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Fullerenol Nanoparticles Decrease Brain Infarction Through Potentiation of Superoxide Dismutase Activity During Cerebral Ischemia-Reperfusion Injury

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Abstract

Background: It has been demonstrated that weakening of the brain antioxidant system and oxidative stress is the main contributor in pathophysiology of ischemic stroke.

Objectives: Since fullerenol nanoparticles have powerful antioxidant effects in biological environments, we aimed to evaluate whether fullerenol administration during cerebral ischemia potentiates the antioxidant defense system of ischemic brain and decreases cerebral infarction.

Methods: Thirty six rats were randomly divided into three groups (n = 12 for each group): sham, control ischemia and ischemic treatment groups. The middle cerebral artery (MCA) was obstructed for 90 minutes in right hemispheres of control ischemia and ischemic treatment groups to achieve the experimental model of ischemic stroke. Treated rats received fullerenol nanoparticles (10 mg/kg, intraperitoneally) 30 minutes before MCA occlusion. Brain infarction, glutathione content and superoxide dismutase (SOD) activity were determined at the end of experiment.

Results: Occlusion of MCA induced considerable infarction and lesion in ischemic hemispheres of control ischemic rats (527 ± 59 mm^3) in accompany with a decrease in the glutathione content (45%), and SOD activity (29%) compared with sham rats. Administration of fullerenol in ischemic treatment group before MCA occlusion reduced the value of infarction (138 ± 67 mm^3) and also increased the value of the SOD activity by 33% compared to control ischemic group.

Conclusions: Our findings indicate that fullerenol nanoparticles decrease the brain infarction through enhancement of the SOD activity during cerebral ischemia-reperfusion injury.

Keywords: Stroke, Fullerenol, Antioxidant, Superoxide Dismutase, Infarction

1. Background

Overproduction of reactive oxygen species (ROS) has been accepted as an important participator in the pathogenesis of cerebral ischemia and ischemic stroke (1). There is growing evidence that these free radicals (ROS) are involved in all stages of the ischemic cascades (1, 2). The cells of cerebral tissue are more susceptible to the toxicity of oxygen free radicals because the cells of central nervous tissue have higher levels of poly-unsaturated fatty acids and weak antioxidant defense system against oxidative damage (3). After occurrence of brain ischemia, the ROS particularly oxygen radicals (superoxide anion) and non-radicals (hydrogen peroxide, H_2O_2) are produced in large quantities that results in neuro-degeneration and also damage to the enzymatic antioxidant system of cerebral tissue such as superoxide dismutase (SOD) and catalase (4). It has been demonstrated that brain ischemia induces an increase in lipid peroxidation and a decrease in the activities of the enzymatic (SOD and catalase) and non-enzymatic (glutathione) systems of the brain antioxidant system (5).

Fullerene nanoparticles, consisted of many carbon atoms (60) organized as a hollow sphere, are suggested as a therapeutic agent in ischemic stroke because they have many neuroprotective effects against neurodegenerative cascades of cerebral ischemia (6-8). Fullerenols are hydroxylated fullerene nanoparticles which may have neuroprotective effects because they scavenge free radicals (9-11). It has been shown that the poly-hydroxyl derivatives of fullerene nanoparticles, fullerenol nanoform ($C_{60}(OH)_n$), can behave as powerful antioxidant agents in biological environments (6). These nanoparticles exert their protective functions through acting as the free radicals sponge (12). The best-known biological characteristic feature of fullerenol nanoparticles is its antioxidative properties (13-15). Fullerenol shows a strong antioxidative activity against induced ROS by ionizing radiation (9-11). It has been shown that the nanoparticles of hydroxylated fullerenes protect

the mitochondria against free radicals by antioxidative activities and direct ROS scavenging properties (15). Other biological activities of fullerenol nanoparticles comprise the anti-proliferative (16), and neuroprotective effects (17). Moreover, it has been reported that fullerenol nanoparticles can offer protection against glutamate-induced intracellular calcium release and cell death by inhibition of glutamate channels (7).

2. Objectives

Based on previous studies, fullerene nanoparticles induce cellular protection against oxidative damage by their scavenging properties. Since brain ischemia weakens the brain antioxidant defense system of brain tissue, we aimed to evaluate whether fullerenol (hydroxyl fullerenes) treatment during brain ischemia potentiates the antioxidant defense system of brain and decreases the cerebral infarction.

3. Methods

3.1. Animals

Thirty six adult male Wistar rats, weighing 280-320 g, were purchased from the experimental animal center of Baqiyatallah University of Medical Sciences, Iran. All the experimental protocols were approved by the institutional animal use and care committee of Baqiyatallah University of Medical Sciences and followed the NIH guidelines for use and care of animals. The rats were kept in standard cages with free access to food and water, temperature of 23 $\pm$ 4°C, humidity of 40% - 60% and 12 hours light/dark cycle throughout the study.

3.2. Middle Cerebral Artery (MCA) Occlusion

The rats were anesthetized with 2.5% isoflorane (Forane, UK) and placed in dorsal recumbent. The temperature was recorded during the surgery using a rectal probe and kept at 37 $\pm$ 1°C with a lamp and heating pad.

The occlusion of middle cerebral artery (MCA) in right hemispheres of ischemic rats was performed by the method designated by Longa et al. (18). In brief, the right common carotid artery was exposed through a midline neck incision, and then, via external carotid artery, a 4-cm poly-l-lysine-coated nylon thread (3-0) was inserted into the internal carotid artery and gently advanced up until a resistance was felt and a sharp decline in the blood flow trace was observed. MCAO was maintained for 90 minutes, and then the thread was gently taken out to reestablish blood flow to the ischemic region. Finally, all the incisions were sutured and the animals were allowed for recovery from anesthesia, and then they were transferred to a warm cage for recuperation during reperfusion period.

3.3. The Protocols and Groups of Experiment

In sham group (n = 12), the animals underwent the surgery at neck areas without being exposed to the occlusion of MCA. The rats of sham group received a single intraperitoneal injection of 1 mL/kg normal saline as the vehicle. Surgery was performed at the neck region of control ischemic group (IR, n = 12) same as sham group. These animals received an intraperitoneal injection of 1 mL/kg normal saline as the vehicle 30 min before MCA occlusion. After 10 minutes rest, induction of cerebral ischemia was established by 90 minutes MCA occlusion followed by 24 hours reperfusion. The rats of ischemic treatment group (IR + Fullerenol, n = 12) received a single intraperitoneal injection of 10 mg/kg fullerenol (Sigma, Germany) in 1 mL normal saline 30 minutes before induction of MCA occlusion, and other procedures were followed same as the control group.

The rats that died during reperfusion period (24 hours after MCA occlusion) were excluded. The number of rats presented in three groups is the number of animals that survived during the reperfusion period.

3.4. Measurement of Cerebral Infarction

Brain infarct volume was measured according to the 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma) staining method (19). In brief, under deep anesthesia using sodium thiopental, the rats were sacrificed. Then, their brains were removed, cleaned, and solidified by immersing in pre-cooled normal saline (4°C) and afterwards, they were kept in the refrigerator for 5 minutes. Then, six slices were prepared from each brain using brain matrix. The slices were stained with TTC solution (2%) and fixed in 10% buffered formalin solution. After staining, color of the non-ischemic areas was red and of ischemic areas was white. The slice images were digitized by a Cannon camera. Images of the stained sections were taken. Grossly visible infarction zones were quantified using image analysis software (NIH image analyzer). Cerebral infarct volume for each hemisphere was calculated by the sum of infarct sizes for six slices and multiplying by 2 (thickness of each slice). Then, the corrected infarct volume for brain edema was calculated and infarct volume finally presented as mm^3.

3.5. Tissue Preparation

After deep anesthesia, the brains were removed and quickly washed in ice-cold PBS (phosphate buffer saline) for determination of glutathione (GSH) content and superoxide dismutase (SOD) activity. Then, the brain tissues

were quickly placed in liquid nitrogen and kept at - 80°C. On the day of biochemical analysis, the brain samples were rapidly weighed and homogenized in ice-cold PBS (1:10). Then, the homogenates were centrifuged for 15 minutes at 4°C (14000 × g). Finally, for assessment of protein and GSH contents as well as SOD activity, the supernatants were separated.

3.6. Measurement of Glutathione (GSH) Content

The content of brain GSH was determined by the method of Tietz (20). The precipitation of cellular protein was done by adding 5% sulfosalicylic acid and the separation by centrifugation for 10 minutes (2000 g). GSH content in the supernatant was determined as follows: 100 μL of the protein-free supernatant of the cell lysate, 800 μL of 0.3 mM Na_2HPO_4 and 100 μL of 0.04% 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1% sodium citrate. The absorbance of DTNB was monitored at 412 nm for 5 minutes. A standard curve of GSH was produced and sensitivity of measurement was determined to be between 1 and 100 μM. Eventually the GSH content of brain tissue was presented as μg/mg protein.

3.7. Determination of SOD Activity

Using Winterbourn et al. method, the SOD activity of brain hemispheres was measured according to the ability of SOD to prevent the decline of nitroblue tetrazolium (NBT) by superoxide (21). For analysis, 0.067 M potassium phosphate buffer, pH 7.8 was added to 0.1 M EDTA containing 0.3 mM sodium cyanide, 1.5 mM NBT and 0.1 mL of sample. Then, 0.12 mM riboflavin was added to each sample to initiate the reaction and was incubated for 12 minutes. The absorbance of samples was read on a Genesys 10 UV spectrophotometer at 560 nm for 5 minutes. The amount of enzyme required to produce 50% inhibition was taken as 1 U, and then the results were normalized relative to data of sham rats and expressed as percent (%).

3.8. Determination of Protein Content

The content of protein in homogenate samples were determined based on the Bradford method using bovine serum albumin (BSA) as a standard (22).

3.9. Statistical Analysis

The values are expressed as mean ± SEM. The comparison of data between groups was analyzed using ANOVA (analysis of variance). In the case of significant difference ($P < 0.05$), the values were analyzed by Tukey Post-hoc test. All states, $P < 0.05$ was considered to be statistically significant.

4. Results

4.1. Cerebral Infarction

Figure 1 shows the qualitative assessment of cerebral infarction. Observation of red color stained with TTC in the slices of left and right hemispheres of sham rats indicates that the surgery in the neck areas did not induce infarction. The presence of different magnitudes of white color zones in the lesioned (right) hemispheres of ischemic control and ischemic treatment groups indicated that 90- minutes obstruction of right MCA has provoked infarctions in different sizes in the right hemispheres of ischemic rats. The qualitative assessments of the ischemic regions (white color zones) in right hemispheres of ischemic animals indicate that fullerenol nanoparticles have attenuated the infarct size in treatment group.

The graph of Figure 2 shows the quantification of infarction in the ischemic (right) hemispheres. There was no infarction in the right hemispheres of sham rats. MCA occlusion considerably induced brain infarction in right hemispheres of control ischemic rats (527 ± 59 mm^3). Treatment with fullerenol significantly attenuated the infarction of ischemic treatment group (138 ± 67 mm^3) compared to the control ischemic rats ($P < 0.01$).

4.2. Glutathione (GSH) Content

Figure 3 shows the GSH contents of right (ischemic) hemispheres after 90- minutes MCA occlusion followed by 24 hours reperfusion. Occlusion of the MCA decreased the mean value of GSH content in control ischemic group (31 ± 2 μg/mg protein) compared with sham (57 ± 6 μg/mg protein), ($P < 0.05$). However, treatment with fullerenol did not significantly increase the value of GSH content in ischemic treated rats (42 ± 15 μg/mg protein) compared to the control ischemic group.

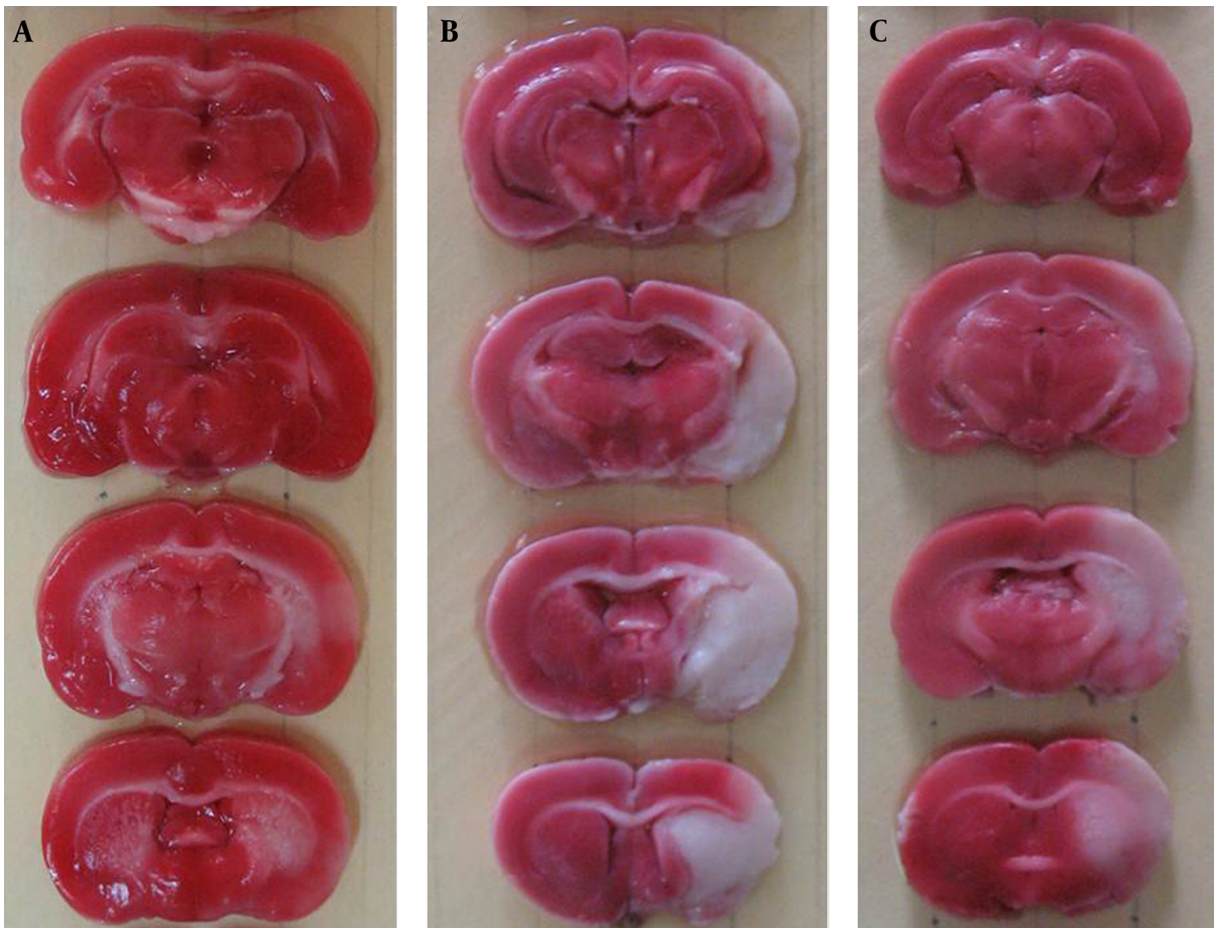
4.3. Superoxide Dismutase (SOD) Activity

The graph in Figure 4 shows the SOD activity of right (ischemic) hemispheres after 90- minutes MCA occlusion followed by 24 hours reperfusion. Occlusion of the MCA decreased the activity of SOD in control ischemic group by 29% compared with sham ($P < 0.05$). Treatment with fullerenol significantly increased the activity of SOD in ischemic treated rats by 33% compared to the control ischemic group ($P < 0.05$).

5. Discussion

Previous studies have shown that water-soluble fullerene derivatives like fullerenol nanoparticles are able to abolish various free radicals in biological environments

Figure 1. Effect of Fullerenol Nanoparticles on Cerebral Infarction During Cerebral Ischemia

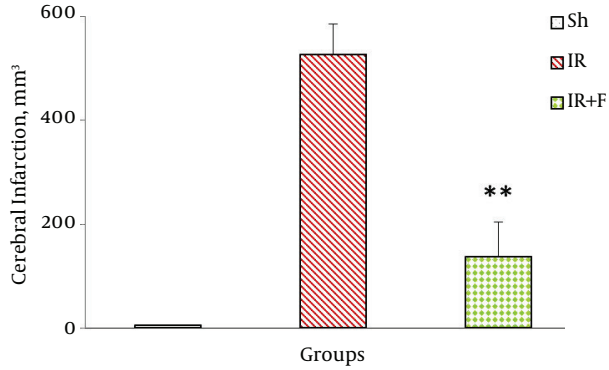


The coronal sections of rat's brain stained with triphenyltetrazolium chloride at termination of experiment in sham (A), control ischemic (B) and ischemic treatment (C) groups. Ischemic areas are white and non-ischemic areas are red.

and protect the cells against oxidative and nitrosative damages (6, 23, 24). In current study, we showed the neuroprotective roles of fullerenol nanoparticles against cerebral ischemia-induced damages in rat model of ischemic stroke. Our results indicated that administration of these nanoparticles before induction of brain ischemia attenuated the infarction and increased the activity of SOD in ischemic brain. It is concluded that fullerenol nanoparticles have various neuroprotective effects in addition to their scavenging properties of free radicals and therefore they protect the brain cells against ischemia-induced brain injuries.

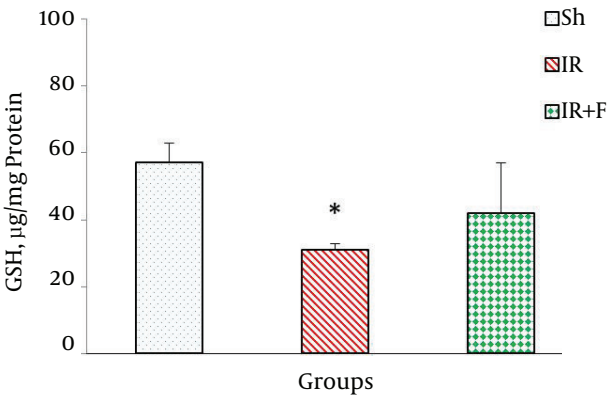
The antioxidant properties of water-soluble fullerene derivatives like fullerenol nanoparticles have been confirmed by previous findings (6, 23). Based on previous studies, fullerene derivatives quench various free radicals in biological environments and act as a free radical sponge (25, 26). Since oxygen free radicals (ROS) are involved in the pathophysiology of ischemic stroke (2), fullerenol nanoparticles may be helpful to attenuate the injuries of brain ischemia. According to our results, fullerenol nanoparticles noticeably decreased the infarction of ischemic hemispheres during occlusion of MCA (Figure 1 and 2). Hence, it is concluded that fullerenol nanoparticles are able to improve the ischemia-induced neuronal damage and brain injury possibly through reduction of oxidative damages. Additionally, the antioxidant defense system of brain is inactivated when brain ischemia is happened (2). Based on our findings, the activity of SOD and glutathione content of ischemic brain were significantly decreased after induction of MCA occlusion. It has been shown that the activity of SOD, as an important enzy-

Figure 2. Effect of Fullerenol Nanoparticles on Total Infarction (mm^3) During Cerebral Ischemia



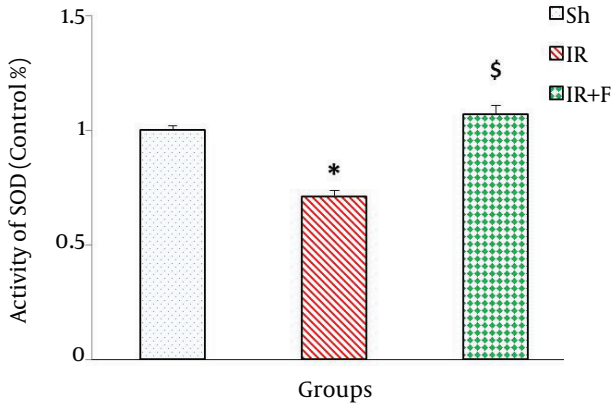
The graph shows the quantification of cerebral infarction in right hemispheres at termination of experiment in sham (Sh), control ischemic (IR) and ischemic treatment (IR + F) groups. All values are presented as mean ± SEM; ** As significant difference compared with IR ($P < 0.01$).

Figure 3. Effect of Fullerenol Nanoparticles on Glutathione (GSH) Content of Ischemic Brain During Cerebral Ischemia



The graph shows the glutathione (GSH) contents of right hemispheres (μg/mg protein) at termination of experiment in sham (Sh), control ischemic (IR) and ischemic treatment (IR + F) groups. All values are presented as mean ± SEM; * As significant difference compared with sham ($P < 0.05$).

Figure 4. Effect of Fullerenol Nanoparticles on Superoxide Dismutase (SOD) Activity of Ischemic Brain During Cerebral Ischemia



The graph shows the SOD activity (control %) of right hemispheres at termination of experiment in sham (Sh), control ischemic (IR) and ischemic treatment (IR + F) groups. All values are presented as mean ± SEM; * As significant difference compared with sham ($P < 0.05$); $ As significant difference compared with IR ($P < 0.05$)

matic antioxidant, and glutathione content, as an important non-enzymatic antioxidant, have been reduced after cerebral ischemia (5). SOD is an important antioxidant enzyme that quenches the superoxide anions in mitochondrial matrix (27). Also, glutathione is an intracellular element that has a crucial function in protection of the cells against free radicals and oxidative damage (1). Additionally, it has been reported that glutathione attenuates the infarction and decreases the brain cell death after cerebral ischemia (28). In the present study, fullerenol administration before induction of MCA occlusion could potentiate the antioxidant defense system of brain (an increase in the SOD activity and glutathione content). Therefore, it is suggested that reduction of brain infarction may be related to improving the antioxidant defense system of ischemic brain. However, more studies are needed to elucidate the direct neuroprotective effects of fullerenol nanoparticles through changes of other enzymes of brain antioxidant system such as catalase and glutathione peroxidase during brain ischemia-reperfusion injury.

It is concluded that fullerenol nanoparticles, as a potent scavenger of free radicals, protect the brain cells against ischemia-induced brain injury through potentiation of antioxidant defense system. Our results suggest the effective therapeutics of these nanoparticles for reduction of brain damage after ischemic stroke.

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Footnote

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