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Induction of Inflammatory Responses by Carbon Fullerene (C60) in Cultured RAW264.7 Cells and in Intraperitoneally Injected Mice

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As the use of carbon fullerene increases in the chemical industry, the concern over its biological and toxicological effects is also increasing. In this study, the suspension of carbon fullerene (C60) in phosphate buffered saline was prepared and toxicity was investigated using cultured RAW 264.7 and in intraperitoneally injected mice, respectively. The average size of carbon fullerene in the suspension was 53.7 ± 26.5 nm when determined by particle size analyzer. Cell viability was significantly decreased by the exposure of carbon fullerene (0.25~2.00 μg/m*l*) for 96 hrs in the cultured RAW 264.7 cells. Intracellular reduced glutathione (GSH) level was also decreased compared to the level of the non-treated control group during the exposure period, while the level of nitric oxide was increased. When mice were intraperitoneally injected with carbon fullerene, serum cytokine levels of IL-1 and IL-6 were increased with the increased expression of inflammatory genes in peritoneal macrophage and T cell distribution in blood lymphocytes. The results suggested inflammatory responses were induced by carbon fullerene.

***Key words*:** Carbon fullerene, Inflammation, RAW264.7, Mice

INTRODUCTION

Fullerene was discovered in 1985 by Robert Curl and is a carbon allotrope that is composed entirely of carbon. The most abundant form is buckminster fullerene (C60) with 60 carbon atoms arranged in a spherical structure (Kroto *et al*., 1985). C60s occur in the environment due to natural and anthropogenic sources such as volcanic eruption, forest fires, and the combustion of carbon-based materials (e.g., coal, soot). Recently, applications of the engineered C60s have been rapidly increased in wide industrial fields and biomedicines due to its unique physico-chemical properties. With the wide applications, the needs of toxicity tests have also been increased.

Some researchers have reported the positive health effect of fullerene (Bogdanoviæ *et al*., 2008; Hu *et al*., 2007; Lens *et al*., 2008; Tykhomyrov *et al*., 2008). The administration of aqueous solutions of hydrated fullerenes as a drinking water during the chronic alcoholization of rats protected the tissues of central nervous system from damage caused by oxidative stress and it prevented the pathological damages in astrocytes and malformation in astrocytes marker protein (glial fibrillary acidic proteins). It significantly improved the behavioral response and eliminated emotional deficits induced by chronic alcohol uptake (Tykhomyrov *et al*., 2008). Pretreatment with fullerenol enhanced the enzymatic activity of superoxide dismutase and glutathione peroxidase in irradiated K562 cells, the human erythroleukemia cell line (Bogdanoviæ *et al*., 2008). In addition, a novel cystine fullerene derivative can penetrate through the cell membrane and has played a distinct role in protecting PC 12 cells (rat pheochromocytoma cell line) against hydrogen peroxide-induced cytotoxicity (Hu *et al*., 2007).

However, the other researchers have suggested the negative health effect of fullerene (Folkmann *et al*., 2009; Fujita *et al*., 2009; Han and Karim, 2008; Kolosnjaj *et al*., 2007; Usenko *et al*., 2008). The levels of 8-oxo deoxy Guanosine (8-oxo-dG) increased in the colon mucosa, liver, and lung of rats after intragastric administration of pristine fullerenes (Folkmann *et al*., 2009). The expressions of a few genes involved in the inflammatory response, oxidative stress, apoptosis, and metalloendopeptidase activity were up-regulated at both 3 days and 1 month after inhalation in the whole-body chamber (Fujita *et al*., 2009). In the mean time, Kovochich *et al*. suggested that the role of nC60 in the cellular responses is likely not due to the direct effect of the

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nC60 material surface on the cells, but is rather related to the conversion of the solvent for suspension into a toxic byproduct during the preparation of the suspension (Kovochich *et al*., 2009).

In this study, the water-suspended carbon fullerene was prepared and was investigated using in *in vitr*o and *in vivo* test systems to get more information on the toxicity controversy.

MATERIALS AND METHODS

Preparation of the C60 suspension in phosphate buffered saline. C60s were purchased from Tokyo Kasei Kogyo Co., Ltd. A C60-water suspension was prepared using the method proposed by Andrievsky *et al*. (1995). A solution of 1 g/*l* C60 in toluene was made, and 50 m*l* of this solution was added to 500 m*l* of double-distilled water. This layered mixture was sonicated using a sonicator until all of the toluene evaporated. The brown suspension was filtered sequentially through a 1-μm pore membrane filter, and then C60s were obtained in the water phase. Finally, the water-suspension was diluted to 1 : 1 by 2X PBS (Phosphate Buffered Saline, 0.15 M, pH 7.2) for the isotonicity. Particle size measurement was performed using a submicron particle sizer (NICOMPTM, CA, USA). Before treatment, the stability of fullerene in PBS was monitored and the average particle size was 53.7 ± 26.5 nm (Fig. 1).

Animals. ICR male mice (25 ± 1 g) were purchased from the Orient-Bio Animal Company (Seongnam, Gyunggi-do, Korea), and were allowed to adapt to the animal room conditions for 1 week prior to initiation of the study. The environmental conditions were a temperature of 23 ± 1°C, relative humidity of 55 ± 5%, and a 12 hrs light/dark cycle. All of the animals used in this study were cared for in accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals" issued by the Animal Care and Use Committee of the National Veterinary Research and Quarantine Service (NVRQS).

Five mice per treated group were intraperitoneally injected in a dose of 0.25~2 mg/kg bw and the control group was treated with vehicle solvent which was made by the same process without C60s. The animals were sacrificed at the designated time after treatment and blood was collected about 1 m*l* per mouse from the retro-orbital venous plexus using heparinized capillary tubes. Whole blood of 50 μ*l* was used for cell phenotype and the rest of coagulated blood was centrifuged at 3,000 rpm for 20 min. Peritoneal fluid harvested from mice were used for the analysis of cell cycle, cell phenotype, cytokines, and gene expression including interleukin-1.

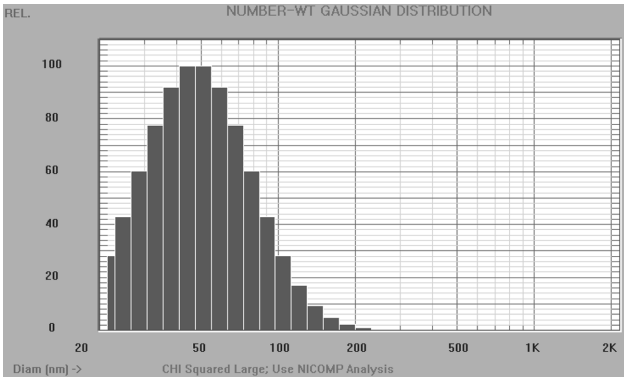

Fig. 1. Particle size distribution of carbon fullerenes (C60s).

Measurement of cell viability. RAW264.7 cell line, which is derived from a mouse peritoneal macrophage cell, was purchased from Korean Cell Line Bank (Seoul, Korea). RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin 100 IU/m*l*, and streptomycin 100 μg/m*l*. Cells were grown in a cell culture dish at 37°C in a 5% CO_2 humidified incubator.

Cell viability was measured using the MTT (3-(4-5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded on 96-well tissue culture plates with 2 × 10^3~1 × 10^4 cells in 100 μ*l* media per well. After a 24 hrs stabilization of the cells, they were treated with 0.25, 0.5, 1, and 2 μg/m*l* concentrations of the particles for 24, 48, 72, and 96 hrs, respectively. At the end of exposure, 40 μ*l* of MTT solution (2 mg/m*l*) was added and the cells were incubated for 4 hours at 37°C. Cells were solubilized with 150 μ*l* of DMSO and absorbance was quantified in 540 nm using a microplate spectrophotometer system (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The viability of the treated group was expressed as a percentage of the control group, which was assumed to be 100%.

Measurement of GSH. The cells treated with nanoparticles (0.25, 0.5, 1, 2 μg/m*l*) in 6-well plates for 24 hrs were washed with PBS, and 1% perchloric acid was added to the cells and cells were maintained on ice for 10 min. The soluble cell lysate fraction was centrifuged at 13,000 rpm at 4°C for 5 min prior to analysis in order to remove the precipitated protein. Cell lysate fraction, KH_2PO_4/EDTA buffer, and *o*-phthaldialdehyde were put in 96-black well plates, and incubated in the dark at room temperature for 30 min. Fluorescence was measured using a fluorescence multi-well plate reader with excitation and emission wavelengths of 350 and 420 nm, respectively. Results were calculated as nmol of glutathione per mg of protein and presented as a percentage of the control group. Protein assays in the cell lysate were performed using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Measurement of nitric oxide (NO). The cells treated with nanoparticles (0.25, 0.5, 1, 2 μg/m*l*) in 6-well plates

for 24 hrs and NO production in the cell culture medium was quantified spectrophotometrically using the Greiss reagent (1% sulfanilamide, 2.5% H_3PO_4, 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance was measured at 540 nm and the nitric oxide concentration was determined using a calibration curve with sodium nitrite as a standard chemical.

Measurement of cytokines. The blood was collected at 6, 24, 48, and 72 hrs, respectively after a single treatment of C60 (2 mg/kg bw) from the retro-orbital venous plexus using heparinized capillaries. The blood was clotted and serum was obtained by centrifugation. Serum concentration of each cytokine was determined using ELISA kits commercially available from eBioscience (San Diego, CA, USA). Briefly, microplates were coated with 100 μ*l* of capture antibody, and incubated overnight at 4°C. After washing and blocking with assay diluent, serum and standard were added to each well, and the plates were maintained for 2 hrs at room temperature. The plates were washed and biotin-conjugated detecting mouse antibody was added to each well and incubated at room temperature for 1 hour. The plates were washed and further incubated with avidin-HRP for 30 min before detection using the TMB solution. Absorbance was measured at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amounts of cytokines were calculated from the linear portion of the standard curve.

Immunophenotyping. Blood lymphocytes distribution was analyzed at 6, 24, 48 and 72 hrs, respectively, after a single treatment of C60 (2 mg/kg-bw). All monoclonal antibodies were purchased from eBioscience (San Diego, CA, USA). T cells (CD3, 1 : 50), B cells (CD19, 1 : 50), NK cells (DX5, 1 : 100), CD4+ T cells (CD4+, 1 : 160), and CD8+ T cells (CD8+, 1 : 50) were identified using directly conjugated anti-mouse antibodies. Briefly, splenocytes of $3\sim5 \times 10^3$ cells/m*l* were blocked with Fc-block (eBioscience, San Diego, CA, USA) to reduce non-specific antibody binding. Cells were then incubated in the dark with 10 μ*l* of the appropriate fluorochrome-conjugated antibody for 20 min at 4°C. Cells were then washed with 500 μ*l* FACS (Fluorescence Activated Cell Sorter) buffer. The blood was lysed for 5 min with FACS lysis buffer (BD Bioscience, Franklin Lakes, NJ, USA) at room temperature and then rewashed with FACS buffer. Finally, each sample was fixed with 1% paraformaldehyde until further analysis. Flow cytometry analysis was performed on the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). Control samples were matched for each fluorochrome. Data were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Gene expression analysis. Gene expression was analyzed in peritoneal macrophage at 6, 24, 48 and 72 hrs, respectively, after a single treatment of C60 (2 mg/kgbw). The RT-PCR reaction was carried out with 1 μg of total RNA, 1 μ*l* of 20-μM oligo-dT primer, and 18 μ*l* of reaction mixture, which was provided by *AccuPower* RT/PCR PreMix (Bioneer, Daejeon, Korea) at 42°C for 60 min. Then PCR was performed in a 20 μ*l* total mixture volume for 25~28 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplified cDNA products were separated on 1.5% agarose gel by electrophoresis. The primer sequences of amplified genes are shown in Table 1. Actin mRNA was also amplified as a loading control.

Table 1. Primer list used in this study

Primer	Sequence
Actin	(L) : GGAGGAAGAGGATGCGGCAGT (R) : GTGGGCCGCCTAGGACCAG
HSP 8	(L) : TCACAGTGCCCGCTTACTTC (R) : GCAGCAGCAGTTGGTTCATT
IL-1	(L) : CTCTGCAGACTCAAACTCCAC (R) : CAGGATGAGGACATGACACC
iNOS	(L) : ACGCTGAGTACCTCATTGGC (R) : AGCTCCTCCCAGGACCACAC
COX-2	(L) : TGACTGTGGGAGGATACATCTCTC (R) : AAGAAGAAAGTTCATTCCTGATCCC

Statistical analysis. The results of the treated groups were compared to those of the control group using the Student's *t*-test and levels of significance were represented for each result.

RESULTS

Cytotoxicity. For the investigation of cytotoxicity, cultured RAW264.7 cells were treated with carbon fullerene at the concentration of 0.25, 0.5, 1, and 2 μg/ml. As shown in Fig. 2, the cellular viability was decreased to 90% level of the non-treated control group after 24 hrs after treatment of carbon fullerene at a concentration of 2 μg/m*l*. At the lower concentrations, viability was not affected. However, the viability decreased as the exposure time increased. At the exposure time of 96 hrs, the cellular viability was decreased to 70.4 ± 1.4, 68 ± 1.2, 65 ± 1.2, and 53.3 ± 0.6% of the non-treated control group by the treatment of 0.25, 0.5, 1, and 2 μg/m*l* of carbon fullerene, respectively. The cytotoxicity by carbon fullerene was increased in a concentration-dependent manner.

Decrease of GSH. The intracellular GSH (antioxidant reduced form) level in RAW264.7 cells were decreased to 97.3 ± 6.5, 95.9 ± 3.4, 91.1 ± 6.6, and 82.9 ± 5.1% of the level of non-treated control group (control level was set as 100% at each measurement) by treatment of 0.25, 0.5, 1, and 2 μg/m*l* for 24 hrs exposure, respectively. The decrease was not statisti-

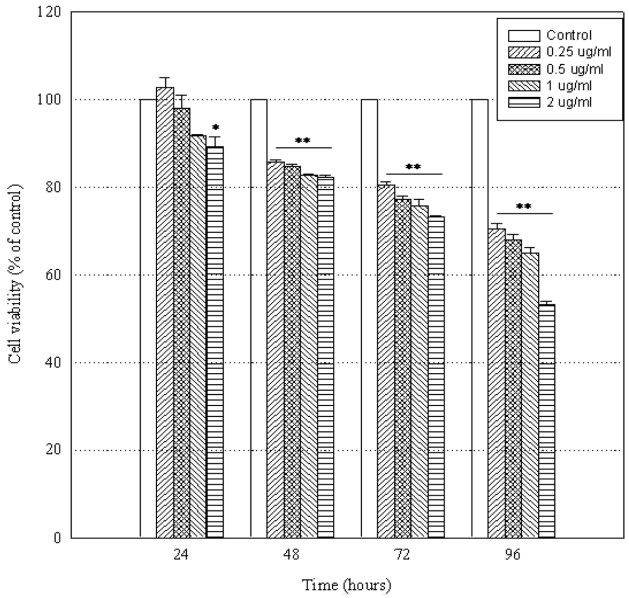

Fig. 2. Effects of C60s on the cellular viability of cultured RAW264.7 cells. Cell viability was assessed by MTT method and the results are presented as a percentage of the viability of control group viability. Cells (2×10^3~1×10^4 cells) were treated with the indicated concentrations of carbon fullerene for 24, 48, 72, and 96 hrs. Results represent the means of three separate experiments, and error bars represent the standard error of the mean. *: $p < 0.05$, **: $p < 0.01$.

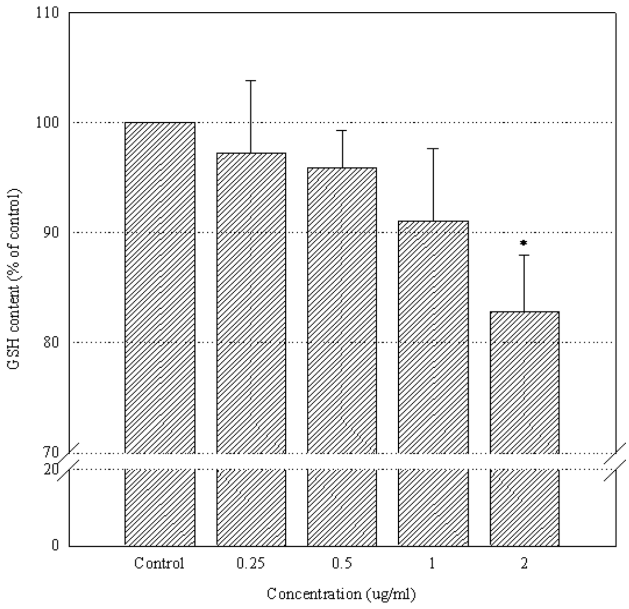

Fig. 3. Effects of C60s on the intracellular level of GSH in cultured RAW264.7 cells. A fluorometric method using *o*-phthaldialdehyde was used to measure GSH. GSH was calculated as nmol of glutathione per mg of protein and then was presented as a percentage of control. Results represent the means of three separate experiments, and error bars represent the standard error of the mean. Data are represented as the percentage of the GSH level in the control group. *: $p < 0.05$.

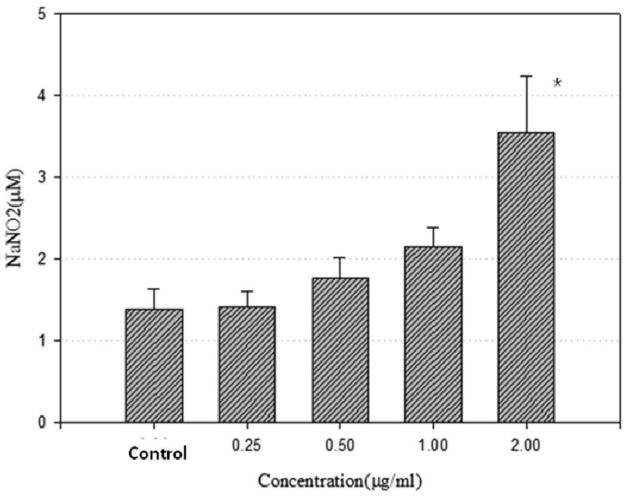

Fig. 4. Effects of C60s on the levels of NO secreted from the cultured RAW264.7 cells. Cells were treated with fullerene at 0.25, 0.5, 1 and 2 μg/ml for 24 hrs. The NO in the culture media was detected using Greiss reagent and absorbance was determined at 540 nm. Results represent the means of five separate experiments, and error bars represent the standard error of the mean. *: $p < 0.05$.

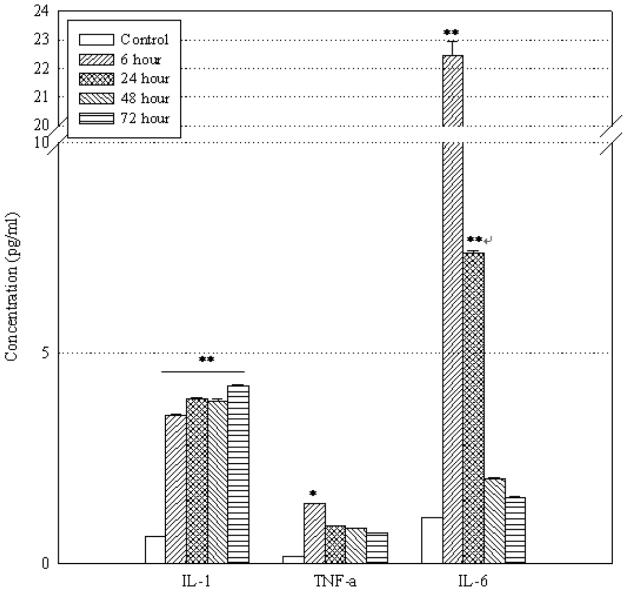

Fig. 5. Effect of C60s on the serum level of IL-1β, TNF-α, and IL-6 in mice treated by a single peritoneal injection. Mice were intraperitoneally injected with fullerene 2 mg/kg bw and were sacrificed at 6, 24, 48, and 72 hrs after injection (n = 5). Results represent the means of four separate experiments, and error bars represent the standard error of the mean. *: $p < 0.05$, **: $p < 0.01$.

cally significant at low concentrations but significance was shown at the highest concentration of 2 μg/m*l* (Fig. 3).

Increase of nitric oxide. Nitric oxide (NO) in the culture media was measured at 24 hrs after fullerene treatment. As

shown in Fig. 4, NO secretion from the cultured RAW264.7 cells increased to 1.42 ± 0.19, 1.77 ± 0.24, 2.15 ± 0.23, 3.54 ± 0.7 μM by fullerene at the concentrations of 0.25, 0.5, 1, and 2 μg/m*l*, respectively. The NO level released from the non-treated control group was 1.38 ± 0.25 μM. Statistical significance was shown only in a highest concentration although average values in the treated group were elevated.

Increase of cytokine secretion. The levels of pro-inflammatory cytokines in serum were shown in Fig. 5 after intraperitoneal injection of 2 mg/kg of fullerene. Levels of IL-1, IL-6, and TNF-α were significantly elevated at 6 hr-exposure when it was compared to the level of control group, which was detected before the treatment. The elevated level of IL-1 was maintained from 6 to 72 hrs after injection. The level at 72 hrs after injection was 4.21 ± 0.03 pg/ml, while that of the control was 0.64 ± 0.0 pg/m*l*. The level of IL-6 reached the maximum level (22.4 ± 0.5 pg/m*l*) at 6 hrs after injection while the level of the control group was 1.1 ± 0.01 pg/m*l*. After early increase of IL-6 and TNF-α, the levels were down to normal level after 72 hr-exposure.

Change of cell phenotype. After a single treatment of a C60s (2 mg/kg), the T cells distribution in blood lymphocytes increased slightly about 10% compared to that of control, and this trend was maintained until 48 hrs after treatment. The proportion of T cells in blood lymphocytes was 30.09% in the control group and it was 33.37, 32.69, and 31.31% at 6, 24, and 48 hrs after C60s treatment, respectively. Figure 6 shows the representative data of control group (Fig. 6A) and data at 24 hrs after treatment (Fig. 6B). The ratio of CD4+/CD8+ T cells in blood lymphocytes of control group was 3.56 (Fig. 6C), while it was 4.00 at 24 hrs after treatment (Fig. 6D), which means a significant increase in CD4+ distribution by carbon fullerene. The increase of CD4+ T cells was observed until 72 hrs after

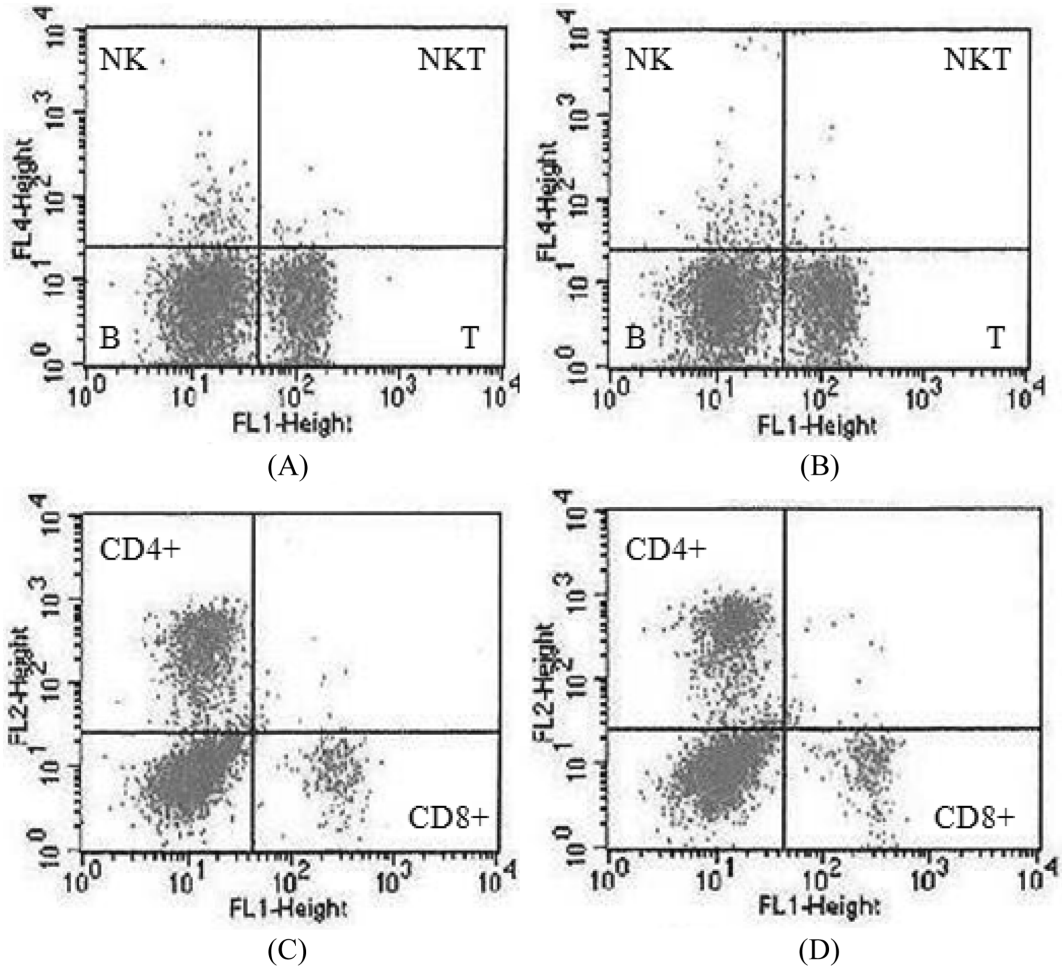

Fig. 6. Analysis of lymphocyte phenotypes in the blood of mice treated by a single peritoneal injection of C60s. Five blood samples were pooled and three separate experiments were done. Control samples were matched for each fluorochrome. (A): lymphocyte of control, (B): lymphocyte of treated group at 24 hrs after injection, (C): T subtype of control, (D): T subtype of treated group at 24 hrs after injection

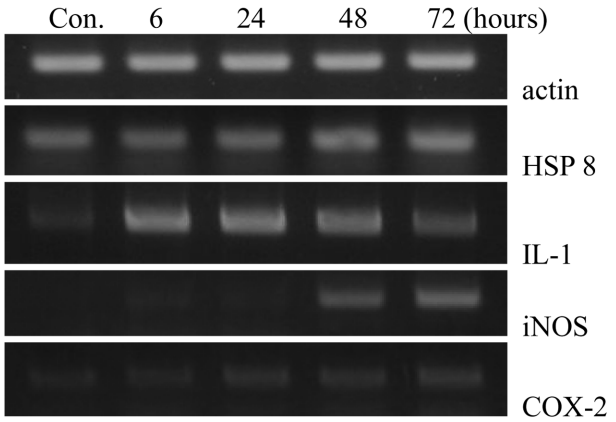

Fig. 7. Effects of C60s on the expression of cytokine genes by a single peritoneal injection. Mice were intraperitoneally injected with fullerene 2 mg/kg bw and were sacrificed at 6, 24, 48, and 72 hrs after injection (n = 6). The peritoneal fluid was harvested and pooled by 2 mice to make 3 test samples (total 6 mice per group at each time point) at the designated time after injection. After isolation of peritoneal macrophage, RNA was extracted from the cells and amplified by RT-PCR using the respective primers described in Table 1. The results were confirmed by several separate experiments and representative images are shown.

exposure (data not shown).

Change of gene expression. To investigate the expression of genes related to the inflammatory responses, mice were intraperitoneally injected with carbon fullerene by dosage of 2 mg/kg bw. Gene expression in peritoneal macrophage was compared between control group and treated group at 6, 24, 48, and 72 hrs after injection (Fig. 7). The expression of heat shock protein 8 (HSP 8) increased time-dependently. IL-1 reached the maximum at 6 hrs after injection, and decreased time-dependently. The expressions of iNOS and COX-2 were induced at 72 hrs after injection.

DISCUSSION

The toxicity of nanoparticles depends on the many physico-chemical factors including size, shape, chemical composition, solubility, surface area, and surface charge (Aillon *et al*., 2009; Oberdörster *et al*., 2005; Snopczyński *et al.*; 2009). It is general that nanoparticles made from the same material show different biological responses.

The carbon atoms in C60 join to form a hexagonal structure resembling a soccer ball. Regarding the biological properties, it has electron-donating or electron-accepting characteristics. Based on the previously published reports, it is not clear yet whether C60s act as anti- or pro-oxidant in biological systems (Ryan *et al*., 2007; Zhu *et al*., 2006). C60 is extremely insoluble in hydrophilic media and it is not easy to find bio-compatible media to make homogeneous state of the solution to test biological function. The organic solvent utilized to prepare water-soluble suspension may modify the properties of C60s and the modified C60s can alter the toxicity of pristine C60s. Sayes *et al*. investigated the pulmonary toxicity effects after instillation of C60s dispersed by tetrahydrofuran (THF) in rats. At the experiment, C60s showed little or no difference in lung toxicity when compared to the control group (Sayes *et al*., 2007).

In this study, we used toluene as the dispersing agent and finally prepared the PBS soluble-C60s by complete removing the toluene. We chose toluene because the solvent dissolve C60s to 3 mg/m*l*, a higher concentration than THF does (0.006 mg/m*l*) (Bezmel'nitsyn *et al*., 1998).

In this study, we identified the decrease of intracellular GSH and the increase of NO production with the decrease of cell viability in vitro. GSH is an important protective antioxidant against free radicals and other oxidants and it has been implicated in immune modulation and inflammatory responses. The antioxidant GSH is critical to the lungs' antioxidant defenses, particularly in protecting the airspace epithelium from oxidative/free radical–mediated injury and inflammation (Rahman and Adcock, 2006; Rahman *et al*., 2005; Rahman and MacNee, 2000). The decreased of intracellular GSH by carbon fullerene seemed to be one of key factors to show the pro-oxidant effect and inflammatory responses. For more direct evidences, carbon fullerene induced the increase of IL-1 and IL-6, and the expression of genes including HSP 8, iNOS, and COX-2 *in vivo* in this study. Previously, a significant increase in apoptosis was observed in the BAL cells at day 14 after the intratracheal instillation of C60s and G1 arrest was also observed in BAL cells at day 28 (Park *et al*., 2010). IL-6 is mainly secreted by macrophages, T cells, and endothelial cells, and plays an important role in the acute phase response. IL-1 stimulates the expression of genes associated with inflammation and autoimmune diseases. IL-1's most salient and relevant properties also include the initiation of COX-2 and iNOS (Dinarello, 2002). In addition, COX-2 remains undetectable in most mammalian tissues under basal conditions. The expression of COX-2 is induced by several extra-cellular signals, including pro-inflammatory and growth-promoting stimuli. COX-2 can be also affected directly in its enzymatic activity by NO and iNOS (Tsatsanis *et al*., 2006). As shown in Fig. 5 and Fig. 7, many evidences were observed that cytokine level may be increased by carbon fullerene. T cell distribution was also increased and CD4+/CD8+ ratio was increased by intraperitoneal injection. In case of intratracheal instillation, carbon fullerene showed the similar results (Park *et al*., 2010). When we compared the induction pattern of cytokines between two different routes of intratracheal instillation and intraperitoneal injection, the elevated level was maintained longer in the intratracheally instilled mice than in the intraperitoneally injected mice. It

seemed that the maintenance of the elevated level of cytokines depends on the disposition and kinetics of carbon fullerene according to the different administration routes.

In summary, carbon fullerene may induce an inflammatory response with pro-oxidant effects in biological systems.

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