Evaluation of the Effects of Fullerene C₆₀ Nanoparticles on Oxidative Stress Parameters in Normal Rats Liver and Brain

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ABSTRACT
Background & Objective: The potent antioxidant property of fullerene C₆₀ nanoparticles and their derivatives has been demonstrated in a wide range of in vitro and in vivo studies. Hence, we examined the effects of fullerene C₆₀ on the oxidative stress parameters in brain and liver of the rats in normal situation.

Materials & Methods: The study was performed in two groups of Wistar rats (each group, n = 6): normal and fullerene-treated normal animals. Treated rats received fullerene via oral gavage at dose of 1 mg/kg/day for 60 days. At termination of the study, the oxidative stress parameters were determined in brain and liver tissues, including the contents of glutathione (GSH) and malondialdehyde (MDA), and the activities of catalase (CAT) and superoxide dismutase (SOD). The t-test was used to analyze the data between two groups.

Results: Fullerene C₆₀ treatment did not change blood glucose level in treated rats compared to untreated rats. Fullerene C₆₀ significantly increased the value of CAT activity by 66% and MDA levels by 68%, while decreased SOD activity by 33% at liver of treated rats compared to untreated animals (P<0.05). Fullerene administration increased significantly only CAT activity of brain in the treated rats (0.34±0.10 U/mg protein) compared to untreated animals (0.12±0.04 U/mg protein), (P<0.05).

Conclusion: Our findings indicated that oral administration of fullerene C₆₀ nanoparticles differently changed the oxidative stress parameters in liver and brain in normal condition. It is suggested that these effects be considered for the application of these nanoparticles in various therapeutic purposes.

Keywords: Antioxidant, Fullerene, Oxidative stress, Nanomaterials

Introduction
An important role of reactive oxygen species (ROS) accumulation has been demonstrated in the pathogenesis of numerous chronic diseases (1,2). In physiological situations, these free radicals are frequently produced in the tissues. They are removed by the antioxidant defense systems to keep a redox balance (3,4). It is believed that to keep the balance between oxidation and antioxidation, the endogenous antioxidant defense system is not sufficient, and therefore, the exogenous antioxidants are continuous demand to prevent stress and age- and disease-related disorders (5). Nonetheless, changes in the antioxidant capacity of the tissues are the main reason of ROS accumulation and oxidative damage and thus antioxidant therapy may diminish the lesions induced by oxidative stress (4,6,7). Hence, application of many natural or synthetic materials with anti-ROS properties determines the attractiveness of antioxidants for pharmacotherapy. For example, using several antioxidants prevented progression of chronic neurodegenerative disorders for instance Parkinson’s disease (PD) or Alzheimer’s disease (AD) as well as delayed aging (5,8).

Fullerene C₆₀ or buckminsterfullerene is a molecular compound, made exclusively from carbon atoms with a cage-like structure in hollow sphere-shapes resembles a “soccer cage” with a diameter of 0.72 nm (8). Fullerene C₆₀ is characterized as a remarkably stable compound with capability to incorporate into cell membrane and powerful reactions with various biochemical compounds (9,10). Distinct redox chemistry of fullerene C₆₀ due to highly delocalized π double bond arrangement has caused this nanoparticle to behave as a powerful antioxidant several hundred-
folds higher than common antioxidants (11). Previous findings have demonstrated several beneficial properties of this nanoparticle and its derivatives in the biological environments (12). The antioxidant properties of fullerene C60 and its derivatives have been shown in a wide range of studies in vitro and in vivo (5,8,13,14). It has been demonstrated that these nanoparticles are localized in the mitochondria, the main site of ROS production, through passing the cell membrane (8). Tokuyama et al., reported that fullerene C60 derivatives protected the cells against photo-induced cytotoxicity as well as photo-induced DNA damage (15). The findings of Basso et al., revealed that application of fullerene compounds improved the axonal loss and disability and induced neuroprotective effects in an experimental multiple sclerosis model, which may be useful in the treatment of neurodegenerative diseases (16). Promising results have shown that fullerene C60 nanoparticles and their derivatives among the new nanomaterials are beneficial against skin damage, and therefore, numerous manufacturers are enthusiastic to apply them in the topical preparations and cosmetics (14). Also, the neuroprotective functions of fullerene C60 have been reported in ischemic stroke. Administration of fullerene during ischemic stroke decreased the injuries of cerebral ischemia by inhibition of oxidative damage and inflammation (17-19). Furthermore, repeated oral administration of fullerene C60 prolonged the lifespan in CCl4-mediated toxicity in rats (12).

Numerous studies have demonstrated that fullerene C60 nanomaterials behave as a strong scavenger of ROS and an excellent antioxidant in preclinical in vitro and in vivo studies. These nanoparticles have also been suggested for the extensive applications in various physiological and pathological conditions. Therefore, we examined the effects of oral administration of fullerene C60 nanoparticles on antioxidant status and the oxidative stress parameters of brain and liver tissues in normal situations in rats.

**Materials and Methods**

**Animals**

All experimental protocols used in the current study were approved by the Institutional Animal Ethics Committee of the University of Baqiyatallah Medical Sciences (Tehran, Iran). The ethical code for the present study was IR.BMSU.REC.1397.029. Adult male Wistar rats, weighting about 220±20 gram (aging 8-10 weeks old), were employed. The rats were acclimatized in the institutional animal house and were given standard food and water ad libitum. The animals were housed under controlled conditions of light exposure (12 h light/dark cycles), temperature (22-24°C), and humidity (40-60%).

**Fullerene Nanoparticles**

Fullerene C60 was obtained from Sharif University of Technology (Tehran, Iran). The degree of purity of this compound was more than 85%. Fullerene was dissolved in sesame oil and administered via oral gavage at dose of 1 mg/kg/day according to the previous studies (12,20).

**Experimental Design and Grouping**

Twelve rats were randomly divided into two untreated and treated normal rats (each group, n=6). Treated rats received fullerene C60 nanoparticles via oral gavage at dose of 1 mg/kg/day for 60 days. Untreated rats received orally sesame oil per day without fullerene C60 nanoparticles in the same volume of the treated rats. During the test, blood glucose of rats and their body weight were determined at the defined times.

**Tissue Preparation**

At termination of the study, the tissues (brains and livers) were quickly removed under deep anesthesia for examination of the oxidative stress parameters. The tissues were homogenized in ice-cold phosphate buffered saline (PBS) and centrifuged at 14000 g for 15 min at 4°C. Afterwards, the supernatants were separated to analyze the oxidative stress parameters as well as protein concentrations.

**Determination of Protein Concentrations**

The Bradford method was used to quantify the protein levels in brains and livers. Bovine serum albumin (Sigma, Germany) was used as the standard (21).

**Determination of Catalase (CAT) Activity in Brain and Liver**

The method of Aebi was used to determine the activity of catalase in the brain and liver tissues homogenates (22). In brief, the reaction mixture was prepared and allowed at room temperature for 10 min. The reaction mixture contained 0.85 ml potassium phosphate buffer (50 mM, pH 7.0) and 0.1 mL homogenate solution. At the next stage, 0.05 ml H2O2 (30 mM prepared in 50 mM potassium phosphate buffer, pH 7.0) was added. Then, using a spec-trophotometer (UV 7500, Spectro Lab, England) the reduction absorbance was recorded at 240 nm for 3 min. The specific activity of catalase in the brain and liver tissues was calculated as U/mg protein. One unit of catalase was defined as 1 nMol H2O2 decomposed per min.

**Determination of Malondialdehyde (MDA) Content in Brain and Liver**

The malondialdehyde (MDA) level in brain and liver tissues was measured as an excellent index of oxidative stress in the tissues, according to the following protocol. In brief, protein precipitation was conducted by adding 1.5 mL trichloroacetic acid (TCA, 10%) to 0.5 mL of tissue homogenate. After vortexing the solution, the sample was incubated at room...
temperature for 10 min. The supernatant (1.5 mL) was incubated in boiling water bath for 30 min after adding thiobarbituric acid (2 mL and 0.67%). The sample was cooled at room temperature and again vortexed after adding n-butanol (1.25 mL). The supernatant was separated following centrifugation at 2000 g for 5 min. Thereafter, the absorbance of the solution was recorded at 532 nm wavelength using spectrophotometer. Ultimately, standard curve was obtained using 1, 1, 3, 3-Tetraethoxypropane to compute the MDA levels (23). The MDA levels in the brain and liver tissues were calculated as nMol/mg protein.

**Determination of Superoxide Dismutase (SOD) Activity in Brain and Liver**

The SOD activity was determined using the method of Winterbourn et al. (1975), based on the nitroblue tetrazolium (NBT) reduction by SOD (24). To determine the enzyme activity, potassium phosphate buffer (0.067 M and pH 7.8), EDTA (0.1 M) and 0.1 mL of sample, NBT (1.5 mM) and sodium cyanide (0.3 mM) were mixed. Then, riboflavin (0.12 mM) was added to the mixture to start the reaction. The solution was incubated for 12 min at room temperature. Thereafter, using a spectrophotometer the absorbance was determined at 610 nm wavelength after 5 min. The extent of enzyme that developed 50% inhibition was considered as 1 U, and finally, SOD activity of the samples was computed as U/mg protein (23).

**Determination of Glutathione (GSH) Content in Brain and Liver**

The glutathione levels in the brain and liver tissues were assessed according to the method of Tietz (1969) (25). Briefly, protein precipitation of the samples was performed using sulfosalicylic acid (5%). Then, the solution was centrifuged at 2000 g for 10 min to separate the supernatant. Afterwards, the GSH content of the samples was measured by adding the protein-free supernatant (100 µL) to 800 mL of 0.3 mM Na₂HPO₄ and 100 mL of 0.04% 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1% sodium citrate. Thereafter, the absorbance of solution was recorded at 412 nm wavelength after 5 min. The glutathione contents of the brain and liver tissues were calculated as nMol/mg protein.

**Statistical Analysis**

To perform the statistical analysis, the SPSS 21 (SPSS Inc., Chicago, IL., USA) was used. The t-test was used to analyze the data between two groups (treated and untreated normal rats). All data were expressed as mean ± SEM. A P-value<0.05 was considered statistically significant.

**Results**

**Effect of Fullerene on Blood Glucose and Body Weight**

The changes in blood glucose (A) and body weight (B) during the study in normal and treated groups are shown in Figure 1. Blood glucose (Figure 2A) in the normal animals was 111±11 mg/dL at beginning of the test. This value did not significantly change during the test on days 30 (118±11 mg/dL) and 60 (125±11 mg/dL). Additionally, blood glucose of the fullerene-treated normal rats was 118±6 mg/dL at beginning of the test. This value did not change in treated group during the test on days 30 (120±6 mg/dL) and 60 (122±6 mg/dL). Finally, there were no significant differences in the blood glucose levels of the two groups at different times during the test. As shown in Figure 2B, the mean value of the body weight in normal and treated groups was 219±7 g and 203±1 g, respectively, at the beginning of the test. The rats of both groups showed progressive weight gain during the study. The body weights of untreated normal animals on days 30 and 60 were 257±7 g and 289±8 g, respectively. Also, the body weights of fullerene-treated animals on days 30 and 60 were 268±7 g and 297±8 g, respectively. There were no significant differences in the body weight values of two groups at different times during the test.

**Figure 1.** The representative changes of blood glucose (A) and body weight (B) during the study in the normal and fullerene-treated rats. There were no significant differences in the blood glucose levels of two groups during the test at different times. All values are presented as mean ± SEM (n=6).
Effect of Fullerene on the Oxidative Stress Parameters of Liver

Catalase activity (CAT): As shown in Figure 2A, the mean value of CAT activity in liver of the untreated normal rats was 2.07±1.43 U/mg protein at the end of the experiment. Treatment with fullerene nanoparticles increased significantly the value of CAT activity in liver of treated normal rats (6.14±0.76 units/mg protein) compared to the normal animals (P<0.05).

Superoxide Dismutase (SOD) Activity: As shown in Figure 2B, SOD activity of liver in untreated normal rats was 6.15±1.19 U/mg protein. Fullerene administration decreased significantly SOD activity in the liver tissues of treated normal rats (4.08±0.36 U/mg protein) compared to the normal animals (P<0.05).

Glutathione (GSH) Content: Figure 2C expresses the GSH content of liver at the end of the test. GSH content of liver in untreated normal group was 938±185 nMol/mg protein. Fullerene did not cause any significant change in GSH content in the liver tissues of treated normal rats (1039±139 nMol/mg protein) compared to the normal animals.

Malondialdehyde (MDA) Content: As shown in Figure 2D, MDA content of liver in untreated normal rats was 0.42±1.23 nMol/mg protein. Fullerene administration increased significantly MDA content in the liver tissues of treated normal rats (13.35±7.37 nMol/mg protein) compared to the normal animals.

Effect of Fullerene on the Oxidative Stress Parameters of Brain

Catalase activity (CAT): Figure 3A shows catalase activity of brain at termination of the experiment. The mean value of catalase activity in the brain tissues of the untreated normal rats was 0.12±0.03 U/mg protein. Fullerene treatment increased the catalase activity significantly in the brain tissues of the treated normal rats (0.34±0.10 U/mg protein) compared to the untreated normal animals (P<0.05).

Superoxide Dismutase (SOD) Activity: As shown in Figure 3B, SOD activity of brain in untreated normal rats was 0.35±0.08 U/mg protein. Fullerene treatment did not change SOD activity significantly in the brain tissues of the treated normal rats (0.34±0.10 U/mg protein) compared to the untreated normal animals (P<0.05).

Glutathione (GSH) Content: Figure 3C presents the GSH content of brain at termination of the study. The GSH content of brain in untreated normal rats was 94±26 nMol/mg protein. Fullerene did not change GSH content in the brain tissues of treated normal rats.
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(77±19 nMol/mg protein) compared to the normal animals.

**Malondialdehyde (MDA) Content:** As shown in Figure 3D, MDA content of brain in untreated normal rats was 1.98±1.31 nMol/mg protein. Fullerene administration did not change MDA content in the brain tissues of treated normal rats (2.15±0.92 nMol/mg protein) compared to the normal animals.

**Discussion**

Fullerene C₆₀ nanoparticles have shown powerful antioxidant activities in the biological environments (5,8,18). In this regard, fullerene nanoparticles are applied to prevent ROS accumulation and development of tissue oxidative stress in various pathophysiological states. These free radicals play a critical role in the pathogenesis of the diseases (1,2). Therefore, the current study examined the effects of fullerene C₆₀ nanoparticles on the oxidative stress parameters in brain and liver tissues in normal animals. The results of the present study indicated that fullerene C₆₀ administration in treated normal rats only changed catalase activity significantly in brain among the oxidative stress parameters. Fullerene also increased the catalase activity, but decreased SOD activity in liver in the treated normal rats. Therefore, the MDA levels of liver in fullerene-treated rats increased. Hence, the results of present study revealed that fullerene C₆₀ nanoparticles could change the antioxidant capacity of brain and liver as well as the capability of resistance against oxidative stress in these tissues.

According to the previous studies, oxidative stress induced by various endogenous and exogenous compounds might play a crucial role in the incidence of hepatotoxicity (26-28). Hence, potentiation of the liver antioxidant capacity might be helpful for prevention of liver damage and hepatotoxicity induced by ROS accumulation. The results of present study indicated that administration of fullerene C₆₀ nanoparticles increased the catalase activity in liver of treated animals noticeably, while decreased the SOD activity which is a key enzyme in antioxidant defense system. In agreement with the findings of present study, fullerene C₆₀ could act as the catalase mimetic in an in vitro experiment (8,29). Catalase enzyme in accompany with SOD protects the hepatic cells against damage caused by free radicals such as hydroperoxides.

![Figure 3. The effects of fullerene (C₆₀) nanoparticles on the oxidative stress parameters (CAT [A]; catalase, SOD [B]; superoxide dismutase, GSH [C]; glutathione and MDA [D]; malondialdehyde) of brain tissue in the fullerene-treated normal rats at termination of the study. All values are presented as mean ± SD (n = 4). * (P<0.05): significant difference compared to the normal group.](image-url)
and lipoperoxides (30). Hydrogen peroxide (H$_2$O$_2$), as a toxic and main oxygen free radical, is generated endogenously or produced by the action of SOD on superoxide anions (31). Catalase inhibits cellular oxidative damage through neutralizing these hydroperoxides by metabolizing them to water and oxygen (31). In the lack of catalase activity, the hydrogen peroxide can be converted to hydroxyl radicals through Fenton reactions. Since H$_2$O$_2$ works physiologically as a signal transduction molecule, fullerene C$_{60}$ can control H$_2$O$_2$-dependent signaling pathways by changing the catalase activity of the cells (32). On the other hand, in the lack of SOD activity, superoxide anion cannot be eliminated in liver tissue (1). Therefore, this very toxic radical is accumulated in liver and then, oxidative stress is occurred. For this claim, the content of MDA, as an excellent marker of oxidative stress, increased considerably in liver of treated animals. Therefore, administration of these nanomaterials might exhibit oxidative damage due to ROS accumulation in liver of the treated normal animals through reduction of SOD activity.

According to the previous findings, fullerene C$_{60}$ nanoparticles behave as a free radical sponge which is able to eliminate the various oxygen and nitrogen free radicals in the biological environments (5,8,17). Since ROS is involved in the pathogenesis of tissue degeneration during various disorders as well as aging process (1,2), fullerene might be helpful for reduction of the tissue damages. Based on our results, administration of fullerene C$_{60}$ nanoparticles increased markedly only catalase activity among the oxidative stress parameters in brain of the treated normal rats. This finding has been confirmed by another in vitro study that showed fullerene C$_{60}$ could act as catalase mimetic activity (13). Hydrogen peroxide which is developed by the SOD enzyme is detoxified by the action of catalase (31). Hence, it is concluded that fullerene enhances the antioxidant capacity of the normal brain against ROS accumulation through enhancement of catalase activity. Because of the weaker antioxidant capacity of brain in comparison with other tissues (33), potentiation of the brain antioxidant capacity might be helpful for prevention of brain damage against ROS in several neurodegenerative diseases as well as aging phenomena. Therefore, application of these nanomaterials might be useful for prevention of neurodegeneration in different pathological states and delaying the aging process in brain through enhancement of the brain antioxidant capacity. Fullerenes in the present study did not cause any changes in blood glucose or body weight of animals, suggesting that these nanoparticles do not alter the physiologic parameters of normal animals. These findings have been confirmed by the other studies (34).

**Conclusion**

In conclusion, the oral administration of fullerene C$_{60}$ nanoparticles differently changed the oxidative stress parameters in liver and brain under normal condition. Fullerene C$_{60}$ might exhibit the neuroprotective effects against ROS-induced brain toxicity due to potentiation of the antioxidant defense systems, whereas in liver it induces oxidative stress through reduction of SOD activity, as a key enzyme in antioxidant system. Therefore, according to the findings of current study, it is suggested to appraise the controversial effects of fullerene C$_{60}$ in different tissues for their application in various therapeutic purposes.

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**Conflict of Interest**

Authors declare no conflict of interest.

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