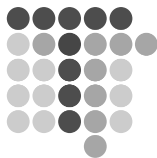

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Neuroprotective Effects of Hydroxyfullerene in Rats Subjected to Global Cerebral Ischemia

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Abstract

Oxidative stress is believed to contribute to the neuronal damage induced by cerebral ischemia/reperfusion injury. The present study was undertaken to evaluate the possible antioxidant neuroprotective effect of hydroxyfullerene (a radical absorbing cage molecule) against neuronal death in hippocampal CA1 neurons following transient global cerebral ischemia in the rat. Transient global cerebral ischemia was induced in male Wistar rats by four vessel- occlusion (4VO) for 10 min. Lipid peroxidation in brain tissues was determined by measuring the concentrations of thiobarbituric acid-reactive substances (TBARS). Furthermore, the apoptotic effects of H_2O_2 on PC12 cells were also investigated. Cell viabilities were measured using MTT [3-(4,5-dimethylthiazolyl-2)-2,-5-diphenyltetrazolium bromide] assays. Hydroxyfullerene, when administered to rats at 0.3-3 mg/kg i.p. at 0 and 90 minutes after 4-VO was found to significantly reduce CA1 neuron death by 72.4% on hippocampal CA1 neurons. Our findings suggest that hydroxyfullerene protects neurons from transient global cerebral injury in the rat hippocampus by reducing oxidative stress and lipid peroxidation levels, which contribute to apoptotic cell death.

Keywords: Ischemia, Neuroprotection, Hydroxyfullerene, Four vessel-occlusion

Pure carbon spheres of buckminsterfullerene (C_{60}, fullerene) have generated considerable interest in a number of scientific fields, which culminated in the award of a Nobel Prize in Chemistry in 1996 to discovery of fullerenes.

Krusic *et al.*[1] characterized fullerene as a 'radical sponge' that is capable of 34 methyl radicals per molecule. However, the potential usages of native C_{60} fullerene as a pharmacological free radical scavenger are severely limited by its poor solubility. In fact, the native material can only be dissolved in a limited number of biologically unattractive solvents, such as, toluene and benzene.

Radical sponges have been demonstrated to decrease, but not eliminate, neuronal death[1]. This neuroprotective effect of fullerenes is based on an ability to react with radical oxygen containing species, such as, superoxide (O_2^-) and hydroxyl (-OH) radicals, which attack lipids, proteins, DNA, and other macromolecules[2].

In particular, poly-hydroxylated fullerenes, named fullerenenol or fullerol [$C_{60}(OH)_n$], have been shown to be excellent antioxidants, and to reduce apoptosis in cortical neuron cultures. Furthermore, they have high solubilities and are able to cross the blood brain barrier. Fullerenol have also been demonstrated to absorb many oxygen radicals per molecule, to reduce free radical damage in neuronal tissues[3], and to prevent hydrogen peroxide and cumene hydroperoxide-elicited damage in hippocampus slices (Figure 1). Transient global or forebrain ischemia arising in humans as a consequence of cardiac arrest or cardiac surgery, or in animals as a result of experimental induction, leads to selective and delayed neuronal cell death[4] of hippocampal CA1 neurons and cognitive deficits[5]. The pyramidal neurons of the CA1 region in the hippocampus are among the cells most vulnerable to loss of blood supply to the brain; cell death occurs days after the initial ischemic insult, a phenomenon termed delayed neuronal death[4].

Moreover, it has been reported that the release of ROS and increases in lipid peroxidation occur rapidly after injury, e.g., 2-3 h post-injury, without evidence of neuronal cell death[6]. ROS can produce a host of

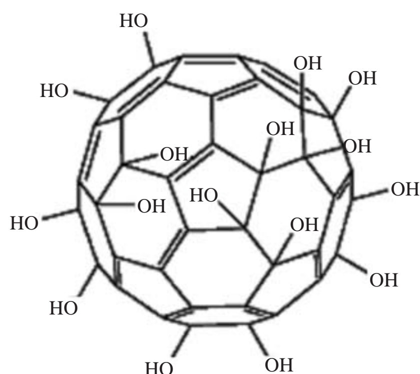

Figure 1. Representative structure of a neuroprotective fullerene derivative functionalized with hydroxy groups attached to the cyclopropane carbons of the C_{60} molecule.

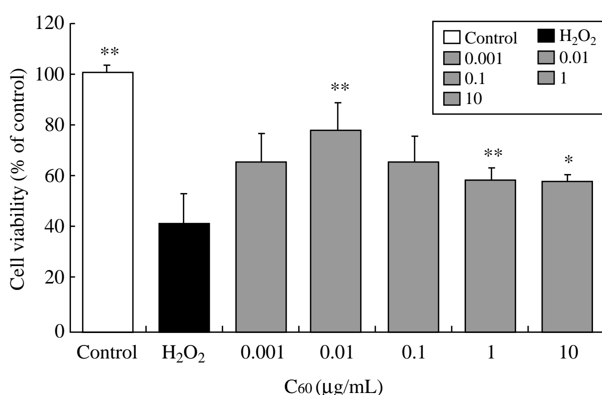

Figure 2. Attenuation of hydrogen peroxide-induced cytoxicity by hydroxyfullerene in PC12 cells. PC12 cells were pretreated with hydroxyfullerene for 1 h and 200 M hydrogen peroxide μM was then added and incubated for 24 h at 37°C. Cell viabilities were determined using MTT reduction assays. Data are presented as means ± SEM. $*P<0.05$ and $**P<0.005$ compared to the cells treated with hydrogen peroxide only ($n=7$).

oxidative mediated deleterious changes in cells, such as, DNA fragmentation, cell membrane lipid peroxidation, decreased mitochondrial energy production, and transporter protein inactivation[7].

Production of reactive oxygen species (ROS) and the subsequent oxidative stress have been thought to play a pivotal role in ischemia/reperfusion (I/R)-induced delayed neuronal death[8]. Accordingly, carbon-60 fullerene-based neuroprotective compounds have attracted considerable attention in this context[9]. Recently, systemic hexa sulfobutylated C_{60} (FC4S) was found to reduce total infarct volumes produced by transient ischemia in the rat brain[10], which suggests that water-soluble derivatives of fullerene act as powerful antioxidants *in vivo*.

Therefore, we undertook the present study to investigate the effects of hydroxyfullerene on neuronal cell death and lipid peroxidation following transient global cerebral ischemia in the rat hippocampus.

Antioxidant Activity on Hydroxyfullerene in PC 12 Cells

The protective role of hydroxyfullerene was examined using hydrogen peroxide-mediated cell death assays (Figure 2). Hydrogen peroxide (200 μM for 24 h) reduced PC12 cell viability to 41.2% of the control value, whereas 78.2% of cells pretreated with hydroxyfullerene at 5 μg/mL before exposure to hydrogen peroxide remained viable. MTT assays demonstrated that hydroxyfullerene at concentration from 0.001 to 10 μg/mL markedly protected PC12 cells.

Neuronal Cell Density in the CA1 Region of the Hippocampus

The neuroprotective effect of hydroxyfullerene on hippocampal neurons was evaluated by measuring neuronal cell densities in the CA1 region at 7 days after ischemia. Body temperatures of animals exposed to ischemia were monitored for 6 h of cerebral reperfusion. No significant difference in body temperatures were observed between saline treated and hydroxyfullerene treated animals at any time (data not shown), which demonstrated that neuroprotective effects of hydroxyfullerene were not due to decreases in body temperature. Control CA1 pyramidal neurons from three hemispherical sections (1 mm square) were counted and averaged (Figure 3A and 3D). The 10 min period of transient ischemia administered caused selective and delayed neuronal cell loss in CA1 (Figure 3B and 3E). Treatment of experimental animals withhydroxyfullerene (3 mg/kg, 0 and 90 min after I/R) markedly reduced the number of damaged pyramidal cells in CA1 subfields (Figure 3C and 3F). Furthermore, this neuroprotective effect of hydroxyfullerene was dose dependent. Moreover, as compared with saline treated ischemic animals, hydroxyfullerene at 0.3, 1, and 3 mg/kg i.p. reduced CA1 cell death by 8.6, 24.2, and 77.3%, respectively. Optimal neuroprotection was achieved at 3 mg/kg, at which level the protection afforded was significant (Figure 4).

Effect of Hydroxyfullerene on Lipid Peroxidation

Malondialdehyde (MDA) is the most abundant reactive carbonyl compound found *in vivo* and is a by product of fatty acid degradation. Thus, MDA levels are widely used as surrogates of lipid peroxidation[11]. Figure 5 shows the effects of I/R ischemia/reperfusion

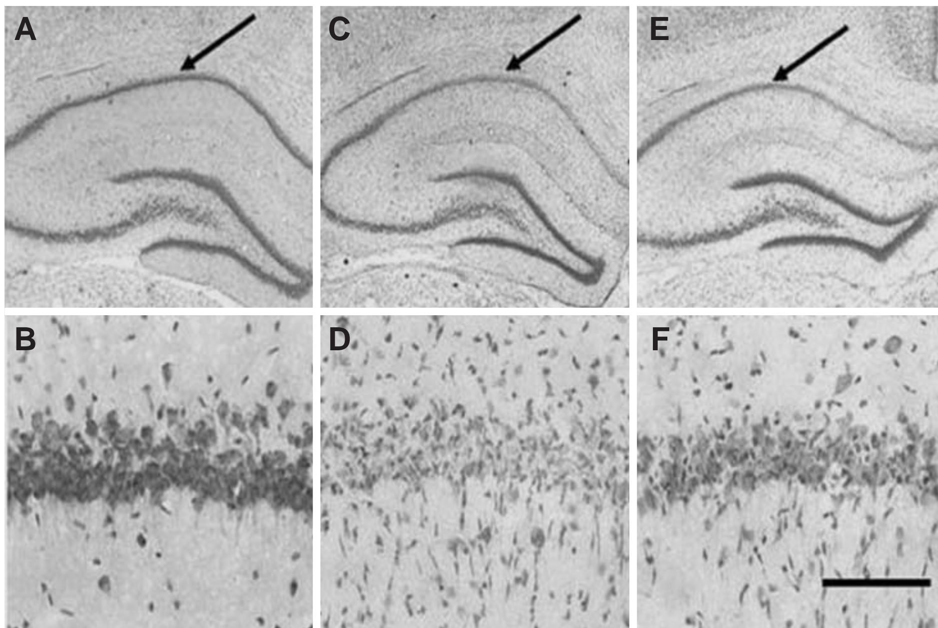

Figure 3. Neuronal densities in the hippocampal CA1 region after hydroxyfullerene treatment. Representative photomicrographs of cresyl violet-stained sections from sham-operated (A, B) or animals that had been subjected to 10 min ischemia and then treated with either saline (C, D) or 3 mg/kg (i.p.) of hydroxyfullerene (E, F). Arrowed regions in A, C, and E are shown in B, D, and F, respectively. Neuronal cell loss was induced 10 min after ischemia in CA1 (C, D). Hydroxyfullerene markedly reduced numbers of damaged pyramidal cells in CA1 (E, F).

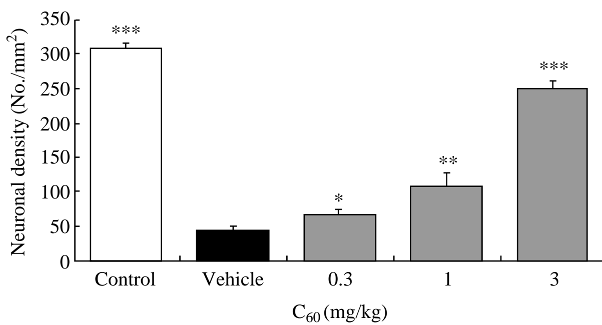

Figure 4. Neuronal density in the CA1 region after hydroxyfullerene treatment. Saline or hydroxyfullerene (0.3, 1, and 3 mg/kg) were administered i.p. to animals immediately after 10 min of ischemia. Values are means ± SEM. Significantly different from the saline group (*P < 0.05, **P < 0.005, ***P < 0.001). control; sham operated group, vehicle; 4VO operated group with saline treatment.

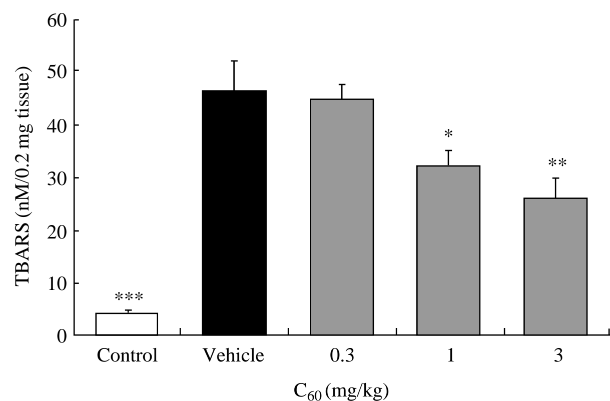

Figure 5. Effect of hydroxyfullerene on lipid peroxidation following 4VO. MDA levels were measured in 10% homogenates of hippocampus portion from rats subjected to 10 min of ischemia. All drugs were administered i.p. 0 and 90 min after reperfusion. The antioxidative effect showed 48.4% protection from tissue damage as compared with untreated animals. Values are means ± SEM. (n=7) (***P < 0.001, **P < 0.005, *P < 0.05).

and treatment with hydroxyfullerene. In sham-operated rats lipid peroxide levels were similar to those of normal control rats, though a substantial increase in TBARs levels was noticed in the hippocampus of rats exposed by 10 min ischemia and reperfusion.

Hydroxyfullerene at 0.3, 1, and 3 mg/kg i.p. reduced TBARs levels to 44.79 ± 2.90, 32.19 ± 2.86, and 25.90 ± 4.14 nmol/g (*P < 0.05, **P < 0.01, Figure 5).

Discussion

Reactive oxygen species (ROS) like hydrogen peroxide, the superoxide anion, and the hydroxyl radical can readily damage biological molecules, and this process can lead to apoptotic or necrotic cell death[12]. Recent studies have indicated that apoptosis is involved in delayed neuronal death of the CA1 region in the hippocampus[13] and that oxidative stress plays a pivotal role in the pathogenesis of acute ischemic stroke[14]. Furthermore, it has been well established that the rat model of transient global cerebral ischemia mimics

transient cardiac arrest in man. In particular, in this model vulnerable cells, such as, hippocampal CA1 pyramidal neurons, display delayed neuronal death[4]. In this rat model, transient cerebral ischemia followed by 1-24 h of reperfusion was found to significantly increase ROS, NO, and lipid peroxidation end-product levels and to markedly reduce levels of the endogenous antioxidant glutathione[15]. Moreover, ROS scavengers and inhibitors of lipid peroxidation protect the brain after ischemia/reperfusion[16].

It has also been proposed that buckminsterfullerene and its derivatives act as inhibitors of lipid peroxidation and free radical-scavengers for the majority of ROS types[17]. Moreover, some C_{60} derivatives can traverse the blood-brain barrier[18,19]. The present study was performed to evaluate the effects of hydroxyfullerene in rat brain tissue exposed to ischemia-reperfusion. The hydroxyfullerenes, a group of polyhydroxylated C_{60} compounds, are powerful free radical scavengers[20], and it has been reported that hydroxyfullerene reduces neuronal apoptosis induced by serum deprivation[3]. Moreover, the present study shows that hydroxyfullerene has a neuroprotective effect, as treatment with hydroxyfullerene (3 mg/kg) markedly reduced neuronal cell death, which suggest that hydroxyfullerene protects against cerebral I/R injury in the rat.

To our knowledge, this is the first report to provide evidence of the antioxidant effects of hydroxyfullerene on brain in a rat model of transient cerebral ischemia.

Although the mechanistics of hydroxyfullerene are not known *in vivo*, prevention of neuronal cell death and the radical absorbing effects of similar species suggest that it reduces neuronal injury by absorbing free radicals, and by preventing lipid peroxidation following global cerebral ischemia. In conclusion, hydroxyfullerene was found to protect neurons in the rat hippocampus from transient global cerebral I/R injury. We suggest that this neuroprotective effect of hydroxyfullerene is attributed to its reducing oxidative stress and lipid peroxidation.

Materials and Methods

Materials

Hydroxyl fullerene ($C_{60}(OH)_{24}$, 99.8% purity) was purchased from MER Corporation (South Kolb Road Tucson, AZ), and was dissolved in saline for *in vivo* studies.

Cell Culture and MTT Assay

PC12 cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37°C. All cells were cultured in poly-d-lysine coated culture dishes for 48 h. Cells were then placed in serum-free medium. Hydroxyfullerene in dimethylsulfoxide was then added to the cells, and incubated for 1 h at 37°C. Hydrogen peroxide solution was then added to the plates for 24 h at 37°C. The final concentration of dimethylsulfoxide in media was 27.5 μg/mL. Cell viabilities were determined using MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenylformazan bromide] assays[21]. Briefly, PC12 cells were plated at 1×10^4 cells per 100 μL in 96-well plates, incubated, treated with hydroxyfullerene and hydrogen peroxide, and then 10 μL MTT solution (final concentration, 0.5 mg/mL) was added for 4 h. The dark blue formazan crystals that formed in intact cells were solubilized with lysis buffer (20% sodium dodecylsulfate in 50% aqueous *N,N*-dimethylformamide) and absorbance at 570/630 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA).

Transient Global Ischemia- 4 Vessel Occlusion

Male Wistar rats weighing between 160 and 180 g were used for the experiments. Rats were divided into five groups (8 per group). Before the experiment, food was withheld overnight, but water was freely available. Animals were anesthetized with isoflurane, initiated at 5% and maintained at 1.5% isoflurane in N_2O : O_2 (70 : 30). Anesthetized animals were surgically prepared for 4VO as described previously[22]. In brief, after an animal was positioned in stereotaxic ear bars (Kopf) with the head tilted downwards at 30° to the horizontal. Paraspinal muscles were separated from midline, and right and left alar foramina of the first cervical vertebra were exposed under an operating microscope. Both common carotid arteries were then isolated via a ventral, midline cervical incision. On the following day, 10 min of 4-VO ischemia was induced by tightening the clasp around the common carotid arteries. To minimize variability, the following criteria were strictly applied for the 10 min ischemic period and during 20 ± 5 min of postischemic coma, which was verified by loss of righting reflex and bilateral pupil dilation. Body temperatures were monitored[22] and maintained at 37 ± 0.5°C using a rectal thermometer coupled to a heating blanket (Harvard Apparatus). Sham-operated animals were used as non-ischemic controls.

Forty male Wistar rat were randomly divided into a control and three dosage groups, and received injections (i.p.) of saline or $C_{60}(OH)_{24}$ (0.3, 1, and 3.0 mg/kg/day), respectively, for 2 times (0 and 10 min after I/R).

Histological analysis was performed as described

previously[23]. Briefly, at 7 days after ischemia, animals were anesthetized with 3.5% chloral hydrate and perfusion-fixed with 4% paraformaldehyde. Fixed brains were cut into 30 μm sections on a sliding microtome. For Nissle staining and cell counting, sections were hydrated with 0.1% Nissle cresyl violet. Neuronal cell densities was measured by counting viable cells in six frames ($1.0 \times 1.0\ mm^2$) of left and right CA1 regions in three coronal sections (about 3.3, 3.5, and 3.7 mm caudal to bregma) in each animal. Cell densities in six regions were averaged to obtain a mean density value for each animal. Cell counting was done by three researchers unaware of the experimental conditions.

The first group was the control group in which only surgery was done without induction of ischemia. The second group was the saline group in which saline solution was given intraperitoneally (i.p.) 0 and 90 min after inducing ischemia. The third, forth and fifth groups were treated with fullerene (0.3, 1, or 3 mg/kg, i.p.) at 0 and 90 min after inducing ischemia.

Lipid Peroxidation

MDA levels in hippocampal tissues were determined using the method described by Zhang *et al.*[24]. Briefly, hippocampi were homogenized in cold 0.1 M phosphate buffer (pH 7.4) to produce a 10% homogenate, which was then centrifuged for 30 min at $3,000 \times g$ and 4°C. An aliquot (100 μL) of supernatant was then added to a reaction mixture containing 100 μL of 8.1% sodium dodecylsulphate, 750 μL of 20 % acetic acid (pH 3.5), 750 μL of 0.8% thiobarbituric acid, and 300 μL distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at $4,000 \times g$ for 10 min. Supernatant absorbances were measured spectrophotometrically at 532 nm and results are expressed as nanomoles of MDA per mg of total protein. Protein concentrations were determined using the Bradford method[25].

Statistical Analysis

All data are presented as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. SPSS version 14.0 (SPSS Inc.) was used throughout, and P values of < 0.05 were considered statistically significant.

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