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

Jing-Ying Xu [a,b], Yuan-Yuan Su [b,c], Jin-Sheng Cheng [d], Shu-Xia Li [e], Ruili Liu [b], Wen-Xin Li [b], Guo-Tong Xu [a,*], Qing-Nuan Li [b,*]

[a] Laboratory of Clinical Visual Sciences of Tongji Eye Institute and Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai 200092, China
[b] Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai 201800, China
[c] Department of Biochemistry, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China
[d] Department of Chemistry, University of Science and Technology of China, Hefei 230026, China
[e] Department of Pathology, Tongji University School of Medicine Affiliated First Maternity and Infant Health Hospital, Shanghai 200040, China

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ABSTRACT

An important biologically-relevant property of fullerenol is its ability to quench free radicals. Carbon tetrachloride (CCl_4)-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 CCl_4 challenge, 24 h following the CCl_4 challenge, all the rats were assessed using serum and tissue homogenates biomarkers as well as the pathological evaluation. All results showed that CCl_4 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 CCl_4. 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 CCl_4-induced oxidative stress by improving the antioxidant ability.

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1. Introduction

Carbon tetrachloride (CCl_4) is a well established hepatotoxin. Previous studies showed that both liver and kidneys are the target organs of CCl_4. Extensive evidence demonstrates that CCl_4 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 CCl_4 are also associated with free radical production [3]. CCl_4-induced damage is also able to alter the antioxidant status of the tissues, which is manifested by abnormal histopathological changes [4]. Histopathologically, exposure to CCl_4 can results in hepatic steatosis, centrilobular necrosis, and cirrhosis in the liver and acute tubular necrosis in the kidney [5,6].

Fullerenol ($C_{60}(OH)_x$, $x = 3$–24), a kind of representative water-soluble derivatives of 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 *Stylonychia mytilus* cells exposed to $^{60}Co\gamma$-rays. Fullerenol was also found to be good radiation protectors for the protozoan *S. mytilus* exposed to γ-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 C_{60}-pretreatment can protect the livers against the acute CCl_4-induced free-radical damage in rats.

Although both of fullerenol and C_{60} have anti-oxidative capabilities [7–15], the stability of fullerene hinders its application in medical therapy. The photosensitization of 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 C_{60}. On the other hand, it is well documented that antioxidants can prevent 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 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 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

CCl_4 was purchased from Sigma. Fullerenol ($C_{60}(OH)_x$, $x = 22,24$) 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:

CCl_4	CCl_4 treatment group
Con	Control group (without treatment)
$Con_{IV\ 1\ mg}$	Control of IV with fullerenol (1 mg/kg)
$Con_{IV\ 1.5\ mg}$	Control of IV with fullerenol (1.5 mg/kg)
$Con_{IV\ 5\ mg}$	Control of IV with fullerenol (5 mg/kg)
$Con_{IP\ 1\ mg}$	Control of IP with fullerenol (1 mg/kg)
$Con_{IP\ 1.5\ mg}$	Control of IP with fullerenol (1.5 mg/kg)
$Con_{IP\ 5\ mg}$	Control of IP with fullerenol (5 mg/kg)
$Fol_{IV\ 1\ mg}$	Fullerenol$_{IV}$ (1 mg/kg) + CCl_4
$Fol_{IV\cdot1.5\ mg}$	Fullerenol$_{IV}$ (1.5 mg/kg) + CCl_4
$Fol_{IV\ 5\ mg}$	Fullerenol$_{IV}$ (5 mg/kg) + CCl_4
$Fol_{IP\ 1mg}$	Fullerenol$_{IP}$ (1 mg/kg) + CCl_4
$Fol_{IP\ 1.5\ mg}$	Fullerenol$_{IP}$ (1.5 mg/kg) + CCl_4
$Fol_{IP\cdot5\ mg}$	Fullerenol$_{IP}$ (5 mg/kg) + CCl_4

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 aminotransferase (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.
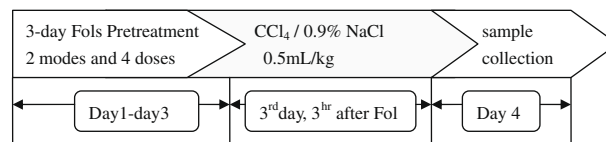

Fig. 1 – The experimental scheme. Fullerenol was given by intravenous injection (IV) and intraperitoneal injection (IP) in four different doses (0, 1, 1.5 and 5 mg/kg d) for continuous 3 days. All the animals except for control groups were injected (IP) with a single dose of CCl_4 (0.5 mL/kg) 3 h after the last administration of fullerenol. The control groups were injected (IP) with 0.9% NaCl (0.5 mL/kg) instead. Twenty-four hours after CCl_4 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 CCl$_4$-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 mon-

itored 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 CCl$_4$-induced hepatotoxicity and nephrotoxicity

CCl$_4$ was given to seven groups of rats, with or without fullerenol-pretreatment, the effects of the fullerenol on the
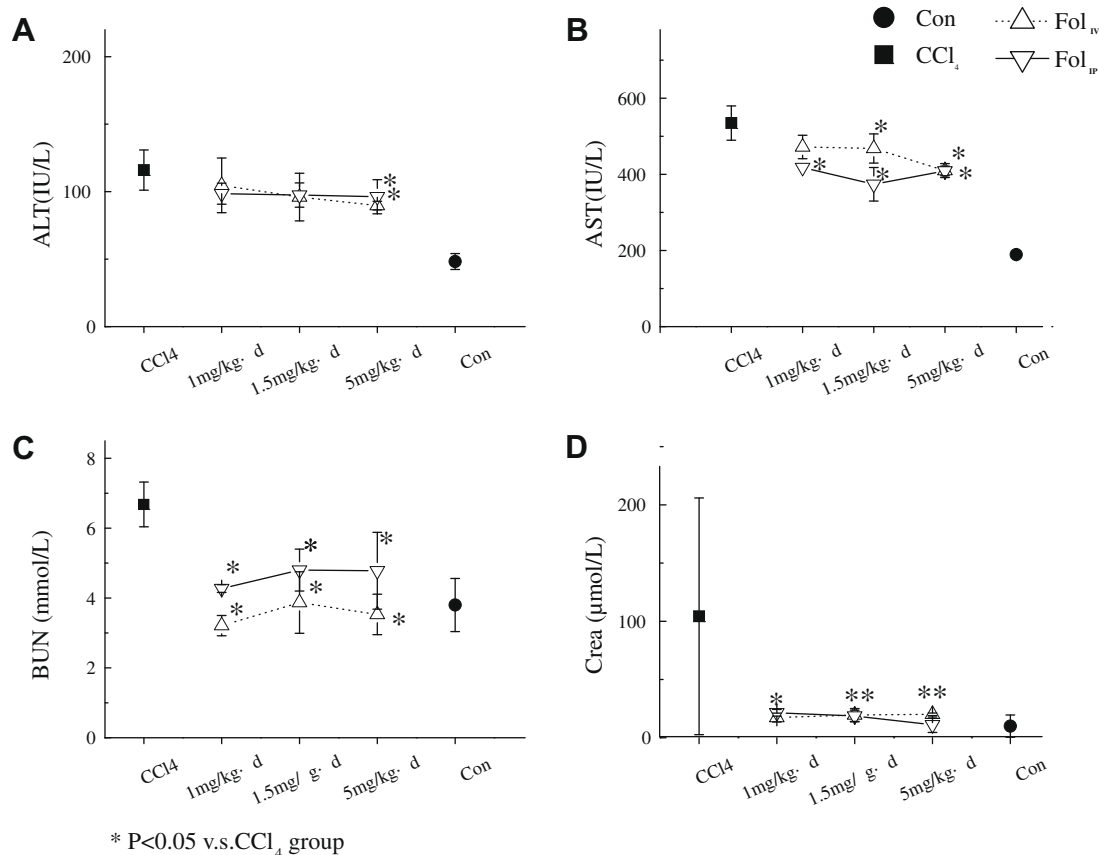

Fig. 2 – The serum markers of protective effect of fullerenol on the CCl$_4$-induced toxicity. (A) Alanine aminotransferase (ALT) activities in serum; (B) Aspartate aminotransferase (AST) activity in serum; (C) Blood urea nitrogen (BUN); and (D) creatinine (Crea) concentration were appraised.

CCl$_4$-induced elevation in the serum ALT and AST activities are shown in Fig. 2A and B. A single dose of CCl$_4$ 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 CCl$_4$ administration, which is indicated by the serum-concentration of BUN and Crea (Fig. 2C and D, $P < 0.05$). Pretreatment with fullerenol before CCl$_4$ 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 CCl$_4$ group ($P < 0.05$). Pretreatment with fullerenol 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 fullerenol 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 fullerenol on CCl$_4$-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 CCl$_4$-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 fullerenol significantly decreased the CCl$_4$-induced lipid peroxidation in dose-dependent tendency. Low dose (1 mg/kg d) did not protect tissues from the CCl$_4$-induced toxicity. As the administrated dose increased, the fullerenol-pretreatment may prevent the CCl$_4$-induced increase of the hepatotoxicity and nephrotoxicity markers in the serum and the CCl$_4$-induced lipid peroxidation ($P < 0.05$). Fullerenol-pretreatment alone did not induce significant lipid peroxidation at any dose.

3.3. Effects of fullerenol on GSH/GSSG level

Being a gauge for the intracellular redox equilibrium, GSH/GSSG ratio was used to appraise the oxidation stress. Since CCl$_4$ may induce lipid peroxidation, the GSH/GSSG ratio in CCl$_4$ group was significantly decreased ($P < 0.05$), being approximately three fold lower compared to the CCl$_4$-untreated group. Pretreatment with fullerenol prevented GSH/GSSG depletion ($P < 0.05$) produced by CCl$_4$ (Fig. 3B and D). Fullerenol-pretreatment alone increased GSH/GSSG ratio in the liver, which suggests that the fullerenol can modulate the intracellular redox status.
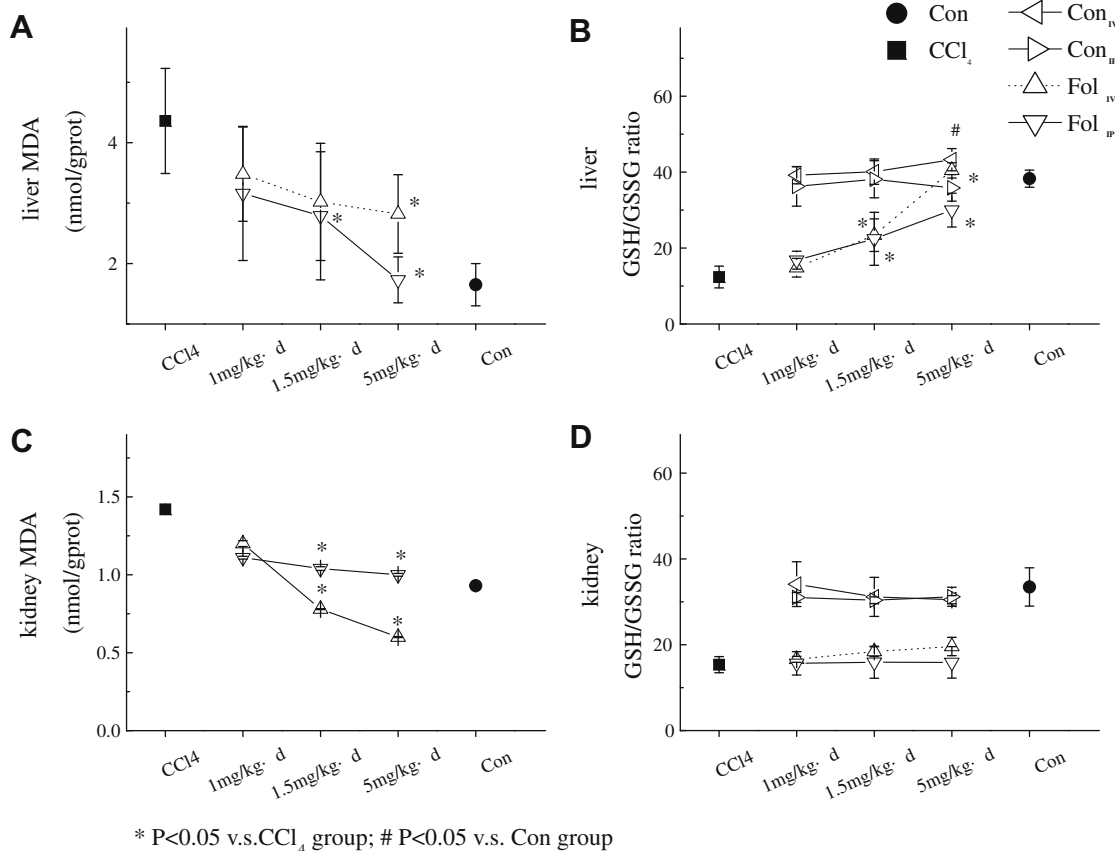

* P<0.05 v.s.CCl$_4$ group; # P<0.05 v.s. Con group

Fig. 3 – The oxidative stress markers of fullerenol protection against CCl$_4$-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.
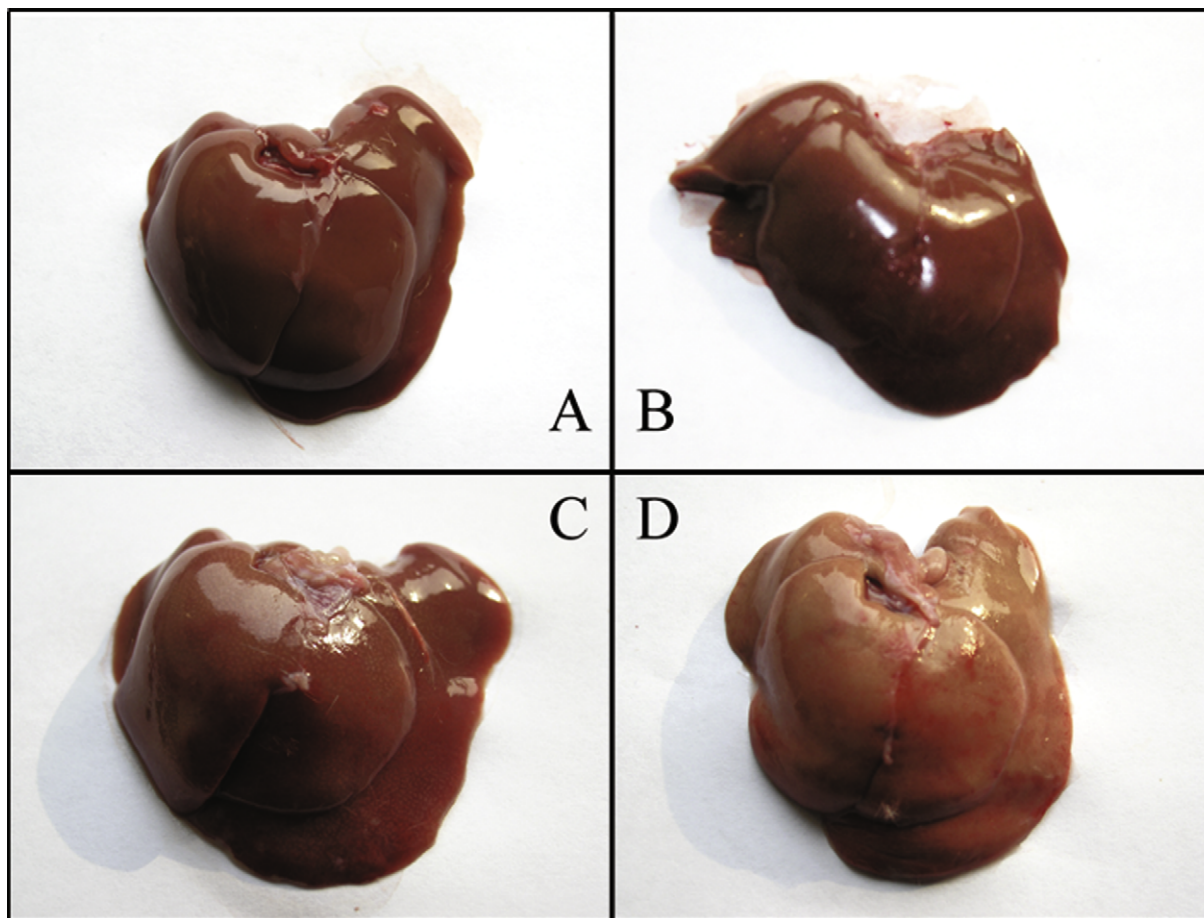
Fig. 4 – Macroscopic changes of rat livers. (A) Control liver; (B) liver after pretreatment with fullerenol (5 mg/kg d); (C) liver of a rat intoxicated with CCl_4(0.5 mL/kg d) after pretreatment with fullerenol (5 mg/kg d); (D) liver of a rat intoxicated with CCl_4 (0.5 mL/kg).

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 CCl_4 group had a significant macroscopic steatosis and necrosis compared to the pretreatment and control groups (Fig. 4).

Histopathological analysis of CCl_4-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 CCl_4.

The administration of CCl_4 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 (CCl_4 group; Fig. 6D). In CCl_4-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 CCl_4-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

CCl_4, a well-known hepatotoxin and nephrotoxin, was used for the purpose of inducing renal damage [25–27]. In our study, CCl_4-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
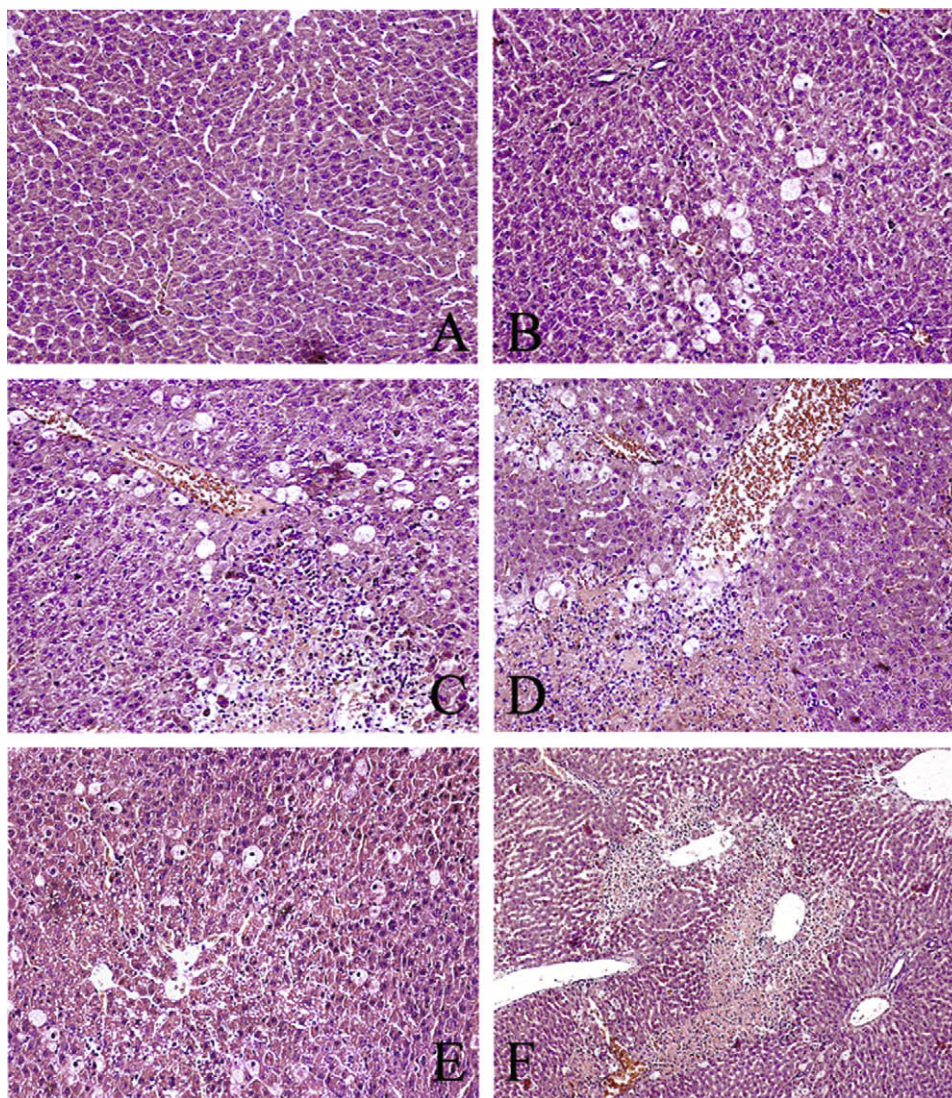
Fig. 5 – Histopathological changes in rat livers, stained with H&E. (A) Control liver; (B) rat liver intoxicated with CCl$_4$ with pretreatment of fullerenol (5 mg/kg d, IP); (C) rat liver intoxicated with CCl$_4$ with pretreatment of fullerenol (1.5 mg/kg d, IP); (D–F) rat liver intoxicated with CCl$_4$. ((A–E), Mag. 200×; (F), Mag. 100×).

crucial determinant of tissue susceptibility to oxidative damage [28]. GSH depletion occurs as a consequence of CCl$_4$-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 CCl$_4$ 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 CCl$_4$-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 CCl$_4$ 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 CCl$_4$ 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 CCl$_4$ 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].
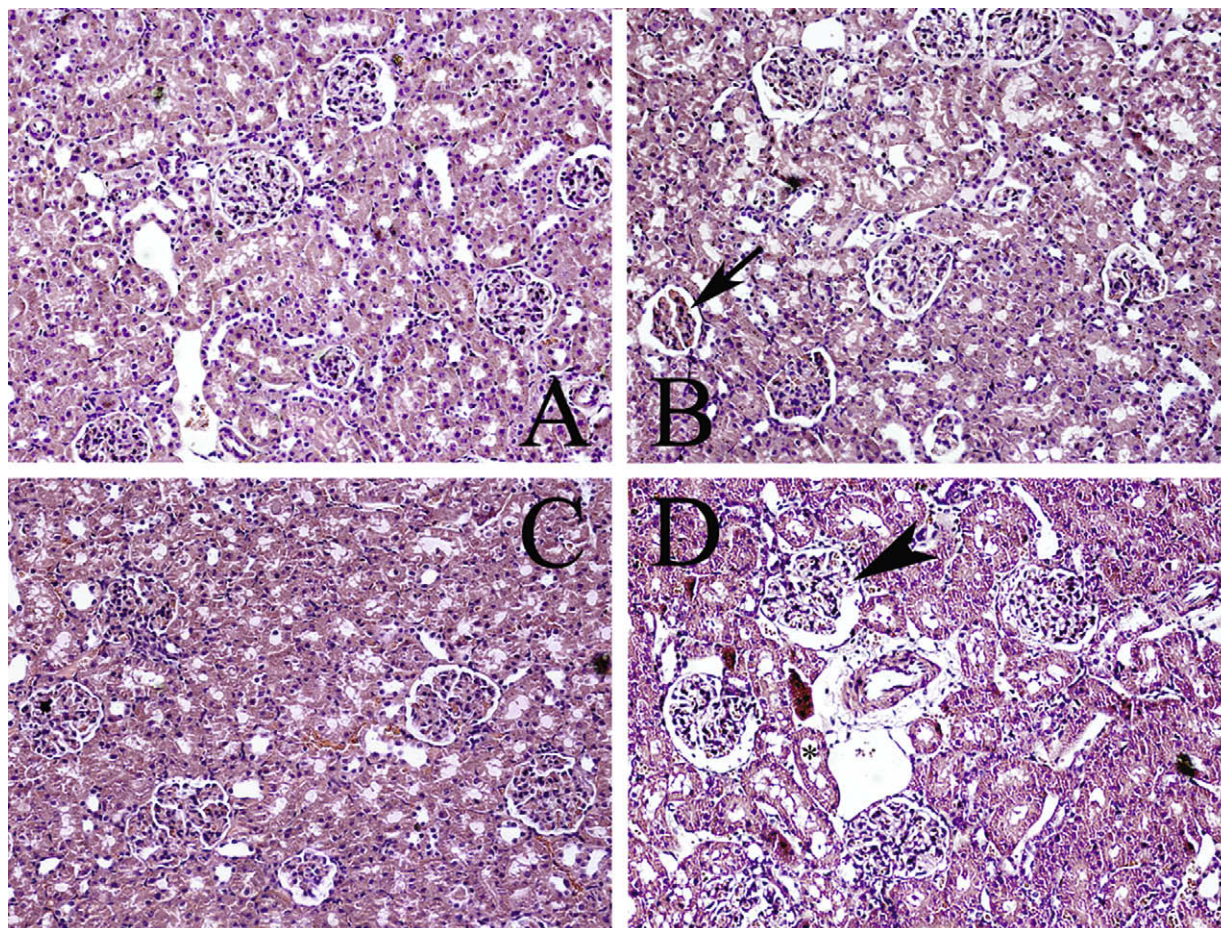
Fig. 6 – Histopathological changes in rat kidneys, stained with H&E. (A) Control kidney; (B) kidney pretreatment with fullerenol (1 mg/kg d, IV) and intoxicated with CCl$_4$. Dilatation of Bowman's space with glomerular atrophy (arrow); (C) kidney pretreatment with fullerenol (5 mg/kg d, IV) and intoxicated with CCl$_4$; (D) kidney intoxicated with CCl$_4$. 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×).

Fullerenol was then found to be a powerful radical-scavenger for superoxide, hydroxyl and lipid radicals [32]. In this study,
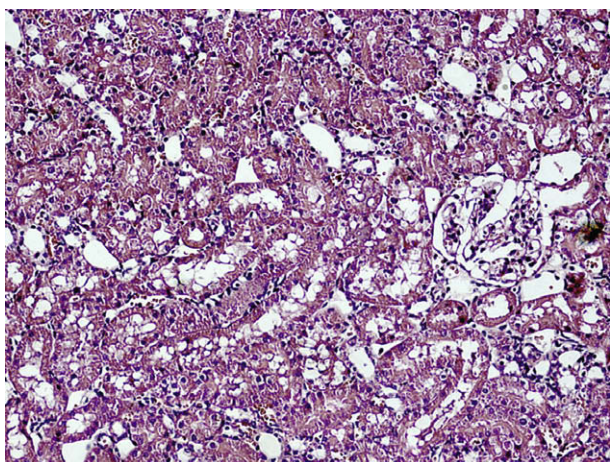

Fig. 7 – Fullerenol treatment on kidney, stained with H&E. (5 mg/kg d, IV; Mag. 400×).

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

biodistribution, it is well documented that fullerenol distributed mostly into reticuloendothelial organs, such as liver, spleen and bone. When the fullerenol 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-fullerenol is mainly excreted by urine [21,22]. Taken together biodistribution and bioavailability characteristics of fullerenol, the high liver accumulation of fullerenol reaches the threshold dose, which is effective in liver-protection by both IV and IP administrations. The fullerenol plasma concentration in IV groups should be higher than that of IP groups, which lead to a high excretion concentration of fullerenol in urine, and made IV more effective than that of IP in the fullerenol protection of CCl_4-induced nephrotoxicity ensue.

Fullerene has been reported to have protective ability in CCl_4-induced liver damages [15]. In contrast to Gharbi's report, the protection effect of fullerenol used in our experiment was more efficient compared to fullerene. Prophylactic treatment with fullerenol (5 mg/kg body weight) for 3 days can alleviate the CCl_4-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, fullerenol 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 fullerenol. Moreover, not only CCl_4-induced hepatotoxicity but also nephrotoxicity were effectively alleviated by the fullerenol-pretreatment showed by our experiments. With the properties of water-solubility and urine excretion, fullerenol can distributed into the kidney and may scavenge the CCl_4-induced free radicals locally. It is crucial that the IV can prevent the CCl_4-induced damages effectively, which is a convenient and easy application route.

5. Conclusions

In this study, CCl_4-induced hepatotoxicity and nephrotoxicity were used as a model of oxidative stress involved damage. In this model, CCl_4 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 CCl_4-induced liver and renal damage process. Pretreatment with fullerenol 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 fullerenol 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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbon.2009.12.029.

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