RESEARCH PAPER

Rutin protects against cognitive deficits and brain damage in rats with chronic cerebral hypoperfusion

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BACKGROUND AND PURPOSE

Chronic cerebral hypoperfusion is a critical causative factor for the development of cognitive decline and dementia in the elderly, which involves many pathophysiological processes. Consequently, inhibition of several pathophysiological pathways is an attractive therapeutic strategy for this disorder. Rutin, a biologically active flavonoid, protects the brain against several insults through its antioxidant and anti-inflammatory properties, but its effect on cognitive deficits and brain damage caused by chronic cerebral hypoperfusion remains unknown. Here, we investigated the neuroprotective effect of rutin on cognitive impairments and the potential mechanisms underlying its action in rats with chronic cerebral hypoperfusion.

EXPERIMENTAL APPROACH

We used Sprague-Dawley rats with permanent bilateral common carotid artery occlusion (BCCAO), a well-established model of chronic cerebral hypoperfusion. After rutin treatment for 12 weeks, the neuroprotective effect of rutin in rats was evaluated by behavioural tests, biochemical and histopathological analyses.

KEY RESULTS

BCCAO rats showed marked cognitive deficits, which were improved by rutin treatment. Moreover, BCCAO rats exhibited central cholinergic dysfunction, oxidative damage, inflammatory responses and neuronal damage in the cerebral cortex and hippocampus, compared with sham-operated rats. All these effects were significantly alleviated by treatment with rutin.

CONCLUSION AND IMPLICATIONS

Our results provide new insights into the pharmacological actions of rutin and suggest that rutin has multi-targeted therapeutical potential on cognitive deficits associated with conditions with chronic cerebral hypoperfusion such as vascular dementia and Alzheimer's disease.

Abbreviations

AD, Alzheimer's disease; BCCAO, bilateral common carotid artery occlusion; CBF, cerebral blood flow; ChAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein; GPX, glutathione peroxidase; MCI, mild cognitive impairment; MDA, malondialdehyde; PD, Parkinson's disease; SOD, superoxide dismutase

Introduction

Reduction of cerebral blood flow (CBF) is a very common event in the brain of elderly subjects and is an important prominent risk factor for geriatric cognitive dysfunction (Tanaka *et al.*, 2002; Hanyu *et al.*, 2010). A large body of evidence from clinical studies has shown that the decreased CBF is often observed in patients with mild cognitive impairment (MCI) (Staffen *et al.*, 2006; Kume *et al.*, 2011), vascular dementia (Schuff *et al.*, 2009; Gao *et al.*, 2013) and Alzheimer's disease (AD) (Nobili *et al.*, 2009; Schuff *et al.*, 2009; Gao *et al.*, 2013), indicating a significant association between CBF deficits in the brain and subsequent cognitive deterioration. Also, the decrease in CBF caused by chronic cerebral hypoperfusion is associated with cognitive decline in ageing and contributes to the onset of clinical cognitive impairments and the progression of dementia (Ruitenberg *et al.*, 2005; Farkas *et al.*, 2007; Hanyu *et al.*, 2010). Thus, chronic cerebral hypoperfusion is thought to be a pivotal contributing factor of cognitive impairments that occurs in human ageing, MCI, vascular dementia and late-onset AD (Ruitenberg *et al.*, 2005; de la Torre, 2012). Although significant advances have been made in understanding the pathophysiology underlying chronic cerebral hypoperfusion-induced cognitive deficits, current treatments are limited in terms of their effectiveness and utility (Levine and Langa, 2011; Baskys and Cheng, 2012). There is a need for investigating potentially neuroprotective ccompounds for pharmacological prevention and treatment of cognitive deficits and dementia associated with chronic cerebral hypoperfusion, in order to determine whether clinical investigation is justified.

Rutin (quercetin-3-rutinoside hydrate; Figure 1), a flavonoid present in many foods and plants (such as buckwheat seed, passion flower, onion, oranges, apple, lemon, grapes, tea and red wine), exhibits neuroprotective effects via its antioxidative and anti-inflammatory properties (Sharma *et al.*, 2013). *In vitro,* rutin decreased Aβ42-induced cytotoxicity, oxidative stress and pro-inflammatory cytokines in SH-SY5Y neuroblastoma cells (Wang *et al.*, 2012), and protected pheochromocytoma (PC12) cells against 6-hydroxydopamine-induced neurotoxicity by improving antioxidant activities (Magalingam *et al.*, 2013). Moreover, *in vivo*, rutin protected rats against brain injury by several insults, such as trimethyl-

tin (Koda *et al.*, 2009), streptozotocin (Kamalakkannan and Stanely Mainzen Prince, 2006), 6-hydroxydopamine (Khan *et al.*, 2012) and focal ischaemia (Khan *et al.*, 2009; Annapurna *et al.*, 2013; Rodrigues *et al.*, 2013), via its anti-oxidative and anti-inflammatory properties. Importantly, rutin treatment attenuated cognitive deficits in rats with repeated cerebral ischaemia (Pu *et al.*, 2007), trimethyltin treatment (Koda *et al.*, 2008), kindled with pentylenetetrazole (Nassiri-Asl *et al.*, 2010), given i.c.v infusions of streptozotocin (Javed *et al.*, 2012) or aged rats (Pyrzanowska *et al.*, 2012). This treaemtn was also effective in scopolamine-injectedzebrafish (Richetti *et al.*, 2011) and dexamethasone-treated mice (Tongjaroenbuangam *et al.*, 2011). All these findings indicate that rutin is a neuroprotective polyphenol, and that, after systemic administration (p.o. or i.p.) ,rutin did pass through the blood–brain barrier to play its neuroprotective roles (Kamalakkannan and Stanely Mainzen Prince, 2006; Pu *et al.*, 2007; Koda *et al.*, 2008; 2009; Khan *et al.*, 2009; 2012; Nassiri-Asl *et al.*, 2010; Richetti *et al.*, 2011; Tongjaroenbuangam *et al.*, 2011; Javed *et al.*, 2012; Pyrzanowska *et al.*, 2012; Annapurna *et al.*, 2013; Rodrigues *et al.*, 2013).

Chronic cerebral hypoperfusion is a critical causative factor for the development of cognitive decline and dementia in the elderly, involving many pathophysiological processes. Therefore, inhibition of multiple pathophysiological pathways should be an attractive therapeutic strategy for cognitive deficits and dementia (Levine and Langa, 2011; Baskys and Cheng, 2012). However, it is not known if rutin has neuroprotective effects against cognitive impairments and brain injury induced by chronic cerebral hypoperfusion. The objective of the present study was to address these issues and investigate the potential mechanisms underlying its action in rats with chronic cerebral hypoperfusion.

Methods

Animals and surgical procedures

All animal care and experimental procedures were in accordance with the University Policies on the Use and Care of Animals and were approved by the Institutional Animal Experiment Committee of Fourth Military Medical University, China. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 40 animals were used in the experiments described here.

Adult male Sprague-Dawley rats (aged 6 months; weighing 380 ± 30 g) were obtained from the Laboratory Animal Center of Fourth Military Medical University (Xi'an, China). Each rat was housed in a single cage and maintained in temperature- and humidity-controlled rooms with *ad libitum* access to food and water throughout the experimental period. Chronic cerebral hypoperfusion was produced in rats with permanent bilateral common carotid artery occlusion (BCCAO), as described earlier (Cechetti *et al.*, 2012; Xi *et al.*, 2014). In brief, after rats were anaesthetized with i.p. administration of sodium pentobarbital (50 mg·kg^{-1}), the bilateral common carotid arteries were ligated with 5-0 type surgical
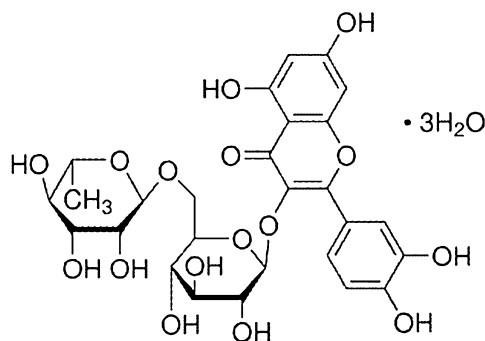
Figure 1
Chemical structure of rutin.

silk suture in BCCAO rats, whereas the sham-operated control rats received the same surgical procedures without common carotid artery ligation., Rats were placed on a heating pad to maintain body temperature at $37.5 \pm 0.5°C$ during the surgical procedure and after surgery until they had recovered from anaesthesia.

Drug administration

Three days after surgery, the BCCAO rats and the sham-operated rats were randomly assigned into the following four groups ($n = 10$, each group): vehicle-treated BCCAO rats, rutin-treated BCCAO rats, vehicle-treated sham-operated rats and rutin-treated sham-operated rats. Each rat with rutin treatment received rutin hydrate (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline once daily for 12 weeks by i.p. injection (50 mg·kg^{-1} in 0.5 mL of saline), whereas each rat with vehicle treatment received equal volume of saline injection (0.5 mL) as control according to the same schedule. The dose of rutin used in this study was chosen based on previous studies (Pu et al., 2007; Richetti et al., 2011; Rodrigues et al., 2013).

Morris water maze test

After the treatment for 12 weeks, the hippocampus-dependent spatial learning and memory of all rats were evaluated by the Morris water maze test as previously described (Cechetti et al., 2012; Xi et al., 2014). In brief, for the acquisition training trials (four trials per day for 5 consecutive days), the rats were required to find the hidden platform (10 cm in diameter, submerged 1.5 cm below the water surface). In each trial, the rats were given a maximum of 120 s to find the submerged platform. If a rat failed to find the platform within 120 s, the training was terminated and a maximum score of 120 s was assigned. The time that an individual rat took to reach the hidden platform was recorded as the escape latency for its spatial learning score.

Twenty-four hours after the acquisition phase, a probe test was conducted by removing the platform from the tank to assess the spatial memory. Rats were allowed to swim freely for 60 s. The percentage of time that an individual rat spent in the target quadrant previously containing the platform was recorded as a measure of spatial memory. Trajectories of all animals were monitored and achieved with a computerized tracking system (Hampton Visual Systems Image 2020, Hampton, UK).

Delayed alternation task in the T-maze

Two days after the Morris water maze test, the cortex-dependent working memory of all rats was evaluated by the delayed alternation task in the T-maze, as previously described (Mizoguchi et al., 2009; Xi et al., 2014). Briefly, all rats were food restricted and maintained at approximately 90% of normal intake per day throughout the experiment. The rats were habituated to the T-maze apparatus with all of the doors open for 10 min for 4 days until they readily ate the sucrose pellets located at the end of each branch arm. After habituation, the delayed alternation task tests were subsequently started. Each trial consisted of an 'information run' and a 'test run'. During the experimental period of the information run, each rat was placed in the starting box of the

stem arm with one branch arm blocked by a guillotine door and was rewarded when entering either branch arm. For the test run, the rat was placed in the starting box again with both branch arms opened and was only rewarded for entering the branch arm that was not chosen in the information run (correct choice). After completing the training trials (10 trials per session), each rat performed 10 trials for the test run each delay time (0, 30 s). The number of errors per test run was recorded. The delay achieved in a set number of testing sessions can be used as an index of cognitive ability. The T-maze apparatus was wiped with alcohol to remove any olfactory cues between trials.

Passive avoidance test

The contextual memory of all rats was assessed by using the passive avoidance task in the two-way shuttle box as described previously (Ishrat et al., 2009; Sarkaki et al., 2013). Briefly, the apparatus consisted of two adjacent Plexiglas compartments with a guillotine door separated the two compartments. Each rat was allowed a 10 min adaptation period with free access to either the light or dark compartment after being placed in the shuttle box. One day after the adaptation, each rat was placed into the illuminated chamber for the acquisition trial, and 30 s later, the sliding door was opened, and the initial latency of animals to enter the dark chamber was recorded as acquisition latency. Upon entering the dark chamber, the sliding door was closed and a constant electric foot shock (75 V, 1.5 mA, 50 Hz) was given to the floor grids for 3 s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. For testing the retention memory, 24 h after the acquisition trial, the retention latency time was measured in the same way as in the acquisition trial without electric foot shock, and the latency time of entering the dark compartment was recorded again as retention latency. The maximum time considered in this procedure was 300 s.

Brain tissue preparation

After behavioural tests, all animals were deeply anaesthetized with sodium pentobarbital (100 mg·kg^{-1}, i.p.) and perfused transcardially with 200 mL of cold saline. Brains were removed and dissected through the mid-sagittal plane. The right hemisphere was fixed in 4% paraformaldehyde for 24 h and embedded in paraffin following standard methods for immunohistochemical and histological analyses. The cerebral cortex and hippocampus in the left hemisphere was quickly dissected on ice and immediately snap-frozen and stored at $-80°C$ to be used for the biochemical analyses.

Determination of oxidative stress

The oxidative stress markers were measured as described previously (Ruan et al., 2010; Zhang et al., 2012a). In brief, the brain tissues were weighed and homogenized in 9 volumes of ice-cold saline containing protease inhibitor mixture (Sigma-Aldrich) and centrifuged at $3000 \times g$ for 20 min at 4°C to obtain the supernatant. The supernatants were diluted with the appropriate buffer solution for determination of the relative biochemical index. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) as well as the contents of malondialdehyde (MDA) and protein

carbonyls were determined spectrophotometrically by using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The total protein concentration in the brain tissue supernatants was determined by using a standard commercial kit (Bio-Rad Laboratories, Hercules, CA, USA). All samples were run in triplicate.

Assay of cholinergic markers

The cholinergic markers were measured as described previously (Ruan *et al*., 2010; Zhang *et al*., 2012a). Briefly, each brain tissue was weighed and homogenized with homogenizer in 9 volumes of ice-cold saline containing protease inhibitor mixture (Sigma-Aldrich) and centrifuged at $3000 \times g$ for 10 min to obtain supernatant. The supernatant was further diluted with an appropriate buffer solution for the determination of the relevant biochemical index. The activities of choline acetyltransferase (ChAT) and AChE, as well as the content of ACh in the brain supernatants were measured spectrophotometrically with commercial assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The total protein concentration in the brain tissue supernatants was determined by using a standard commercial kit (Bio-Rad Laboratories). All samples were run in triplicate.

Determination of pro-inflammatory cytokines

The levels of pro-inflammatory cytokines were assessed as described previously (Bossù *et al*., 2012). Briefly, brain tissue samples were weighed and homogenized in an ice-cold lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg·mL^{-1} aprotinin, 1 μg·mL^{-1} leupeptin and 0.5 mM sodium vanadate, containing protease inhibitor mixture (Sigma-Aldrich). The tissue homogenates were centrifuged with $14\,000 \times g$ for 25 min at 4°C, and the supernatants were collected and stored at –80°C until analysis. The levels of IL-1β, IL-6 and TNF-α in the samples were determined using commercial ELISA kits (Invitrogen, Camarillo, CA, USA) in strict accordance with the manufacturer's instructions. The concentration of total protein in the brain supernatants was measured by the Bradford assay (Bio-Rad Laboratories). All samples were run in triplicate.

Assessment of neuroinflammatory cells

Immunohistochemical staining was performed as described previously (Zhang *et al*., 2012a,b). Briefly, sections were cut in the sagittal plane of 5 μm thick and mounted on slides. Sections at the level of hippocampus were deparaffinized and rehydrated. Antigen retrieval was performed by treatment with proteinase K (0.2 mg·mL^{-1}) for 10 min at room temperature for astrocyte staining, and by 10 mM sodium citrate solution (pH 6.0) for 20 min at 90°C in a water bath for activated microglia staining. Sections for non-specific binding were blocked by incubating in PBS containing 0.1% Triton X-100 and 2% BSA (Sigma-Aldrich) for 20 min at room temperature. The sections were incubated with the following primary antibodies overnight at 4°C for immunohistochemical analysis: mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) for the detection of astrocytes (1:500; Millipore, Billerica, MA, USA) and rabbit anti-Iba1 monoclo-

nal antibody for the detection of activated microglia (1:300; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Primary antibodies were detected with HRP-conjugated secondary antibodies and visualized with either a stable diaminobenzidine solution (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations.

For quantification of inflammatory cells, image analysis was performed using Image-Pro Plus imaging software (version 5.0; Media Cybernetics, Bethesda, MD, USA) as described previously (Zhang *et al*., 2012b). In brief, images were acquired from the series of adjacent sections stained for immunohistochemistry by using an Olympus microscope connected to a digital camera (Olympus BX50 microscope, DP50 digital camera; Olympus, Tokyo, Japan). The GFAP-positive astroglial cells and Iba1-positive microglial cells in the cerebral cortex and hippocampal CA1 region were counted at a 400× magnification in five visual fields chosen randomly from each rat. The number of immunopositive cells per mm^2 in each rat was recorded as the mean of the six sections.

Assessment of neuronal loss

The Nissl staining was performed as described previously (Annaházi *et al*., 2007). In brief, the sections adjacent to those used for immunohistochemical staining were deparaffinized, hydrated and dyed with 0.5% cresyl violet solution for about 1 min until the desired depth of staining was achieved. For quantification of the intact neurons, photographs of the cerebral cortex and hippocampus CA1 region were taken with a computerized image analysis system (Olympus BX50 microscope, DP50 digital camera; Olympus). Cell counting restricted to the cerebral cortex and hippocampus CA1 region was performed using Image-Pro Plus imaging software (version 5.0; Media Cybernetics). The number of intact neurons in the cerebral cortex and hippocampal CA1 region was quantitatively counted at a 400× magnification in five visual fields chosen randomly from each animal. The number of intact neurons per mm^2 in each rat was recorded as the mean of the six sections.

Data analysis

All data are presented as means ± SEM. Data of the escape latency and swimming speed in the Morris water maze test were analysed using two-way repeated-measures ANOVA followed by Fisher's least significant difference (LSD) *post hoc* test for multiple comparisons. Data of the delayed alternation performance were initially analysed using two-way ANOVA followed by *post hoc* LSD test for individual between-group comparisons. The other data were analysed using one-way ANOVA followed by *post hoc* LSD test for multiple comparisons. All analyses were performed with SPSS statistical package (version 17.0 for Windows, SPSS Inc., Chicago, IL, USA). For all statistical analyses, the *P* value < 0.05 was considered statistically significant.

Results

General observation

No significant difference in the body weight between the four groups of rats was found after the surgery for 12 weeks (*P* >

0.05; data not shown). In addition, no neurological symptoms (i.e. hemiplegic paralysis and seizure) and behavioural abnormalities (i.e. poor feeding, irritability and abnormal locomotor activity) in the four groups of rats were observed throughout the experimental period.
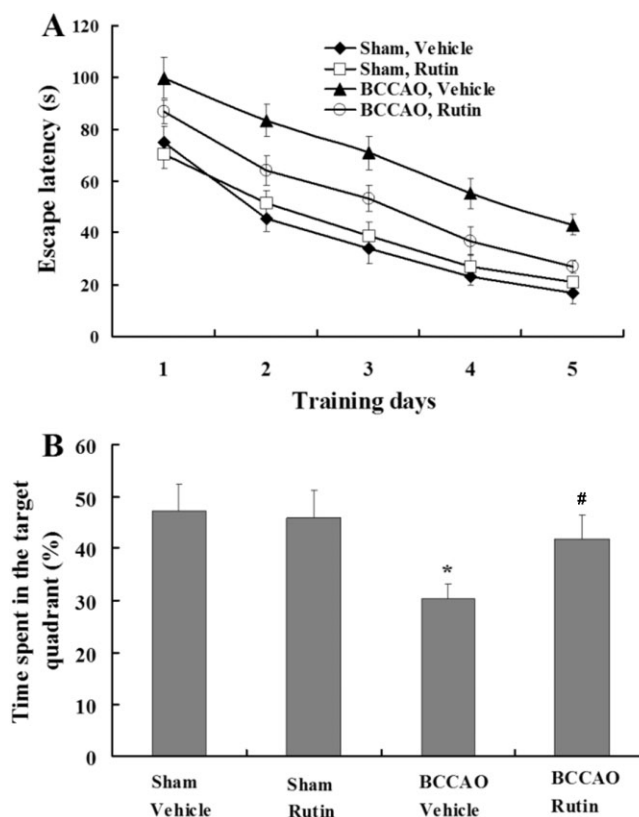
Rutin improves hippocampus-dependent spatial learning and memory impairments induced by BCCAO

The hippocampus-dependent spatial learning and memory functions of all rats were assessed by Morris water maze. Figure 2A shows the results of the time required to find the hidden platform (escape latency) of all rats during the water maze acquisition training. *Post hoc* analyses for multiple group comparisons showed that vehicle-treated BCCAO rats had significantly longer escape latency compared with the vehicle- and rutin-treated sham-operated rats ($P < 0.001$), showing that chronic cerebral hypoperfusion induced a learning deficit in vehicle-treated BCAAO rats. Repeated-measures ANOVA revealed a significant main treatment effect on the overall escape latency data among the four groups [$F_{(1,3)} = 13.593$, $P < 0.001$] and rutin treatment of BCAAO rats significantly decreased escape latency, compared with vehicle-treated BCAAO rats ($P < 0.01$). suggesting that rutin did improve the learning deficit in BCCAO rats.

The performances of spatial memory in rats are shown in Figure 2B. *Post hoc* analyses for multiple group comparisons showed that vehicle-treated BCAAO rats spent less time in the target quadrant compared to vehicle- and rutin-treated sham-operated rats ($P < 0.001$), indicating that there was spatial memory impairment in vehicle-treated BCAAO rats. One-way ANOVA revealed a significant treatment effect on the time spent in the target quadrant among the four groups [$F_{(1,3)} = 9.631$, $P < 0.001$], and rutin-treated BCAAO rats spent more time in the target quadrant, compared with vehicle-treated BCAAO rats ($P < 0.01$), suggesting that rutin treatment was effective in attenuating memory deficit in the BCAAO rats.

Rutin alleviates cortex-dependent memory deficits in BCCAO rats

Given that our recent study demonstrated that chronic cerebral hypoperfusion may cause cortex-dependent working memory dysfunction in the BCCAO rats (Xi *et al.*, 2014), we next examined the effect of rutin on the working memory function of all rats in the delayed alternation task using a T-maze apparatus. As shown in Figure 3, two-way ANOVA revealed a significant delay time effect [$F_{(3,18)} = 16.531$, $P < 0.001$]. *Post hoc* test revealed that the performances under no-delay (0 s) condition did not differ among the four groups of rats ($P > 0.05$). However, the performances under 30-s-delay condition in vehicle-treated BCCAO rats were significantly decreased compared with the vehicle- and rutin-treated sham-operated rats ($P < 0.001$), indicating impaired working memory in vehicle-treated BCCAO rats. Rutin treatment significantly attenuated the decreased performances in vehicle-treated BCCAO rats ($P < 0.01$), indicating improved cortex-dependent working memory in rutin-treated BCCAO rats.
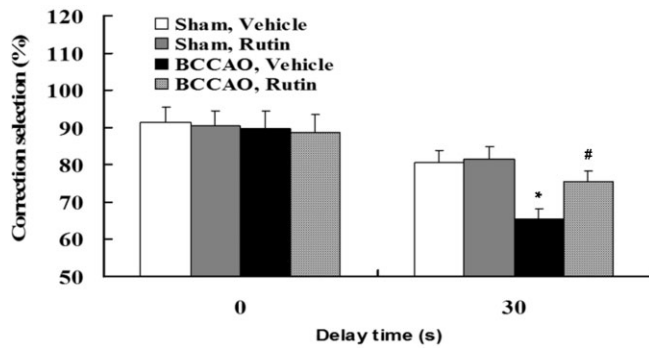

Figure 2

Effect of rutin on the spatial learning and memory deficits induced by BCCAO in rats. The hippocampus-dependent spatial learning and memory in rats treated with vehicle or rutin were assessed by the Morris water maze. (A) Mean escape latency during acquisition training trials of the hidden-platform water maze task. Vehicle-treated BCCAO rats took longer to find the hidden platform (escape latency) than the vehicle- and rutin-treated sham-operated rats ($P < 0.001$), whereas rutin treatment in BCCAO rats significantly decreased the escape latency compared with the vehicle-treated BCCAO rats ($P < 0.01$). No significant difference in the escape latency was observed between vehicle- and rutin-treated sham-operated rats ($P > 0.05$). (B) Spatial memory was evaluated 24 h after the acquisition training trials. Vehicle-treated BCCAO rats had significantly less time spent in the target quadrant compared with vehicle- and rutin-treated sham-operated rats, whereas rutin-treated BCCAO rats had significantly more time spent in the target quadrant compared with vehicle-treated BCCAO rats. No significant difference in the time spent in the target quadrant was found between vehicle- and rutin-treated sham-operated rats. Data are expressed as mean ± SEM ($n = 10$ rats per group). *$P < 0.001$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.01$ versus vehicle-treated BCCAO rats.
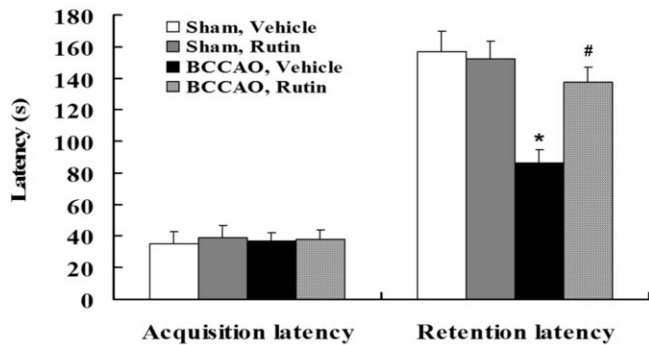
Rutin attenuates the contextual memory deficits in BCCAO rats

To further confirm the improvement of memory impairment by rutin, the contextual memory performance of all rats was assessed using the step-through passive avoidance task, which is widely used to evaluate drug effects on the contextual memory in rodents (Meunier *et al.*, 2006; Yamamoto *et al.*, 2009). As shown in Figure 4, the acquisition latency in the acquisition trail did not differ among the four groups of
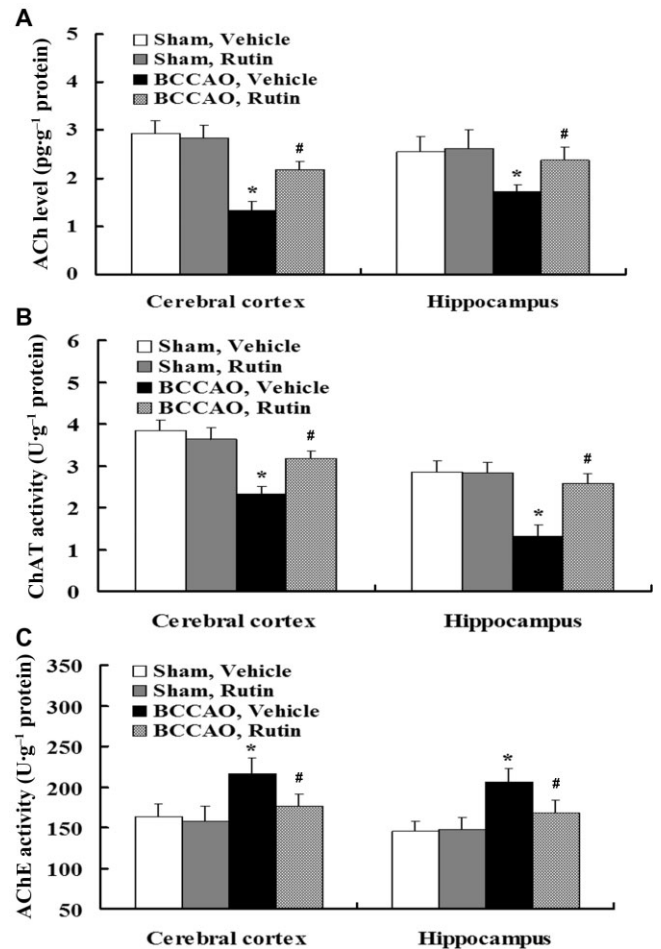
Figure 3

Effect of rutin on the working memory deficits induced by BCCAO in rats. The cortex-dependent working memory in rats treated with vehicle or rutin was evaluated by the delayed alternation task in the T-maze. No significant differences in the performance of the delayed alternation task with no delay (0 s) were observed among the four groups of rats. However, vehicle-treated BCCAO rats made significantly more errors compared with the vehicle- and rutin-treated sham-operated rats in T-maze performance with 30 s delay, whereas rutin-treated BCCAO rats had significantly less errors compared with vehicle-treated BCCAO rats. Data are expressed as mean ± SEM ($n = 10$ rats per group). *$P < 0.001$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.01$ versus vehicle-treated BCCAO rats.

Figure 4

Effect of rutin on the contextual memory deficits induced by BCCAO in rats. The contextual memory in rats treated with vehicle or rutin was assessed by using the step-through passive avoidance task. No significant differences in the acquisition latency in the passive avoidance task were observed among the four groups of rats. However, the retention latency in the passive avoidance test was significantly decreased in vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats, whereas rutin treatment in BCCAO rats significantly prolonged the retention latency compared with the vehicle-treated BCCAO rats. Data are expressed as mean ± SEM ($n = 10$ rats per group). *$P < 0.001$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.01$ versus vehicle-treated BCCAO rats.

rats ($P > 0.05$). However, the retention latency was significantly decreased in the vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats ($P < 0.001$), indicating that BCCAO in rats may result in impaired contextual memory in the passive avoidance task. Whereas, rutin treatment significantly restored the reduction in reten-
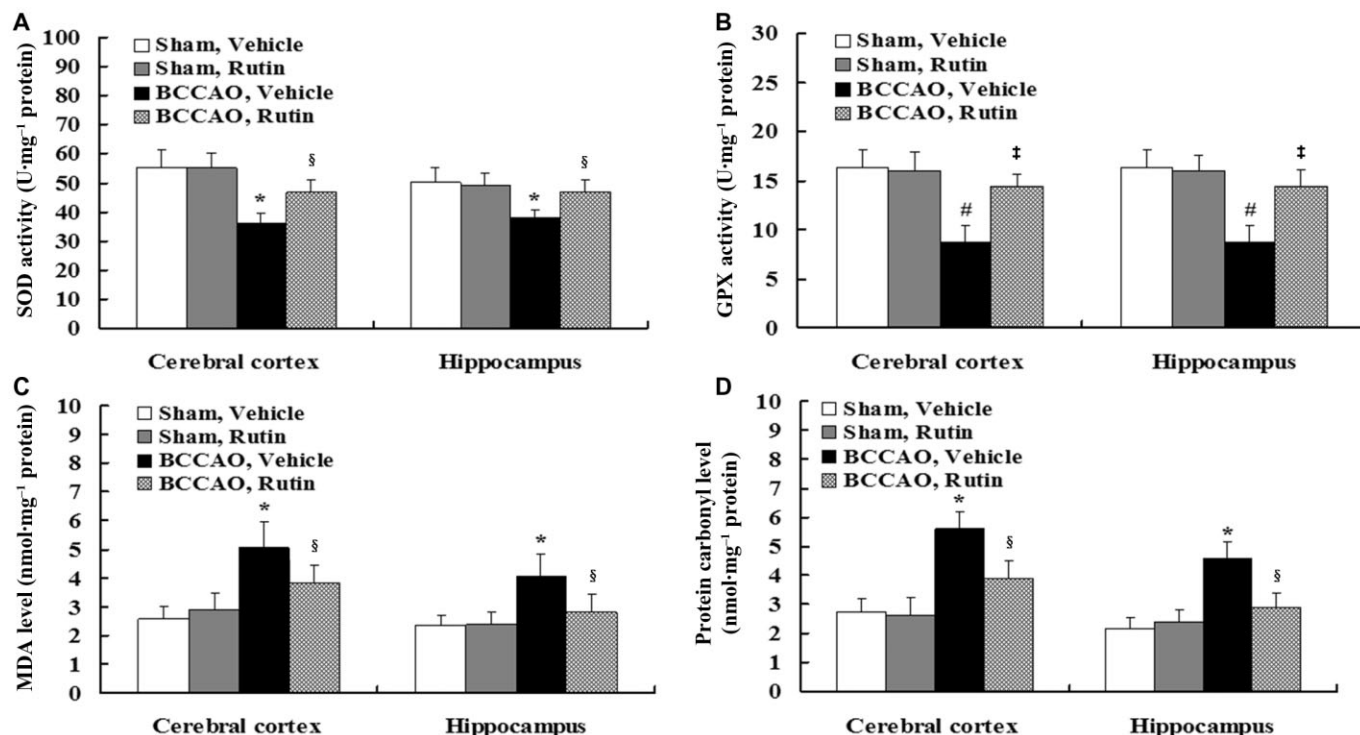

Figure 5

Effect of rutin on the central cholinergic dysfunction induced by BCCAO in rats. The levels of ACh, ChAT and AChE in the cerebral cortex and hippocampus were spectrophotometrically measured with assay kits. Vehicle-treated BCCAO rats had lower ACh level (A) and ChAT activity (B) and higher AChE activity (C) in the cerebral cortex and hippocampus compared with vehicle- and rutin-treated sham-operated rats. Whereas, rutin treatment in BCCAO rats significantly alleviated the decrease of ACh level (A) and ChAT activity (B) as well as the increase of AChE activity (C) compared with vehicle-treated BCCAO rats. Data are expressed as mean ± SEM ($n = 10$ rats per group). *$P < 0.01$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.01$ versus vehicle-treated BCCAO rats.

tion latency time of vehicle-treated BCCAO rats ($P < 0.01$), indicating improved the contextual memory impairment in rutin-treated BCCAO rats.

Rutin alleviates the central cholinergic dysfunction caused by BCCAO

Biochemical analyses revealed that vehicle-treated BCCAO rats showed a significant decrease of ACh level (Figure 5A; $P < 0.01$) and ChAT activity (Figure 5B; $P < 0.01$) as well as a significant increase of AChE activity (Figure 5C; $P < 0.01$) in the cerebral cortex and hippocampus compared with vehicle- and rutin-treated sham-operated rats. Because no significant

Figure 6

Effect of rutin on increased oxidative stress induced by BCCAO in rats. The contents of endogenous antioxidant enzymes, lipid oxidation and protein oxidation in the cerebral cortex and hippocampus were spectrophotometrically measured by using assay kits. Vehicle-treated BCCAO rats had the decreased contents of SOD activity (A) and GPX activity (B) as well as the increased levels of MDA (C) and protein carbonyls (D) in the cerebral cortex and hippocampus compared with vehicle- and rutin-treated sham-operated rats, whereas rutin treatment in BCCAO rats markedly attenuated the changes in SOD activity (A), GPX activity (B), MDA (C), and protein carbonyls (D) compared with vehicle-treated BCCAO rats. Data are expressed as mean ± SEM (n = 10 rats per group). *$P < 0.01$, #$P < 0.001$ versus vehicle- and rutin-treated sham-operated rats; §$P < 0.01$, ‡P < 0.001 versus vehicle-treated BCCAO rats.

differences in ACh level, ChAT activity and AChE activity were observed between vehicle- and rutin-treated sham-operated rats (Figure 5A–C; $P > 0.05$,), this indicates that the central cholinergic dysfunction was caused by chronic cerebral hypoperfusion. Interestingly, rutin treatment significantly alleviated the decrease of ACh level (Figure 5A; $P < 0.01$) and ChAT activity (Figure 5B; $P < 0.01$) as well as the increase of AChE activity (Figure 5C; $P < 0.01$) induced by cerebral hypoperfusion compared with vehicle-treated BCCAO rats. These findings suggest that rutin may significantly improve central cholinergic dysfunction caused by BCCAO.

### Rutin attenuates the oxidative damage induced by BCCAO

Biochemical analyses showed that the contents of SOD activity (Figure 6A; $P < 0.01$) and GPX activity (Figure 6B; $P < 0.001$) were significantly decreased and the levels of MDA (Figure 6C; $P < 0.01$) and protein carbonyl (Figure 6D; $P < 0.01$) were significantly elevated in the cerebral cortex and hippocampus of vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats. Given that no significant differences in SOD, GPX, MDA and protein carbonyl were observed between vehicle- and rutin-treated

sham-operated rats (Figure 6A–D; $P > 0.05$, respectively), this indicates that increased oxidative damage was induced by chronic cerebral hypoperfusion. However, rutin treatment markedly attenuated the decreased SOD activity (Figure 6A; $P < 0.01$) and GPX activity (Figure 6B; $P < 0.001$) as well as increased MDA (Figure 6C; $P < 0.01$) and protein carbonyl (Figure 6D; $P < 0.01$) compared with the vehicle-treated BCCAO rats. These findings suggest that rutin may significantly attenuate the oxidative damage induced by BCCAO.

Rutin inhibits glial activation in BCCAO rats

Immunohistochemical staining showed a few of GFAP-positive astrocytes in the cerebral cortex and hippocampus of vehicle-treated sham-operated rats (Figure 7A and E) and rutin-treated sham-operated rats (Figure 7B and F). In contrast, reactive astrocytes were markedly increased in the cerebral cortex and hippocampus of vehicle-treated BCCAO rats (Figure 7C and G), whereas reactive astrocytes were markedly decreased in rutin-treated BCCAO rats compared to vehicle-treated BCCAO rats (Figure 7D and H). Quantitative analysis revealed that vehicle-treated BCCAO rats had a significant increase in the amount of reactive astrocytes in the cerebral cortex and hippocampus compared with vehicle- and rutin-treated sham-operated rats (Figure 7I; $P < 0.001$), whereas
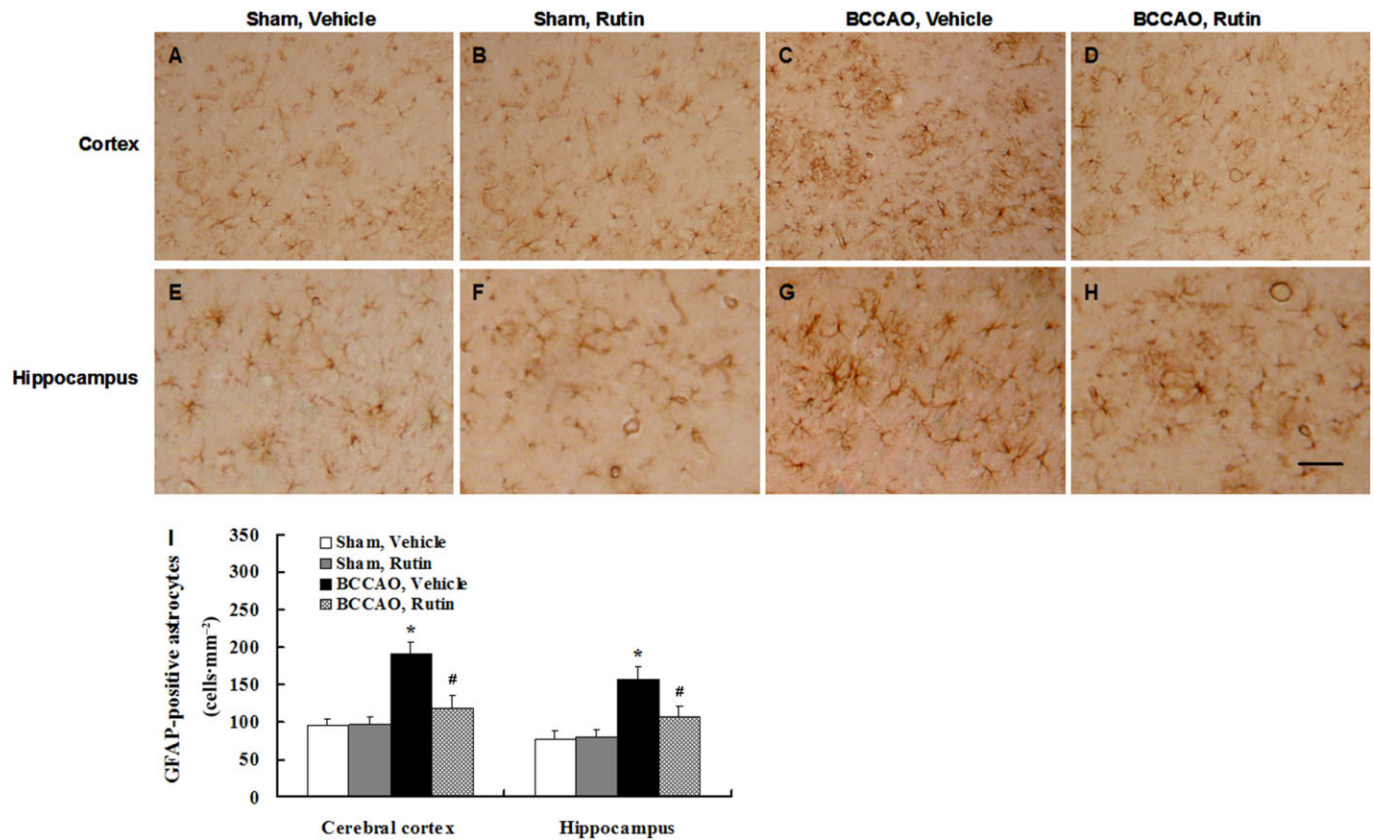
Figure 7

Effect of rutin on the reactive astrocytes induced by BCCAO in rats. Astrocytes were labelled by immunohistochemical staining with GFAP antibody. (A–H) Representative photomicrographs of GFAP-positive immunostaining in each group. A few of GFAP-positive astrocytes were observed in the cerebral cortex and hippocampus of vehicle-treated sham-operated rats (A, E) and rutin-treated sham-operated rats (B, F). In contrast, robust reactive astrocytes were seen in vehicle-treated BCCAO rats (C, G), whereas a marked reduction of reactive astrocytes was found in rutin-treated BCCAO rats (D, H). Quantitative analysis disclosed that the amount of reactive astrocytes in the cerebral cortex and hippocampus was significantly increased in vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats (I), whereas rutin treatment in BCCAO rats significantly reduced the number of reactive astrocytes in the cerebral cortex and hippocampus compared with vehicle-treated BCCAO rats (I). Data are expressed as mean ± SEM (n = 10 rats per group). *$P < 0.001$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.01$ versus vehicle-treated BCCAO rats. Scale bar = 200 μm.

chronic administration of rutin in BCCAO rats substantially reduced the amount of reactive astrocytes in the cerebral cortex and hippocampus compared with vehicle-treated BCCAO rats (Figure 7I; $P < 0.01$).
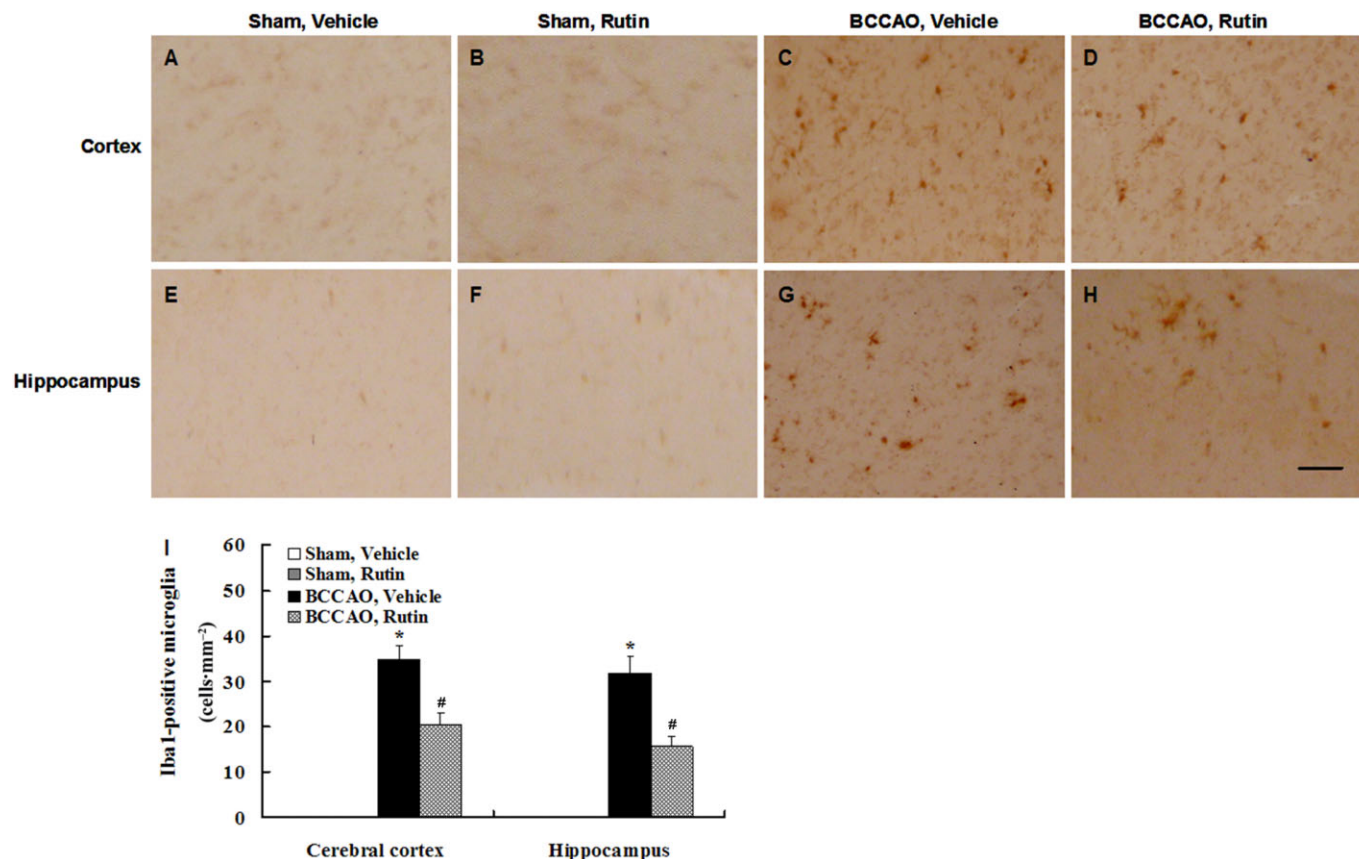
Immunohistochemical staining showed no Iba1-positive microglia in the cerebral cortex and hippocampus of vehicle-treated sham-operated rats (Figure 8A and E) and rutin-treated sham-operated rats (Figure 8B and F). However, robust activated microglia were observed in the cerebral cortex and hippocampus of vehicle-treated BCCAO rats (Figure 8C and G), whereas markedly decreased activated microglia were seen in rutin-treated BCCAO rats compared with vehicle-treated BCCAO rats (Figure 8D and H). Quantitative analysis revealed that vehicle-treated BCCAO rats had a significant increase in the amount of activated microglia in the cerebral cortex and hippocampus compared with vehicle- and rutin-treated sham-operated rats (Figure 8I; $P < 0.001$), whereas chronic rutin treatment in BCCAO rats resulted in a significant decrease in the amount of activated microglia in the cerebral cortex and hippocampus compared with vehicle-treated BCCAO rats (Figure 8I; $P < 0.01$). These findings demonstrate that chronic cerebral hypoperfusion may induce glial cell activation, which is suppressed by rutin.

Rutin reduces the levels of pro-inflammatory cytokines in BCCAO rats

ELISA analyses revealed that the levels of IL-1β (Figure 9A; $P < 0.001$), IL-6 (Figure 9B; $P < 0.001$) and TNF-α (Figure 9C; $P < 0.01$) were significantly elevated in the cerebral cortex and hippocampus of vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats, whereas rutin treatment in BCCAO rats significantly reduced these elevated levels of IL-1β (Figure 9A; $P < 0.01$), IL-6 (Figure 9B; $P < 0.01$) and TNF-α (Figure 9C; $P < 0.05$) compared with vehicle-treated BCCAO rats. These findings demonstrate that chronic cerebral hypoperfusion may induce increased release of pro-inflammatory cytokines, which are suppressed by rutin.

Figure 8

Effect of rutin on the activated microglia induced by BCCAO in rats. Microglia were labelled by immunohistochemical staining with Iba1 antibody. (A–H) Representative photomicrographs of Iba1-positive immunostaining in each group. No Iba1-positive microglia were observed in the cerebral cortex and hippocampus of vehicle-treated sham-operated rats (A, E) and rutin-treated sham-operated rats (B, F), whereas numerous Iba1-positive microglia were seen in vehicle-treated BCCAO rats (C, G), and a marked reduction of activated microglia was found in rutin-treated BCCAO rats (D, H). Quantitative analysis revealed that the amount of activated microglia in the cerebral cortex and hippocampus was significantly increased in vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats (I), whereas rutin treatment in BCCAO rats significantly reduced the amount of activated microglia in the cerebral cortex and hippocampus compared with vehicle-treated BCCAO rats (I). Data are expressed as mean ± SEM ($n = 10$ rats per group). *$P < 0.001$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.01$ versus vehicle-treated BCCAO rats. Scale bar = 200 μm.

Rutin prevents neuronal damage in BCCAO rats

Nissl staining showed that no damaged neurons were seen in the cerebral cortex and hippocampal CA1 region of vehicle-treated sham-operated rats (Figure 10A and E) and rutin-treated sham-operated rats (Figure 10B and F). In contrast, a few damaged neurons were observed in the cerebral cortex and hippocampal CA1 region of vehicle-treated BCCAO rats (Figure 10C and G), whereas rutin treatment in BCCAO rats markedly reduced the damaged neurons compared with vehicle-treated BCCAO rats (Figure 10D and H). Quantitative analysis revealed that vehicle-treated BCCAO rats had a significant decrease in the number of intact neurons compared with vehicle- and rutin-treated sham-operated rats (Figure 10I; $P < 0.01$), whereas rutin treatment in BCCAO rats resulted in a significant increase in the number of intact neurons compared with vehicle-treated BCCAO rats (Figure 10I; $P < 0.05$). These results demonstrate that rutin

may protect against cerebral hypoperfusion-induced neuronal damage.

Discussion

The major finding of this study is that rutin effectively alleviates cognitive deficits in BCCAO rats, which is likely to be attributable to its neuroprotective effects on central cholinergic dysfunction, oxidative damage, neuroinflammation and neuronal loss induced by chronic cerebral hypoperfusion. Our results provide new insight into the pharmacological actions of rutin, indicating that rutin has multi-targeted therapeutical potential on cognitive deficits associated with chronic cerebral hypoperfusion.

Previous studies have demonstrated that chronic cerebral hypoperfusion following BCCAO in rats induced deficits in spatial learning and memory (Vicente *et al.*, 2009; Cechetti
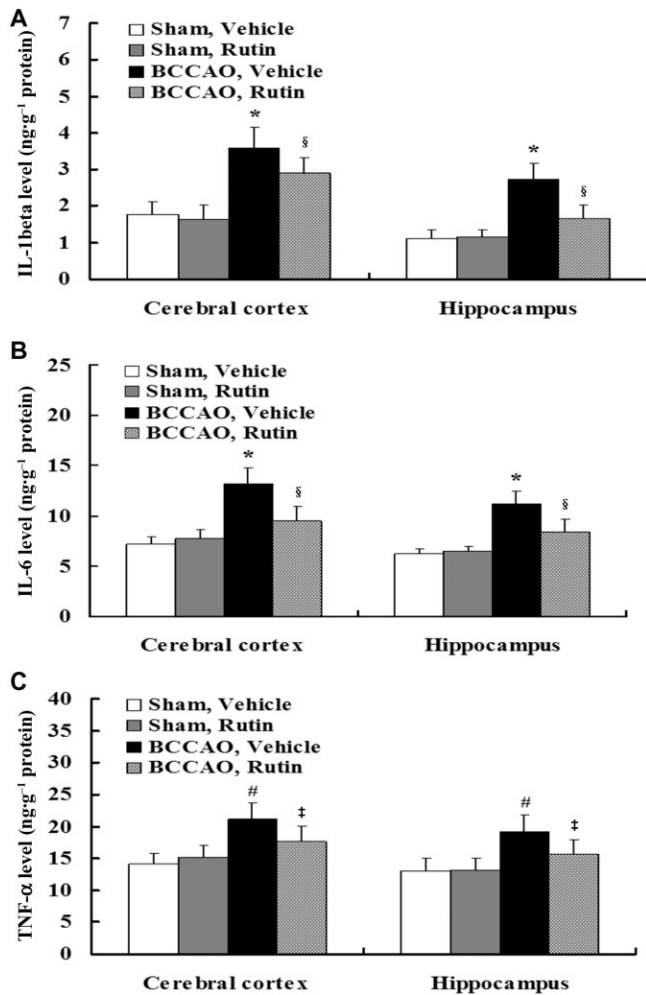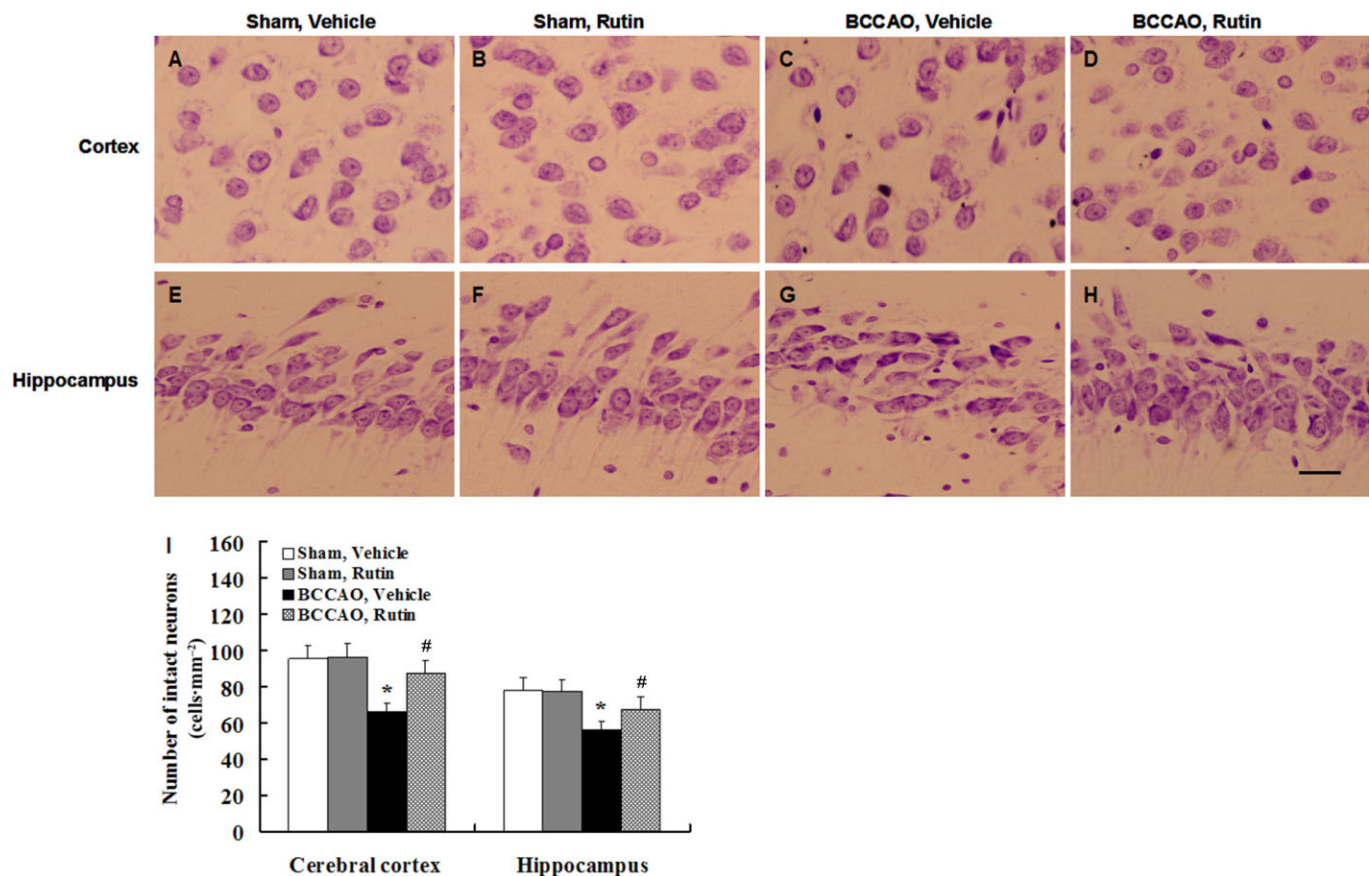
Figure 9

Effect of rutin on proinflammatory cytokines induced by BCCAO in rats. Pro-inflammatory cytokine levels in the cerebral cortex and hippocampus were assessed using ELISA kits. Quantitative analysis disclosed that the levels of IL-1β (A), IL-6 (B) and TNF-α (C) were significantly elevated in the cerebral cortex and hippocampus of vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats, whereas rutin treatment in BCCAO rats significantly reduced the levels of IL-1β (A), IL-6 (B) and TNF-α (C) compared with vehicle-treated BCCAO rats. Data are expressed as mean ± SEM ($n = 10$ rats per group). $*P < 0.001$, $\#P < 0.01$ versus vehicle- and rutin-treated sham-operated rats; $\S P < 0.01$, $\ddagger P < 0.05$ versus vehicle-treated BCCAO rats.

et al., 2012; Li et al., 2012; Xi et al., 2014) and dysfunction og non-spatial memory (Sarti et al., 2002; Xi et al., 2014). Consistent with these previous observations, we show here that vehicle-treated BCCAO rats had deficits in the hippocampus-dependent spatial learning and memory as assessed by Morris water maze test, cortex-dependent working memory as assessed by the delayed alternation task, and contextual memory as assessed using the step-through passive avoidance task. Interestingly, rutin treatment significantly ameliorated all these cognitive deficits in BCCAO rats. Moreover, no significant differences in the performances of spatial learning and memory, working memory and contextual memory were

observed between vehicle- and rutin-treated sham-operated rats, implying that rutin, by itself, did not directly affect the learning and memory functions. Thus, our results demonstrated that rutin was effective in alleviating not only the spatial learning and memory impairments but also non-spatial memory dysfunction caused by decreased CBF in vivo. The findings support the hypothesis that the improvement of cognitive deficits following rutin treatment may be due to the attenuation of biochemical and neuropathological impairments caused by chronic cerebral hypoperfusion.

Although cognitive deficits may occur in rats with chronic cerebral hypoperfusion, the underlying mechanisms are not fully understood (Farkas et al., 2007). Oxidative stress, an imbalance between free radicals and the antioxidant system, is well known to contribute to the pathogenesis of cognitive disorders, notably vascular dementia and AD (Ishrat et al., 2009; Crichton et al., 2013). In this study, a marked decrease in activity of the antioxidant enzymes (SOD and GPX) and a marked increase in lipid peroxidation (MDA) and protein oxidation (protein carbonyls) were observed in vehicle-treated BCCAO rats, indicating that chronic cerebral hypoperfusion may substantially increase oxidative damage. Moreover, rutin treatment significantly counteracted all the changes in the markers of oxidative damage in the hippocampus and cerebral cortex of BCCAO rats, suggesting that rutin has free radical-scavenging properties, as found earlier (Kamalakkannan and Stanely Mainzen Prince, 2006; Khan et al., 2009; 2012; Javed et al., 2012; Wang et al., 2012). As the measures of oxidative damage have been demonstrated to be directly correlated with the cerebral hypoperfusion-induced cognitive deficits by our recent study (Xi et al., 2014) and the earlier report (Peng et al., 2007), the present results indicate that the beneficial effect of rutin on cognitive deficits is associated with its antioxidant activity.

It is well known that the central cholinergic system plays a crucial role in learning and memory formation (Richetti et al., 2011). Chronic cerebral hypoperfusion decreased ChAT activity and ACh levels in the brain (Tanaka et al., 1996; Kumaran et al., 2008; Choi et al., 2011; Xi et al., 2014), which correlated with cognitive disabilities in the hypoperfused rats (Tanaka et al., 1996; Xi et al., 2014). In this study, we further confirmed that chronic cerebral hypoperfusion induced central cholinergic dysfunction, as shown by decreased levels of ACh level and ChAT activity, as well as a significant increase in AChE activity in the cerebral cortex and hippocampus of vehicle-treated BCCAO rats. Treatment with rutin for 12 weeks, however, restored the decreased ACh level and ChAT activity as well as increasing AChE activity in BCCAO rats. Given that our recent study demonstrated that spatial learning and memory impairments and working memory deficits significantly correlated with the indices of central cholinergic dysfunction in BCCAO rats (Xi et al., 2014), our present results indicated that the beneficial effect of rutin on the cerebral hypoperfusion-induced cognitive dysfunction could be due to its improving central cholinergic function to enhance ACh synthesis and release. Moreover, recent studies have shown that central cholinergic dysfunction is associated with increased oxidative stress in rat models of focal cerebral ischaemia (Ahmad et al., 2012) and chronic cerebral hypoperfusion (Xi et al., 2014). Particularly, in our recent study, correlational analyses revealed that central cho-

Figure 10

Effect of rutin on the neuronal damage induced by BCCAO in rats. Brain sections were stained with cresyl violet solution for Nissl staining. (A–H) Representative photomicrographs of cerebral cortical and hippocampal neurons in each group. No damaged neurons were observed in the cerebral cortex and hippocampal CA1 region of vehicle-treated sham-operated rats (A, E) and rutin-treated sham-operated rats (B, F), whereas numerous damaged neurons were seen in vehicle-treated BCCAO rats (C, G), and a marked reduction of damaged neurons was found in rutin-treated BCCAO rats (D, H). Quantitative analysis revealed that the number of intact neurons in the cerebral cortex and hippocampal CA1 region was significantly decreased in vehicle-treated BCCAO rats compared with vehicle- and rutin-treated sham-operated rats (I), whereas rutin treatment in BCCAO rats significantly increased the number of intact neurons as compared with vehicle-treated BCCAO rats (I). Data are expressed as mean ± SEM ($n = 10$ rats per group). *$P < 0.01$ versus vehicle- and rutin-treated sham-operated rats; #$P < 0.05$ versus vehicle-treated BCCAO rats. Scale bar = 300 μm.

linergic function (as ACh, ChAT and AChE) in the cerebral cortex and hippocampus were highly correlated with the oxidative state (as SOD, GPX, MDA and protein carbonyls) in BCCAO rats, indicating that the central cholinergic dysfunction could be attributed to increased oxidative stress in BCCAO rats (Xi *et al.*, 2014). Therefore, the recovery of central cholinergic dysfunction in rutin-treated BCCAO rats may result from the antioxidant effects of rutin.

Several studies have shown that chronic cerebral hypoperfusion may cause glial activation and overproduction of pro-inflammatory cytokines, which are considered harmful for neurons (Peng *et al.*, 2007; Vicente *et al.*, 2009; Zhang *et al.*, 2011; Jeon *et al.*, 2012). In this study, we further demonstrated increased neuroinflammation in vehicle-treated BCCAO rats, as shown by increased activated glial cells (astrocytes and microglia) and elevated levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the cerebral cortex and hippocampus. Moreover, rutin treatment markedly reduced

the neuroinflammatory responses in BCCAO rats, indicating that rutin not only suppressed glial activation but also reduced cytokine production, which was compatible with earlier reports showing that rutin was neuroprotective via its anti-inflammatory capacity (Koda *et al.*, 2009; Javed *et al.*, 2012; Wang *et al.*, 2012).

A number of studies have demonstrated that chronic cerebral ischaemia may lead to selective neuronal damage or loss in vulnerable regions of the brain, especially in the cerebral cortex and hippocampal CA1 regions (Farkas *et al.*, 2007; Peng *et al.*, 2007). In this study, histopathological examination revealed markedly damaged neurons in the cerebral cortex and hippocampal CA1 region of vehicle-treated BCCAO rats compared with sham-operated rats, whereas rutin treatment significantly attenuated the cerebral hypoperfusion-induced neuronal damage in BCCAO rats. Our findings are in accordance with previous observations showing rutin is effective in protecting the neuronal damage

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and loss induced by focal cerebral ischaemia in rats (Pu *et al.*, 2007; Khan *et al.*, 2009; Rodrigues *et al.*, 2013). As learning and memory functions are known to be dependent upon the integrity of the cerebral cortex and hippocampus (Li *et al.*, 2011) and the severity of neuronal damage was strongly correlated with cortex- and hippocampus-dependent cognitive impairments in BCCAO rats (Xi *et al.*, 2014), the improvement of cerebral hypoperfusion-induced cognitive deficits by rutin, observed in this study is likely to be attributable to its neuroprotective effect on neuronal cells.

Several study limitations should be considered in this work. Firstly, there was no information on the changes of CBF in our cohort of the BCCAO rats. Because CBF measurement requires anaesthesia, we did not make these measurements as we wanted to minimize the effects of anaesthesia on the behavioural performance and histopathological findings. Further studies should be made of CBF in BCCAO rats after rutin treatment. Secondly, although we examined the effect of post-treatment with rutin on the rat model with chronic cerebral hypoperfusion, we did not assess the effects of rutin before the BCCAO surgery. It I spossible that rutin could prevent the development of cognitive impairment and brain injury induced by chronic cerebral hypoperfusion. Finally, other potential molecular mechanism(s) responsible for amelioration of cerebral hypoperfusion-induced damage by rutin was not examined in this study. Therefore, it cannot be excluded that the brain-protective effects of rutin observed in this study might be partly attributed to other mechanisms. Further study is needed to elucidate the detailed molecular mechanisms involved in rutin-induced neuroprotection.

In conclusion, the current study demonstrates that rutin can effectively improve cognitive deficits induced by chronic cerebral hypoperfusion via its multi-target pharmacological actions on multiple pathophysiological processes in the brain. Our results suggest that rutin could be a promising new candidate for the therapy of cognitive deficits and brain injury associated with decreased cerebral blood flow in vascular dementia and AD.

Acknowledgements

Author contributions

J. Qu, Q. Zhou and M. Bai took part in surgery and contributed to the behavioural tests. Q. Zhou, Y. Du and W. Zhang prepared the samples and performed the biochemical analysis. W. Zhang, Z. Zhang and Y. Xi prepared the samples and performed the histopathological experiments. J. Qu, Q. Zhou and Y. Du collected the data, undertook the statistical analyses and wrote the first draft of the manuscript. Z. Li and J. Miao designed and supervised the study, including editing of the manuscript, from the first draft to the final version. All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

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