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C_{70}-Carboxyfullerenes as Efficient Antioxidants to Protect Cells against Oxidative-Induced Stress

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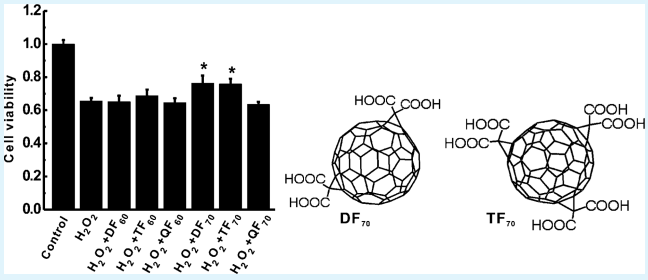
Supporting Information

ABSTRACT: Oxidative stress induced by excessive production of reactive oxygen species (ROS) has been implicated in the etiology of many human diseases. Acquiring a highly efficient antioxidant with good biocompatibility is of significance in eliminating the deleterious effect induced by the oxidative stress. Herein, we address our efforts on investigating the cytoprotective effect of carboxyfullerenes on H_2O_2-injured cells. Meanwhile, the uptake and intracellular location of carboxyfullerenes were studied. The results show that C_{70}-carboxyfullerenes (dimalonic acid C_{70} fullerene (DF_{70}) and trimalonic acid C_{70} fullerene (TF_{70})) exhibit an obviously protective effect against oxidative stress on C2C12 cells at concentrations as low as 2.5 μmol L^{-1}, whereas C_{60}-carboxyfullerenes (dimalonic acid C_{60} fullerene (DF_{60}) and quadrimalonic acid C_{60} fullerene (QF_{60})) show a protective effect at relatively higher concentration (40 μmol L^{-1}). The molecular structure of carboxyfullerenes and the physiological state of cells play an important role in the different cytoprotective capability. Further study reveals that DF_{70} and TF_{70} could enter into cells and mainly localize into the lysosome, which possibly involves the protective mechanism by stabilizing lysosome. The use of a significantly low concentration of C_{70}-carboxyfullerene as the antioxidative agent will benefit the therapeutic approaches aiming at alleviating ROS-induced injuries such as muscle disorder and arthritis.



KEYWORDS: *carboxyfullerene, oxidative stress, cell viability, intracellular location, C2C12 cell*

INTRODUCTION

Oxidative stress induced by excessive production of reactive oxygen species (ROS) has been implicated in the etiology of many human diseases, including muscle disorders, arthritis, cancer, cardiovascular disease, and a number of neurodegenerative disorders.[1,2] Harmful effects of ROS generally arise when cells are exposed to a hostile environment, such as UV, heavy metal ions, and X-ray irradiation.[3−5] As a result, cell damage and tissue inflammation occur under the oxidative stimulation. It is therefore necessary to look for an efficient ROS scavenger to counteract the detrimental effects and maintain the ROS formation/reduction balance which is very important in biological processes.

Over the past several years, nanomaterials have become a new class of antioxidative agent for therapeutic approaches aiming at alleviating ROS-induced injuries and shown great expectation in the reduction of ROS damage in biological systems.[6] Many nanoparticle-based antioxidants including cerium oxide nanoparticles,[7,8] fullerene,[9] and gold and platinum diamond nanoparticles[10] have exhibited high antioxidative capability. In particular, fullerene and its derivatives possess unique biological significance as antioxidants,[11−18] due to the conjugated π-system of the cage which can efficiently absorb electrons.[19]

Currently, various fullerene derivatives have been utilized as the antioxidants to help solve many modern medical limitations.[20,21] C_{60} fullerene modified with polymers exhibited good antioxidant activities and could attenuate the increase in intraneuronal superoxide induced by angiotensin II (Ang II) stimulation.[22] Poly/*N*-ethyl C_{60} fullerenes and C_{70} fullerene-tetraglycolate can prevent allergic responses both in vitro and in vivo.[23,24] Interestingly, it has been reported that amphiphilic fullerene derivatives can induce hair regeneration.[25] In addition, the amphiphilic C_{70} fullerene liposome can inhibit radical-induced lipid peroxidation and maintained the integrity of the lipid bilayer structure.[26] Other fullerene derivatives, such as metallo-fullerene compounds, were capable of decreasing organophosphate-induced acetylcholinesterase inhibition in primary murine neuronal cultures and human neuroblastoma cells.[27] Among the available water-soluble fullerenes, the carboxylic group modified C_{60} fullerene (e.g., dimalonic acid or trimalonic acid modified C_{60} fullerene) is one of the most popular fullerene-based antioxidants that has been extensively studied both in vitro and in vivo,[28,29] for its simple preparation,

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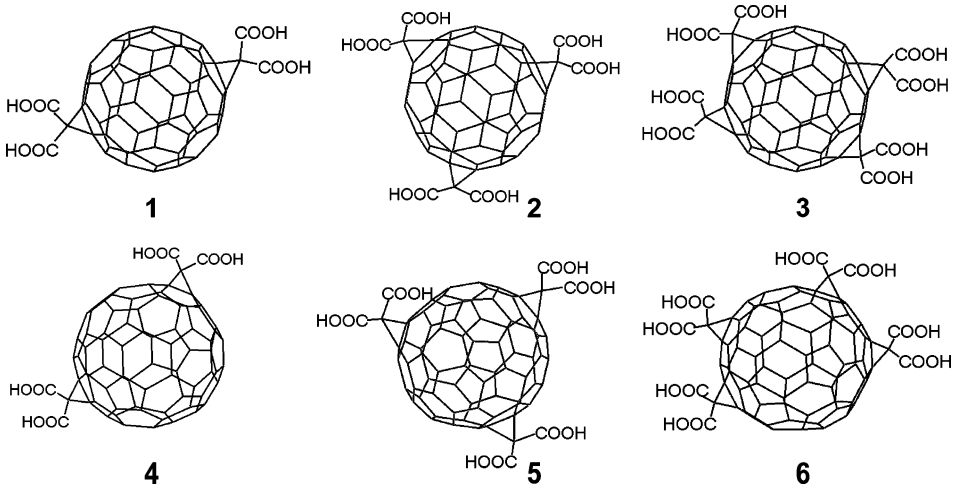

Figure 1. Schematic chemical structures of six carboxyfullerenes. DF_{60}, TF_{60}, QF_{60}, DF_{70}, TF_{70}, and QF_{70} denote dimalonic acid C_{60} fullerene (1), trimalonic acid C_{60} fullerene (2), quadri-malonic acid C_{60} fullerene (3), dimalonic acid C_{70} fullerene (4), trimalonic acid C_{70} fullerene (5), and quadri-malonic acid C_{70} fullerene (6), respectively.

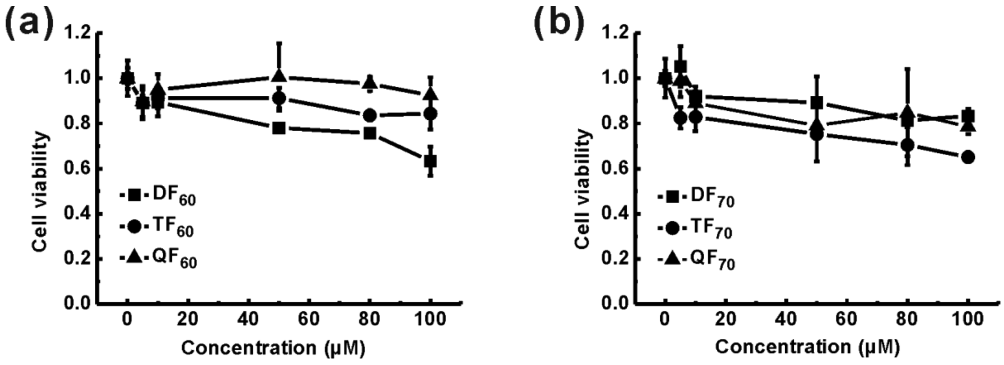

Figure 2. Cell viability of C2C12 cells incubated with C_{60}-carboxyfullerenes (a) and C_{70}-carboxyfullerenes (b) at various concentrations (0−100 μmol L^{-1}) for 24 h in the culture medium.

good biocompatibility, and more importantly, the definite molecular composition.[30]

Recently, we investigated the cytoprotective capability of carboxyfullerenes with different cage size (C_{60} fullerene vs C_{70} fullerene) and different number of carboxylic groups modified on the cage.[31] We found that quadri-malonic acid C_{70} fullerene (QF_{70}) is much superior in preventing postmitotic muscle cells from oxidative-induced stress in comparison with other carboxyfullerenes. Since the aim of this work is to reduce the ROS-induced injury, healthy cells damaged by H_2O_2-induced oxidative stress were used as a model, and a variety of carboxyfullerenes were investigated to seek for a highly efficient cytoprotective agent to alleviate the side effect induced by the excessive ROS.

Herein, we investigate the cytoprotective capability of several different carboxyfullerenes on H_2O_2-injured C2C12 cells and the uptake and intracellular location of carboxyfullerenes to reveal the possible protective mechanism. The results suggest that dimalonic acid C_{70} fullerene (DF_{70}) and trimalonic acid C_{70} fullerene (TF_{70}) exhibit an obviously protective effect at the concentration as low as 2.5 μmol L^{-1}, which is more efficient than the currently used C_{60} fullerene-based cytoprotective agent. That is to say, C_{70}-carboxyfullerenes (DF_{70} and TF_{70}) are more powerful in protecting cells with minimization of toxicity due to the low concentration and may exhibit great potential for therapeutic approaches aiming at alleviating ROS-induced injuries.

EXPERIMENTAL METHODS

Preparation and Characterization of Six Carboxyfullerenes. Six carboxyfullerenes with different cage size and carboxylic groups were prepared according to previous reports.[31,32] The absorption spectra were obtained by a UV−visible (UV−vis) spectrophotometer (UV-1601 PC, SHIMADZU). The size distribution of 2.5 μmol L^{-1} carboxylfullerenes in Dulbecco's modified Eagle's medium (DMEM) and the zeta potential in pure water was measured by Nano-ZS ZEN3600 (Malvern Instruments, Germany).

Cell Culture and Treatment. C2C12 cells (a mouse myoblast cell line, purchased from Shanghai cell bank, type culture collection committee, Chinese Academy of Sciences) were used to investigate the antioxidative ability of carboxyfullerenes. C2C12 cells were cultured with DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone Company, South Logan, UT), penicillin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1}) (Gibco, Grand Island, NY, USA) in 5% CO_2 at 37 °C in a humidified incubator.

To evaluate the protective effect of carboxyfullerenes in H_2O_2-induced damage, cells were first incubated with 100 μmol L^{-1} of H_2O_2 for 1 h. Then, the medium (H_2O_2 solution) was replaced with various carboxyfullerenes at different concentrations. The carboxyfullerenes were incubated with cells for 3 h in the dark at 37 °C. After that, the medium was removed, and cells were cultivated in a culture medium for 24 h in a humidified incubator. For the dark cytotoxicity examination, cells were incubated with carboxyfullerenes at various

concentrations (0−100 μmol L^{-1}) in the dark at 37 °C for 24 h; afterward, the cell viability was detected.

Cell Viability Assay and Analysis of Cellular Uptake toward Carboxyfullerenes. As reported in our previous study, cell viability was evaluated using a WST-8 assay with a Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan).[33] Similar to our previous reported method,[34] the UV−vis absorption of the six carboxyfullerenes (10 μmol L^{-1}) dispersed in DMEM culture medium was detected. All spectra were measured at 25 °C in a 1 cm quartzose cell. The results were reported as mean values of triplicates.

Labeling the Fullerene with Fluorescein Cadaverine. To visualize the location of C_{70}-carboxyfullerenes, C_{70}-carboxyfullerenes were labeled with a fluorescent dye, fluorescein cadaverine, by a routine amido linkage method.[35] The details can be found in our previous report.[32]

Internalization of Fluorescein-Labeled Fullerene into the C2C12 Cell. To examine the internalization of fluorescein cadaverine labeled C_{70}-carboxyfullerenes, C2C12 cells were treated with 100 μmol L^{-1} of H_2O_2 for 1 h and then incubated with fluorescent C_{70}-carboxyfullerenes in DMEM culture medium for 3 h at 37 °C in 5% CO_2 in the dark. After that, the cells were washed with PBS three times before fluorescence imaging.

Fluorescent Dye Staining and Confocal Microscope Imaging. C2C12 cells were stained by LysoTracker Red (Invitrogen) to detect the intracellular localization of fluorescent C_{70}-carboxyfullerenes as previously reported.[31,33] For fluorescence and light microscopic images, cells stained by fluorescent dye were imaged by an FV 1000-IX81 confocal laser scanning microscope (Olympus, Japan) according to our previous study.[32]

Statistical Analysis. All the experiments were done in triplicate. Results are expressed as means ± standard deviation of the mean value (SD). The statistical significance of the observed differences was analyzed by t test. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Preparation and Characterization of Carboxyfullerenes. For the purpose of clarity, we focused our studies primarily on carboxylated C_{60} and C_{70} fullerene and compared their cytoprotective activities with different substituent degree (dimalonic acid, trimalonic acid, and quadri-malonic acid) on the fullerene cage. Herein, a series of carboxyfullerenes with different cage size and adduct numbers were first prepared as previously reported.[31−33] Briefly, C_{60} or C_{70} fullerene was converted into fullerene malonic ester by the commonly used Bingel−Hirsch reaction.[36] The products were separated and purified by silica gel column chromatography. After hydrolysis, these carboxyfullerenes show good solubility in both water and culture medium.

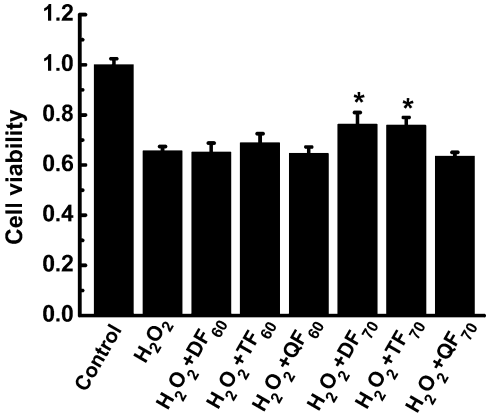

Figure 3. Protective effect of six carboxyfullerenes against H_2O_2-induced damage on C2C12 cells. The cells were treated with 100 μmol L^{-1} of H_2O_2 for 1 h before the incubation of carboxyfullerenes (2.5 μmol L^{-1}) for 3 h in the dark. Cells without treatment were used as control. Statistical difference was set at $^*P < 0.05$ vs H_2O_2-treated cells.

The schematic molecular structures of carboxyfullerenes were illustrated in Figure 1. DF_{60}, TF_{60}, QF_{60}, DF_{70}, TF_{70}, and QF_{70} denote dimalonic acid C_{60} fullerene (1), trimalonic acid C_{60} fullerene (2), quadri-malonic acid C_{60} fullerene (3), dimalonic acid C_{70} fullerene (4), trimalonic acid C_{70} fullerene (5), and quadri-malonic acid C_{70} fullerene (6), respectively. These carboxyfullerenes tend to form aggregations in the culture medium with the size range of 100−200 nm, as confirmed by dynamic light scattering (Supporting Information (SI), Figure S1). Furthermore, these carboxyfullerenes have negative charge in pure water at the concentration of 5 μmol L^{-1} as verified by zeta potential examination (SI, Table S1).

To test the biocompatibility of these carboxyfullerenes, C2C12 cells were incubated with six different carboxyfullerenes at concentrations ranging from 0 to 100 μmol L^{-1} for 24 h, respectively. The cell viability was detected by Cell Counting Kit-8 (CCK-8). The results show similar cell viability for both C_{60}-carboxyfullerene- and C_{70}-carboxyfullerene-treated cells. As shown in Figure 2, all carboxyfullerenes exhibit negligible cytotoxicity on C2C12 cells at concentrations below 10 μmol L^{-1}. However, a slight inhibitory effect on cell viability can be observed for the concentrations above 50 μmol L^{-1}. Since the negligible cytotoxicity of carboxyfullerenes is a prerequisite for the biological application, lower concentration is preferable to avoid the risk of cytotoxicity.

Protective Effect of Carboxyfullerenes against Oxidative-Induced Cellular Stress. To investigate the protective effect of carboxyfullerenes, C2C12 cells were first treated with 100 μmol L^{-1} of H_2O_2 for 1 h and then incubated with carboxyfullerenes with different cage size and adduct numbers at the concentration of 2.5 μmol L^{-1} for 3 h in the dark. The cell viability was detected by CCK-8 after 24 h postcultivation.

As shown in Figure 3, a statistical difference of cell viability can be observed for the cells incubated with DF_{70} and TF_{70} as compared with H_2O_2-treated cells, indicating the treatment of DF_{70} and TF_{70} could efficiently protect cells against oxidative-induced cellular stress. Especially, DF_{70} and TF_{70} exhibit an apparent cytoprotective effect at the concentration as low as 2.5 μmol L^{-1}, which has not been reported in the previous study. The available low concentration of C_{70}-carboxyfullerenes is very important in their antioxidative application since high concentration may initiate side effects.

It is worth noticing that C_{60}-carboxyfullerenes (DF_{60}, TF_{60}, and QF_{60}), however, do not show the protective effect at such low concentration (2.5 μmol L^{-1}). In contrast, a previous study revealed that dimalonic acid modified C_{60} fullerene ($C_{60}(C(COOH)_2)_2$) has a protective effect on H_2O_2-treated cerebral microvessel endothelial cells at the concentration of 50 μmol L^{-1}.[13] To further verify the protective ability of these carboxyfullerenes, we tested the protective effect of carboxyfullerenes at a series of gradient concentrations (0−50 μmol L^{-1}).

As illustrated in Figure 4, all carboxyfullerenes exhibit a protective effect on cell viability but at different concentrations. The minimal concentration required for a statistical difference is 40 μmol L^{-1} for DF_{60}, 10 μmol L^{-1} for TF_{60}, 40 μmol L^{-1} for QF_{60}, 40 μmol L^{-1} for QF_{70}, and 2.5 μmol L^{-1} for DF_{70} and TF_{70} under the same conditions. This result is well consistent with a previous report that $C_{60}(C(COOH)_2)_2$ (termed DF_{60} in this study) exhibited a protective effect on H_2O_2-damaged cells

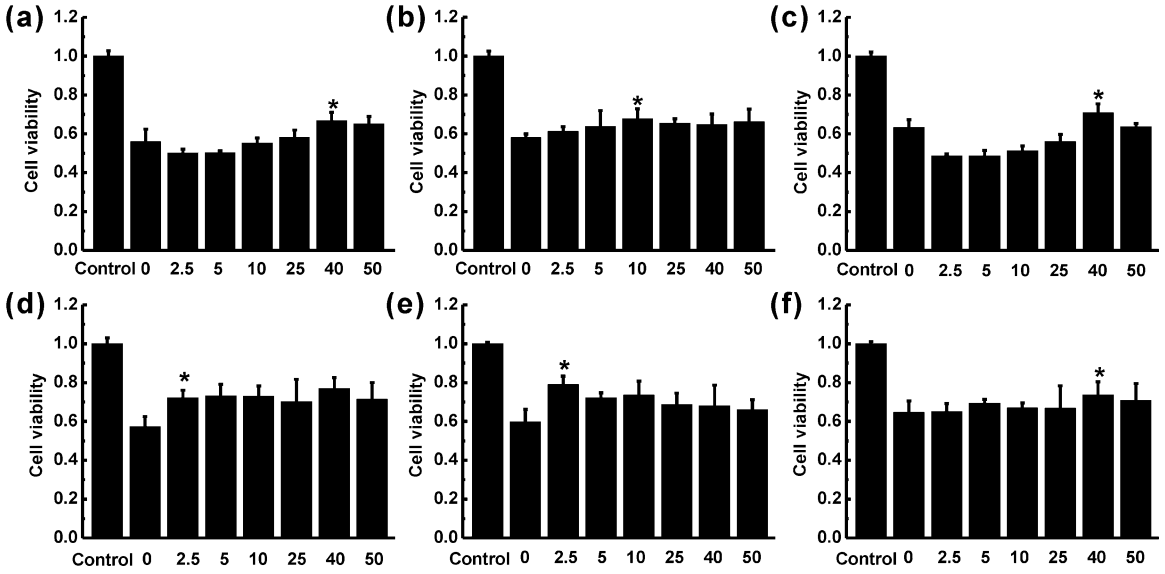

Figure 4. Cell viability of C2C12 cells treated with 100 μmol L^{-1} of H_2O_2 for 1 h and then incubated with DF_{60} (a), TF_{60} (b), QF_{60} (c), DF_{70} (d), TF_{70} (e), and QF_{70} (f) at various concentrations (2.5−50 μmol L^{-1}) for 3 h in the dark. Cell without treatment was used as control. Statistical difference was set at $^*P < 0.05$ vs H_2O_2-treated cells.

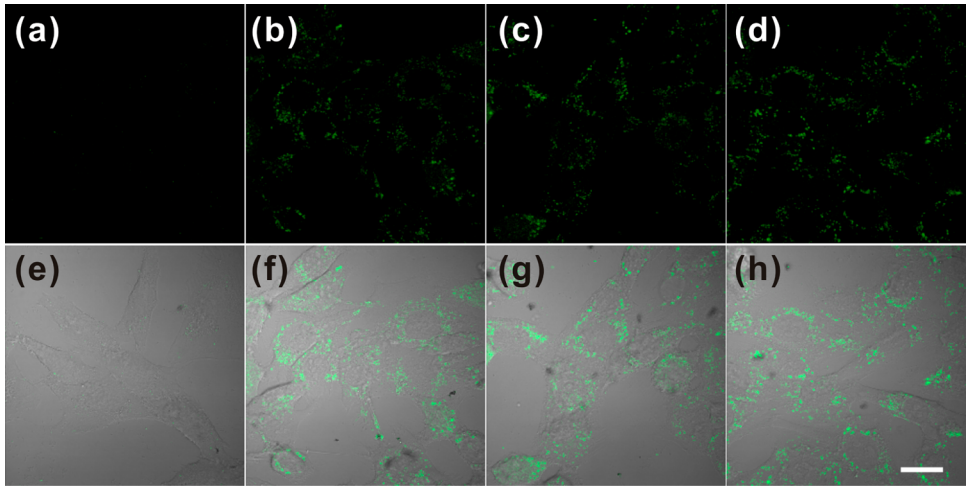

Figure 5. Confocal images of H_2O_2-treated cells incubated with fluorescein-labeled DF_{70} (b), TF_{70} (c), and QF_{70} (d) for 3 h. Untreated cells were used as control (a). (a)−(d) were fluorescence images, and (e)−(h) were the overlay images of the optical and fluorescence images. The scale bar is 20 μm.

at the concentration of 50 μmol L^{-1}.[13] Therefore, to achieve a similar cytoprotective effect, more C_{60}-carboxyfullerenes are required as compared to their C_{70}-carboxyfullerene counterparts. The higher protective ability of C_{70}-carboxyfullerenes is possibly due to the extended π system and lower symmetric structure of the C_{70} fullerene cage, which possesses higher activated electron-deficient areas on the cage surface to quench various free radicals.[16,37,38]

Interestingly, QF_{70} does not show apparently cytoprotective effect at low concentration (e.g., 2.5 μmol L^{-1}) among these C_{70}-carboxyfullerenes (DF_{70}, TF_{70}, and QF_{70}), though they have the same cage size. As we know, QF_{70} containing more carboxyl groups is subject to a deteriorated conjugated degree as compared to DF_{70} and TF_{70} that contain few functional groups. It is possible that the inferior protective effect of QF_{70} is due to the introduction of more sp^3 hybridization component which reduces the conjugated degree of the cage and results in weakened efficiency in ROS elimination. The surface modification on the fullerene cage has been implicated in the antioxidative activity of fullerene-based antioxidant.[39−42] The antioxidative ability of hydroxylated C_{60} could be altered by the numbers of hydroxyl groups on the cage.[41] Moreover, the superoxide quenching activities of fullerene derivatives in xanthine/xanthine oxidase assays also indicated that the addition pattern, lipophilicity, number, and type of charge have a close relationship with their antioxidative activity.[42]

In addition to the molecular structure which determines the intrinsic antioxidative property of carboxyfullerene, the physiological state of cells is also responsible for the different cytoprotective capability of carboxyfullerene. In our previous work, we found that QF_{70} showed cytoprotective capability on protecting C2C12 cells from oxidative-induced stress.[31] However, in this study, DF_{70} and TF_{70} exhibit an obviously protective effect on oxidative-stressed C2C12 cells. The main difference between the two studies is the physiological state of the cells. The cells used in a previous study are the healthy cells,

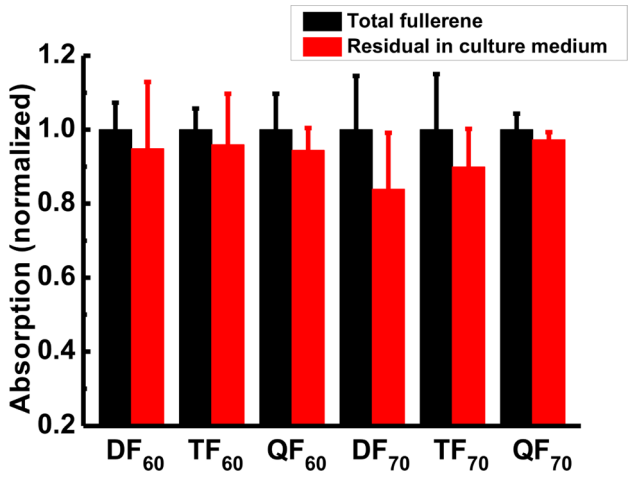

Figure 6. Normalized UV−vis absorption of carboxyfullerenes in the culture medium at 470 nm. The absorption of 10 μmol L^{-1} of carboxyfullerenes dispersed in a culture medium was shown as "total fullerene" (black column). After the incubation with H_2O_2-treated cells for 3 h, the absorption of carboxyfullerenes in the culture medium was measured and shown as "residual in culture medium" (red column).

and those used in this study are H_2O_2-injured cells. The physiological state of cells possibly determines the cellular response and uptake of carboxyfullerene, which may also account for the different cytoprotective abilities. Accordingly, we examined the cellular uptake of carboxyfullerenes as described below.

Cellular Uptake of Carboxyfullerenes. To investigate if C_{70}-carboxyfullerenes act as intracellular antioxidants, herein, we labeled C_{70}-carboxyfullerenes (DF_{70}, TF_{70}, and QF_{70}) with a fluorescent dye, fluorescein cadaverine, by a routine amido linkage method.[35] The conjugation of fluorescein cadaverine to C_{70}-carboxyfullerenes was accomplished by cross-linking of the −COOH group on the fullerene cage and $-NH_2$ group on fluorescein cadaverine in the presence of EDC and sulfo-NHS. Therefore, the internalization of fluorescein-labeled C_{70}-carboxyfullerenes could be monitored by tracing the fluorescence signal.

Briefly, C2C12 cells were treated with 100 μmol L^{-1} of H_2O_2 for 1 h and then incubated with fluorescein-labeled DF_{70}, TF_{70}, and QF_{70} separately in a culture medium for 3 h. Afterward, the treated cells were observed under a confocal microscope. As shown in Figure 5b−d, intracellular green fluorescence can be observed in cells incubated with the fluorescein-labeled C_{70}-carboxyfullerenes, which unambiguously indicated the internalization of C_{70}-carboxyfullerenes after the incubation. In addition, no fluorescence signal from the nucleus can be detected, indicating that C_{70}-carboxyfullerenes cannot enter into the nucleus. This result is consist with a previous report that no nuclear or secretory granule localization was observed for Texas red conjugated C_{70} fullerenes.[43]

Since carboxyfullerenes could enter into cells, the amount of internalized carboxyfullerenes possibly plays an important role in their different cytoprotective effect. Accordingly, the cellular uptake of C_{70}-carboxyfullerenes (DF_{70}, TF_{70}, and QF_{70}) was quantified by monitoring the UV−vis absorption of C_{70}-carboxyfullerenes dispersed in culture medium.[34]

In the absence of C2C12 cells, the characteristic UV−vis absorption spectra of C_{70}-carboxyfullerenes in the culture medium were demonstrated (SI, Figure S2). Their absorption value at 470 nm was detected and normalized in Figure 6 (black column). After the incubation with H_2O_2-treated cells for 3 h, different degrees of the decreased absorption of C_{70}-carboxyfullerenes in the culture medium were detected due to the different cellular uptake (Figure 6, red column). Interestingly, DF_{70} and TF_{70} are preferable to be uptaken by H_2O_2-treated cells, which is consistent with their better cytoprotective effect (Figure 4d, e). Although we do not know the mechanism for the cellular uptake selectivity, the elevated uptake of DF_{70} and TF_{70} should account for their enhanced antioxidative effect.

Intracellular Location of Carboxyfullerenes. To identify the intracellular localization, the colocalization of fluorescein-labeled DF_{70} and TF_{70} with lysosome-specific fluorescent

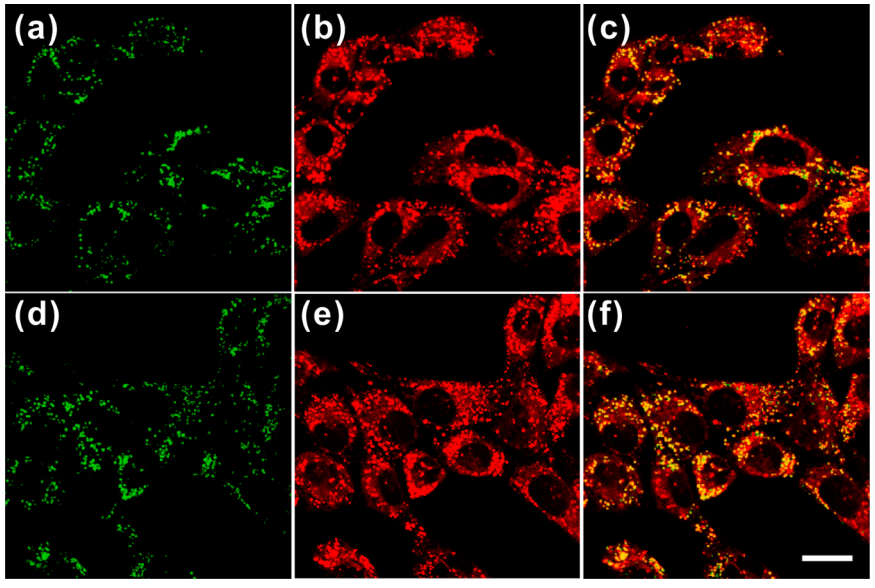

Figure 7. Confocal microscopic images showing the intracellular localization of fluorescein-labeled DF_{70} (a) and TF_{70} (d) (green fluorescence) costained with LysoTracker Red (red fluorescence, (b) and (e)). The merged fluorescent images (yellow fluorescence, (c) and (f)) reveal the lysosomal localization of fluorescein-labeled DF_{70} and TF_{70}. The scale bar is 20 μm.

probes (LysoTracker Red) was carried out. By detecting the fluorescence signal, we can observe that most of the DF_{70} and TF_{70} are present in a lysosome, as strong yellow fluorescence could be observed in the merged image of the cells (Figure 7c,f). No significant difference in the intracellular distribution was observed for those cells incubated with either fluorescein-labeled DF_{70} or TF_{70}. This result is consistent with a previous report that C_{60}-carboxyfullerene was localized in endosome-like vesicles after oxidative stimulation using confocal microscopy and transmission electronic microscopy.[13,43] It was reported that the internalized small nanoparticles of C_{60}-carboxyfullerene would insert into lysosomal membranes and form fullerene bilayer membranes, which could stabilize lysosomes and prevent the release of lysosomal enzymes and the activation of lytic proteins that triggered cell death.[44,45] We propose that C_{70}-carboxyfullerenes may play a similar role to C_{60}-carboxyfullerene, since their lysosome localization and the smaller particle size in the acidic environment have been confirmed in our previous study.[33]

The implication of the present findings is promising for the possible application of fullerene-based antioxidant to protect the oxidative-stressed cells. Additionally, the diverse cytoprotective capability of these carboxyfullerenes implies that differences in molecular structure (cage size and adduct numbers), the physiological state of cells, as well as dosage and incubation time are critical to induce the cytoprotective effects. These factors should be carefully considered to achieve the best cytoprotective effect.

CONCLUSIONS

Six carboxyfullerenes with different cage size and carboxylic groups were investigated for their cytoprotective effect on H_2O_2-injured C2C12 cells. C_{70}-carboxyfullerenes (DF_{70} and TF_{70}) exhibit an efficiently protective effect at a lower concentration (2.5 μmol L^{-1}) as compared to the previously reported. The molecular structure of carboxyfullerenes and the physiological state of cells may play an important role in the different cytoprotective capability. This study would provide an important insight into optimizing the antioxidative agents for therapeutic approaches aiming at alleviating ROS-induced injuries and promote the application of fullerene in nanomedicine.

ASSOCIATED CONTENT

Supporting Information

The particle size characterization (Figure S1), zeta potential (Table S1), and UV−vis absorption spectra (Figure S2) of carboxyfullerenes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium
DF_{60}, dimalonic acid C_{60} fullerene
TF_{60}, trimalonic acid C_{60} fullerene
QF_{60}, quadri-malonic acid C_{60} fullerene
DF_{70}, dimalonic acid C_{70} fullerene
TF_{70}, trimalonic acid C_{70} fullerene
QF_{70}, quadri-malonic acid C_{70} fullerene
ROS, reactive oxygen species
CCK-8, Cell Counting Kit-8
EDC, 1-ethyl-3-(3-dimethylaminopropyl carbodiimide)-hydrochloride
sulfo-NHS, *N*-hydroxysulfosuccinimide

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