Protective effects of fullerenol on carbon tetrachloride-induced acute hepatotoxicity and nephrotoxicity in rats

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A B S T R A C T

An important biologically-relevant property of fullerenol is its ability to quench free radicals. Carbon tetrachloride (CCl4)-induced hepatotoxicity and nephrotoxicity model was used to investigate the possible mechanisms of fullerenol protection in Sprague–Dawley rats in this study. Rats were administrated with fullerenol (0, 1, 1.5 and 5 mg/kg d) by intravenous or intraperitoneal injection for 3 days before CCl4 challenge, 24 h following the CCl4 challenge, all the rats were assessed using serum and tissue homogenates biomarkers as well as the pathological evaluation. All results showed that CCl4 caused significant increasing serum activity of alanine aminotransferase and aspartate aminotransferase, as well as the concentration of blood urea nitrogen and creatinine. Malondialdehyde level was also increased significantly, whereas the ratio of reduced glutathione to oxidized glutathione was decreased in tissue homogenates. The pathological evaluation indicated the liver and kidney were damaged by CCl4. Fullerenol-pretreatment alleviated biomarker changes as well as histological changes significantly, and fullerenol-pretreatment alone can increase the ratio of reduced glutathione to oxidized glutathione, which indicated fullerenol could protect tissues against CCl4-induced oxidative stress by improving the antioxidant ability.

1. Introduction

Carbon tetrachloride (CCl4) is a well established hepatotoxin. Previous studies showed that both liver and kidneys are the target organs of CCl4. Extensive evidence demonstrates that CCl4 is activated in the liver to highly reactive trichloromethyl radical which initiates free radical-mediated lipid peroxidation of the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane, which leading to accumulation of lipid-derived oxidants causing liver injury [1,2]. Meanwhile, some studies show that the nephrotoxic effects of CCl4 are also associated with free radical production [3]. CCl4-induced damage is also able to alter the antioxidant status of the tissues, which is manifested by abnormal histopathological changes [4]. Histopathologically, exposure to CCl4 can results in hepatic steatosis, centrilobular necrosis, and cirrhosis in the liver and acute tubular necrosis in the kidney [5,6].

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Fullerenol \((\text{C}_{60}(\text{OH})_x; x = 3–24)\), a kind of representative water-soluble derivatives of \(\text{C}_{60}\), is an effective radical-scavenger and antioxidant both in vivo and in vitro [7–9]. Previously, researchers have found the protective effect of fullerenol on DOX-induced toxicity might be mediated by its hydroxyl-radical-scavenger activity through investigating on the effects of fullerenol on its modulating activity on doxorubicin (DOX)-induced toxicity in vivo and in vitro [10–13]. Similarly, we have studied the protective effects of fullerenol on Stylophora mytilus cells exposed to \(60\text{Co}\gamma\)-rays. Fullerenol was also found to be good radiation protectors for the protozoan \(S.\) mytilus exposed to \(\gamma\)-rays, and the anti-oxidative and radical scavenging activities of fullerenol were responsible for this radioprotection [14]. In 2005, Gharbi et al. [15] reported that the \(\text{C}_{60}\)-pretreatment can protect the livers against the acute \(\text{CCl}_4\)-induced free-radical damage in rats.

Although both of fullerenol and \(\text{C}_{60}\) have anti-oxidative capabilities [7–15], the stability of fullerene hinders its application in medical therapy. The photosensitization of \(\text{C}_{60}\) leads to its transition to a long-lived triplet excited state. The subsequent energy or electron transfers to molecular oxygen, yielding highly reactive singlet oxygen or superoxide anion, respectively, which may lead to serious adverse effect [16–18]. It is well-known that compound and its metabolites need to be removed from the body via excretion in drug discovery research. Accumulation of foreign substances can adversely affect normal metabolism unless excretion is complete. Fullerenol showed different ADME (absorption, distribution, metabolism and excretion) properties compared to fullerene due to a better water-solubility. Especially, fullerenol can be excreted (eliminated) in urine, which makes it a better candidate for application as a drug or a drug-carrier compared with \(\text{C}_{60}\). On the other hand, it is well documented that antioxidants can prevent \(\text{CCl}_4\) toxicity to liver and kidney by inhibiting lipid peroxidation and increasing antioxidant enzyme activities [19,20]. Fortunately, none kidney protection was found in Gharbi’s work [15] probably due to poor solubility of \(\text{C}_{60}\). Thus, our hypothesis is that and fullerenol may have both liver and kidney protective capability. Herein, we conducted this study to evaluate the effects and probable mechanisms of fullerenol in protecting acute liver and kidney injuries induced by the \(\text{CCl}_4\). In order to know whether it can be used as a route administration mode, we administrated-fullerenols both by intravenous and by intraperitoneal injection.

2. Experimental details

2.1. Chemicals

\(\text{CCl}_4\) was purchased from Sigma. Fullerenol \((\text{C}_{60}(\text{OH})_x; x = 22,24)\) used in this study was synthesized as previously described [21–23]. All other chemicals used for the study were of analytical grade.

2.2. Animals and treatment and sample collection

A total of 84 male Sprague–Dawley (SD) rats weighing 200 ± 10 g were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., China. The rats were randomly divided into 14 groups (six rats per group). Absence of infection was confirmed 1 week before experiment. The rats were housed in plastic cages, fed with commercial diet, and given water ad libitum. The cages were placed in a conventional room, which was air conditioned at 23 °C and 55–70% humidity with a 12 h light/12 h dark cycle. All animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare (See Fig. 1).

Animals were grouped as follows:

<table>
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<tr>
<th>CCl4 Treatment Group</th>
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<tr>
<td>Con</td>
<td>Control group (without treatment)</td>
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<tr>
<td>CoIV 1 mg</td>
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<td>CoIV 1.5 mg</td>
<td>Control of IV with fullerenol (1.5 mg/kg)</td>
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<tr>
<td>CoIV 5 mg</td>
<td>Control of IV with fullerenol (5 mg/kg)</td>
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<tr>
<td>CoIP 1 mg</td>
<td>Control of IP with fullerenol (1 mg/kg)</td>
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<td>CoIP 1.5 mg</td>
<td>Control of IP with fullerenol (1.5 mg/kg)</td>
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<td>FOLIV 1.5 mg</td>
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<td>Fullerenol (5 mg/kg) + CCl4</td>
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Blood samples were collected in routine biochemical test tubes and centrifuged at 1000g for 10 min. The sera were collected for further determination of aspartate transaminase (AST) and alanine aminotransferase (ALT) activities, creatinine (Crea) and blood urea nitrogen (BUN) contents. Liver and kidney sections from rats were homogenized rapidly in ice-cold 0.9% NaCl and centrifuged at 1500g for 30 min at 4 °C, the supernatants were immediately for biological analysis. The protein concentration was determined using Bradford Protein Assay Kit (Beyotime, China) using crystalline bovin serum albumin (BSA) as standard.

Fig. 1 – The experimental scheme. Fullerenol was given by intravenous injection (IV) and intraperitoneal injection (IP) in four different doses (0, 1, 1.5 and 5 mg/kg) for continuous 3 days. All the animals except for control groups were injected (IP) with a single dose of CCl4 (0.5 mL/kg) 3 h after the last administration of fullerenol. The control groups were injected (IP) with 0.9% NaCl (0.5 mL/kg) instead. Twenty-four hours after CCl4 challenge, rats were anesthetized by an overdose of pentobarbital (70 mg/kg BW, IP) and killed by exsanguination via the abdominal aorta.
2.3. **Hepatotoxicity and nephrotoxicity studies**

Serum levels of AST and ALT activities, Crea and BUN concentrations were determined using a HITACHI 7020 Automatic Analyzer (Hitachi, Tokyo, Japan) according to the diagnostic kit obtained from the Sysmex (SYSMEX Shanghai Ltd., China). AST and ALT activities were expressed as IU/L and Crea and BUN were expressed as µmol/L and mmol/L, respectively.

2.4. **Estimation of lipid peroxidation products**

Liver and kidney lipid peroxidation was measured by the formation of the thiobarbituric acid-reactive material, malondialdehyde (MDA) [24]. The determination of MDA values were measured by using the spectrophotometric diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China). The absorbance value was monitored at 540 nm on a microplate reader (Model 680 Microplate Reader, Bio-Rad, USA). The results were expressed as nmol mg⁻¹ protein.

2.5. **Effect on the GSH/GSSG ratio in CCl₄-treated rats**

The ratios of reduced and oxidized glutathione (GSH and GSSG) were measured by a commercially available kit (GSH&GSSG Assay Kit, Beyotime, China) according to the manufacturer’s instructions. The absorbance value was monitored at 412 nm on a microplate reader (Model 680 Microplate Reader, Bio-Rad, USA).

2.6. **Histopathological analysis**

Histopathological analysis was performed using the standard laboratory procedure. Liver and kidney sections were fixed with 4% polyformaldehyde-PBS (0.1 M, pH 7.4) and embedded in paraffin. After routine processing, slices of 4 µm thickness were sectioned and stained with hematoxylin-eosin (HE), and examined by a pathologist who was blind to the grouping of rats.

2.7. **Statistics**

Data were expressed as mean ± S.D. The statistical analysis was carried out by one-way analysis of variance (ANOVA) and made the comparison by Dunnett’s t-test. P < 0.05 was considered statistically significant.

3. **Results**

3.1. **Effect of fullerenol on CCl₄-induced hepatotoxicity and nephrotoxicity**

CCl₄ was given to seven groups of rats, with or without fullerenol-pretreatment, the effects of the fullerenol on the...
CCl₄-induced elevation in the serum ALT and AST activities are shown in Fig. 2A and B. A single dose of CCl₄ caused a significant rise in serum levels of ALT and AST in the rats (P < 0.05), demonstrating a marked liver damage. In addition to hepatotoxicity, the nephrotoxicity has also been induced during the CCl₄ administration, which is indicated by the serum-concentration of BUN and Crea (Fig. 2C and D, P < 0.05). Pretreatment with fullerol before CCl₄ challenge significantly protected liver cell injury on ALT and AST serum-release in dose-dependent tendency, as well as the BUN and Crea serum-concentration which indicate renal damage is lower than the CCl₄ group (P < 0.05). Pretreatment with fullerol in both application routes and all doses resulted in no changes in the serum ALT and AST activities, neither the concentration of BUN and Crea, compared with the control. All fullerol control groups showed no difference in ALT, AST, BUN and Crea.

### 3.2. Effect on hepatic and renal MDA levels

In order to evaluate the effect of the pretreatment with fullerol on CCl₄-induced liver lipid peroxidation, the levels of MDA, an indicator of oxidative damage, was monitored. Fig. 3A and C showed the production of MDA in the CCl₄-treated groups increased when compared to the control in liver and kidney, respectively. Considering the same administration mode and consistent with the levels of serum markers, pretreatment with fullerol significantly decreased the CCl₄-induced lipid peroxidation in dose-dependent tendency. Low dose (1 mg/kg d) did not protect tissues from the CCl₄-induced toxicity. As the administrated dose increased, the fullerol-pretreatment may prevent the CCl₄-induced increase of the hepatotoxicity and nephrotoxicity markers in the serum and the CCl₄-induced lipid peroxidation (P < 0.05). Fullerenol-pretreatment alone did not induce significant lipid peroxidation at any dose.

#### 3.3. Effects of fullerol on GSH/GSSG level

Being a gauge for the intracellular redox equilibrium, GSH/GSSG ratio was used to appraise the oxidation stress. Since CCl₄ may induce lipid peroxidation, the GSH/GSSG ratio in CCl₄ group was significantly decreased (P < 0.05), being approximately three fold lower compared to the CCl₄-untreated group. Pretreatment with fullerol prevented GSH/GSSG depletion (P < 0.05) produced by CCl₄ (Fig. 3B and D). Fullerol-pretreatment alone increased GSH/GSSG ratio in the liver, which suggests that the fullerol can modulate the intracellular redox status.

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**Fig. 3** The oxidative stress markers of fullerol protection against CCl₄-induced toxicity. Liver and kidney lipid peroxidation was measured by the formation of the thiobarbituric acid-reactive material, malondialdehyde (MDA); GSH/GSSG ratio (reduced and oxidized glutathione ratio) was used to appraise the oxidation stress. (A) MDA content in liver; (B) GSH/GSSG ratio in liver; (C) MDA content in kidney; (D) GSH/GSSG ratio in kidney.
3.4. Pathological analysis

After the exsanguinations, the liver and kidney were separated to observe the macroscopic changes. The observable macroscopic changes were found in livers but not in kidneys. Liver tissue samples of the CCl₄ group had a significant macroscopic steatosis and necrosis compared to the pretreatment and control groups (Fig. 4).

Histopathological analysis of CCl₄-treated rats showed focal necrosis (Fig. 5F), microvesicular steatosis (Fig. 5E) and ballooning degeneration (Fig. 5D and E). These changes were alleviated with pretreatment with fullerenol. In fullerenol control groups, neither necrosis nor steatosis has been observed. Only few of ballooning degeneration was found sparsely. The histopathological changes in different groups indicated that the fullerenol-pretreatment protected the liver injury induced by CCl₄.

The administration of CCl₄ caused significant morphological damage to the kidneys, especially to the renal cortex. The histopathologically observed changes ranged in severity from none (control group; Fig. 6A) to mild (Fig. 6B) to severe (CCl₄ group; Fig. 6D). In CCl₄-treated kidneys, the affected glomeruli were observed exhibiting different forms of degeneration, in addition to the renal corpuscles with normal appearance. Some glomeruli showed mild dilatation of Bowman’s space with glomerular atrophy (Fig. 6B), whereas a small number of others exhibited congestion in the capillary loops with an adhesion between visceral and parietal layers of Bowman’s capsule (Fig. 6D). Some of renal tubules were dilated and their epithelial cells tended to be vacuolated with a foamy appearance (Fig. 7). These findings in the renal cortices of CCl₄-treated rats were spread throughout the subcortical areas as well. In the fullerenol-pretreatment groups, these pathological changes were alleviated (Fig. 6C). In fullerenol administration groups, histological appearances of the glomeruli and tubules were normal (Fig. 7).

4. Discussion

CCl₄, a well-known hepatotoxin and nephrotoxin, was used for the purpose of inducing renal damage [25–27]. In our study, CCl₄-induced a severe liver and renal damage as represented by significantly elevated levels of serum markers (ALT, AST, BUN and Crea) coupled with a marked hepatic oxidative stress and decrease of GSH/GSSG. It is well-known GSH plays very important role in cellular antioxidant defense, which is a
crucial determinant of tissue susceptibility to oxidative damage [28]. GSH depletion occurs as a consequence of CCl4-induced toxicity [29–31]. During the radical stress, GSH is catalyzed by glutathione peroxidase and transferred into its oxidized form, GSSG, which lead to the depletion of intracellular GSH and the decrease of GSH/GSSG ratio.

Since the damaging effects of CCl4 involve oxidative stress, numerous investigations have used this model to investigate protective effects of antioxidants. Several experimental studies have demonstrated the beneficial effects of antioxidant treatment on CCl4-induced tissue injury [19,20,27]. Thanks to recent development in nanoscience and nanotechnology, carbon-based nanomaterials (e.g., fullerenes, nanotubes, nanodiamonds) attract great interests. Owing to the antioxidant property and good water-solubility, we postulated that fullerenol might protect both liver and kidney against chemical induced toxicity.

Our results showed that prophylactic treatment with fullerenol (5 mg/kg body weight) for continuous 3 days before CCl4 challenge resulted in considerable protection for both of liver and kidney. We examined the serum levels of ALT, AST, BUN and Crea, which are well defined as serum markers indicating the damage of liver and kidney. The change of serum markers indicated that pretreatment with fullerenol ameliorated the hepatotoxicity and nephrotoxicity after CCl4 treatment in a dose-dependent tendency. Furthermore, these finding were confirmed by histopathological analysis. The GSH/GSSG ratio provides the estimation of the oxidative stress as a direct consequence of CCl4 challenge, while MDA analysis revealed the assessment of lipid peroxidation. Both of these radical stress markers were confirmed the serum examination results. The results from MDA content analysis indicated that the lipid peroxidation in liver and kidney was alleviated by fullerenol-pretreatment. Such effect may come from antioxidant and free-radical scavenging capabilities of fullerenol, which can protect liver and kidney either directly or indirectly in this study. We have proved fullerenol may act as an extracellular antioxidant in oxidation stress in another study [14].

Fig. 5 – Histopathological changes in rat livers, stained with H&E. (A) Control liver; (B) rat liver intoxicated with CCl4 with pretreatment of fullerenol (5 mg/kg d, IP); (C) rat liver intoxicated with CCl4 with pretreatment of fullerenol (1.5 mg/kg d, IP); (D–F) rat liver intoxicated with CCl4((A–E), Mag. 200×; (F), Mag. 100×).
Fullerenol was then found to be a powerful radical-scavenger for superoxide, hydroxyl and lipid radicals [32]. In this study, fullerenol was potent in increasing the GSH/GSSG ratio in rat liver but not kidney, which may be resulted from the biodistribution nature of fullerenol. The uptake of fullerenol in liver was relatively high, while the clearance from liver was rather slow [21,22,33]. This may eventually lead to the relatively long retention of fullerenol in liver and consequently be absorbed into liver cells and acted as an antioxidant there. Fullerenol was mainly excreted unbounded by urine and its retention in kidney is transitory. Therefore, this transient interaction with kidney cells disables fullerenol uptake by the cells and manifestation of its antioxidant activity.

Considering the difference between the two administration routes, not only the biodistribution but also the bioavailability of the administration modes must be taken into account. Bioavailability is a pharmacological parameter which is used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation. By this definition, when a drug is administered intravenously, its bioavailability is 100%. If a drug is administered via other routes, its bioavailability decreases due to the incomplete absorption. According to fullerenol bioavailability in current study, IP should be lower than that of IV. Regarding

Fig. 6 – Histopathological changes in rat kidneys, stained with H&E. (A) Control kidney; (B) kidney pretreatment with fullerenol (1 mg/kg d, IV) and intoxicated with CCl₄; (C) kidney pretreatment with fullerenol (5 mg/kg d, IV) and intoxicated with CCl₄; (D) kidney intoxicated with CCl₄. The congestion in the capillary loops (arrowhead), and renal tubules were dilated and their epithelial cells tended to be vacuolated with a foamy appearance (*). (Mag. 200×).

Fig. 7 – Fullerenol treatment on kidney, stained with H&E. (5 mg/kg d, IV; Mag. 400×).
biodistribution, it is well documented that fullerol distributed mostly into reticuloendothelial organs, such as liver, spleen and bone. When the fullerol were administrated by IP, it can be distributed into liver in two ways, from the circulation and the hepatic portal vein [21,22]. Meanwhile, as shown in our previous study, a big proportion of administrated-fullerol is mainly excreted by urine [21,22]. Taken together biodistribution and bioavailability characteristics of fullerol, the high liver accumulation of fullerol reaches the threshold dose, which is effective in liver-protection by both IV and IP administrations. The fullerol plasma concentration in IV groups should be higher than that of IP groups, which lead to a high excretion concentration of fullerol in urine, and made IV more effective than that of IP in the fullerol protection of CCl₄-induced nephrotoxicity ensue.

Fullerol has been reported to have protective ability in CCl₄-induced liver damages [15]. In contrast to Gharbi’s report, the protection effect of fullerol used in our experiment was more efficient compared to fullerene. Prophylactic treatment with fullerol(5 mg/kg body weight) for 3 days can alleviate the CCl₄-induced hepatotoxicity effectively, whereas the dosage used in their experiment was as high as 2 g/kg. Fullerene and its derivatives are all prone to aggregating. The specific surface area decreased with the aggregation, and the biological activity of them decreased as well. On the premise of a better solubility, the particle specific surface can be relatively stable. Therefore, the biological activity can be effectively brought into play. With better solubility, fullerol may possess better biological activity than fullerene. Besides, plenty of fullerene accumulated in liver made the liver macroscopic appearance (color and luster) different from the normal one in that study [12]. None of such changes were observed in our experiment, which may be owing to the water-solubility of fullerol. Moreover, not only CCl₄-induced hepatotoxicity but also nephrotoxicity were effectively alleviated by the fullerol-pretreatment showed by our experiments. With the properties of water-solubility and urine excretion, fullerol can distributed into the kidney and may scavenge the CCl₄-induced free radicals locally. It is crucial that the IV can prevent the CCl₄-induced damages effectively, which is a convenient and easy application route.

5. Conclusions

In this study, CCl₄-induced hepatotoxicity and nephrotoxicity were used as a model of oxidative stress involved damage. In this model, CCl₄ not only significantly increased serum hepatotoxicity and nephrotoxicity markers, but also altered MDA and GSH/GSSG ratio in tissues, which indicate that free radical plays an important role in CCl₄-induced liver and renal damage process. Pretreatment with fullerol can normalized serum markers levels, improved the antioxidant ability. Histopathological analysis is also consistent with these results. Our study provided basic understanding for the application of fullerol in health sciences and pharmaceuticals in the future. Further studies are in progress in vitro to clarify the mechanism of its action, especially the influence on the increase of GSH/GSSG ratio.

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Appendix A. Supplementary data


References


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