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Protective Effect of C_{70}-Carboxyfullerene against Oxidative-Induced Stress on Postmitotic Muscle Cells

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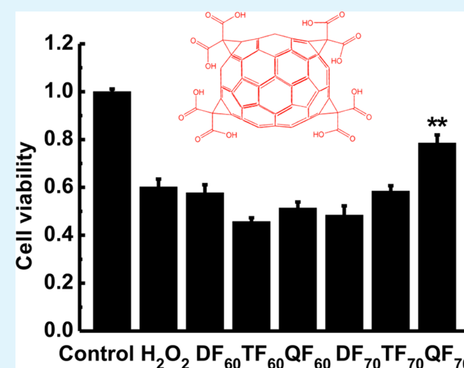
Supporting Information

ABSTRACT: Satellite muscle cells play an important role in regeneration of skeletal muscle. However, they are particularly vulnerable to oxidative stress. Herein, we address our efforts on the cytoprotective activities of carboxyfullerenes with different cage size (C_{60} vs C_{70}) and adduct number on postmitotic muscle cell (C2C12 cell). The correlation of the structural effect on the cytoprotective capability of carboxyfullerenes was evaluated. We find that quadri-malonic acid C_{70} fullerene (QF_{70}) exhibits higher capability on protecting cells from oxidative-induced stress among these tested carboxyfullerenes. The accumulation of intracellular superoxide dismutase (SOD) is proposed to play an important role in their diverse antioxidative ability. Moreover, the pretreatment of QF_{70} could also obviously enhance the viability of myotubes originated from oxidative-stressed C2C12 cells, which facilitates the future application of carboxyfullerenes in tissue engineering and nanomedicine.



KEYWORDS: *carboxyfullerene, oxidative stress, chemical structure, cytoprotective capability, C2C12 cell*

INTRODUCTION

Satellite muscle cells play an important role in regeneration of skeletal muscle.[1] It can be released and activated to become myoblast, which eventually differentiate into myotube and mature muscle fiber.[2] However, these cells are particularly vulnerable to oxidative stress, which will affect the differentiation capability and morphological normality.[3] The excess oxidative stress not only is harmful to muscle cells but also can induce muscle related diseases such as muscle disorders, muscle aging, and muscle wasting.[4−7] It is therefore significant to seek an effective cytoprotective agent to counteract the detrimental effects of oxidative stress on satellite muscle cells.

Regulation of cell oxidant−antioxidant balance by nanomaterials has shown great potential for antioxidant therapies. It has been well established that the fullerene and its derivatives possess biological significance as free radical scavengers or antioxidants due to the unique cage-like π-system.[8−16] Especially, carboxyfullerene as one of the most popular reactive oxygen species (ROS) scavengers has been widely investigated both in vitro and in vivo,[17−19] for its desirable water solubility, simple preparation, good biocompatibility, and more importantly, the controllable molecular structure.[20]

However, current knowledge is still restricted to particular aspects of fullerenes' capability to reduce oxidative stress on mitotic cells. In contrast, the investigation of carboxyfullerenes as cytoprotective agent on postmitotic muscle cells (e.g., satellite muscle cells) is less studied. As those cells are responsible for the physiological cell turnover and tissue repair, it is important to maintain their antioxidant microenvironment which is fundamental to cell differentiation.[21]

The present study aims to optimize carboxyfullerenes with different cage size (C_{60} vs C_{70}) and addition pattern to correlate with the structural effect and the cytoprotective capability of carboxyfullerenes on postmitotic C2C12 cells. We find that quadri-malonic acid C_{70} fullerene (QF_{70}) exhibits higher cytoprotective capability against H_2O_2-induced oxidative stress among these tested carboxyfullerenes, and as a result, it could obviously enhance the cell viability of differentiated cells (myotubes) originated from H_2O_2-stressed C2C12 cells. Our result also reveals that the accumulation of intracellular superoxide dismutase (SOD) plays an important role in the diverse cytoprotective ability. Moreover, QF_{70} does not alter the myogenic differentiation, suggesting a rationale for their application in tissue engineering and nanomedicine.

EXPERIMENTAL METHODS

Preparation and Characterization of Six Carboxyfullerenes. Carboxyfullerenes were prepared according to the methods previously reported.[22,23] Briefly, C_{60} or C_{70} fullerene was converted into fullerene malonic ester following the treatment with diethyl bromomalonate (Sigma-Aldrich) and 1,8-diazobicyclo[5,4,0]undec-7-ene (Alfa-Aesar) in dry toluene under nitrogen. The malonate derivatives of fullerenes with two to four additional groups could be separated by silica gel column chromatography. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra (AXIMA Assurance) verified the product with different ester groups on the fullerene cage. Hydrolysis of the purified products with NaH in CH_3OH led to

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the formation of the corresponding acids, as confirmed by Fourier transform infrared spectroscopy (FTIR, iN10 MX IR, Nicolet). The particle size distributions in DMEM culture medium were ranging from 150 to 200 nm, as confirmed in a previous report.[23]

Cell Culture and the Treatment of Carboxyfullerenes. C2C12 cells obtained from Shanghai cell bank (type culture collection committee, Chinese Academy of Sciences) were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone Company, South Logan, UT), penicillin (100 μg/mL), and streptomycin (100 μg/mL) (Gibco, Grand Island, N. Y. USA) in 5% CO_2 at 37 °C in a humidified incubator.

Cells were cultured in 96-well plates for 24 h and then treated as follows. To evaluate the protective effect of carboxyfullerenes against H_2O_2-induced oxidative stress, cells were incubated with carboxyfullerenes at different concentrations (0−80 μM) for 3 h and then treated with 100 μM H_2O_2 for 1 h. After that, the medium was replaced with culture medium and the cells were cultivated for 24 h in the dark at 37 °C. For the blocking experiment, diethyldithiocarbamic acid (DDC) was used to inhibit the intracellular SOD. C2C12 cells were incubated with 1 mM of DDC and 40 μM QF_{70} for 3 h and then treated with 100 μM H_2O_2 for 1 h.

Cytotoxicity of Carboxyfullerenes. For the cytotoxicity examination, C2C12 cells were incubated with carboxyfullerenes at the concentrations of 40 μM in the dark at 37 °C for 3 h; then, the cell viability was detected by Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan).

Cell Viability Assays. C2C12 cell viability was evaluated using a WST-8 assay with a Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan). The absorption value at 450 nm was read with a 96-well plate reader (iMark microplate reader, Bio-RAD, USA) to determine the viability (cell viability = $(OD_{tre} - OD_{medium})/(OD_{con} - OD_{medium})$), where OD_{tre} is the absorption value at 450 nm of treated cells, OD_{con} is that of control cells, and OD_{medium} is that of the culture medium.[23]

Analysis of Cellular Uptake toward Carboxyfullerenes. For analysis of the uptake of carboxyfullerenes, C2C12 cells were incubated with six different carboxyfullerenes (10 μM) in DMEM culture medium for 3 h at 37 °C in 5% CO_2 in the dark. Then, the culture medium was collected and the UV−vis absorption was detected. The UV−vis absorption of the six carboxyfullerenes (10 μM) dispersed in DMEM culture medium was used as a control. All spectra were measured at 25 °C in a 1 cm quartzose cell. The results were reported as mean values of triplicates.

Assay of Intracellular SOD. C2C12 cells were treated with 40 μM of C_{60}-carboxyfullerenes or C_{70}-carboxyfullerenes in DMEM culture medium for 3 h. Then, cells were collected and washed with PBS buffer for 3 times. Cell number of each sample was calculated by flow cytometry (Accuri C6 cytometer, BD Immunocytometry systems). The enzyme analysis was performed with the supernatant of cytosolic fraction as in a previous report.[24] Briefly, after the incubation, cells were collected and the cytosolic fraction was obtained by ultrasonification. The supernatant solution of cytosolic fraction was collected by centrifugation and kept at −80 °C until use. The enzyme activity of superoxide dismutase (SOD) is determined by a hydroxylamine method according to the recommendation (KGT00150-1, KeyGEN Biotech, China).

Myogenic Differentiation and Analysis. An in vitro myotube differentiation protocol was followed as previously developed.[25,26] Briefly, C2C12 cells with 90% confluence were treated with 40 μM of QF_{70} in DMEM culture medium for 3 h. Then, the culture medium was replaced with 2% heat-inactivated horse serum (differentiation medium) to induce myogenic differentiation. The differentiation medium was changed every other day throughout the entire period (7 days). Control cells without pretreatment of QF_{70} were treated by the same procedures and used as a control.

For the analysis of myotubes, we define the fusion index as the ratio of nuclei number in the myotube having more than 2 nuclei to the total number of nuclei according to the literature.[27] Furthermore, the maturation index to quantify the myoblast differentiation was calculated by measuring the percent of myotubes with more than 5 nuclei.[27] More than 1800 cell nuclei were calculated for fusion index evaluation, and more than 150 myotubes were calculated for maturation index evaluation for both untreated and QF_{70}-treated cells.

Cell Viability of Myotubes. C2C12 cells with 90% confluence were treated with 40 μM of either C_{60}-carboxyfullerenes or C_{70}-carboxyfullerenes in DMEM culture medium for 3 h. Then, 100 μM of H_2O_2 in the culture medium was added and incubated with cells for 1 h. After that, the medium was replaced with 2% heat-inactivated horse serum (differentiation medium) to induce myogenic differentiation. The differentiation medium was changed every other day throughout the entire period (7 days). Cells without preincubation of QF_{70} were treated by the same procedures and used as a control. Cell viability was detected by Cell Counting Kit-8 (CCK-8; DOJINDO, Kumamoto, Japan).

Fluorescence Staining and Imaging. MitoTracker Red CMXRos (Invitrogen) and Hoechst 33258 (Invitrogen) was used to stain mitochondrion and nucleus according to the manufacture's instructions. Cells were stained with MitoTracker Red CMXRos for 1 h and then with Hoechst 33258 for 15 min in the dark as previously reported.[23] For fluorescence imaging, cells stained by fluorescent dye were washed and imaged by a FV 1000-IX81 Confocal Laser Scanning Microscope (Olympus, Japan). The cells were excited at 405 nm for Hoechst 33258 and 561 nm for MitoTracker Red CMXRos, respectively. Fluorescence signals were collected from 420 to 480 nm in the blue fluorescence channel for Hoechst 33258 and from 570 to 670 nm in the red fluorescence channel for MitoTracker Red CMXRos.

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* tests. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Preparation of C_{60}-Carboxyfullerenes and C_{70}-Carboxyfullerenes. C_{60}-carboxyfullerenes and C_{70}-carboxyfullerenes with three different adduct numbers were used in this study. Herein, we defined them as DF_{60}, TF_{60}, QF_{60}, DF_{70}, TF_{70}, and QF_{70} which denotes dimalonic acid C_{60}, trimalonic acid C_{60}, quadri-malonic acid C_{60}, dimalonic acid C_{70}, trimalonic acid C_{70}, and quadri-malonic acid C_{70}, respectively. The preparation procedure of these carboxyfullerenes followed our previously study.[23] Briefly, C_{60} or C_{70} fullerene was converted into fullerene malonic ester by the commonly used Bingel-Hirsch reaction.[28] The products were separated and purified by silica gel column chromatography, and their components were evaluated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). After hydrolysis, the carboxyfullerenes show excellent solubility in water. The schematic chemical structures of C_{60}-carboxyfullerenes and C_{70}-carboxyfullerenes are illustrated in Figure 1.

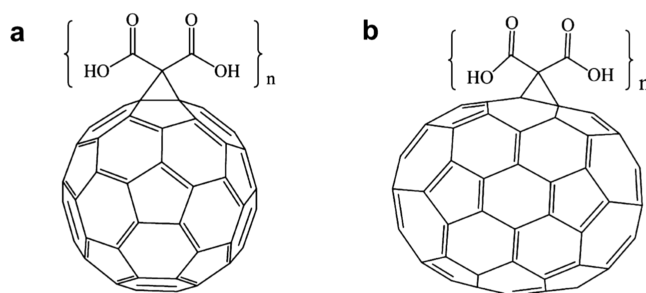

Figure 1. Schematic chemical structures of C_{60}-carboxyfullerenes (a) and C_{70}-carboxyfullerenes (b) with different adduct numbers (n = 2−4).

Carboxyfullerenes Protect C2C12 Cells against Oxidative Stress. To test the protective ability of carboxyfullerenes, mouse skeletal myoblasts (C2C12 cells) were incubated with 40 μM carboxyfullerenes for 3 h and then treated with 100 μM H_2O_2 for 1 h. After 24 h postcultivation in culture medium, the cell viability was detected by CCK-8. As shown in Figure 2, decreased cell viability can be observed for H_2O_2-treated cells, which indicated the H_2O_2-induced oxidative effect. However, QF_{70} could protect cells from H_2O_2-induced oxidative stress, as the enhanced cell viability of cells pretreated with QF_{70} can be observed. In contrast, the rest of the carboxyfullerenes (DF_{60}, TF_{60}, QF_{60}, DF_{70}, and TF_{70}) exhibit little positive effect on cell viability (Figure 2).

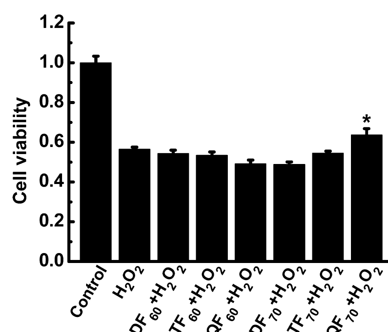

Figure 2. Cytoprotective effects of the six carboxyfullerenes against H_2O_2-induced stress on C2C12 cells. The cells were incubated with carboxyfullerenes at the concentration of 40 μM for 3 h and then treated by 100 μM of H_2O_2 for 1 h. The cell without treatment was used as control. *$P < 0.05$ vs H_2O_2-treated cells.

Furthermore, a series of concentrations for the six carboxyfullerenes (0−80 μM) were also tested on C2C12 cells as described above, and the results confirmed the superior capability of QF_{70} in protecting cells from H_2O_2-induced oxidative stress (Supporting Information, Figure S1). To test if the incubation of carboxyfullerenes could induce cytotoxicity, cells were incubated with carboxyfullerenes at 40 μM for 3 h and the cell viability was detected. The results show good cell viabilities after this treatment, which get rid of the nanotoxicity induced by these carboxyfullerenes (Supporting Information, Figure S2).

It has been demonstrated that C_{60}-carboxyfullerenes could protect cells from oxidative stress induced apoptosis;[8−11] however, it is not known if they can exhibit their protective effect on postmitotic muscle cells against oxidative-induced stress. Moreover, the cytoprotective capability of C_{70}-carboxyfullerenes is still not known. In this study, C_{60}-carboxyfullerenes do not show obvious cytoprotective effect under this experimental condition, while C_{70}-carboxyfullerene (QF_{70}) exhibits the enhanced cell viability of H_2O_2-treated cells. That is to say, the pretreatment of QF_{70} could effectively protect cells from the subsequent H_2O_2-induced oxidative stress. As we know, it is the first report that QF_{70} exhibits a protective effect on postmitotic muscle cells.

It has been suggested that the molecular structure of C_{60} and C_{70} as well as their derivatives could influence their antioxidative ability, a property that was attributed to a delocalized π double bond system of the fullerene cage.[29] As C_{70} fullerene is a more extended π system, it should be more efficient than C_{60} fullerene. On the other hand, as fullerenes are highly hydrophobic, the proper modification with hydrophilic groups is required before the application in the biological system. As we know, carboxyfullerenes with higher carboxylic groups will exhibit better water solubility than those containing less carboxylic groups. Thus, we expected that fullerenes with a larger cage and more hydrophilic groups will exhibit good antioxidative capability.

Carboxyfullerenes Induce the Increased Intracellular SOD. SOD is an antioxidative enzyme that is suggested to be important to ROS elimination.[30,31] A previous report proposed that fullerenes can potentially mediate the expression of genes whose products contribute to a cell's redox status and subsequently several rudimentary cell processes.[32] Thus, we analyzed the alteration of intracellular SOD of C2C12 cells incubated with carboxyfullerenes at the concentration of 40 μM for 3 h. The SOD analysis was performed in the cytosolic fraction of C2C12 cells as previously reported.[24] As shown in Figure 3, all of the carboxyfullerenes can stimulate the production of SOD. However, the highest total intracellular SOD appears in the cells incubated with QF_{70}, which is consistent with its good cytoprotective ability.

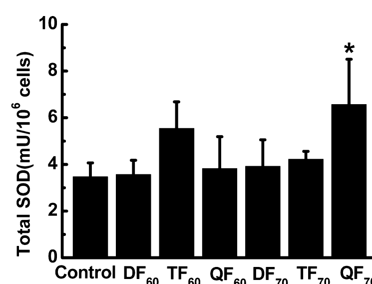

Figure 3. Total intracellular SOD per 10^6 cells incubated with carboxyfullerenes at the concentration of 40 μM for 3 h. A cell without fullerene treatment was used as control. *$P < 0.05$ vs control cells.

It has been reported that preincubation with $C_{60}(C(COOH)_2)_2$ (DF_{60}) at the concentration of 50 μM could protect human lung adenocarcinoma A549 cells or rat brain capillary endothelial cells from H_2O_2-induced oxidative stress.[8] It is worth noticing that a longer incubation time (24 h) is required in that study. We proposed that the adequate incubation time of carboxyfullerenes with cells is possibly a requirement to actively stimulate the intracellular SOD production. The accumulation of intracellular SOD is beneficial to protect cells from the subsequent oxidative stress. Actually, the production of SOD under the stimulation of fullerene derivative has been verified, as fullerenol could evidently enhance the activity of SOD after a 24 h incubation with K562 cells.[24] In this study, little protective effect appears for DF_{60} at the concentration of 40 μM for 3 h. It is probably due to the relatively short incubation time (3 h) which is not enough to effectively stimulate the SOD production.

As we know, the intrinsic antioxidative property of fullerene derivatives was sensitive to their chemical structure, such as the cage size and substitution pattern of the addends to the fullerene core.[33−36] However, the interaction of fullerenes with cells also plays an important role in their cytoprotective ability. In other words, the chemical structure may influence the physicochemical properties of carboxyfullerene, such as the internalization of fullerenes and the subsequent cellular response, and ultimately their cytoprotective capability. Actually, we have first detected the different cellular uptake of carboxyfullerenes. However, QF_{70} was not the favorable one to

be uptaken by cells as compared with other carboxyfullerenes (Supporting Information, Figure S3), indicating the uptake of fullerenes is not the main factor that determines the cytoprotective capability. In contrast, a notable increase of intracellular SOD was observed for cells incubated with QF_{70}. Interestingly, once the cells were treated with diethyldithiocarbamic acid (DDC, an SOD inhibitor),[37,38] QF_{70} could not protect cells against H_2O_2-induced damage (Supporting Information, Figure S4), suggesting the important role of SOD in the cytoprotective process. It is reported that the intracellular SOD plays an important role in regulating cellular oxidative−antioxidative balance;[39,40] our results suggest that the increased intracellular SOD, together with QF_{70}, prevented the deleterious effects of H_2O_2-induced stress. The detailed mechanism underlying the protective effect of QF_{70} still needs further investigation. However, our results unambiguously confirm the great potential of QF_{70} as cytoprotective agent in future application.

Carboxyfullerenes Enhance the Viability of Myotube. As previously mentioned, an important characteristic of C2C12 cells is their ability to fuse together to form multinucleate myotubes when cultured in differentiation media.[41] To further identify the cytoprotective capability of carboxyfullerenes on myotubes originated from C2C12 cells, cells incubated with carboxyfullerenes (40 μM) were subsequently exposed to 100 μM of H_2O_2 for 1 h. After that, cells were cultivated in differentiation medium to induce myogenesis. After 5 days of differentiation, the cell viability was detected.

As illustrated in Figure 4, the enhanced viability of myotubes was demonstrated for QF_{70} pretreated cells. It shows a statistical significance versus cells just treated with H_2O_2. In contrast, the other five carboxyfullerenes (DF_{60}, TF_{60}, QF_{60}, DF_{70}, and TF_{70}) can not enhance the viability of myotubes. Interestingly, decreased cell viability can be observed for the cells treated with TF_{70} and DF_{70}. It is possible that TF_{70} and DF_{70} induce negative effect on cell myogenesis and depress myotube viability. Still, further investigation is necessary to verify the differences. In this study, the pattern of carboxyfullerenes to enhance the viability of myotubes is consistent with that of their undifferentiated C2C12 cells (Figure 2), which indicate that the improved cell viability of C2C12 cells could also be beneficial for the viability of their differentiated myotubes.

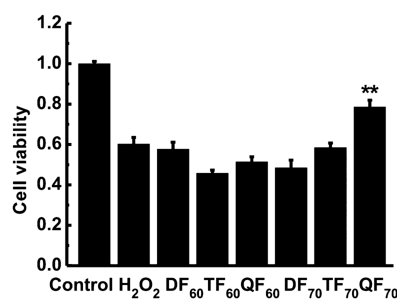

Figure 4. Cell viability of myotubes originated from C2C12 cells pretreated with carboxyfullerenes at the concentration of 40 μM for 3 h and the subsequent exposure of 100 μM H_2O_2 for 1 h. $^{**}P < 0.01$ vs. H_2O_2-treated cells.

Although fullerenes are actively scrutinized for their potentially noxious effects and also for their antioxidative characteristics both in vivo and in vitro,[8−19,42] fewer studies were performed on satellite muscles cells and the differentiated myotubes, which is important in regeneration of skeletal muscle. In this study, QF_{70} shows good cytoprotective effect on both C2C12 cells and the differentiated myotubes. It makes QF_{70} a very promising candidate for the application of regenerative medicine since the presence of QF_{70} would improve the cell viability of differentiated cell (myotubes).

QF_{70} Does Not Alter C2C12 Cell Differentiation. To confirm their abided differentiation potency, C2C12 cells were first incubated with 40 μM of QF_{70} and then cultured in DMEM culture medium containing 2% horse serum. After 7 days, cells were stained by MitoTracker Red CMXRos and Hoechst 33258. The differentiated cells (myotubes) containing multinucleus can be easily distinguished under a confocal microscope.

As shown in Figure 5a−h, no obvious morphological changes of the QF_{70}-treated cells can be found, both the untreated and QF_{70}-treated cells can form myotubes in differentiation media. We calculated the fusion index to quantify the myoblast differentiation by determining the ratio of nuclei number in the myotube having more than 2 nuclei to the total number of nuclei according to the literature.[27] The fusion index was 27.68% for the untreated cell and 25.76% for QF_{70}-treated cells, respectively, demonstrating that the QF_{70} treatment did not interfere with cell ability to proceed toward the myotube (Figure 5i). Furthermore, we calculated the maturation index as a differentiation parameter by measuring the percent of myotubes with more than 5 nuclei.[27] Still, no significant difference in the maturation index can be observed. That is 28.01% for untreated cells and 26.36% for QF_{70}-treated cells (Figure 5j). These results show that QF_{70} alone has negligible effect on cell fusion and myotube maturation.

This finding is promising for the application of fullerene in regenerative medicine since the presence of QF_{70} would not disrupt cell differentiation while protecting cells from oxidative stress. It could keep the oxidant−antioxidant balance and help to create the most favorable artificial microenvironment to maintain postmitotic cells in optimal conditions during in vitro tissue growth.[43] Moreover, the diverse cytoprotective capability of these carboxyfullerenes in this study implies that some requirements must be satisfied before the antioxidant effect emerges. In this respect, differences in molecular structure (cage size and adduct numbers), dosage, incubation time, and the subsequent cell response were suggested to be critical to induce the beneficial effects and to explain some conflicting results so far reported about fullerenes as protective agents in biological systems.

CONCLUSIONS

Utilizing the six carboxyfullerenes (DF_{60}, TF_{60}, QF_{60}, DF_{70}, TF_{70}, and QF_{70}), we have revealed the structural effect of carboxyfullerenes on cytoprotective effect on satellite muscle cells (C2C12 cells). Among these carboxyfullerenes, quadrimalonic acid modified C_{70} fullerenes (QF_{70}) could dramatically protect C2C12 cells against H_2O_2-induced oxidative stress. Different amounts of intracellular SOD stimulated by carboxyfullerene play an important role in their discrepant cytoprotective efficiency. Moreover, QF_{70} has little effect on cell differentiation of the postmitotic cell. Our observations may provide important insights in incorporating the antioxidative nanomaterials into therapeutic approaches aiming at alleviating ROS-induced injuries. In addition, the established structure−activity relationship of carboxyfullerene as antioxidant could

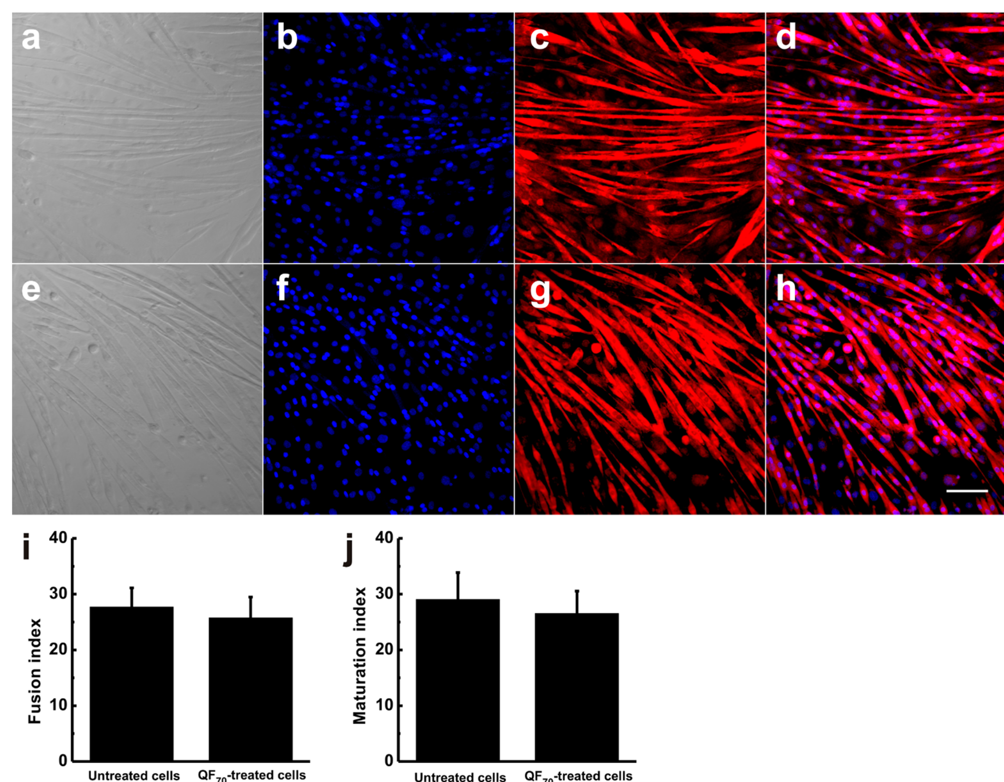

Figure 5. Confocal images of myotubes originated from C2C12 cells. Cells pretreated with 40 μM of QF_{70} for 3 h and then induced myogenesis in differentiation medium for 7 days (a−d). Cells without treatment were used as control (e−h). The optical images were shown in (a) and (e). The fluorescence images of cells stained by Hoechst 33258 (b, f, blue) and MitoTracker Red CMXRos (c, g, red). The overlay images of Hoechst 33258 (blue) and MitoTracker Red CMXRos (red) were shown in (d) and (h). The fusion index (i) was evaluated by calculating more than 1800 nuclei of the observed cells, and the maturation index (j) was evaluated by examining more than 150 myotubes. The scale bar is 100 μm.

promote the future studies in biomedical application for maintaining the oxidant−antioxidant balance.

■ ASSOCIATED CONTENT

Supporting Information

Cytoprotective effect of carboxyfullerenes at various concentrations (2.5−80 μM) (Figure S1). Cell viabilities of C2C12 cells incubated with carboxyfullerenes (Figure S2). The uptake of carboxyfullerenes in C2C12 cells (Figure S3). Blocking experiment with SOD inhibitor (Figure S4). 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
SOD, superoxide dismutase

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