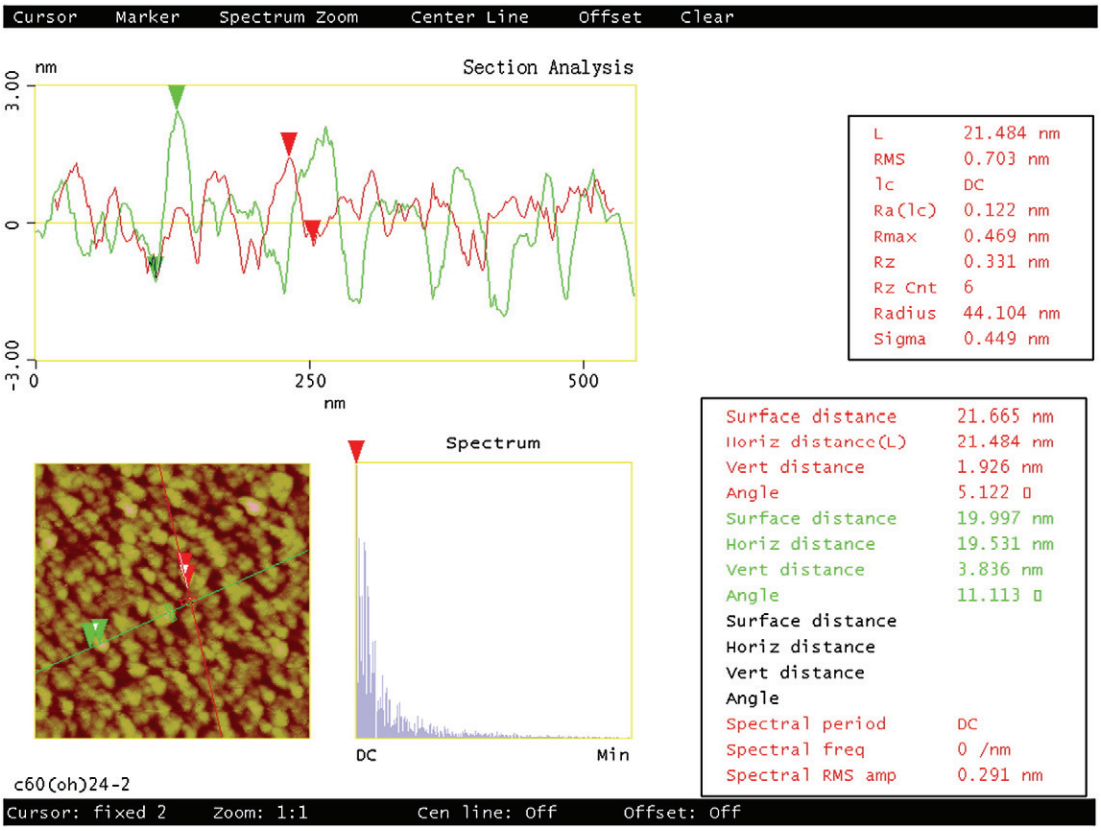

Fig. 1. Characterization of $C_{60}(OH)_{24}$. The AFM images of an aqueous suspension of $C_{60}(OH)_{24}$.

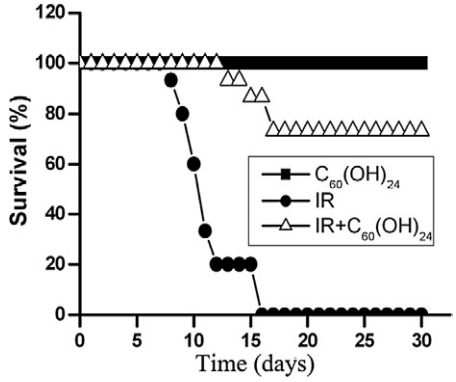

Fig. 2. Effects of $C_{60}(OH)_{24}$ on the survival of mice exposed to ionizing radiation. $C_{60}(OH)_{24}$ control group ($C_{60}(OH)_{24}$, rectangles), ionizing radiation group (IR, closed circles), and IR + $C_{60}(OH)_{24}$ (triangles). The survival rate was observed over 30 days after the radiation. Each group started with 20 animals.

2000). Isolated splenocytes were incubated with 10 μg/ml of JC-1 for 15 min at 37 °C, washed twice with PBS and analyzed by a dual-wavelength/double-beam recording spectrophotometer (Flex Station 384, Molecular Devices, USA).

Detection of apoptosis in splenocytes. The effect of $C_{60}(OH)_{24}$ on the apoptosis rate was determined by flow cytometry after staining the cells with propidium iodide (Park et al., 2007). Isolated splenocytes were washed three times in phosphate buffer saline (PBS, PH 7.4) to about 10^5 cells per ml, and fixed with 70% ethanol at −80 °C. The fixed cells were then centrifuged and washed with PBS. The cells were treated with 1 unit of DNase-free RNase (10^5 cells in 1 ml), and incubated for 30 min at 37 °C. Propidium iodide (50 μl, 1 mg/ml) was added directly to the cell suspension. The percentage of cells containing sub-G1 DNA were measured with a FACStar flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using lysis II and Cellfit software (Becton Dickinson).

Statistical analysis. Results are expressed as means ± SE and statistical comparisons between groups were performed using one-way ANOVA followed by post-hoc comparison using Tukey/Kramer HSD or LSD tests in SPSS.

Results

Characterization of $C_{60}(OH)_{24}$

The molecular formula of fullerenol was found to be $C_{60}(OH)_{24}$ with the following parameters: Fourier Transform Infrared Spectroscopy (FTIR) peaks at 3381, 1595, 1376, and 1063 cm^{-1}; an Atmospheric Pressure Chemical Ionization Quadrupole Mass Spectrometer (APCI-MS) (m/z) of 720 [C_{60}^{+}] and 1128 [$C_{60}(OH)_{24}^{+}$]; high resolution MS (HRMS):target m/z = 1128.0638 ± 0.005; and net charge = +1. The obtained results are very similar to those that were previously published (Friedman et al., 1993; Mirkov et al., 2004). Fullerenol aggregates were observed as shown in Fig. 1. The height of fullerenol aggregates shown by the red arrow was about 1.9 nm and that shown by the blue arrow was about 3.8 nm. DLS measurements indicated that their average diameter was 60.2 nm in our previous publication (Cai et al., 2008).

$C_{60}(OH)_{24}$ increases of the survival of mice exposed to lethal whole body radiation

The animals of pretreated $C_{60}(OH)_{24}$ without irradiation group survived with no apparent adverse effects after 30 days. Pretreatment of mice with 40 mg/kg daily for 2 weeks decreased the radiation-induced mortality when compared with the radiation control group. Pretreatment with $C_{60}(OH)_{24}$ significantly increased the survival of animals (Fig. 2): 73.7% to 8 Gy for $C_{60}(OH)_{24}$ survived against 0 for 30 days.

Effect on hemograms

The hemogram analysis revealed statistically significant changes in white blood cells and platelets as shown in Table 1. The white blood cell and platelet counts were significantly reduced in the irradiated mice. $C_{60}(OH)_{24}$ treatment significantly protected the irradiation-induced reduction of white blood cell counts. Other parameters had no significant changes.

Effect on ROS,SOD, GSH, and MDA in the liver and liver mitochondria

Since mitochondria are both the source and target of ROS (Beckman and Ames, 1998), we tested whether ionizing-radiation-induced mitochondrial dysfunction and oxidative damage are accompanied by an increase in ROS using DCF staining. As shown clearly in Figs. 3A and B, irradiation induced a significant increase in ROS and H_2O_2, while $C_{60}(OH)_{24}$ treatment significantly prevented the irradiation-induced increase in ROS and H_2O_2.

Ionizing radiation caused a significant decrease in the antioxidant system as shown in the reduction of the activity of SOD and the level of GSH. Pretreatment with $C_{60}(OH)_{24}$ prevented the irradiation-induced decrease in SOD activity (Fig. 3C) and GSH levels (Fig. 3D). Ionizing radiation also resulted in a significant increase in the content of MDA, a biomarker of lipid peroxide. $C_{60}(OH)_{24}$ pretreatment leads to a significant inhibition of the irradiation-induced increase in MDA (Fig. 3E).

Effect on protein carbonyls in the liver mitochondria

Protein carbonyl, an index of oxidative protein damage, was detected using the DNPH reaction followed by Western blotting of the soluble protein fraction. We measured the protein carbonyls in the liver mitochondria. Ionizing radiation resulted in a significant increase in protein carbonyls in the liver mitochondria, compared to non-irradiated controls, and pretreatment with $C_{60}(OH)_{24}$ significantly inhibited protein carbonyl increase (Figs. 4A, B).

Effect on cell proliferation, mitochondrial membrane potential (MMP), and apoptosis in splenocytes

As shown in Fig. 5A, ionizing radiation resulted in a significant decrease in spleen cell proliferation, compared to control, suggesting a significant decrease in immune function. Pretreatment with $C_{60}(OH)_{24}$ significantly protected against the ionizing-radiation-induced con A-induced decrease in spleen cell proliferation.

As shown in Fig. 5B, as compared with control, ionizing radiation caused a decrease in mitochondrial membrane potential (MMP). Pretreatment with $C_{60}(OH)_{24}$ significantly prevented the ionizing-radiation-induced MMP decrease.

Table 1
Hematological levels.

	Control group	$C_{60}(OH)_{24}$ group	IR group	$C_{60}(OH)_{24}$ + IR group
WBC (10^9/l)	4.74 ± 0.45	5.24 ± 0.39	0.20 ± 0.03**	1.32 ± 0.1#
PLT (10^9/l)	776 ± 70	839 ± 53	20.2 ± 2.9**	43.7 ± 3.7
RBC (10^{12}/l)	8.09 ± 0.12	7.9 ± 0.13	6.82 ± 0.2	5.8 ± 0.31
HBG (g/l)	146 ± 3.9	152 ± 0.9	122 ± 2.9	102 ± 2.6
MCV (fL)	54.9 ± 0.31	53.5 ± 0.5	53.4 ± 0.3	50.9 ± 0.2
MCHC (g/l)	344 ± 4.5	339 ± 4	330 ± 1.5	334 ± 5.2

WBC, white blood cells; PLT, platelets;, RBC, red blood cells; HGB, hemoglobin concentration; MCV, mean corpuscular volume; MCHC, mean cell hemoglobin concentration. The data are means ± S.E. derived from 8 mice in each group.**$p<0.01$ vs. control group and #$p<0.05$ vs. irradiated control group (ANOVA).

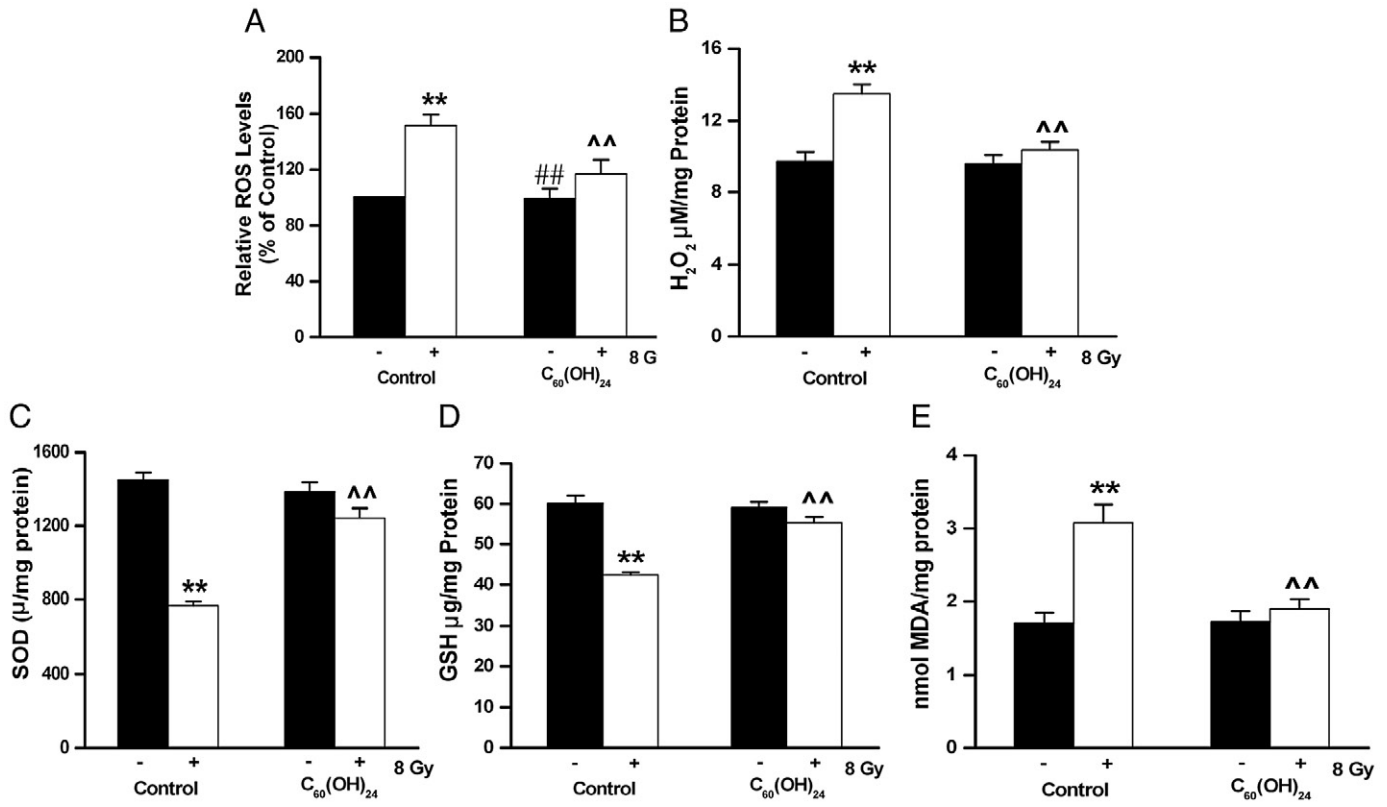

Fig. 3. Effect of $C_{60}(OH)_{24}$ on oxidant generation, SOD, GSH, and MDA in the liver and liver mitochondria. (A) Quantitative results of ROS levels of DCF staining in the liver mitochondria assayed. (B) Production of hydrogen peroxide in the liver mitochondria. (C) Activity of SOD in liver tissue extract. (D) GSH levels in liver tissue extract. (E) MDA levels in the liver tissue extract. The data are means ± SE derived from 8 mice in each group. $^{**}p<0.01$ vs. control group, $\hat{}\hat{}p<0.01$ vs. irradiated control group and $\#\#p<0.01$ vs. irradiated $C_{60}(OH)_{24}$ group (ANOVA).

We performed an *in vitro* apoptosis detection assay to determine whether $C_{60}(OH)_{24}$ could protect ionizing-radiation-induced apoptosis in splenocytes. Representative images of sub-G1 phase cells as quantified using PI staining and flow cytometry were shown in Fig. 5C. As shown in Fig. 5D, ionizing radiation induced a significant increase in the number of cells in sub-G1 phase, while pretreatment with $C_{60}(OH)_{24}$ prevented the ionizing-radiation-induced increase in the number of cells in sub-G1 phase, suggesting that $C_{60}(OH)_{24}$ pretreatment can inhibit ionizing-radiation-induced apoptosis.

Discussion

Because radiation-induced cellular damage is primarily due to the harmful effects of free radicals, compounds with direct free radical scavenging properties are of particular interest as radioprotectors. The best known radioprotectors are sulfhydryl compounds, such as cysteine and cysteamine (Patt et al., 1949). These compounds, however, produce serious side effects due to toxicity at the doses required for radioprotection. Therefore, there is a great need to search for novel radioprotectors. Lee and Park have studied two well-known antioxidants, specifically, alpha-phenyl-*N*-*t*-butylnitrone and manganese (III) tetrakis(*N*-methyl-2-pyridyl)porphyrin, and found that they are effective non-sulfur-containing radiation protectors (Lee and Park, 2003, 2004).

Because of the unique chemical and physical properties of C_{60}, water-soluble fullerene derivatives with various functional groups (e.g., −OH, −COOH, $-NH_2$) attached to the fullerene cage are promising candidates for many biomedical applications (Bosi et al., 2003). C_{60} and its derivatives have been shown to be antioxidants (McEwen et al., 1992; Chiang et al., 1995) and free radical trappers in biological systems (Dugan et al., 1996, 1997; Tsai et al., 1997; Lin et al., 1999). In the present study, the *in vivo* role of the polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ against ionizing radiation was investigated in mice. $C_{60}(OH)_{24}$ significantly increased the survival rate of mice exposed to lethal doses of ionizing radiation. Previously, Trajkovic et al. (2007) reported that one acute injection of 100 or 300 mg/kg $C_{60}(OH)_{24}$ administered 30 min (i.v.) before a lethal and sublethal dose of X-ray irradiation offers radioprotection with a significant prolongation of mean lethal time and tissue-protective effect in rats. But Trajkovic et al. only studied the radioprotective effect of $C_{60}(OH)_{24}$, the protective mechanisms remains unanswered. We used a lower dose 40 mg/kg, but with 2 weeks of chronic pretreatment (i.p.), and γ-irradiation from a ^{60}Co source. We not only report the radioprotective effect of $C_{60}(OH)_{24}$ in mice, but also elucidate that $C_{60}(OH)_{24}$ protects against ionizing-radiation-induced mortality, possibly by enhancing immune function, decreasing oxidative damage and improving mitochondrial function. We administered $C_{60}(OH)_{24}$ via i.p. injection at 40 mg/kg for 14 days, which were much lower than the dose of Trajkovic et. al. (i.v. at 100–300 mg/kg for one time). Our results are consistent with Trajokovic et al. in protective effect but different in the doses and duration. As is known, i.v. route is not a convenient route for clinical application while a systemic route of drug delivery and longer duration of smaller dose will be more desirable in clinical application than the i.v. route.

Ionizing radiation is known to functionally alter the immune system and break self-tolerance (Sakaguchi et al., 1994; Gridley et al., 2002). One of the possible protective mechanisms of $C_{60}(OH)_{24}$ against ionizing-radiation-induced mortality might be enhancement of the immune system. As is already known, white blood cells or leukocytes are the relevant components of the immune system that defend the body against both infectious disease and foreign materials. As shown in Table 1, ionizing radiation caused a significant decrease in white blood cell number, suggesting a marked decrease in immune function. $C_{60}(OH)_{24}$ treatment pro-

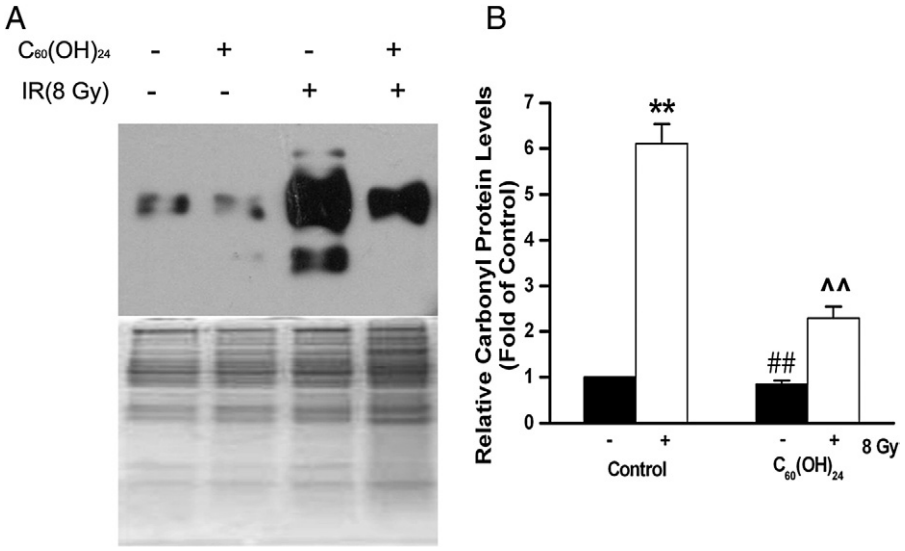

Fig. 4. Effect of $C_{60}(OH)_{24}$ on protein carbonyl levels in the liver mitochondria. (A) Liver mitochondrial protein oxidation. Representative images from one of three independent experiments are shown. Upper panel: Western blot for protein carbonyls. Lower panel: Coomassie blue staining for protein levels. (B) Quantification of the relative protein carbonyl level. Values are mean ± SE of three experiments. $^{**}p<0.01$ vs. control group, $^{\wedge\wedge}p<0.01$ vs. irradiated control group and $\#\#p<0.01$ vs. irradiated $C_{60}(OH)_{24}$ group (ANOVA).

tected mice from an ionizing-radiation-induced decrease in white blood cell counts. Consistent with the blood biochemical data, our cell proliferation results from the spleen strongly support the protective effect of $C_{60}(OH)_{24}$ on immune function. One of the possible mechanisms for the rescue immune cells from radiation-induced death of $C_{60}(OH)_{24}$ may be related to its nanomolecular properties. This polyhydroxylated fullerene, being of nanometer scale, might be immunologically recognized and significantly captured by the reticuloendothelial cells enriched in immune organs (Marina et al., 2007). Bio-distribution studies in mice by radionuclide label indicated that polyhydroxylated fullerene derivative was widely distributed in all tissues, particularly in the liver, spleen and bone (Qingnuan et al., 2002). The high uptake of fullerenols in these key organs remarkably enhances the protective effect of the organs on γ-ray damage, thus increasing the immune function in whole animals.

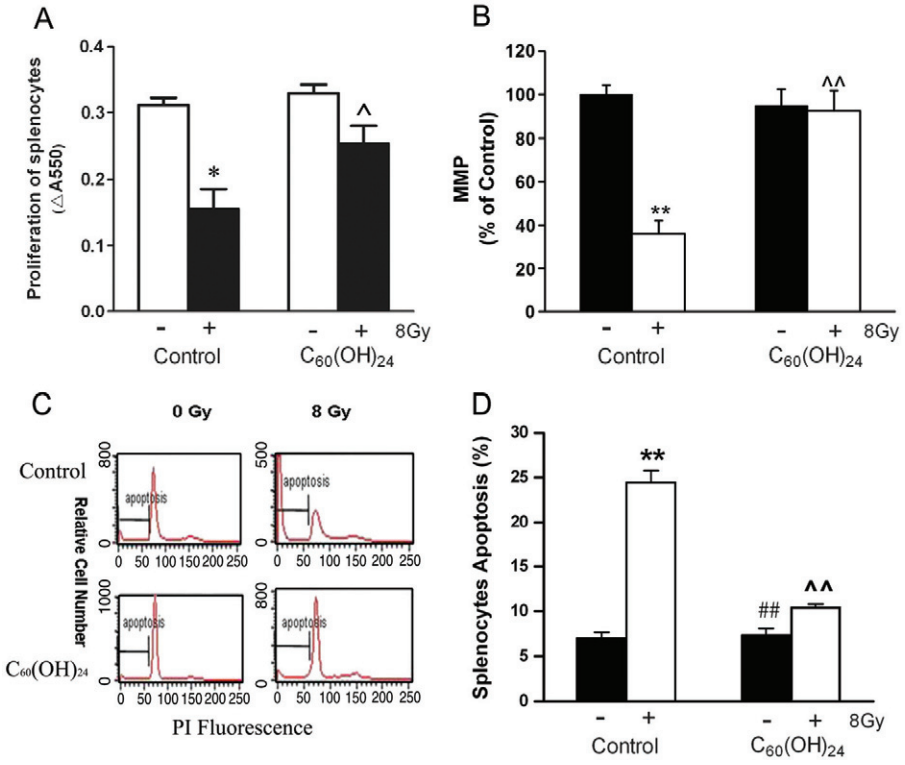

Fig. 5. Effect of $C_{60}(OH)_{24}$ on splenocytes cell proliferation, mitochondrial membrane potential, and apoptosis. (A) Cell proliferation assayed by the MTT method. The background proliferation means and SE for each group were 0.15 ± 0.02,0.13 ± 0.04, 0.16 ± 0.03, and 0.15 ± 0.01 of control, radiation, control plus $C_{60}(OH)_{24}$, and radiation plus $C_{60}(OH)_{24}$, correspondingly. (B) Mitochondrial membrane potential (MMP) assayed by JC-1 staining. Quantitative analysis of JC-1 staining with a dual-wavelength/double-beam recording spectrophotometer: a histogram showing the ratio of the intensity of red to green fluorescence. (C) Apoptosis as determined by flow cytometry after staining the cells with propidium iodide. Representative images of sub-G1 phase cells as quantified using PI staining and flow cytometry. The data are means ± SE derived from 8 mice in each group. $^{*}p<0.05$ and $^{**}p<0.01$ vs. control group, $^{\wedge}p<0.05$ and $^{\wedge\wedge}p<0.01$ vs. irradiated control group and $\#\#p<0.01$ vs. irradiated $C_{60}(OH)_{24}$ group (ANOVA).

The reduction in immune function in the spleen may be caused by ionizing-radiation-induced mitochondrial dysfunction and apoptosis in this organ. Mitochondrial membrane potential reduction and oxidant generation are found when apoptosis is induced by a series of pathogenic agents. As is known, lethal irradiation provokes mitochondrial and nuclear signs of apoptosis, and both of these alterations are absent in mice bearing a p53-null mutation, suggesting that a reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo* (Zamzami et al., 1995). Lymphocyte apoptosis induced by dexamethasone or superantigens is accompanied by the generation of oxidants and reduction of mitochondrial membrane potential that precedes nuclear DNA fragmentation (Castedo et al., 1995; Macho et al., 1995). As we have shown in Fig. 5B and C, radiation significantly decreased mitochondrial membrane potential and increased apoptosis. These results suggest that $C_{60}(OH)_{24}$ enhances immune function possibly by guarding mitochondrial function and decreasing apoptosis. However, it should be pointed out that the conclusion of the result the Con A proliferation assay should be interpreted with cautions because we only utilized a single sub-optimal (off-peak) Con A concentration (10 micrograms/ml) and did not generate curves for controls. We recognize the need for generating curves with varying concentrations of Con A but we did not do so because there were very few cells available following irradiation. In addition, the proliferation was evaluated after only 24 h of mitogen exposure, which serves to further decrease the means obtained. Therefore, further studies are warranted to confirm this preliminary result of the effect of $C_{60}(OH)_{24}$ on the splenocytes proliferation by using different concentrations of Con A and stimulating times in the future.

Mitochondria are vulnerable to oxidants because they are limited in their ability to cope with oxidative stress (de Grey, 2000). Mitochondrial DNA has been shown to be more oxidatively damaged than nuclear DNA after irradiation (Richter et al., 1988). Fullerene derivatives have been shown to preferentially bind to mitochondria (Foley et al., 2002; Chirico et al., 2007). As shown in Figs. 3 and 4 for the spleen and liver, $C_{60}(OH)_{24}$ pretreatment significantly protected mice from ionizing-radiation-induced toxicity by enhancing mitochondrial function, and inhibiting oxidant generation and oxidative damage to lipids and proteins. Our previous studies (Cai et al., 2008) have shown a dose-dependent scavenging effect of $C_{60}(OH)_{24}$ on superoxide radicals, hydroxyl radicals and lipid radicals. These results suggest that $C_{60}(OH)_{24}$ may act as an effective mitochondrial antioxidant to protect animals from lethal irradiation.

Biological systems have evolved to develop an effective and complicated network of defense mechanisms to cope with lethal ionizing radiation (Lee and Park, 2004). One of the most important antioxidant enzymes is SOD, and one of the most important endogenous antioxidants is GSH. Ionizing radiation caused a marked decrease in these antioxidant systems, and pretreatment with $C_{60}(OH)_{24}$ inhibited the ionizing-radiation-induced decrease in the activity of SOD and the level of GSH as compared to the ionizing radiation group. This could also be attributed to its free radical scavenger antioxidant property.

As a new potential radioprotector for ionizing-radiation-induced damage, one must be concerned about the toxicity of fullerene or its derivatives. It was shown that aqueous suspensions of pristine fullerene C_{60} have no acute or sub-acute toxicity *in vivo* (Gharbi et al., 2005). Isakovic et al. (2006) showed that the soluble $C_{60}(OH)_{24}$ has little toxicity to primary or transformed cells, with LD_{50} values as high as 800–1000 μg/ml. Ueng et al. showed that acute toxicity of fullerenol in mice had a LD_{50} value of about 1.2 g/kg (i.p.), pretreatments with 0.5 and 1.0 g/kg fullerenol decreased monooxygenases in liver microsomes, but pretreatments 0.1 g/kg fullerenol had no effects (Ueng et al., 1997). Our present study showed that pretreatments with 40 mg/kg fullerenol for 14 days had no obvious signs of toxicity. Therefore, $C_{60}(OH)_{24}$ should be a potential radioprotecting agent.

In summary, our results demonstrate that chronic pretreatment with $C_{60}(OH)_{24}$ is effective in protecting mice from ionizing-radiation-induced decrease in survival and immune function, and increase in oxidant generation and oxidative damage. The possible underlying mechanisms likely involve mitochondrial protection due to its wide bio-distribution to various organs and its antioxidant activity as a free radical scavenger and/or stimulator of antioxidant defense systems. These typical properties of polyhydroxylated fullerenols make them promising radioprotective agents.

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