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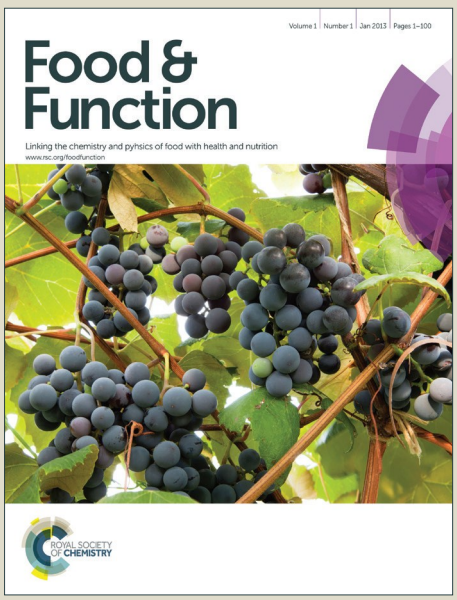

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Quercitrin offers protection against brain injury in mice by inhibiting oxidative stress and inflammation

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Abbreviations:

AChE, Acetylcholine esterase; CAT, Catalase; CYP2E1, Cytochrome P450 2E1; GPX, Glutathione peroxidase; IL-6，Interleukin-6; MAO, Monoamine oxidase; MDA, Malondialdehyde; NR2B, N-methyl-D-aspartate receptor 2B subunit; QE, Quercitrin; ROS, Reactive oxygen species; SOD, superoxide dismutase; t-PA, Tissue-type plasminogen activator; TNF-α, tumor necrosis factor.

ABSTRACT

Quercitrin is one of the primary flavonoid compounds present in vegetable and fruits. The aim of present study was to evaluate the effects of quercitrin against carbon tetrachloride (CCl_4) induced brain injury and further to elucidate its probable mechanisms. ICR mice received intraperitoneally CCl_4 with or without quercitrin co-administration for 4 weeks. Our data showed that quercitrin significantly suppressed the elevation of reactive oxygen species (ROS) production and malondialdehyde (MDA) content, reduced tissue plasminogen activator (t-PA) activity, enhanced the antioxidant enzyme activities and abrogated cytochrome P450 2E1 (CYP2E1) induction in mouse brains. Quercitrin also prevented CCl_4 induced cerebral function disorders associated with its ability to inhibit the activities of monoamine oxidase (MAO), acetylcholine esterase (AChE) and N-methyl-D-aspartate receptor 2B subunit (NR2B). In addition, western blot analysis showed that quercitrin suppressed the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). Taken together, our findings suggested that quercitrin may be potential to be developed as a neuroprotective agent.

Keywords: Quercitrin; CCl_4; Brain; Oxidative stress; Inflammation; Dysfunction

1 Introduction

Flavonoids possess antioxidant and anti-inflammatory activities, readily permeate the blood–brain barrier and afford neuroprotection in a wide array of cellular and animal models of neurological diseases. [1,2] Flavonoids are ubiquitous plant secondary products, which are produced in response to diverse environmental cues. In plants, naringenin was converted into dihydroflavonols. Dihydroflavonols was converted by flavonol synthase into flavonols. Flavonols were further glucosylated to flavonol glycosides.[3] Quercetin is the most abundant bioflavonoid found in vegetable and fruits, which exists primarily in its glycoside form as quercitrin (3-rhamnoside). The sugar portion bound to the aglycone portion in quercitrin (QE) increases its solubility in polar solvents and consequently improves absorption.[1, 2] The quercitrin is known to have biological effects such as anti-oxidative, anti-inflammatory, anti-apoptosis effects.[4,5] Quercitrin might be more potent antioxidant and neuroprotective agent than quercetin due to its high bioavailability in the digestive track. [6,7] Previous report indicated that quercitrin significantly reduced acute systemic inflammation in LPS-induced models as well as attenuate inflammation in the livers of mice.[7] Quercitrin could also reduce inflammation and apoptosis in cytokine-induced models.[8] In streptozotocin (STZ)-induced diabetic models, quercitrin display organ protective properties in pancreas, liver and kidney by improving the antioxidant status.[5]

Carbon tetrachloride (CCl_4) is a well-known toxic compound. Administration of CCl_4 to animal is an accepted experimental model to produce damage to hepatic and other tissues.[10] CCl_4 can cause damage to the brain through lipid peroxidation, resulting in an up-regulation of pro-inflammatory pathways and the alterations of the neurotransmission system.[11,12] Recent studies from our laboratory showed that puerarin (a natural flavonoid) could attenuate CCl_4-induced oxidative stress and inflammation in kidney.[12] However, the molecular mechanisms of CCl_4-induced brain injury and neuroprotective effects of quercitrin are not yet completely understood. In the present study, we aimed to determine whether the dietary uptake of quercitrin can protect mouse brain from CCl_4-induced injury by inhibiting oxidative stress and

inflammation. In addition, we investigated whether the protective mechanism of quercitrin associated with the activities of tissue plasminogen activator (t-PA), monoamine oxidase (MAO), acetylcholine esterase (AChE) and N-methyl-D-aspartate receptor 2B subunit (NR2B).

2 Materials and methods

2.1 Chemicals and reagents

Quercitrin and CCl_4 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). anti-IL-6 antibody, anti-TNF-α antibody and anti-NR2B antibody are from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology (Beverly, MA, USA). All other reagents unless indicated were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Animals and treatment

Male ICR mice (20–25g) were obtained from the Branch of National Breeder Center of Rodents (Beijing). The animals were maintained in an animal facility at 20–22 ℃ under 40–60 % relative humidity and a 12/12 h (light/dark) cycle.

After acclimation for 1 week, forty mice were randomly divided into four groups. Brain injury was induced by intraperitoneal (i.p.) injection of 2 ml of CCl_4 in olive oil (1:1, v/v) per kg body weight twice weekly for up to 4 week [13]. Mice in Group 1 were given twice weekly injections of pernut oil (vehicle control) and received water containing 0.5% carboxymethylcellulose sodium by oral gavage; mice in Group 2 were injected with CCl_4 and received water containing 0.5% carboxymethylcellulose sodium by oral gavage; mice in Group 3 and Group 4 were injected with CCl_4, as in group 2, and administered with quercitrin (50 and 100 mg/kg body-weight, suspended in 0.5% carboxymethylcellulose sodium daily) by oral gavage, respectively. The choice of quercitrin dose is based on previous findings [14]

At the end of treatment, the mouse brains were used for the biochemical analysis and western-blot evaluations. The brain tissue were quickly collected for experiments and placed in ice-cold 0.9% NaCl solution, perfused with the physiological saline solution to remove blood cells, blotted on filter paper. Then, the

removed brain were immediately collected for experiments or stored at -70°C for later use.

This research was conducted in accordance with the Chinese laws on the use and care of laboratory animals. The committees for animal experiments of Jiangsu Normal University and Sichuan University of Science and Engineering approved these experiments.

2.3 Biochemical analysis

ROS was measured as described previously, based on the oxidation of 2'7'-dichlorodihydrofluorescein diacetate to 2'7'-dichloro-fluorescein. [13] ROS formation was quantified from a DCF-standard curve and data are expressed as pmol DCF formed/min/ mg protein. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), monoamine oxidase (MAO) and acetylcholine esterase (AChE) in the hippocampus homogenates were measured using the commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China).

The malondialdehyde (MDA) concentration in the hippocampus homogenates were measured by the thiobarbituric acid reactive substances (TBARS) assay in accordance with the manufacturer's instructions (MDA: A003-1, Jiancheng Biochemical Institute, Nanjing, China) and the absorbance was measured at a wavelength of 532 nm.

2.4 Functionally active t-PA activity assay

Functionally active t-PA was determined using the active t-PA ELISA Kit according to the manufacturer's instructions. [15] The amount of color development is directly proportional to the concentration of active t-PA in the sample.

2.5 Western blot analysis

Western blot analysis was performed by our previous method. [16,17] Nuclear and cytoplasmic extracts for western blotting were obtained by using a nuclear/cytoplasmic isolation kit (Beyotime Institute of Biotechnology, Beijing, China). Protein levels in the hippocampus homogenates were determined using the BCA assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.6 Statistic analysis

All statistical analyses were performed using the SPSS software, version 11.5. A one-way analysis of variance (ANOVA; $P < 0.05$) was used to determine significant differences between groups and the individual comparisons were obtained by Turkey's HSD post hoc test. Statistical significance was set at $P \leq 0.05$.

3 Results

3.1 Quercitrin Inhibited CCl_4-induced Oxidative Stress in Mouse Brains

To investigate whether quercitrin can attenuate the oxidative stress damage in the brain of CCl_4-treated mice, we measured the concentrations of ROS and MDA (Figure 2). The results showed that the levels of ROS and MDA were highly increased by 107.28% and 80.04% in the brains of CCl_4-treated group compared with the control group, respectively ($P < 0.01$). However, treatment with quercitrin dose-dependently decreased the levels of ROS and MDA (by 30.78%, 40.03% and 27.24%, 43.15%, respectively) compared with CCl_4 treatment ($P < 0.01$).

3.2 Quercitrin Inhibited the CYP2E1 Level in Brains of CCl_4-treated Mice

In order to determine whether quercitrin can inhibit CCl_4-inducd the brain injury, we examined the CYP2E1 level of in mouse brains. As shown in Figure 3, CCl_4 treatment caused significant increase in the CYP2E1 level by 363.21% in mouse brains as compared with that of the control group ($P < 0.01$). Interestingly, treatment with quercitrin dose-dependently decreased the CYP2E1 level by 43.23% and 72.19% compared with CCl_4 treatment, respectively ($P < 0.01$).

3.3 Quercitrin Increased the Activities of SOD, CAT and GPX in Mouse Brains

During the pathological process of CCl_4-induced brain injury, reductions in antioxidant enzyme activities play important roles [10]. CCl_4 administration significantly decreased the activities of SOD, CAT and GPX in mouse brains by 24.26%, 43.57% and 24.98%, respectively, when compared to those in control group ($P < 0.01$). However, pretreatment with low-dose and high-dose of quercitrin remarkably ameliorated the depletion of the SOD, CAT and GPX activities (Figure 4).

3.4 Quercitrin Decreased the t-PA Activity in Brains of CCl_4-treated Mice

As shown in Figure 5A, quercitrin also prevented the increase of the t-PA activity in the brain homogenates of the mice treated with CCl_4. The t-PA activity in CCl_4-treated group was significantly higher (by 40.47%) than the control group ($P < 0.01$). However, the treatment with low-dose and high-dose of quercitrin alongside CCl_4 reduced the t-PA activity by 17.68% and 25.96% in the brains of the mice, respectively.

3.5 Quercitrin Inhibited the NR2B level in Brain of CCl_4-treated Mice

NR2B play critical roles in brain functions and diseases. In this study, NR2B protein immunocontent was examined by western blot analysis on the hippocampus homogenates from mice. The results showed that the NR2B level in brain was notably increased in the CCl_4-treated mice as compared with that of the control group (Figure 5B). Interestingly, treatment with low and high dose of quercitrin significantly down-regulated the NR2B level (by 35.53% and 58.89%, respectively) compared with CCl_4 treatment ($P < 0.01$).

3.6 Quercitrin Decreased the Activities of MAO and AChE in Brain of CCl_4-treated Mice

AChE and MAO play an important role in the central nervous system (CNS). In order to determine whether quercitrin can attenuate the neurotransmitter system damage in the brain of CCl_4-treated mice, we measured the activities of AChE and MAO. The results were shown in Figure 6. The CCl_4 markedly increased activities of AChE and MAO in mouse brain hippocampus by 58.17% and 72.29% than the control group ($P < 0.01$). However, treatment with quercitrin dose-dependently decreased the activities of AChE and MAO (by 30.25%, 37.66% and 25.31%, 34.33%, respectively) compared with CCl_4 treatment ($P < 0.01$).

3.7 Quercitrin Reduced the Production of Brain Inflammatory Cytokines of CCl_4-treated Mice

To examine the effect of quercitrin treatment on the CCl_4-induced inflammatory cytokines, the hippocampus protein immunocontents of TNF-α and IL-6 were measured using western blot analysis. As shown in Figure 7, the production of both

TNF-α and IL-6 were significantly increased by in the brains of CCl_4-treated group, compared to those in the control group. However, these increases were attenuated by quercitrin treatment ($P < 0.01$).

4 Discussion

The brain is an organ with a high metabolic rate that relies mainly on aerobic metabolism, which inevitably produced ROS. [15] CCl_4 has been extensively used in experimental models to elucidate the cellular mechanisms behind oxidative damage and inflammation [18, 19]. In the present study, the effect of quercitrin treatment on CCl_4 induced brain injury was investigated using a mouse model. Our results demonstrated that treatment with quercitrin significantly ameliorated brain injury induced by CCl_4. This protective effect was associated with its ability to inhibit oxidative stress, inflammation and the neurotransmitter system damage.

The brain is especially sensitive to oxidative stress. Moreover, neuronal cells are rich in polyunsaturated fatty acid, which are prone to lipid peroxidation by ROS. [15] Cytochrome P450, in particular, CYP2E1, is characterized as a free enzyme with high pro-oxidant activity, with a potential source of oxidative stress. CCl_4 can be activated by cytochrome P450 enzyme, to form the trichloromethyl radical $CCl_3^{\bullet}$ and trichloromethyl peroxy radical $CCl_3OO^{\bullet}$, which leads to lipid peroxidation and subsequent tissue damage. [11] Our previous studies showed that CCl_4 induced oxidative stress in liver and kidney. [13,20] In this research, the results revealed the role of lipid peroxidation in brain hippocampus accompanied with stimulating and induction of CYP2E1 with sever oxidative stress status in CCl_4-treated mice, which is characterized by high levels of ROS and MDA (Figure 2). Reports have shown that quercitrin could suppress oxidative stress. [2,5] The present study showed that quercitrin significant decreased the CYP2E1 level and the peroxidation product MDA confirmed that treatment with quercitrin could effectively protect against the brain oxidative stress induced by CCl_4 (Figure 2 and 3).

Primary antioxidant enzymes involved in direct elimination of free radicals are superoxide dismutase (SOD) which removes $O_2^{\bullet}$, catalase (CAT) which converts

H_2O_2 to water (H_2O) and O_2, and glutathione peroxidase (Gpx) which helps in the removal of H_2O_2, thereby preventing hydroxyl radical ($OH^{-\bullet}$) formation. [10,21] A reduction in the activity of these enzymes is associated with the accumulation of excess ROS, leading to deleterious effects such as loss of integrity and function of cell membranes. [10,19,22] In this study, the increase in ROS and MDA levels in brains induced by CCl_4 suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. [10] Interestingly, treatment with quercitrin restored the activities of these antioxidant enzymes to near-normal levels in CCl_4-intoxicated mice. So, quercitrin possibly confers its protective effect by restoring the activities of SOD, CAT and GPx enzymes to near-normal levels, or by preventing decreases in the activities of these enzymes (Figure 4).

Tissue-type plasminogen activator (t-PA), a serine-protease that activates plasmin, endogenously produced in many types of cells. [23,24] t-PA can convert plasminogen into plasmin to dissolve clots and restore blood flow. t-PA also plays important roles in maintaining the function of the brain blood barrier and has been approved for clinical use. [25,26] Several researches indicated broader and more complex functions of the t-PA/plasmin system, an intriguing one being its role in the central nervous system (CNS). [15,26] Oxidative damage of neuronal cells has been causatively implicated in multiple neurodegenerative diseases, in several of which the PA/plasmin system has been involved. [15,27] The PA/plasmin system has been implicated in localized extracellular matrix remodeling in several pathophysiological events, including CNS diseases, where oxidative stress plays a pivotal role. [27,28] Moreover, t-PA potentiates signaling mediated by glutamatergic receptors by modifying the properties of the NMDA receptor. [15] t-PA mediates cell signaling effects through binding to cell surface molecule NMDA receptor. [15,29] NMDA receptor 2B (NR2B) subunits are essential for neurotransmission, neuronal development and synaptic plasticity, and have been shown to be involved in learning and memory abilities. [26,30] Inhibition of NR2B receptors was found to reverse signaling abnormalities and spine density as well as stereotyped behavior and

depression-like behavior in GluD1 KO mice. [31] In the present study, the results showed that CCl_4 increased the levels of active t-PA in rat brain homogenates (Figure 5A), which were in agreement with previous reports. [13] The injured neurons in CCl_4 treated mice may provoke enhancement of localized t-PA activity through formation of aggregates that facilitate t-PA binding in the injured area. [32] Alternatively, the induction of active t-PA in the brain was probably due to transcriptional activation of the respective genes in neuronal or glial cells. [15] This results that quercitrin, like other flavoinoids, decreased levels of t-PA activity, which may be attributed to inhibited oxidative stress, decreased secretion of t-PA by neuronal cells, decreased transcription of the t-PA gene in neuronal and glial cells, or even to reduction in t-PA binding sites as a result of restricted neuronal damage. [15,33] The results of NR2B in brain hippocampus of CCl_4-treated mice were much higher than these in the control group, suggesting that CCl_4 caused the neurotransmission system dysfunction by affecting the function of NMDA receptors and some glutamate (Glu) transporters. [11] While quercitrin may display the neuroprotective effect associated with decreasing NR2B level in mice (Figure 5B).

Acetylcholine is considered as one of the important neurotransmitter implicated in memory function. One of the major markers for cholinergic function is the determination of AChE activity. [35] AChE hyperactivity can cause memory deficit in animal model. [10] Several studies reported that oxidative stress was related to the AChE activity in the brain. [35,36] In this study, CCl_4 markedly increased the AChE activity and oxidative stress in the mouse brain, which was in agreement with the previous research.[10] Monoamine oxidase (MAO) plays a vital role in the inactivation of neurotransmitters. MAO dysfunction (too much/too little MAO activity) is thought to be responsible for various mental and neurodegenerative disorders. [11,37] MAO, which constitutes the major pathway for inactivation of the catecholamine neurotransmitters, produces hydrogen peroxide and triggers the formation of other forms of ROS as well, which can induce neuronal damage. MAO can therefore be regarded as a key marker of the cellular antioxidant and pro-oxidant status. [38,39] Our results revealed a significant increase in MAO activity in brain tissue along CCl_4

induction, suggesting that CCl_4 induced neurotransmitter dysfunction and oxidative stress. However, quercitrin treatment markedly decreased the activities of AChE and MAO in mouse brain, indicating its potent neuroprotective capacity (Figure 6).

Various inflammatory cytokines play a significant role in the development of an acute or chronic inflammatory response. TNF-α and IL-6 have been widely considered to be the most important inflammatory cytokines in CCl_4 induced brain injury.[11] It was well known that oxidative stress also stimulates the release of these cytokines and amplify the inflammation response to cause further damage.[13,17,39] Moreover, the increased CYP2E1 and produced ROS accompanied with oxidative stress can activate the inflammation process by producing TNF-α, IL-6 and other pro-inflammatory cytokines.[11,40] Our study showed increased levels of CYP2E1 and proinflammatory cytokines in CCl_4-exposed mouse brain hippocampus and quercitrin treatment could effectively suppress them (Figure 3 and 7). These results indicated that quercitrin application markedly inhibited CCl_4-induced oxidative stress and inflammation in mouse brains.

In conclusion, this is the first report that quercitrin has protective effects on brain injury exposed to CCl_4. The primary mechanisms of this effect could be due to attenuating brain oxidative stress, suppressing inflammation, and ameliorating neurotransmitter dysfunction (Figure 8). Quercitrin decreased activities of ROS, CYP2E1, MDA, t-PA, NR2B, AChE and MAO, and increased the activities of antioxidant enzymes in mouse brains exposed to CCl_4. Quercitrin also suppressed levels of key pro-inflammatory cytokines triggered by an inflammatory stimulus. Overall, despite the positive effects of quercitrin on CCl_4-induced brain injury, more indepth mechanistic studies are needed to support these beneficial effects.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Figure captions:

Figure.1. Chemical structure of quercitrin (QE)

Figure.2. Effect of quercitrin (QE) on the oxidative stress markers in CCl4-treated mouse brains. (A) Level of ROS; (B) Level of MDA. Each value is expressed as mean±S.E.M. (n=7). ## P<0.01, compared with the control group; ** $P < 0.01$, vs. CCl_4-treated group.

Figure.3. Quercitrin (QE) reduced the CAP2E1 level in the brains of mice exposed to CCl_4. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Each value is expressed as mean±S.E.M. ** $P < 0.01$, compared with the control group; ## $P < 0.01$, vs. the CCl_4-treated group.

Figure.4. Effect of quercitrin (QE) on the antioxidant enzyme activities in the brains of mice exposed to CCl_4. (A) CAT activity; (B) SOD activity; (C) GPx activity. All values are expressed as mean ± S.E.M. (n=7). ## $P < 0.01$, compared with the control group; **P < 0.01, vs. CCl_4-treated group.

Figure.5. Effect of quercitrin (QE) on the activities of t-PA and NR2B in the brains of mice exposed to CCl_4. (A) t-PA activity; (B) The level of NR2B. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Each value is expressed as mean±S.E.M. ** $P < 0.01$, compared with the control group; ## $P < 0.01$, vs. the CCl_4-treated group.

Figure.6. Effect of quercitrin (QE) on the activities of AChE and MAO in the brains of mice exposed to CCl_4. (A) AChE activity; (B) MAO activity. All values are expressed as mean ± S.E.M. (n=7). ## $P < 0.01$, compared with the control group; *P < 0.05,**P < 0.01, vs. CCl_4-treated group.

Figure.7. Western blot analysis of the protein immunocontent in association with inflammation in the mouse brains. (A) The level of TNF-α; (B) The level of IL-6. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Each value is expressed as mean±S.E.M. ** $P < 0.01$, compared with the control group; ## $P < 0.01$, vs. the CCl_4-treated group.

Figure.8. Schematic diagram showing the protective effects of quercitrin in CCl_4-induced brain injury. The → indicates activation or induction, and ┤ indicates inhibition or blockade.

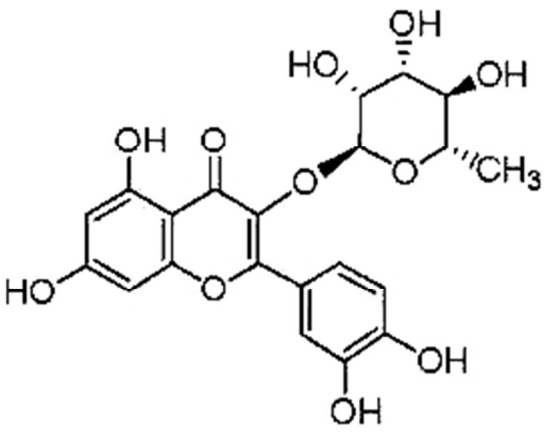

Figure.1. Chemical structure of quercitrin (QE)
73x56mm (96 x 96 DPI)

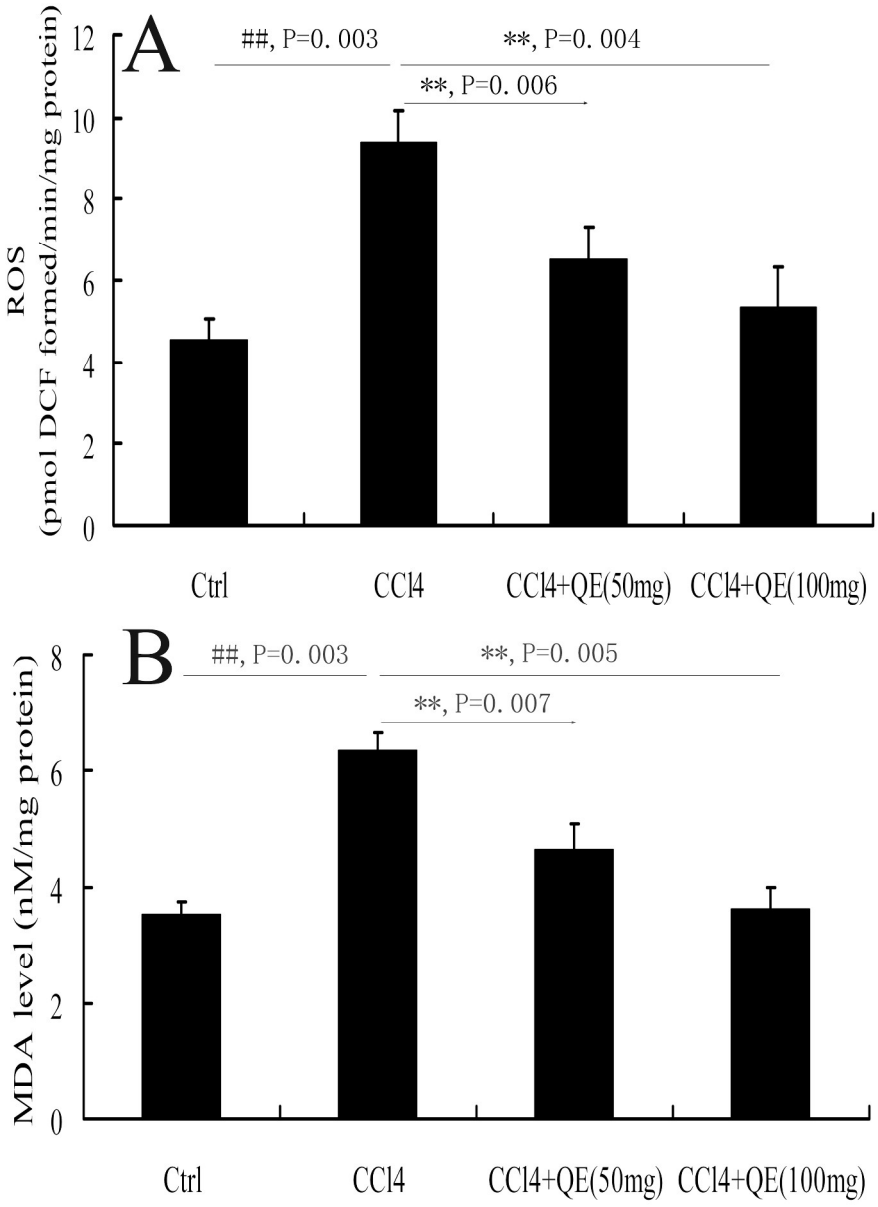

Figure.2. Effect of quercitrin (QE) on the oxidative stress markers in CCl4-treated mouse brains. (A) Level of ROS; (B) Level of MDA. Each value is expressed as mean±S.E.M. (n=7). ## P<0.01, compared with the control group; ** P < 0.01, vs. CCl4-treated group.
122x167mm (600 x 600 DPI)

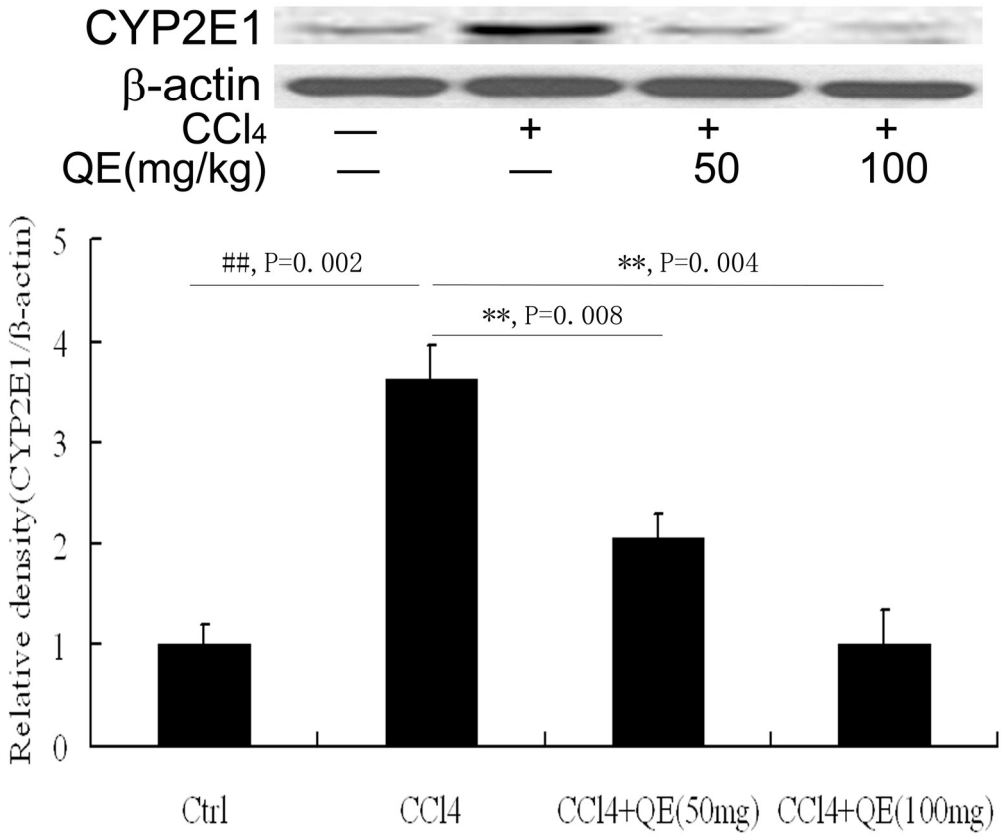

Figure.3. Quercitrin (QE) reduced the CAP2E1 level in the brains of mice exposed to CCl4. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Each value is expressed as mean±S.E.M. ** P < 0.01, compared with the control group; ## P < 0.01, vs. the CCl4-treated group.
84x70mm (600 x 600 DPI)

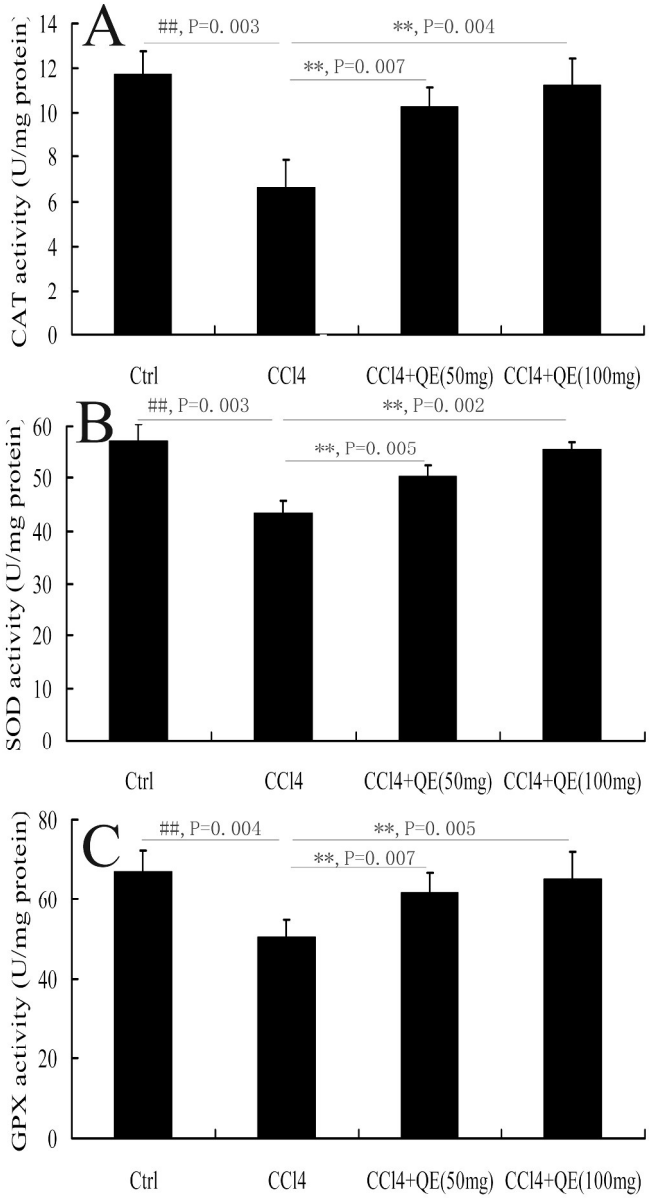

Figure.4. Effect of quercitrin (QE) on the antioxidant enzyme activities in the brains of mice exposed to CCl4. (A) CAT activity; (B) SOD activity; (C) GPx activity. All values are expressed as mean ± S.E.M. (n=7). ## P < 0.01, compared with the control group; **P < 0.01, vs. CCl4-treated group.
165x305mm (600 x 600 DPI)

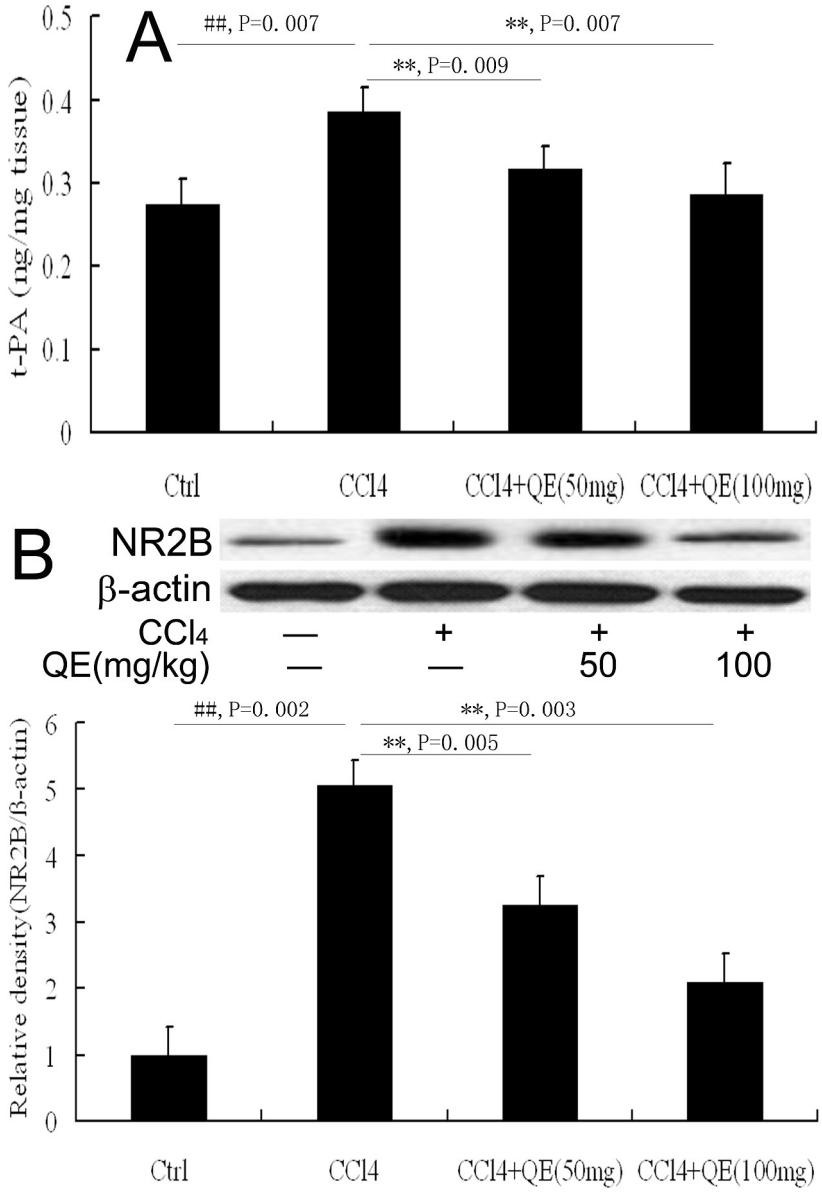

Figure.5. Effect of quercitrin (QE) on the activities of t-PA and NR2B in the brains of mice exposed to CCl4. (A) t-PA activity; (B) The level of NR2B. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Each value is expressed as mean±S.E.M. ** P < 0.01, compared with the control group; ## P < 0.01, vs. the CCl4-treated group.
146x213mm (600 x 600 DPI)

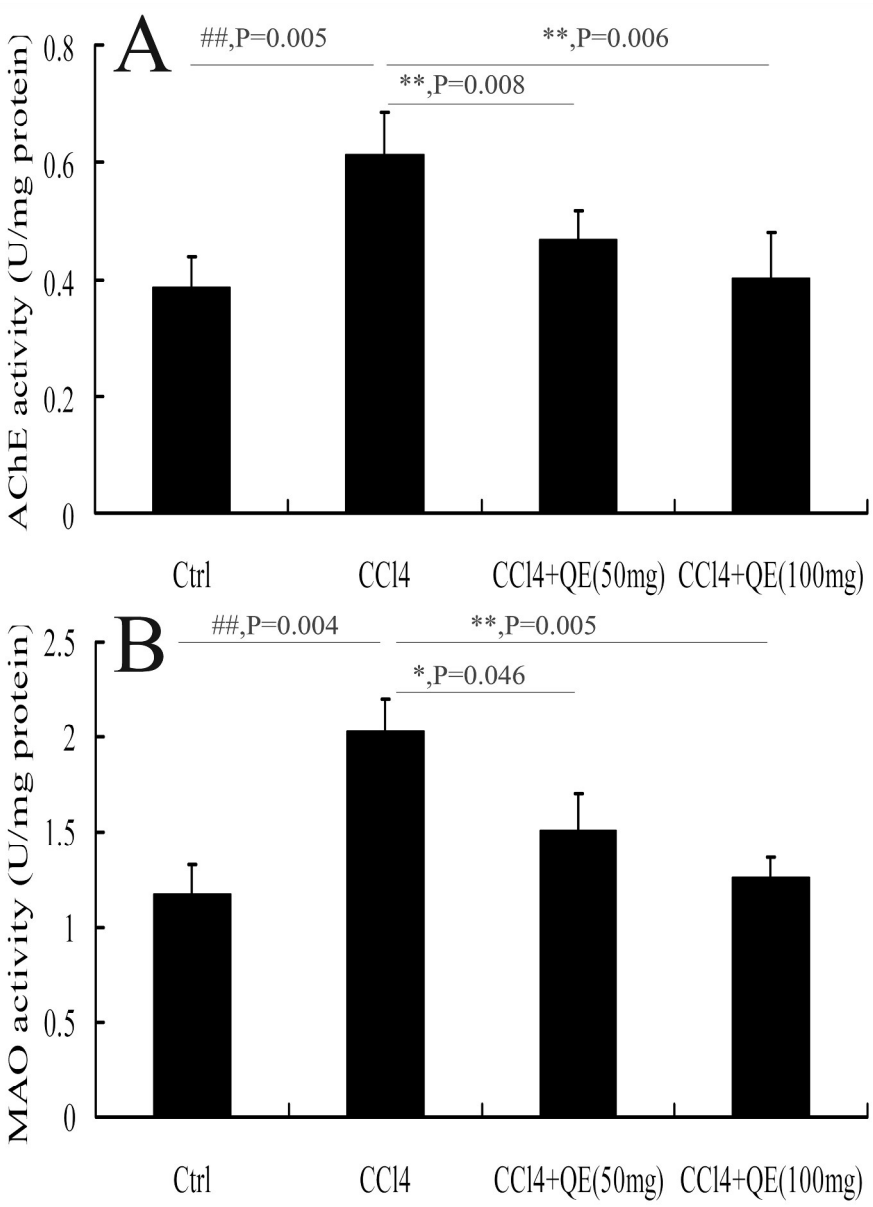

Figure.6. Effect of quercitrin (QE) on the activities of AChE and MAO in the brains of mice exposed to CCl4. (A) AChE activity; (B) MAO activity. All values are expressed as mean ± S.E.M. (n=7). ## P < 0.01, compared with the control group; *P < 0.05,**P < 0.01, vs. CCl4-treated group.
122x167mm (600 x 600 DPI)

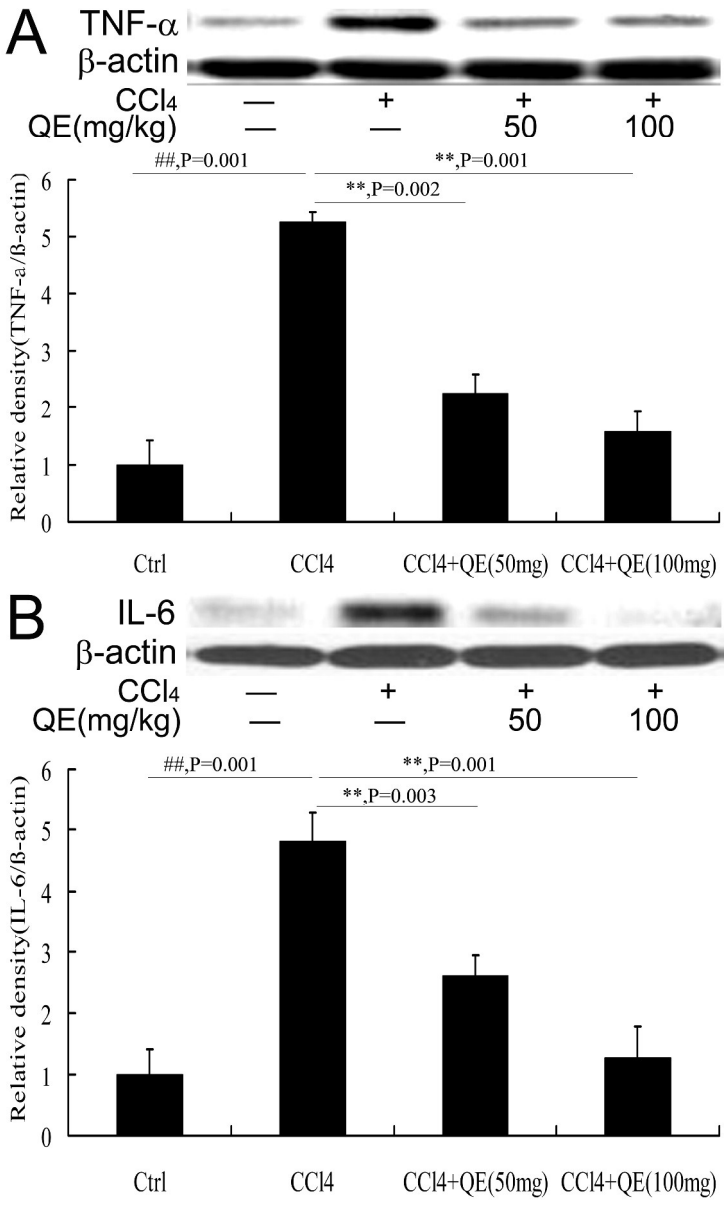

Figure.7. Western blot analysis of the protein immunocontent in association with inflammation in the mouse brains. (A) The level of TNF-α; (B) The level of IL-6. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Each value is expressed as mean±S.E.M. ** P < 0.01, compared with the control group; ## P < 0.01, vs. the CCl4-treated group.
164x272mm (600 x 600 DPI)

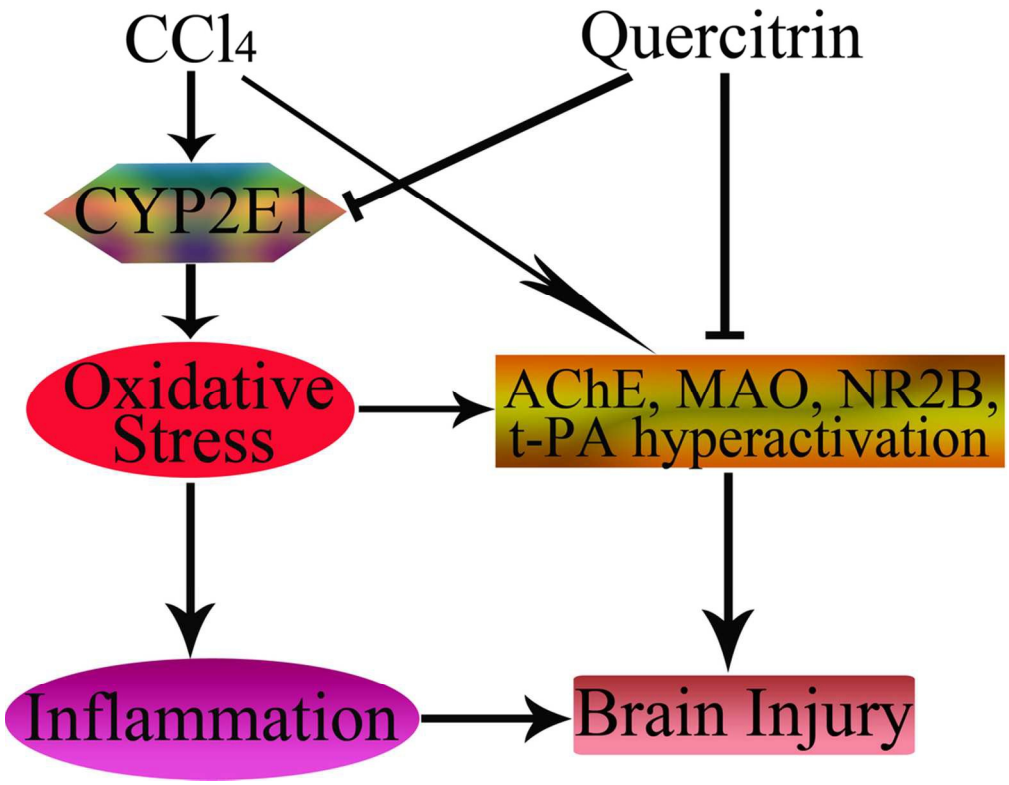

Figure.8. Schematic diagram showing the protective effects of quercitrin in CCl4-induced brain injury. The → indicates activation or induction, and ┤indicates inhibition or blockade.
47x37mm (600 x 600 DPI)