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Formation of Water-Soluble Fullerenes [C_{60},C_{70}] under Ultrasonication and Antioxidant Effect

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Abstract

The water-soluble fullerenes [C_{60}, C_{70}] are prepared with fullerenes [C_{60}, C_{70}] and a mixture of oxidants (v/v) at the ratio of 3:1 under ultrasonic condition at room temperature. The MALDI-TOF MS confirmed that the water-soluble compounds were C_{60} and C_{70}. The antioxidant effect of water-soluble fullerenes [C_{60}, C_{70}] in the PC 12 cells (Rat pheochromocytoma) line following exposure to hydrogen peroxide (H_2O_2) was investigated.

Introduction

Since C_{60} was detected in 1985 [1], the increasing interest and significance of the work in the fullerene field led to the award of the 1996 Nobel prize in the chemistry field [2]. To make a water-soluble fullerene was one of the great challenges in fullerene chemistry and scientific interest in the potential applications of these fascinating molecules [3] in the biochemical, biophysical and biological field. There were some introductions to make fullerene derivatives that were soluble in aqueous condition [4-12]. While a number of fullerene derivatives have been prepared for such a purpose, most of them have been of only poor aqueous solubility, the results of that have limited application in chemical reactions. Here, we introduce somewhat different approach in which fullerene species are solubilized in water by ultrasonic method. The ultrasonic waves were applied during the synthesis of C_{60} [13]. Ultrasonic waves in liquids are known to cause chemical reactions either in homogeneous or in heterogeneous systems [14,15]. The chemical reactions are promoted by cavitation of liquids caused by ultrasonic waves traveling in the liquid. Cavitation implies the formation of microbubbles in a liquid subjected to sonication, which implode and generate high pressures and temperatures in their surrounding [14,15]. There have been a number of studies on the reaction of fullerenes [C_{60}, C_{70}] under ultrasonication [16-18]. The reactivity of the fullerenes [C_{60}, C_{70}] under ultrasonic condition is likely to be an important consideration in any technological application of these substances. We report that the reaction of C_{60} and C_{70} under ultrasonication with a mixture of concentrated sulfuric acid and concentrated nitric acid, give rise to the formation of water-soluble fullerenes [C_{60}, C_{70}] at room temperature. Also, the antioxidant effect of water-soluble fullerenes [C_{60}, C_{70}] in the PC 12 cells (Rat pheochromocytoma) line following exposure to hydrogen peroxide (H_2O_2) was investigated.

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Experimental

The fullerenes [C_{60}, C_{70}] used in this work was Golden grade from the Hoechest and Southern chemical group Inc. PC12 cells were obtained from ATCC (American Type Culture Collection) and the cells were grown in a (5% CO_2)/(95% air) humidified atmosphere at 37°C with exchange of medium three times a week. Cells were seeded into dish RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, horse serum and 100 μg/ml penicillin, 100 μg/ml streptomycin from Gibco RBL (Grand Island, NY, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and H_2O_2 were purchased from Sigma Chemical Co. All the solvents and chemical reagents were from Aldrich and Fluka. The ultrasonication of all the samples was conducted in pulse mode with an ultrasonic generator UG 1200 made by Hanil Ultrasonic Co, Ltd. Ultrasonic equip-

ment employed in this research having frequency 20 kHz, power 750 W, the configuration of equipment is horn system, and the size of the horn tip is 13mm in diameter. All the samples were analyzed by MALDI-TOF MS (Voyager-DE STR) and the matrix was a cyano-4-hydroxy cinnamic acid.

Preparation water-soluble fullerenes [C_{60}, C_{70}] under ultrasonication: The immiscible solutions of C_{60} (20 mg, 0.028 mmol) and C_{70} (20 mg, 0.024 mmol) were added to each of the mixture solutions, 10ml of concentrated sulfuric acid and concentrated nitric acid (v/v) at the ratio of 3:1 and reacted under ultrasonication for 3 days in air at room temperature. The immiscible solution changed into a mixture solution, also the color of solution changed from colorless to yellow-green. The mixture solution was neutralized with 1M-sodium hydroxide solution. The color changed from yellow-green to dark brownish-orange. Then, each of the resulting solution was evaporated, so that the remaining solid material was dried in a vacuum oven.

Investigation of antioxidant effect by water-soluble fullerenes [C_{60}, C_{70}]: all experiments were performed on PC12 cells (Rat pheochromocytoma) during their phase of growth. PC 12 cells were grown in 100 mm^2 dish in RPMI 1640 medium supplemented with 10% HS (Horse Serum), 5% FBS (Fetal Bovine Serum) and 1% streptomycin/penicilline and incubated in a humidified 5% CO_2 chamber at 37°C. After add Trypsin (500 μl/dish), PC 12 cells were detached by washing, centrifuged at 1200rpm for 5 min and resuspended in RPMI with 10% HS (Horse Serum), 5% FBS (Fetal Bovine Serum) and 1% streptomycin/penicilline. Cell was seeded at 3×10^4 cells per well in 96 well plates with 200 μl of medium per well. The cells were subcultured every three days, and the medium was replaced every days. Fullerenes [C_{60}, C_{70}] were dissolved and diluted with phosphate-buffered saline (PBS). To produce oxidative stress, H_2O_2 was freshly prepared from 30% dilution prior to each experiment, and after 24hours exposure cells were washed and kept in serum-free medium. Preincubation with fullerenes [C_{60}, C_{70}] was conducted 90 minutes before H_2O_2 added. To evaluate cytotoxicity, modified MTT method [19] was performed. The medium containing MTT (0.5 mg/ml) were added to each well and after 4 hours of exposure, the medium was removed and 100 μl of isoprophyl alcohol were added to each well to solubilize the precipitates and then shaken for 20 minutes. The plates were transferred to an ELIZA E09090 (Molecular Device, U.S.A.) reader to measure absorbance at 570 nm. The results are expressed as percentage of the untreated control. Standard curve was constructed utilizing different concentrations of cells.

MTT reduction assay: MTT reduction assay is one of the most widely used assay for determining cell viability [20,21], it detects living, but no dead cells, and the signal generated is dependent on the degree of activation of the cells [22]. In the MTT assay, viable cells convert the soluble dye MTT to insoluble (in aqueous medium) blue formazan crystals. 50% inhibitory concentration (IC_{50}) value, which means 50% inhibition of cell growth, was calculated by regression analysis (plotting the viability versus the concentration of the test compound). Statistical analysis: Experiments were done in triplicate and graphic results are shown as mean ± SEM corresponding to 3-7 separate experiments.

Results and Discussion

The MALDI-TOF MS analysis revealed that the water-soluble compounds were C_{60} and C_{70}. MALDI-TOF MS analysis reported in the Fig. 1 (a), (b) shows the formation of water-soluble fullerenes [C_{60}, C_{70}], because MALDI-TOF MS spectrum shows the peaks of fullerenes [C_{60}, C_{70}], at m/z=720, m/z=840 correspond to C_{60} and C_{70} in water solvent. Even though fullerenes [C_{60}, C_{70}] are hydrophobic, it may be possible to model the hydration of fullerenes [C_{60}, C_{70}] by water molecules, because it is the consequence of spherical layer of the formation of H-bonded H_2O molecules around the fullerenes [C_{60}, C_{70}] shell in according to the electron –accepting properties of fullerenes [C_{60}, C_{70}] and the electron-donating properties of the oxygen atoms of water [34] under ultrasonic condition. It may be probable that water-soluble fullerenes [C_{60}, C_{70}] are [$C_{60}@(H_2O)_n$], [$C_{70}@(H_2O)_n$]. Ultrasonic treatment, which produces high pressure during the cavitation [24] may facilitate the inclusion of fullerenes into cavities in the water structure [25] and formation of chlathrate-like networks of water molecules [26] around fullerenes and it may be due to donor-acceptor interaction of fullerenes [C_{60}, C_{70}] with polar solvent (etc, distilled H_2O) in aqueous systems. Geometrical matching between the structures, which may be formed by hydrogen bonding of water molecules in the clathrate and covalent bonds of the fullerenes carbon atom [27-29]. In parallel with geometrical factors, the electronic properties of fullerenes [C_{60}, C_{70}] may lead to the possibil-

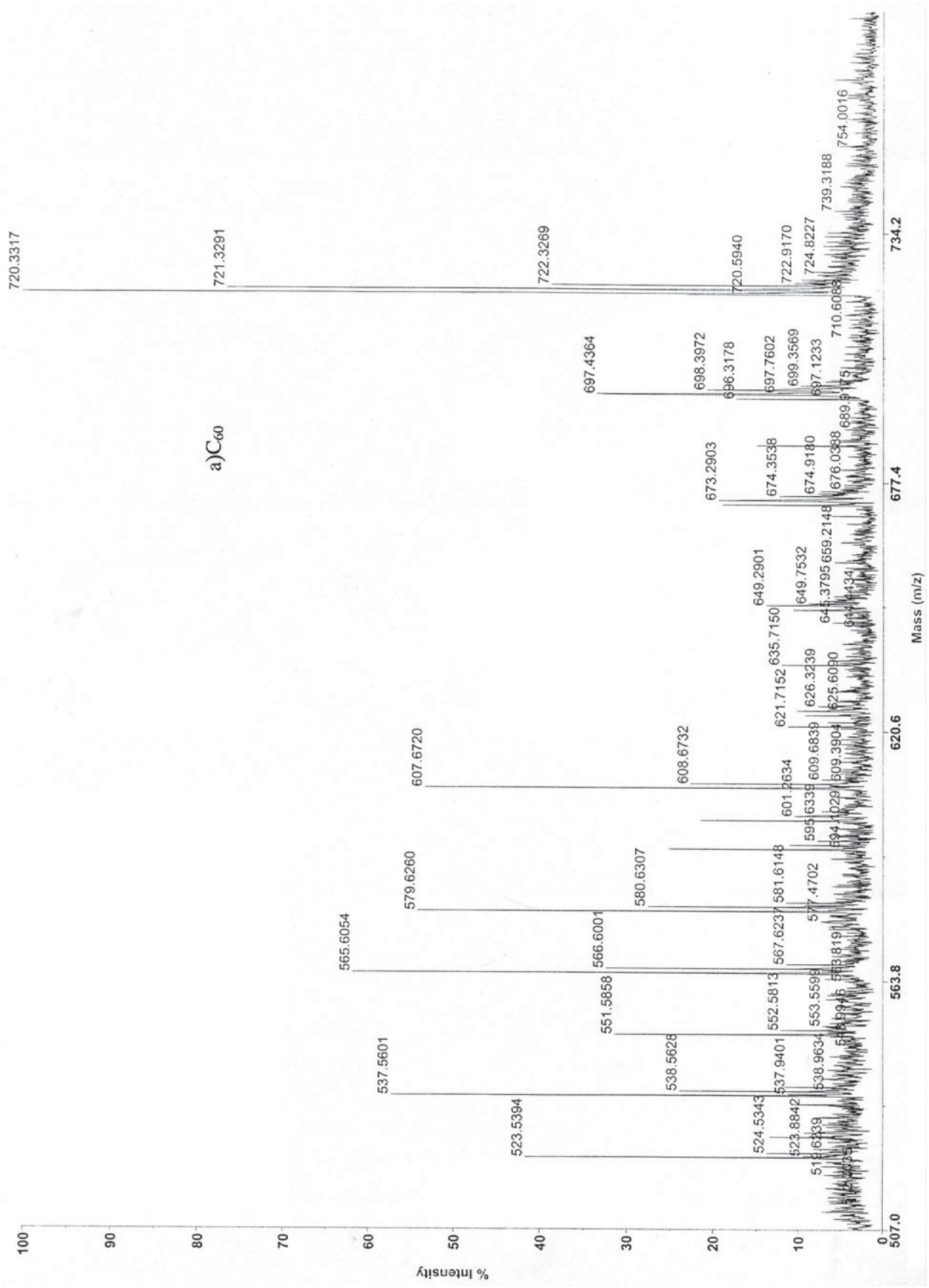

Fig. 1. (a) MALDI-TOF mass spectrum of water-solubilized fullerene [C_{60}].

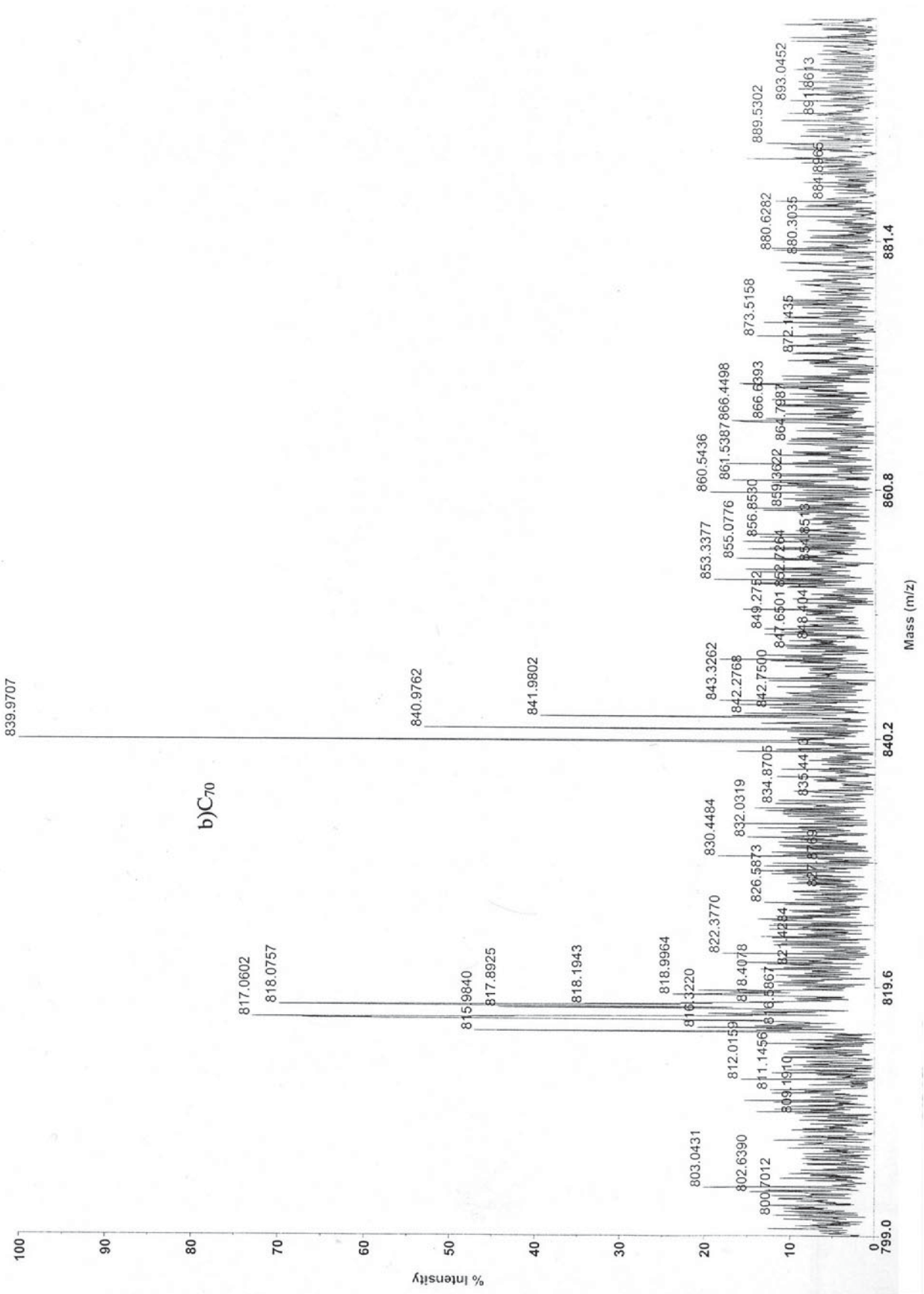

Fig. 1. (b) MALDI-TOF mass spectrum of water-solubilized fullerene [C_{70}]

ity of donor-acceptor and charge-transfer interactions [30-32] which may promote weak intermolecular water-fullerene interactions. Such interactions have been invoked to explain peculiarities of fullerene behavior in other solvens [31,32]. Also, we could observe the degradation of fullerenes [C_{60}, C_{70}] each C_2 unit, for molecular weight of fullerenes [C_{60}, C_{70}] decreased each m/z = 24 in the MS spectrum.

Water-soluble fullerenes [C_{60}, C_{70}] protect H_2O_2 induced cell apoptosis: MTT reduction, which measures metabolic activity dependent of endocytosis [20] were used to evaluate cell damage. Active mitochondria of living cells can cleave MTT to produce formazan the amount of which is directly related to the living cell number. Cell viability was markedly decreased after PC12 cells were exposed to H_2O_2 (Fig. 2 (a), (b)). When H_2O_2 concentrations were increased, PC12 cell showed a 40% and 80% decrease in MTT reduction at 0.001 μg/ml and 10 μg/ml, respectively. As estimated by the concentration of H_2O_2 need to decrease MTT reduction by 50%. The best concentration of H_2O_2 was 150μM. The cells were pretreated with increasing concentrations of fullerenes [C_{60},C_{70}] 0.01 μg/ml (1.0×10 μM) – 10 μg/ml (1.0×10^4 μM) before addition of 150 μM H_2O_2 for an additional 24 h. PC12 cells were dramatically reduced by H_2O_2 as assessed by MTT test, while pretreatment with fullerenes [C_{60}, C_{70}] tend to increase the viability of the cells respectively, when compared to vehicle (PBS) in the Fig. 2 (a),(b). But the cytoprotective effects were not always dependent of dose quantity by fullerenes [C_{60}, C_{70}] as a antioxidant agent in the range from 0.001 μg/ml (1.0 μM) to 10 μg/ml (1.0×10^4 μM). According to the Fig. 2 (a), (b), the moderate concentration of fullerenes [C_{60}, C_{70}] in antioxidative effect showed 0.1 μg/ml (1.0×10^2 μM) at C_{60}, 0.01 μg/ml (1.0×10 μM) at C_{70}. The results of present study showed that fullerenes [C_{60}, C_{70}] protects PC12 cells against H_2O_2- induced oxidative stress. PC12 cells exposed to 150 μM H_2O_2 demonstrated a typical oxidative stress by MTT reduction assay. Consistantly, cellular damage induced by H_2O_2 is inhibited by antioxidants [33]. The greater resistance to oxidative stress-generating fullerenes [C_{60}, C_{70}] in PC12 cell is probably related to a higher content of antioxidant factors. Although a large number of studies have shown a wide spectrum of analytical method for fullerenes, only few studies have been conducted on the antioxidative effects of fullerene [34]. The exactly specific cell targets of fullerenes [C_{60}, C_{70}] and its mechanism of action are still unrevealed. Now, further study is going on progress to analyze the potentiation effect by fullerenes [C_{60}, C_{70}] on this cell line and comparing various positive control with fullerenes [C_{60}, C_{70}].

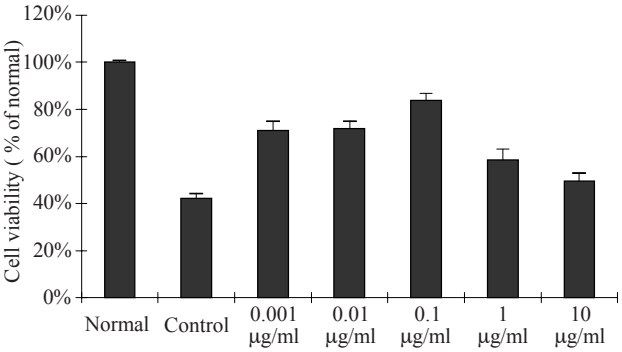

Fig. 2. (a) Cytoprotective effects of fullerene [C_{60}] on PC12 cells injured by hydrogen peroxide. After pretreatment of PC12 cells with C_{60} (0.001, 0.01, 0.1, 1,0, 10 μg/ml) for 90 min, cells were exposed to hydrogen peroxide (150 μM) for 24 hrs, then cytotoxicity measured by MTT assay. Viability of cell untreated hydrogen peroxide was set to 100%. Values are mean ± S.E.M. of percentages of normal group.

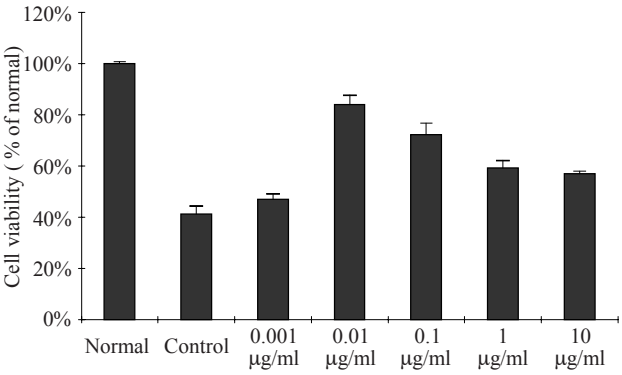

Fig. 2. (b) Cytoprotective effects of fullerene [C_{70}] on PC12 cells injured by hydrogen peroxide. After pretreatment of PC12 cells with C_{70} (0.001, 0.01, 0.1, 1,0, 10 μg/ml) for 90 min, cells were exposed to hydrogen peroxide (150 μM) for 24 hrs, then cytotoxicity measured by MTT assay. Viability of cell untreated hydrogen peroxide was set to 100%. Values are mean ± S.E.M. of percentages of normal group.

Conclusion

This work has demonstrated the formation of water-soluble fullerenes [C_{60}, C_{70}] and the possibility of utilization of such solutions in biological field studies. The water-soluble fullerenes [C_{60}, C_{70}] are prepared with fullerenes [C_{60}, C_{70}] and a mixture of oxidants (concentrated H_2SO_4, concentrated HNO_3,

v/v) at the ratio of 3:1 under ultrasonic condition at room temperature. By MALDI-TOF MS, the water-soluble compounds identified C_{60} and C_{70}, respectively. The antioxidant effect of water-soluble fullerenes [C_{60}, C_{70}] in the PC 12 cells (Rat pheochromocytoma) line following exposure to hydrogen peroxide (H_2O_2) was investigated, the moderate concentration of water-soluble fullerenes [C_{60}, C_{70}] as a antioxidant agent showed 0.1 μg/ml (1.0×10^2 μM) at C_{60}, 0.01 μg/ml (1.0×10 μM) at C_{70}. Investigation of photodynamic activities of the water-soluble fullerenes [C_{60}, C_{70}] are in progress.

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