

# Concentration-dependent effects of fullereneol on cultured hippocampal neuron viability

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**Background:** Recent studies have shown that the biological actions and toxicity of the water-soluble compound, polyhydroxyfullerene (fullereneol), are related to the concentrations present at a particular site of action. This study investigated the effects of different concentrations of fullereneol on cultured rat hippocampal neurons.

**Methods and results:** Fullereneol at low concentrations significantly enhanced hippocampal neuron viability as tested by MTT assay and Hoechst 33342/propidium iodide double stain detection. At high concentrations, fullereneol induced apoptosis confirmed by Comet assay and assessment of caspase proteins.

**Conclusion:** These findings suggest that fullereneol promotes cell death and protects against cell damage, depending on the concentration present. The concentration-dependent effects of fullereneol were mainly due to its influence on the reduction-oxidation pathway.

**Keywords:** fullereneol, nanomaterial, neurotoxicity, neuroprotection, hippocampal neuron

## Introduction

Fullerene (C<sub>60</sub>) has attracted great interest since it was discovered in soot by laser ablation of graphite in 1985.<sup>1</sup> Fullerene represents a special group of compounds and has a number of applications due to its distinctive material properties. Its low solubility initially limited its use in biomedical science.<sup>2</sup> This has now been overcome by the development of water-soluble suspensions of fullerenes. There is an increased interaction between linked hydrophilic functional groups on fullereneol molecule and water. Fullerene derivatives including fullereneol have become increasingly available for numerous applications.<sup>3</sup>

Fullerene and its derivatives have been found variously to have beneficial properties or harmful tendencies in many different fields of science.<sup>4</sup> At low concentrations, fullerene and its derivatives might have a protective effect on cells or organs. It has been proposed that fullerene might play a role in treating drug abuse, because intraperitoneal administration of fullerene 100 mg/kg was found to prevent liver and kidney damage caused by morphine.<sup>5</sup> In addition, intracerebroventricular administration of fullerene 0.3 mg/kg has been found to have a protective effect against ischemia-reperfusion injury.<sup>6</sup> In other studies, intraperitoneal fullerene 10–100 mg/kg was found to prevent self-injurious behavior in mice, which might cause DNA and protein damage.<sup>7</sup> Perhaps the most important property of fullerene derivatives is their ability to scavenge reactive oxygen species and free radicals. These properties were shown to be related to the antitumor effect observed after injection of  $2 \times 10^{-7}$  mol/kg [Gd@C<sub>82</sub>(OH)<sub>22</sub>]<sub>n</sub> nanoparticles of fullerene to tumor-bearing mice.<sup>8</sup>

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However, recent studies have raised concerns about their potential toxicity because, to a certain extent, the properties which preserve some biomedical functions may damage others.<sup>9</sup> Generation of reactive oxygen species plays a critical role in this respect because it can result in damage to the cell membrane, lipid peroxidation, and necrotic cell death. Some scientists have suggested that hydroxylated fullerenes may be markedly less toxic than their parent compounds, but can still cause apoptosis at high concentrations.<sup>10</sup> Hydroxylated fullerene at a concentration of 100 mg/mL was found to cause protein polyubiquitination in human umbilical vein endothelial cells, killed 58% of cells, and caused conspicuous leakage from the cell membrane.<sup>11</sup> Other studies have shown that exposure of pregnant mice to fullerene 50 mg/kg resulted in placental transfer to the embryos and that maternal exposure to a higher dose (137 mg/kg) resulted in embryo death in all cases.<sup>12</sup> The growing likelihood of accidental or inadvertent release of these substances into the environment calls for a better understanding of their properties, fate, and impact on biological systems.

In this paper, we focus on the concentration-dependent effects of fulleranol, a fullerene derivative, on cultured hippocampal neurons. Molecular dynamics have confirmed that fullerene derivatives have unusually high permeability into lipid membranes and are able to cross the blood-brain barrier.<sup>13,14</sup> It has also been proposed that Buckminsterfullerene may have neuroprotective properties and cause lower glutamate receptor-induced elevation of  $Ca^{2+}$  levels.<sup>15,16</sup> These earlier studies suggest that the central nervous system may be a potential target of fullerene derivatives.

It has been shown that the toxicity of a nanomaterial can be approximately determined by more than ten factors and one of them is dose.<sup>17</sup> The present study was undertaken to investigate the effects of different concentrations of fullerene on the central nervous system. We wanted to test if fulleranol at low concentrations had a repair function on lead-induced neuron damage in rats and if fulleranol at high concentrations induced neuronal apoptosis. We also analyzed the redox level of neurons exposed to different concentrations of fulleranol in order to determine its mechanism of action.

## Materials and methods

### Experimental animals

Wistar rats were obtained from the Shanghai SLAC Laboratory Animal Center (Shanghai, China) and housed in the animal care facility at the University of Science and Technology of China. All rats were maintained according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals,

and procedures for the care and use of rats were approved by the Animal Care Unit and Use Committee of the University of Science and Technology of China.

### Hippocampal neuron culture

Hippocampal neurons were derived from newborn Wistar rats. Prior to culturing, 6-well plates (Costar, Cambridge, MA) were prepared for Comet assay, caspase-3 and caspase-9 activity assays, and measurement of oxidative stress; 24-well plates (Costar) were prepared for Hoechst 33342/propidium iodide double stain detection; and 96-well plates (Costar) were prepared for MTT assay. The plates were precoated with 0.1 mg/mL of poly-L-lysine (Solarbio, Beijing, China).

Hippocampal tissue was dispersed at a cell density of  $10^4$ – $10^5$ /mL in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium (Gibco, Grand Island, NY) and 10% fetal bovine serum (HyClone, Logan, UT). The suspension was incubated for 16–18 hours at 37°C in 5%  $CO_2$ , and the cells were transferred into Neurobasal medium (Gibco) and 2% B27 supplement (Gibco) with glutamine 0.5% added. On day 3, cytosine arabinoside 0.5% was added to the culture medium in order to arrest the growth of non-neurons. The neurons were cultured at 37°C in a 5%  $CO_2$  incubator for 7 days, to obtain mature hippocampal neurons.

### Preparation of fulleranol

Water-soluble polyhydroxyfullerene, obtained from MER Corporation (Tucson, AZ), was dissolved in Neurobasal medium. The resulting compound had the molecular formula  $C_{60}(OH)_x(ONa)_y$  ( $y \approx 6 - 8$ ,  $x + y \approx 24$ ).

### Transmission electron microscopy imaging

Field emission transmission electron microscopy imaging was performed on an FEI (Hillsboro, OR) Tecnai G2 F20 operated at 200 kV to determine the physical properties of fulleranol at different concentrations.

### Neuron and astrocyte staining

Cytosine arabinoside (Ara-C), which interferes with cell proliferation by inhibiting the cell DNA synthesis process, was added to the medium to arrest the growth of non-neuronal cells. Neurons and astrocytes were distinguished using mouse antineuronal nuclei monoclonal antibody (Chemicon, Temecula, CA), rabbit antigial fibrillary acidic protein antibody (Chemicon), fluorescein isothiocyanate conjugate-goat antimouse IgG and tetramethylrhodamine isothiocyanate conjugate-goat antirabbit IgG.

## Cell viability assay

An MTT cell proliferation and cytotoxicity assay kit was purchased from Beyotime (Jiangsu, China). Concentrations of fullerenol ranged from 0  $\mu\text{M}$  to 100  $\mu\text{M}$ . Hippocampal neurons were cultured in 96-well plates for 7 days and then exposed to fullerenol for 24, 48 and 72 hours. A 200  $\mu\text{L}$  fresh medium sample was added to each well, together with 10  $\mu\text{L}$  of freshly prepared MTT 5 mg/mL and 100  $\mu\text{L}$  of dissolved formazan liquid. Absorbance was measured at 570 nm by a microplate reader.

## Hoechst 33342/propidium iodide detection

Lead-induced damage was used to produce oxidative damage. In this study, a Hoechst 33342/propidium iodide detection kit from KeyGen BioTech (Nanjing, China) was used to measure the toxicity of lead in neurons and to assess the potential protective scavenging effects of fullerenol on the damage caused by lead exposure. It has been shown that lead significantly decreases hippocampal neuron survival at concentrations  $>20 \mu\text{M}$ .<sup>18</sup> Seven-day-old cells cultured in 24-well plates were exposed to lead at 20  $\mu\text{M}$  in the presence or absence of fullerenol for 24 and 48 hours. The cells were collected by enzymolysis and suspended ( $10^5$ – $10^6$  cells) in 1 mL of medium to which 10  $\mu\text{L}$  Hoechst 33342 dye was added. The mixture was then left to react at 37°C for 10 minutes and was centrifuged at 1000 rpm for 5 minutes at 4°C. The precipitate was mixed with 1 mL of buffer and 5  $\mu\text{L}$  of propidium iodide dye for 10 minutes at room temperature. Fluorescence was measured at 352 nm and 488 nm (excitation wavelength) using a microscope (Zeiss, Oberkochen, Germany).

## Comet assay

The extent of cell damage from exposure to high concentrations (50, 100, and 200  $\mu\text{M}$ ) of fullerenol was assessed. Neurobasal medium (control) or fullerenol was added to the cells which have been cultured for 7 days. After 24 or 48 hours, a suspension of neurons was mixed 1 to 1 with molten low-melting-point agarose at 37°C and was spread onto a glass microscope slide. Individual cells were embedded in a thin agarose gel on a clean slide, and the DNA was allowed to unwind in the dark under alkaline conditions for one hour at 4°C. DNA electrophoresis was undertaken at 25 V for 25 minutes at 4°C, allowing the broken DNA fragments and damaged DNA to migrate away from the nucleus. The reaction mixture was then neutralized and washed three times with 0.4 M Tris-HCl (pH 7.5). The neurons were stained with a DNA-

specific fluorescent dye such as ethidium bromide. Using this technique the resulting images resemble a “comet” with a distinct head and tail. The size of the comet and the distribution of DNA correlate with the extent of DNA damage.<sup>19</sup>

## Caspase-3 and caspase-9 activity

Caspase-9 and caspase-3 activity assay kits (Beyotime) were used to validate generation and to find the possible pathway for apoptosis. Cells cultured for 7 days were exposed for 24 hours to fullerenol 50, 100, or 200  $\mu\text{M}$ . The broken cells were collected and washed. The homogenate was centrifuged at 16,000 rpm for 20 minutes at 4°C, and the supernatant was used or stored at  $-80^\circ\text{C}$ . A reaction mixture containing 50  $\mu\text{L}$  of detection buffer liquid, 40  $\mu\text{L}$  samples, and 10  $\mu\text{L}$  Ac-DEVD-pNA (2 mM), was incubated for 120 minutes at 37°C. Absorbance was recorded at 405 nm on a microplate reader (Ex1800; BioTek, Seattle, WA).

## Measurement of oxidative stress

Assay kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used to measure superoxide dismutase (SOD) activity, glutathione (GSH) levels, and maleic dialdehyde (MDA) concentrations. Cells cultured in 6-well plates for 7 days were exposed to fullerenol for 24 hours. Neurons were collected, centrifuged at 16,000 rpm for 20 minutes at 4°C, and the supernatant was collected to provide samples for assay.

SOD reagent was added to 0.2 mL samples to assess whether removal of superoxide anions protected cells from damage. The reaction mixture contained 1.2 mL of sodium pyrophosphate buffer 0.052 mM (pH 7.0), 0.1 mL of phenazine methosulfate 186  $\mu\text{M}$ , and 0.3 mL of nitro blue tetrazolium 300  $\mu\text{M}$ .<sup>18</sup> Absorbance was measured at 550 nm.

GSH reagent containing 5,5-dithiobis-2-nitrobenzoic acid was mixed with 0.1 mL of the assay sample for 5 minutes to assess molecular clearance and oxidation resistance. The optical density value was obtained at an absorbency of 405 nm.

MDA assay to evaluate the extent of cell damage involved exposing 0.1 mL of the sample to thiobarbituric acid at 95°C for 40 minutes. The heated supernatant was cooled with running water and absorbance at 532 nm was recorded on a spectrophotometer.

## Data analysis

Data were collected using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD) for the Hoechst 33342/propidium

iodide assay and CASP 1.2.2 (University of Wroclaw) for the Comet assay. Data were analyzed using Origin 8.0 software (University of Northampton, Northampton, MA). Values were expressed as the mean  $\pm$  standard error. One-way analysis of variance and Tukey tests were used to identify treatment differences. Values of  $P < 0.05$  were to be considered statistically significant.

## Results

### Characterization of fullereneol

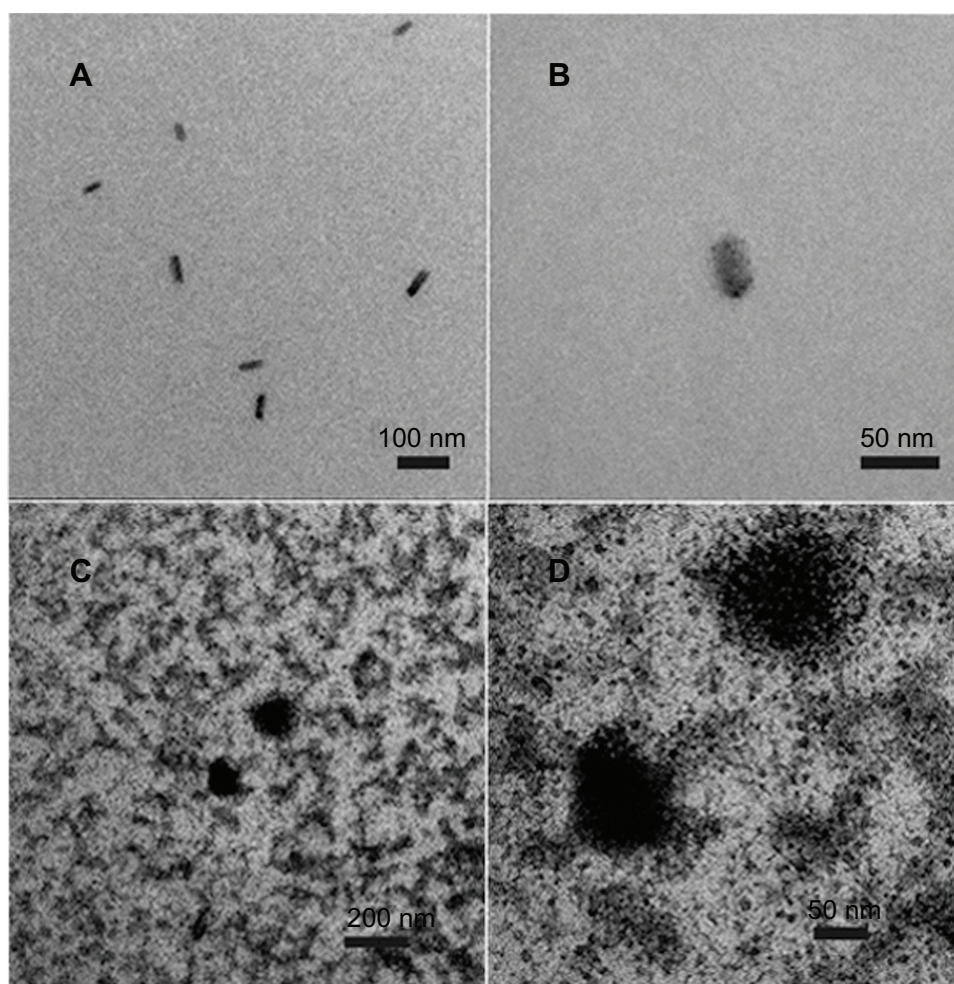
Because the culture medium composition was too complex to enable electron microscopy to be undertaken, the molecular structure of fullereneol was observed after dissolution in water. As shown in Figure 1A and B, fullereneol at a low concentration (1  $\mu$ M) appeared as a monomer in water, and in Figure 1C and D, at the highest concentration used in our experiments (200  $\mu$ M), it was also predominantly in the monomer form with only a small amount of aggregation seen.

### Neuronal and astrocyte differentiation

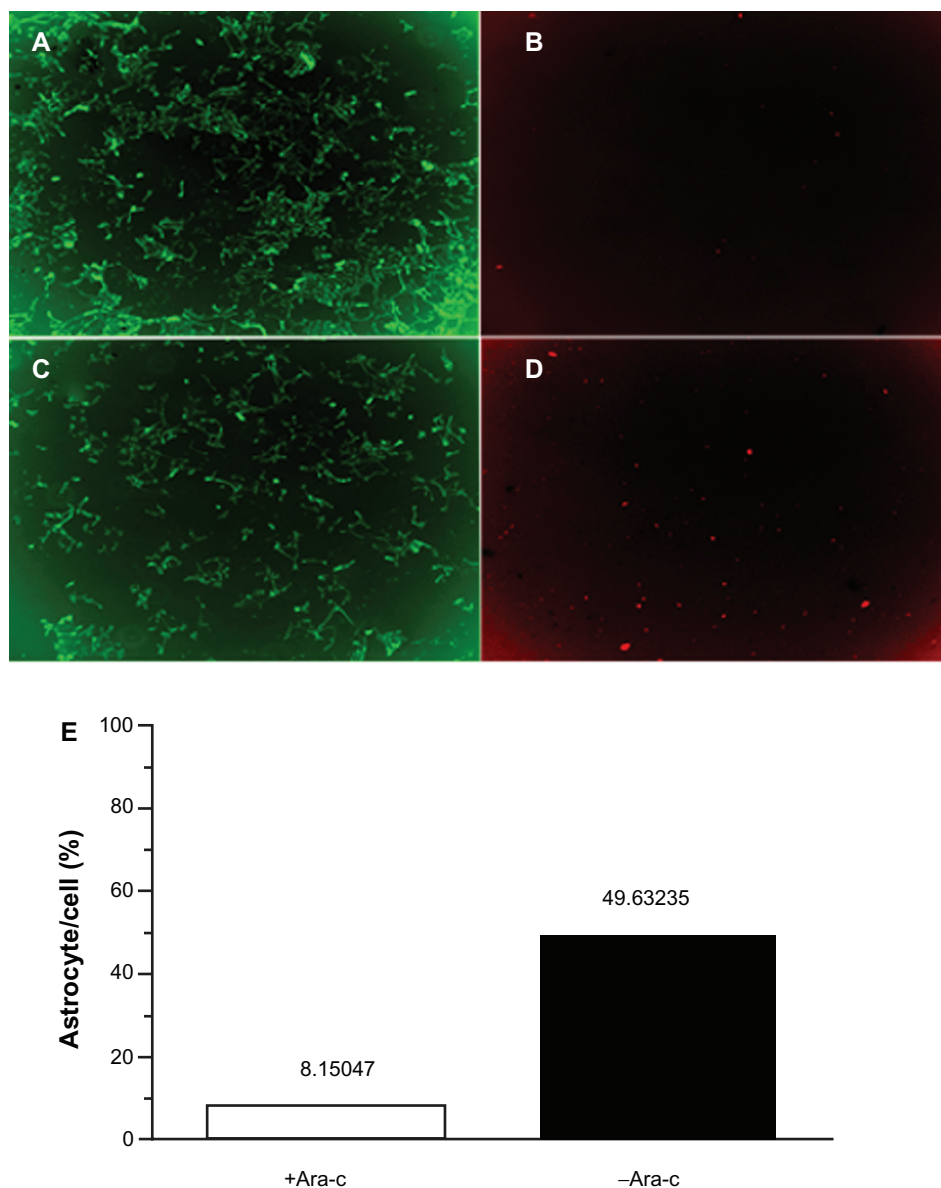
Figure 2 shows the effects of Ara-C on astrocyte and neuron cell numbers. Administration of Ara-C interfered with astrocyte growth by arresting cell proliferation. In Figure 2E, astrocytes accounted for 49.6% of the cultured cells prior to Ara-C administration but accounted for only 8.2% of cells after exposure to Ara-C.

### Effects of fullereneol on neuron viability based on concentration

Cell viability was measured in order to estimate the effect of fullereneol on cultured hippocampal neurons. As shown in Figure 3A, exposure to fullereneol for 24 hours at concentrations of 1  $\mu$ M and 5  $\mu$ M increased hippocampal neuron viability by  $111.55\% \pm 0.53\%$  ( $P < 0.01$ ) and  $110.33\% \pm 1.82\%$  ( $P < 0.05$ ), respectively. Fullereneol at concentrations  $>5 \mu$ M had no effect on cell viability. In Figure 3B, exposure of cells to fullereneol 100  $\mu$ M for 48 hours reduced neuron viability to



**Figure 1** Transmission electron microscopy image of the physical state of  $C_{60}(OH)_x(ONa)_y$ . (A) Image of 1  $\mu$ M fullereneol in water. (B) Amplified image of 1  $\mu$ M fullereneol in water. (C) Image of 200  $\mu$ M fullereneol in water. (D) Amplified image of 200  $\mu$ M fullereneol in water.



**Figure 2** Double staining of neurons and astrocytes. (A) Neurons in the +Ara-C group. (B) Astrocytes in the +Ara-C group. (C) Neurons in the -Ara-C group. (D) Astrocytes in the -Ara-C group. (E) Histogram showing the ratio of astrocyte to cell numbers.

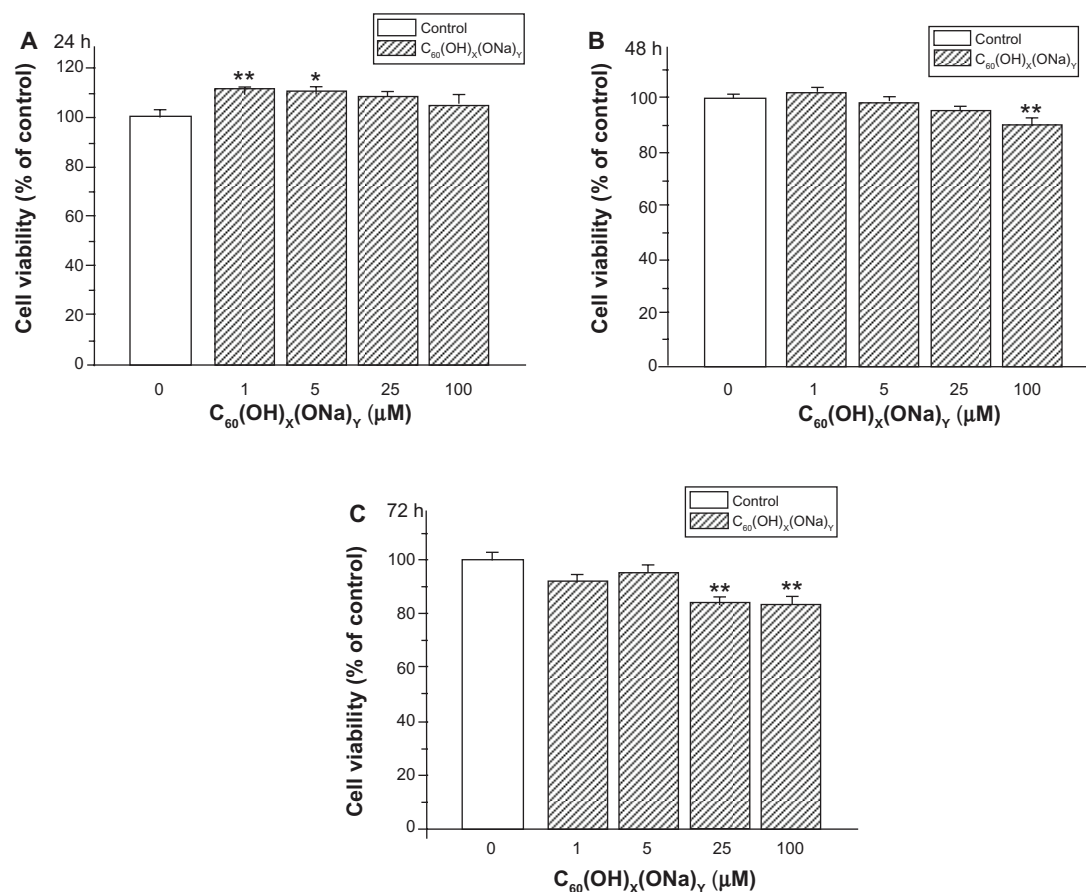
**Abbreviation:** Ara-C, cytosine arabinoside.

$89.62\% \pm 2.90\%$  ( $P < 0.01$ ), whereas no significant changes were seen at concentrations  $<25 \mu\text{M}$ . Exposure of cells to fullerenol  $25 \mu\text{M}$  and  $100 \mu\text{M}$  for 72 hours (Figure 3C) reduced neuron viability to  $83.59\% \pm 2.26\%$  ( $P < 0.01$ ) and  $84.21\% \pm 2.45\%$  ( $P < 0.01$ ), respectively.

### Effect of fullerenol on lead-induced hippocampal neuronal damage

The possible protective effect of fullerenol against lead-induced cytotoxicity was assessed by Hoechst/propidium iodide assay. Hoechst dye entered into living cells, producing blue fluorescence, and propidium iodide dye entered into

dead cells, producing red fluorescence. Figure 4A shows three bright-field images detected with a high-power optical microscope. The left image shows normal cells; the middle image shows necrotic cell after exposure to lead; and the right image shows partially viable cells exposed to lead and to fullerenol. Propidium iodide fluorescence intensity increased in cells exposed to lead, whereas no notable change was seen after exposure to lead and fullerenol (Figure 4B). Figure 4C and D show the effect of fullerenol on hippocampal neuron survival rate after exposure to lead. Survival was reduced after exposure to lead acetate for 24 hours ( $82.61\% \pm 5.84\%$ ;  $P < 0.01$ ) or 48 hours ( $72.52\% \pm 5.49\%$ ;  $P < 0.01$ ). Concurrent exposure to



**Figure 3** Effect of  $C_{60}(OH)_x(ONa)_y$  on viability of cultured hippocampal neurons. **(A)** Hippocampal neurons treated with fullereneol 0, 1, 5, 25, and 100  $\mu\text{M}$  for 24 hours. **(B)** Hippocampal neurons treated for 48 hours. **(C)** Hippocampal neurons treated for 72 hours.

**Notes:** The results show mean  $\pm$  standard error ( $n = 8$ ) values from a representative experiment that was repeated at least three times. \* $P < 0.05$  versus control group; \*\* $P < 0.01$ , versus control group. One-way analysis of variance with Bonferroni post hoc tests.

**Abbreviation:** PI, propidium iodide.

fullerenol provided significant protection against lead-induced neurotoxicity. After exposure for 24 hours to fullereneol 1, 5, and 25  $\mu\text{M}$ , cell viability was, respectively, increased  $95.87\% \pm 1.29\%$  ( $P < 0.05$ ),  $97.93\% \pm 0.92\%$  ( $P < 0.01$ ), and  $95.55\% \pm 2.05\%$  ( $P < 0.05$ ) of control values. However, this protective effect becomes inconspicuous in Figure 4D.

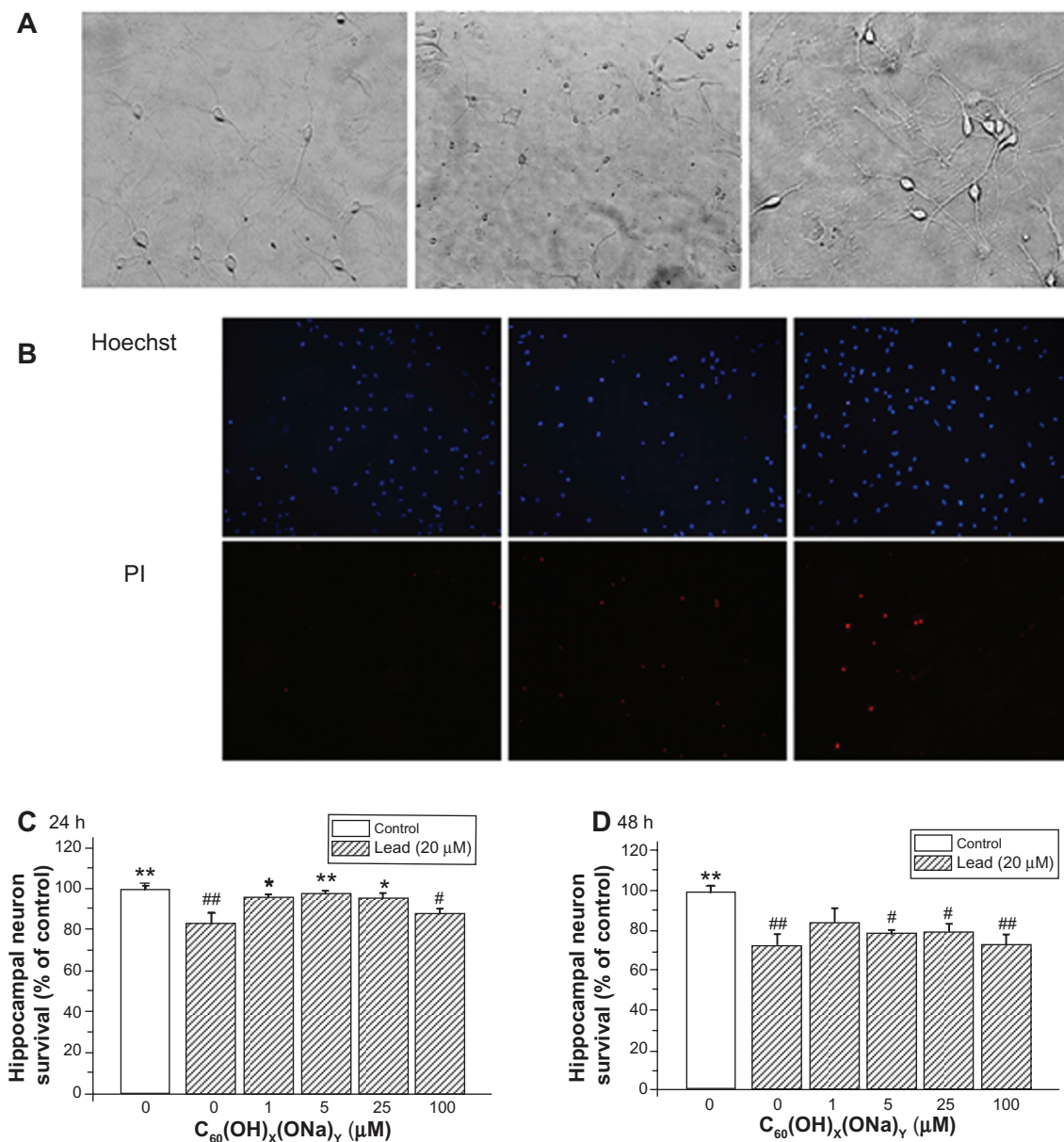
### Influence of high concentrations of fullereneol in Comet assays

The influence of fullereneol at high concentrations on hippocampal neuronal apoptosis is shown in Figure 5. A normal cell comprises a single head (Figure 5A) while an apoptotic cell has both a head and a long tail (Figure 5B). In analytic, images normal cells are characterized by one peak and apoptotic cells by two peaks. Figure 5C shows mean percentage changes in DNA. Following exposure to fullereneol 50  $\mu\text{M}$  for 24 hours, there was no notable change in the mean percentage of tail DNA relative to the control group; however, DNA content increased at concentrations  $>100$   $\mu\text{M}$ . The mean

DNA content also increased after exposure for 48 hours. After exposure for 24 hours, the DNA content was  $2.01\% \pm 0.36\%$  in the control group and  $17.88\% \pm 1.76\%$  in the 200  $\mu\text{M}$  group ( $P < 0.01$ ). Corresponding values after 48 hours of exposure were  $2.65\% \pm 0.46\%$  and  $28.20\% \pm 2.74\%$ , respectively ( $P < 0.01$ ). As shown in Figure 5D, there was evidence of a positive association between the numerical value of the olive tail moment and concentration of fullereneol. In the control group, all values were  $<1$  or  $<5$ , whereas in the group exposed to fullereneol 200  $\mu\text{M}$  for 48 hours, some values were  $>10$  or  $>20$ .

### Changes in caspase protein content in hippocampal neurons

Compared with control neurons, there were no significant changes in caspase protein in the 50  $\mu\text{M}$  group, whereas neurons exposed to fullereneol 100  $\mu\text{M}$  or 200  $\mu\text{M}$  for 24 hours had significantly increased caspase-3 activity (Figure 6A) and caspase-9 activity (Figure 6B). Exposure to fullereneol 100  $\mu\text{M}$  and



**Figure 4** Protective effect of  $C_{60}(OH)_x(ONa)_y$  against lead-induced neurotoxicity. Hippocampal neurons were treated with various concentrations of fullereneol 0, 1, 5, 25, and 100  $\mu\text{M}$  and lead 20  $\mu\text{M}$  for 24 or 48 hours. The Hoechst dye entered into normal cells and emitted blue fluorescence, while the propidium iodide dye entered into necrotic cells and emitted red fluorescence. **(A)** Three typical images of random fields per treatment group detected with a high-power optical microscope. Left, control; middle, 20  $\mu\text{M}$  lead-exposed for 24 hours; right, 20  $\mu\text{M}$  lead and 1  $\mu\text{M}$  fullereneol exposed for 24 hours. Magnification is  $10 \times 20$ . **(B)** Three typical images detected with fluorescence microscopy. **(C)** Survival rate of hippocampal neurons after coculturing for 24 hours. **(D)** Survival rate after coculturing for 48 hours.

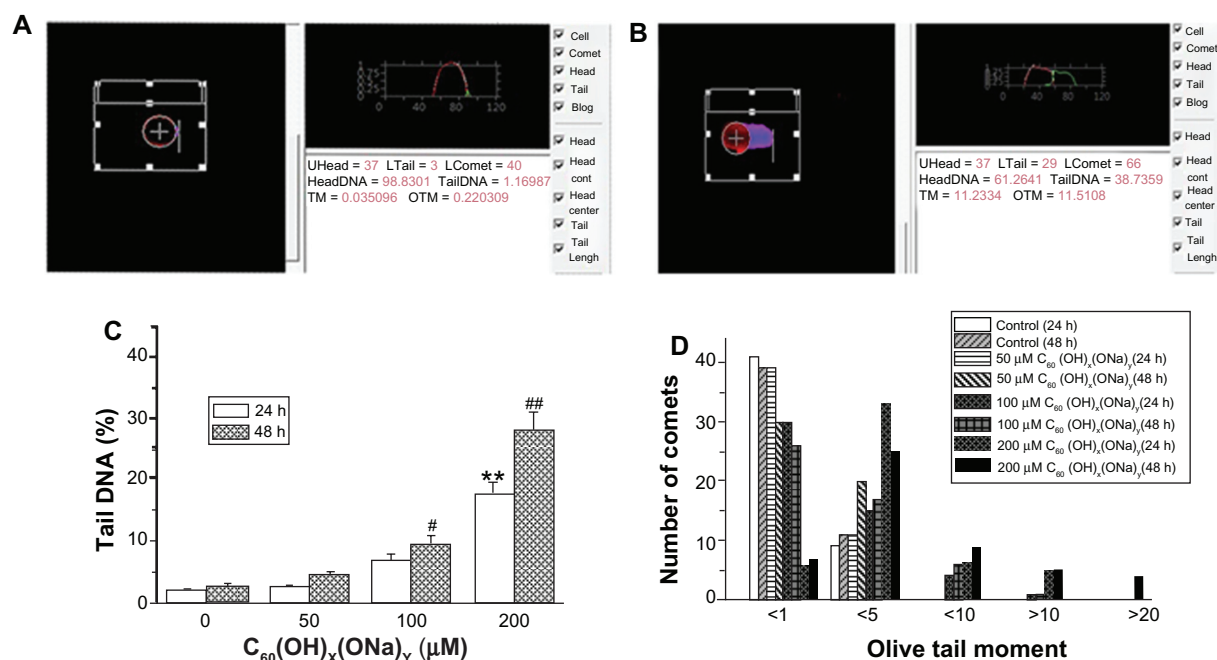
**Notes:** \* $P < 0.05$  versus lead-exposed group without fullereneol. \*\* $P < 0.01$  versus lead-exposed group without fullereneol. # $P < 0.05$  versus control group; ## $P < 0.01$  versus control group. One-way analysis of variance with the Bonferroni post hoc tests.

200  $\mu\text{M}$ , respectively, increased caspase-3 to  $130.24\% \pm 4.77\%$  and  $150.77\% \pm 7.10\%$  (both  $P < 0.01$ ) of control levels. Corresponding values for caspase-9 were  $128.67\% \pm 2.34\%$  and  $136.23\% \pm 1.11\%$ , respectively (both  $P < 0.01$ ).

### SOD, GSH, and MDA activity in hippocampal neurons

Figure 7A shows that the activity of SOD significantly increased from  $121.91 \pm 1.57$  U/mprot in the control group

to  $152.98 \pm 1.60$  U/mprot ( $P < 0.01$ ) in cells exposed to fullereneol 1  $\mu\text{M}$ . In the 100  $\mu\text{M}$  group, there was a significant decrease to  $88.09 \pm 0.61$  U/mprot, respectively ( $P < 0.01$ ). There were no notable changes in the 5  $\mu\text{M}$  and 25  $\mu\text{M}$  groups. As shown in Figure 7B, there was a significant increase in GSH from  $0.92 \pm 0.13$  mg/gprot in controls to  $2.10 \pm 0.40$  mg/gprot after exposure to fullereneol 5  $\mu\text{M}$  ( $P < 0.05$ ), and there was a decline in GSH with fullereneol at 100  $\mu\text{M}$  ( $0.92 \pm 0.15$  mg/gprot). In Figure 7C, MDA



**Figure 5** High concentrations of  $C_{60}(OH)_x(ONa)_y$  can induce apoptosis. **(A)** Comet image of a normal neuron detected with CASP software. **(B)** Comet image of an apoptosis neuron. **(C)** Mean percentage of tail DNA in hippocampal neurons treated with fullereneol 0, 50, 100, and 200  $\mu M$ . **(D)** Olive tail moment distribution (50 observations) in hippocampal neurons treated with fullereneol 0, 50, 100, and 200  $\mu M$ .

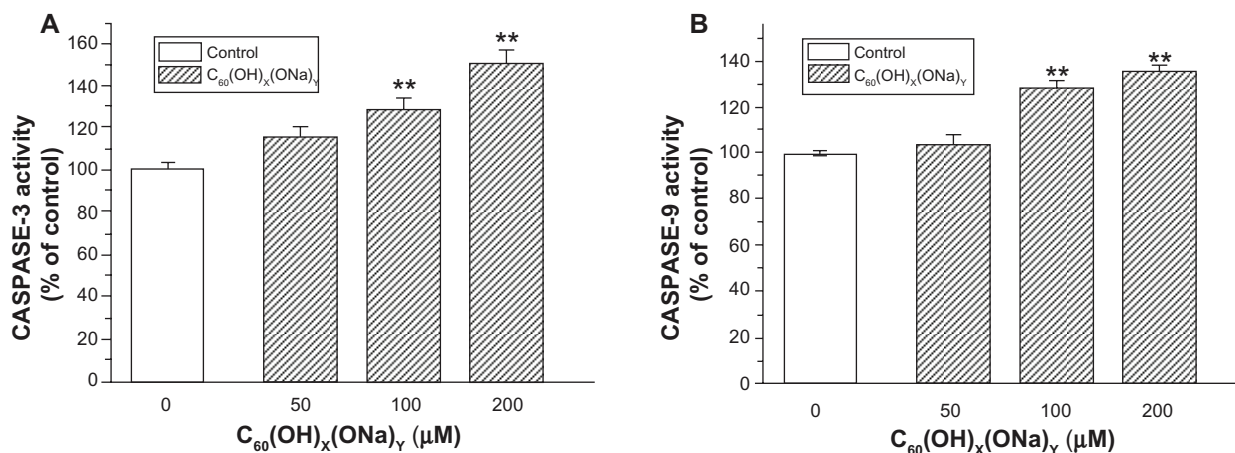
**Notes:** The results are shown as the mean  $\pm$  standard error ( $n = 50$ ) of a representative experiment that was repeated at least three times.  $**P < 0.01$  versus control group in 24 hours.  $^{\#}P < 0.05$  versus control group in 48 hours;  $^{\#\#}P < 0.01$  versus control group in 48 hours. One-way analysis of variance with Bonferroni post hoc tests.

levels showed no significant decrease from control values ( $2.03 \pm 0.01$  mg/gprot) after exposure to 5  $\mu M$  fullereneol ( $1.85 \pm 0.11$  mg/gprot) and no significant increase after exposure to fullereneol 100  $\mu M$  ( $2.15 \pm 0.04$  mg/gprot).

## Discussion

Our own extensive experience and study of the mechanism of lead poisoning has shown that its effects are not readily reversed. It has been reported that cell damage

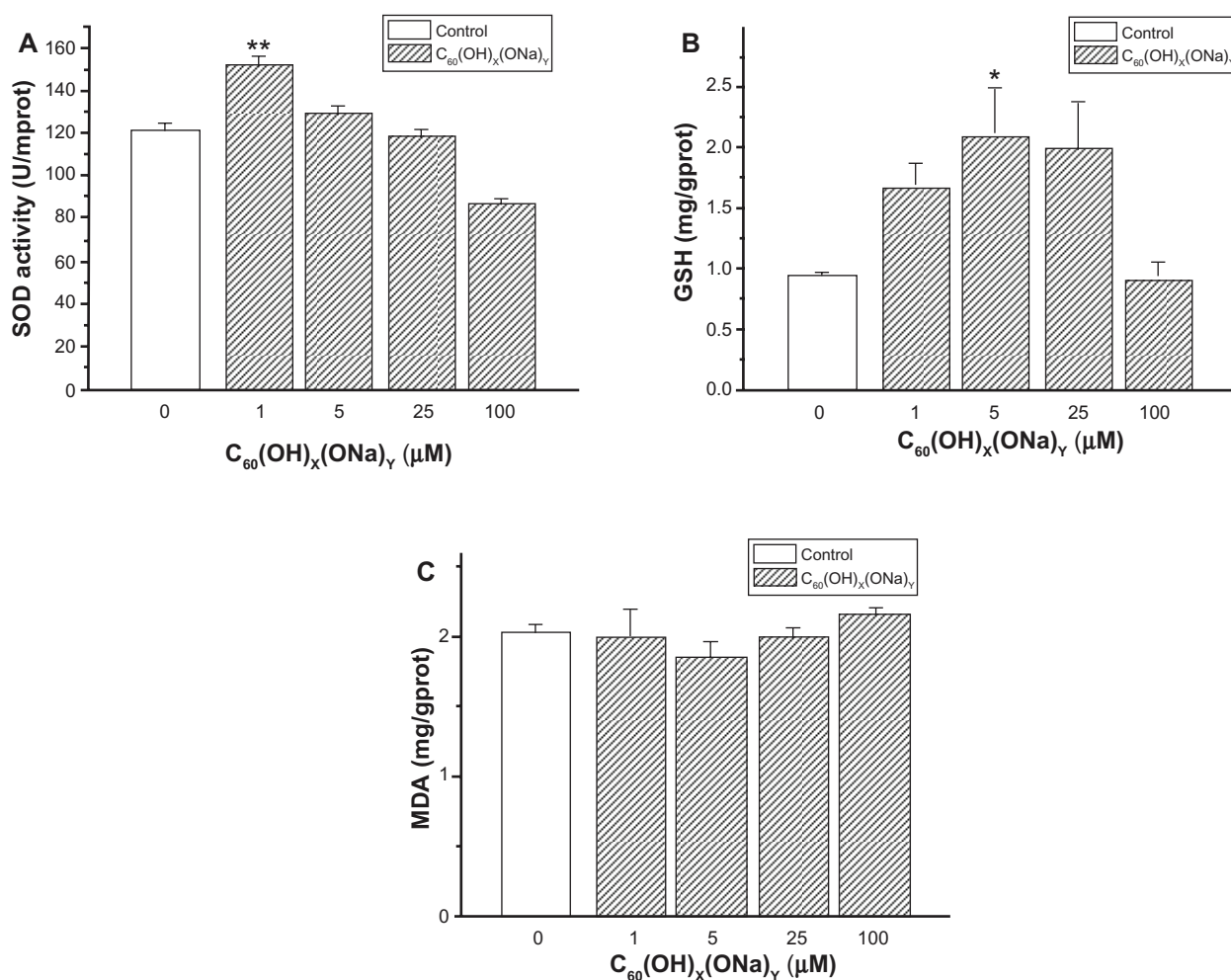
following exposure to lead is caused by oxidative damage.<sup>20</sup> For these reasons, we chose lead toxicity for our current experiments in order to investigate whether fullereneol had the ability to restore oxidative damage, or whether it induced oxidative damage in itself. Our results show that, at low concentrations, fullereneol significantly increased hippocampal neuronal viability and protected neurons from oxidative damage, improving the survival rate of cells exposed to lead. However, at high concentrations, fullereneol decreased



**Figure 6** Effect of  $C_{60}(OH)_x(ONa)_y$  on caspase content of cultured hippocampal neurons. Hippocampal neurons were treated with fullereneol 0, 50, 100, and 200  $\mu M$  for 24 hours. **(A)** Caspase-3 activity was detected using a protein assay kit. **(B)** Caspase-9 activity was detected.

**Notes:** The results are the mean  $\pm$  standard error ( $n = 8$ ) of a representative experiment that was repeated at least three times.  $**P < 0.01$  versus control group. One-way analysis of variance with Bonferroni post hoc tests.





**Figure 7** Extent of oxidative stress in cultured hippocampal neurons of control and fullereneol-exposed groups (1, 5, 25, and 100 μM). **(A)** Effects of fullereneol on superoxide dismutase activity in cultured hippocampal neurons. **(B)** Effects of fullereneol on glutathione level in neurons. **(C)** Effects of fullereneol on maleic dialdehyde content in neurons. **Notes:** Values are mean ± standard error (n = 26). \**P* < 0.05 versus corresponding value of control group; \*\**P* < 0.01 versus corresponding value of control group. One-way analysis of variance with the Bonferroni post hoc tests.

**Abbreviations:** GSH, glutathione; MDA, maleic dialdehyde; SOD, superoxide dismutase.

hippocampal neuron viability and induced apoptosis. These findings support the notion that water-soluble fullerene may be an effective agent against lead poisoning with an antioxidant mechanism, and appears to be involved in the process of apoptosis caused by mitochondrial damage.

The first finding of our study was that low concentrations of fullereneol significantly increased the viability of cultured hippocampal neurons. Cell viability was assessed by MTT assay, which measures cell proliferation rate by measuring reduction in mitochondria. As neurons cannot proliferate or regenerate, increased cell viability indicates enhancement of reducing power. It is interesting in this context to note that treatment with *e,e,e*-C<sub>60</sub>(COOH)<sub>2</sub><sub>3</sub> has been shown to reduce superoxide radical formation in brain mitochondria and increase the lifespan of mice.<sup>14</sup> Fullereneol is known to have scavenging ability, but the role of this property remains unknown. Our data from Hoechst 33342/propidium iodide

detection studies indicate that fullereneol is able to increase the survival neurons exposed to lead and, therefore, suggest that fullereneol might protect neurons against lead-induced damage.

Another significant finding of our study was that fullereneol at high concentrations decreased the viability of cultured hippocampal neurons. Other studies have shown that, at high concentrations, the toxicity of fullerene derivatives induces apoptosis.<sup>10</sup> Our findings support this hypothesis in part. Using Comet assays, we found evidence of apoptosis in hippocampal neurons exposed to high concentrations of fullereneol. In our experiments, the magnitude of tail DNA in experimental groups changed according to length of exposure and concentration, and numerical values for the olive tail moment were also characteristic of apoptosis.

Caspases are a family of proteases that plays an important role in the process of apoptosis. These agents selectively

remove specific peptide bonds and thereby promote apoptosis. Some caspases, such as caspase-9, act as initiators, and are activated by foreign protein signals to remove downstream proteins. Other caspases, such as caspase-3, induce apoptosis by hydrolyzing target proteins. In our experiments, we identified both caspase-9 and caspase-3. Caspase-9 is linked to the mitochondrial death pathway, and caspase-3 interacts with caspase-9 and some other caspases. Therefore, the concentrations of caspase-3 and caspase-9 proteins are an important index of apoptosis induced through the cytochrome-c signaling pathway. An elevation of both caspases as was apparent in our experiments, suggests that apoptosis is related to mitochondrial damage.

The ability of fullereneol to increase cell viability in MTT experiments may be the consequence of increased reduction reactions within neurons. We consider the increasing of cell viability to be the enhancement of reducing power. The Hoechst 33342/propidium iodide double stain detection experiments suggest that reduction reactions ease oxidative stress. We propose that at low concentrations fullereneol increases reducing power and thereby enhances hippocampal neuron viability and improves survival rate in the lead-exposed groups. The Comet and caspase-3 and caspase-9 assays suggest that the mean percentage of tail DNA and the content of caspase protein in cultured hippocampal neurons both increase in oxidant-induced apoptosis. Thus, at high concentrations, fullereneol may increase oxidative stress and induce apoptosis via the caspase-9 pathway. To prove these points, we tested SOD, GSH, and MDA levels. SOD is a critical component of the oxidant defense system, and in our experiments its levels showed a significant decrease. GSH, a molecular clearing agent, increased at low concentrations of fullereneol and decreased at high concentrations. MDA levels, which are an index of lipid peroxidation, decreased at low concentrations and increased at high concentrations of fullereneol. These findings suggest that there was an increase in reduction reactions in neurons at lower concentrations of fullereneol, and that there was increasing oxidative stress in neurons with higher concentrations of fullereneol. Based on these findings, we propose that the fate of neurons depends on the redox level within neurons, and this in turn depends on the concentration of fullereneol that they are exposed to.

In our experiments, we used transmission electron microscopy to detect the physical state of fullereneol at different concentrations. Other workers have shown that fullereneol is present as a monomer state at concentration of  $<4 \times 10^{-5}$  M and as an aggregate state at concentrations  $>2 \times 10^{-4}$  M.<sup>21</sup> Our transmission electron microscopic imaging studies

showed that, at lower concentrations, the compound was in the form of monomer in water, and at higher concentrations, both monomer and aggregate states were found. Thus we have demonstrated that at lower concentrations fullereneol in the monomer state protected neurons against oxidative stress, and at higher concentrations, fullereneol in both monomer and aggregate states induced oxidant-induced apoptosis. Hydroxyl groups attached to the fullerene core might contribute to free radical scavenging, while aggregates might cause oxidative damage. Thus, one of the reasons for the redox level changes might be the state of fullereneol.

Our current findings have some practical significance for human life. Due to their special material and bonding properties, fullerenes have found a use not only in a range of technologies, and also in living systems.<sup>22</sup> A recent study using fluorescence analysis showed that water-soluble fullereneol was able to destroy amyloid fibrils of the brain peptide A $\beta$  (1–42) in vitro and had a potential role in treating Alzheimer disease.<sup>23</sup> Other reports indeed have paid more attention to studies in vivo. For instance, it has been shown that fullerene altered the vertical migration response of *Daphnia magna* to the addition of food, and reduced swimming speed.<sup>24</sup>

In summary, our study has clearly shown that fullereneol can be deemed a promoter of cell death as well as a protector against oxidative risk, depending on the concentration used. Further in vivo and in vitro studies are needed to understand better the underlying mechanisms of these dual effects. Because of their growing uses, nanoparticles are widely regarded as new types of chemical pollutants.<sup>25</sup> Recent air samples from urban atmospheres have been shown to contain fullerene and its derivatives, demonstrating that humans are exposed to environmental fullerene and its derivatives via inhalation.<sup>26</sup> The increasing use of fullerene materials therefore warrants toxicological investigation.

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## Disclosure

The authors report no conflicts of interest in this work.

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