Original Article
Protective effect of carnosine after chronic cerebral hypoperfusion possibly through suppressing astrocyte activation

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Abstract: Aim: Subcortical ischemic vascular dementia (SIVD) induced by chronic hypoperfusion is a common cause of vascular dementia. The aim of this study was to determine whether the protective effect of carnosine on white matter lesion after chronic cerebral hypoperfusion through suppressing astrocyte activation. Methods: Adult male mice (C57BL/6 strain) were subjected to permanent occlusion of the right unilateral common carotid arteries (rUCCAO) and treated with carnosine or histidine. Open field test, freezing test, Klüver-Barrera staining, immunohistochemical analyses and western blot were performed after rUCCAO. Results: We found that carnosine ameliorated white matter lesion and cognitive impairment after rUCCAO. Carnosine suppressed the activation of astrocyte in both wide type mice and histidine decarboxylase knockout mice. However, administration of histidine did not show the same effect. We found that there were no differences between rUCCAO group and sham group for the expression of glutamate transporter-1 (GLT-1) and glutamate/aspartate transporter (GLAST). Furthermore, carnosine significantly attenuated the increase of inflammatory cytokine interferon gama. Conclusion: These data suggest carnosine induced neuroprotection during SIVD in mice is not dependent on the histaminergic pathway or the regulation of the expression of GLT-1 and GLAST, but may be due to a suppression of astrocyte activation and inflammatory cytokine release.

Keywords: Astrocyte, carnosine, subcortical ischemic vascular dementia, white matter injury, interferon gama

Introduction

Subcortical ischemic vascular dementia (SIVD) induced by chronic hypoperfusion as a result of small-artery disease is a common cause for vascular dementia (VaD), which is recognized as the second most prevalent type of dementia [1]. Its characteristic damage includes progressive demyelination, white matter rarefaction, cognitive impairment, glial activation and oligodendrocyte deficiency [2]. However, pathogenetic mechanisms of SIVD are far from understood. The therapy of SIVD has received extensive attention and there are several compounds with different mechanisms showing mild efficacy in SIVD patients [3]. So far no drug has been approved to potentially prevent the progress of SIVD [4].

Astrocytes, because of their diverse and significant roles, especially in glutamatergic signaling regulation in VaD, have become the focus of attention [5]. In recent years, it has been shown that glutamate can be toxic to white matter oligodendrocytes and to myelin by sustained activation of glutamate receptors [6]. A line of study demonstrated that astrocytes play an important role in maintaining low extracellular glutamate levels and eliminating and recycling glutamate in brain. Astrocytic glutamate transporter-1 (GLT-1) and glutamate/aspartate transporter (GLAST) are the primary controllers of extracellular glutamate levels in brain [7]. The inhibition of GLT-1 and/or GLAST by pharmacological blockers, antisense oligonucleotides or transgenic knockout elevates extracellular glutamate levels and induces neuronal death [8, 9], which may also affect the oligodendrocytes after hypotension. In addition, astrocytes release inflammatory cytokines and activate metalloproteases that contribute to blood-brain barrier disruption after ischemia [10]. Although

the exact cause of white matter lesion after SIVD has not been conclusively established, white matter lesions have frequently been suggested to have the relationship with glia activation [11]. Activated astrocyte is the major sources of proinflammatory cytokines, including TNF-α, IL-6 and IFN-γ, which may result in the degeneration of myelin and apoptosis of oligodendrocyte [12]. This evidence suggests that astrocyte plays an important role in SIVD.

Carnosine (b-alanyl-L-histidine) is a natural dipeptide that is highly expressed in the central nervous system, and can easily enter the brain from the periphery. Carnosine can converse to histidine and then histamine, and serves as a non-mast-cell reservoir for histamine [13]. Many evidences suggest that histaminergic neurotransmission plays an important role in brain ischemia. Carnosine ameliorates acute renal failure induced by ischemia/reperfusion in rats and NMDA-induced excitotoxic injury in differentiated PC12 cells through its conversion to histidine and histamine [14]. Besides it has been assigned many putative roles, such as anti-inflammatory agent, free radical scavenger and mobile organic pH buffer. Carnsoine protects against SIVD induced by permanent occlusion of the right unilateral common carotid arteries (rUCCAO) through its anti-oxidative effect [15]. However, few reports have demonstrated significant relations between brain carnosine/histamine and astrocyte function in SIVD.

Therefore, we investigated the effect of carnosine on astrocyte function in SIVD induced by rUCCAO and whether its action involves the histaminergic pathway.

Materials and methods

Animal preparation

All experiments using animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old wild-type (WT, C57BL/6 strain) and HDC-KO male mice weighing 22-30 g were used [16].

Experimental design

After administered with sodium pentobarbital (60 mg/kg) for anesthesia, the right common carotid artery was isolated from the adjacent vagus nerve and double-ligated with 6-0 silk sutures to perform rUCCAO. Sham-operated mice were subjected to the same procedure, except for carotid ligation.

Carnosine and histidine (Sigma, USA) were dissolved in sterile saline and administered by intraperitoneal injection. Adult male WT mice received rUCCAO, and were administered with saline, carnosine (200 mg/kg) and histidine (200 or 500 mg/kg), 30 min before surgery and every other day until the mice were sacrificed. HDC-KO mice received rUCCAO and were administered with saline or carnosine (200 mg/kg) at the same way. On day 29 after rUCCAO, the mice were subjected to open filed test. On day 30 mice started freezing task for two days. During the behavorial evaluation, carnosine or histidine was administered after the tests every other day. On day 32 after rUCCAO, the mice were sacrificed and brain tissues were removed for immunohistochemical staining, and western blot.

Open field test

Locomotor activity was measured using an open field test. Each subject was placed in the center of the open field box (30×45×30 cm) [17]. Time spent in the center was recorded. Data were collected for 180 minutes.

Fear conditioning tests

Freezing behavior is an indicator of aversive memory that is measured when subject mice are exposed to the conditional stimulus. Mice were allowed to familiarize themselves with the surroundings (context) for 120 seconds, followed by 30-seconds, 2800 Hz, 84-dB tone (conditional stimulus). This contextual interval was terminated by an unconditional stimulus, a 0.30-mA electrical foot shock for 2 seconds. The training allowed the mice to disengage from the process of association before a new set of stimuli was introduced. After 24 h of conditional-unconditional stimuli, the mice were placed back in the original conditioning chamber, where no tone or shock was presented, to assess recall of context and/or environment. Then the mice were placed in a new environment chamber, followed by a 0.30-mA electrical foot shock for 360 seconds after 120 seconds adaptation. Our measure of associative learning was the percentage of time spent not moving (percentage freezing time).
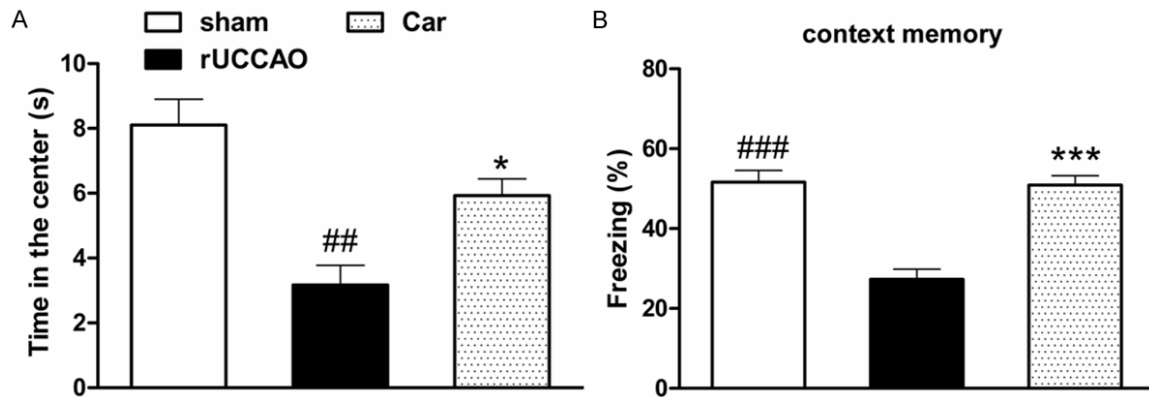
Figure 1. Effect of carnosine on cognitive ability in open filed test (A) and fear conditioning test (B) after rUCCAO. n = 10-12. ###P < 0.001, ##P < 0.01, vs. the sham group; ***P < 0.001, *P < 0.05, vs. the rUCCAO group.
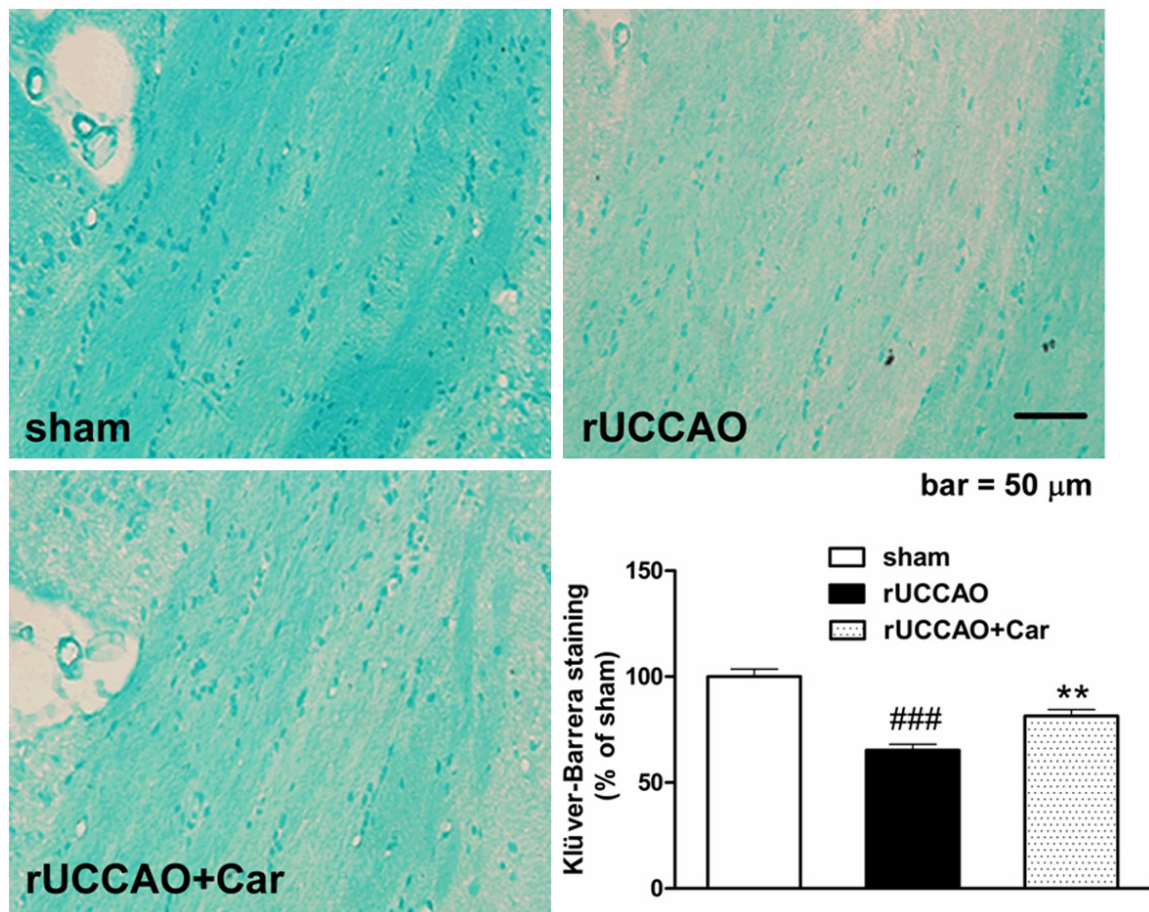

Figure 2. Effect of carnosine on white matter damage in the truncus corporis callosi. Scale bar, 50 mm. n = 10~12. ###P < 0.001, vs. the sham group; **P < 0.01, vs. the rUCCAO group.

Histochemical staining

Mice were deeply anesthetized with sodium pentobarbital (60 mg/kg), and perfused trans-cardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were separated and stored in 4% paraformaldehyde at 4°C for 24 h, and then in 30% sucrose for 3
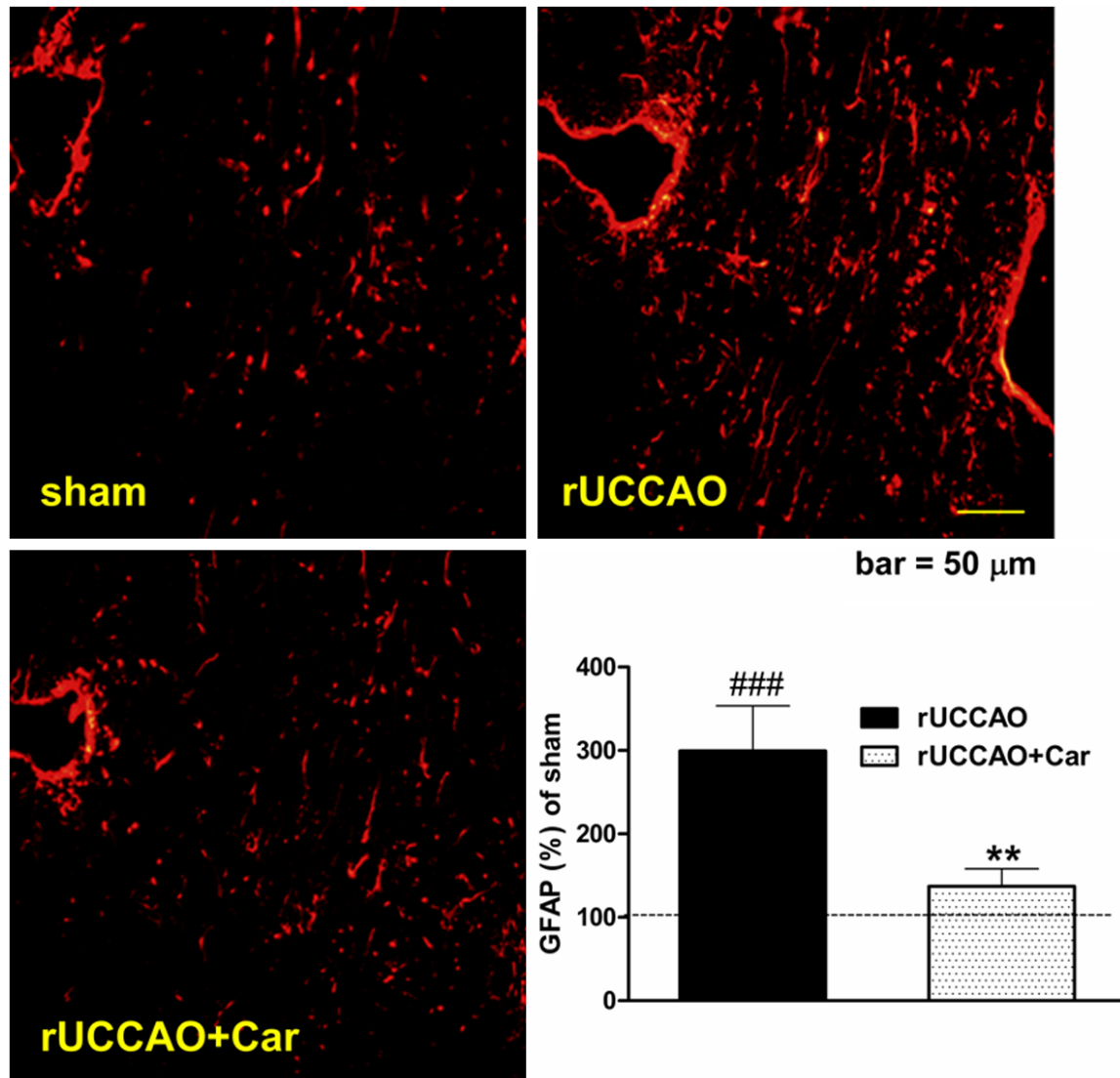
Figure 3. Effect of carnosine on astrocyte activation in the truncus corporis callosi. Scale bar, 50 mm. n = 10~12. ###P < 0.001, vs. the sham group; **P < 0.01, vs. the rUCCAO group.

d. Frozen brain sections (10 μm thick) were made by a cryostat (SM2000R, LEICA, Germany).

The severity of white matter lesions was evaluated by the fiber density of Klüver-Barrera staining as in Wakita's report [18]. Individual brain sections were incubated with PBS containing 3% normal donkey serum, 0.3% Triton X-100 for 2 h, and then with the appropriate primary antibodies overnight as following: mouse monoclonal anti-glial fibrillary acidic protein (anti-GFAP; 1:500; Sigma-Aldrich, USA); rabbit anti-interferon gama (anti-IFN-γ; 1:50; Boster, China). Then the sections were washed in PBS and incubated with Cy3-conjugated anti-mouse IgG antibody (1:400) for 2 h at room tempera-

ture. Finally, the sections were observed under a fluorescence microscope (Olympus BX51; Japan).

Western blots

The corpus callosum was dissected and homogenized in ice-cold lysis buffer containing (in mM): 50 Tris-HCl, 150 NaCl, 1% NP-40, 2 EDTA, 1 Na_3VO_4, pH 7.4) by a homogenizer (Bertin Precellys 24). Protein samples were separated on 12% SDS-polyacrylamide gels and electro-transferred onto a nitrocellulose membrane. After being blocked with 5% fat-free milk, the membranes were incubated with primary antibodies against GLT-1 and GLAST (1:50; Santa Cruz Biotechnology) and mouse monoclonal
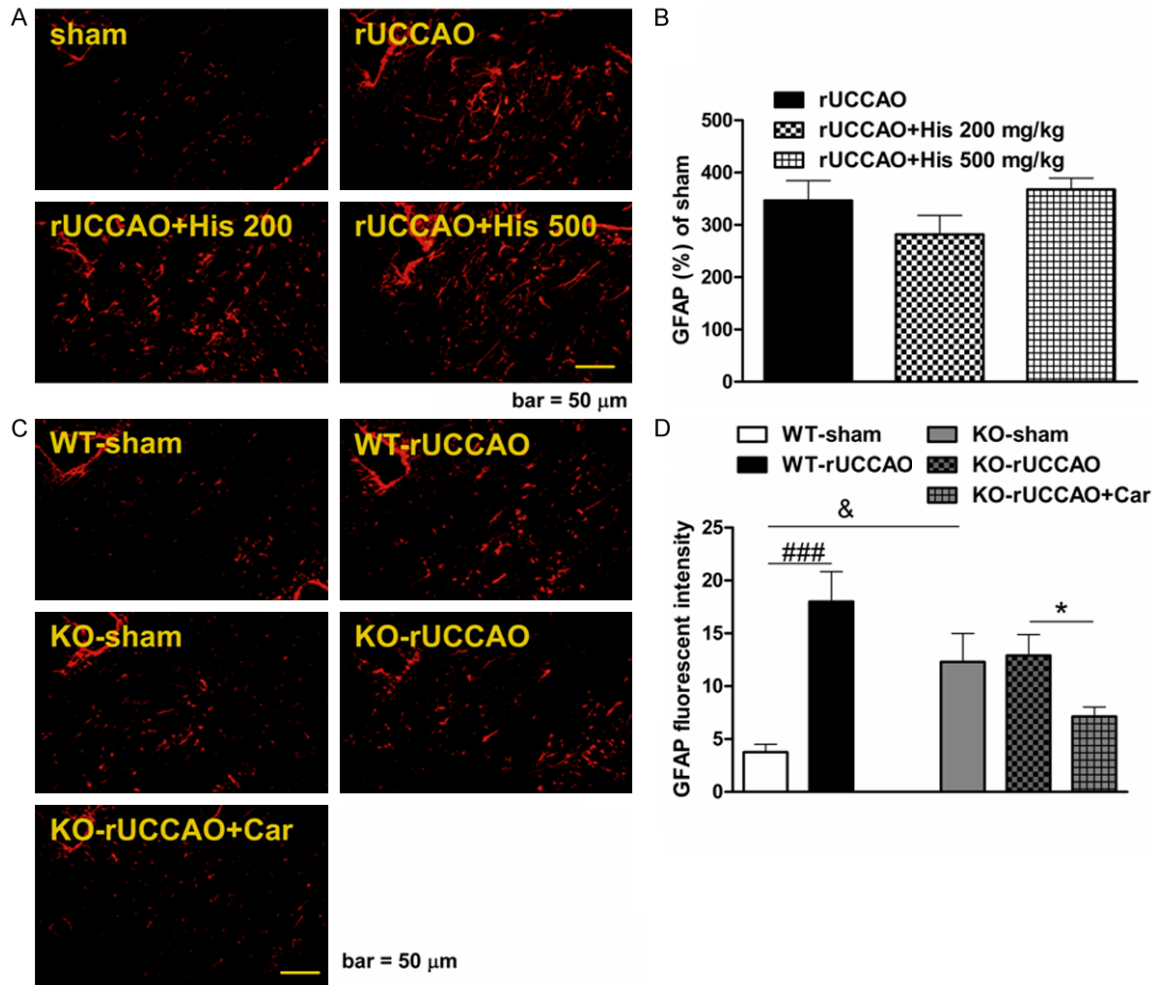
Figure 4. Effect of carnosine on astrocyte in corpus callosum is independent of the histaminergic pathway. The astrocyte activation in corpus callosum in WT mice was analyzed by GFAP staining after histidine (B) treatment in rUCCAO or sham groups. In HDC-KO mice, the astrocyte activation in corpus callosum was also analyzed after carnosine treatment (D). The representative photomicrographs of astrocyte activation in corpus callosum at day 32 after rUCCAO are shown in (A and C) Scale bar, 50 mm. n = 8. ###$P < 0.001$, vs. the sham group; *$P < 0.05$, vs. the KO-rUCCAO group; & $P < 0.05$, vs. the WT-sham group.

antibody against glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 1:5000; Kangchen) for 2 h at room temperature. Membranes were washed three times with TBST buffer and incubated with IRDye 800 anti-rabbit Molecular Probe (1:8000; LI-COR Biosciences, USA) or IRDye 700 anti-mouse Molecular Probe (1:3000; LI-COR Biosciences, USA) for 2 h at room temperature. Images were acquired with the Odyssey infrared imaging system and analyzed by Odyssey software [19].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were done with SPSS 11.5 for Windows. One-way analysis of variance (ANOVA) followed by the LSD or Dunnett's T3 post-hoc test (where equal variances were not assumed) was used for multiple comparisons.

Results

Effect of carnosine on cognitive impairment induced by the rUCCAO

In this experiment, the cognitive ability was evaluated by open field test and fear conditioning test. After rUCCAO, time in the center was decreased in the open field test (**Figure 1A**), while carnosine (200 mg/kg) significantly ele-
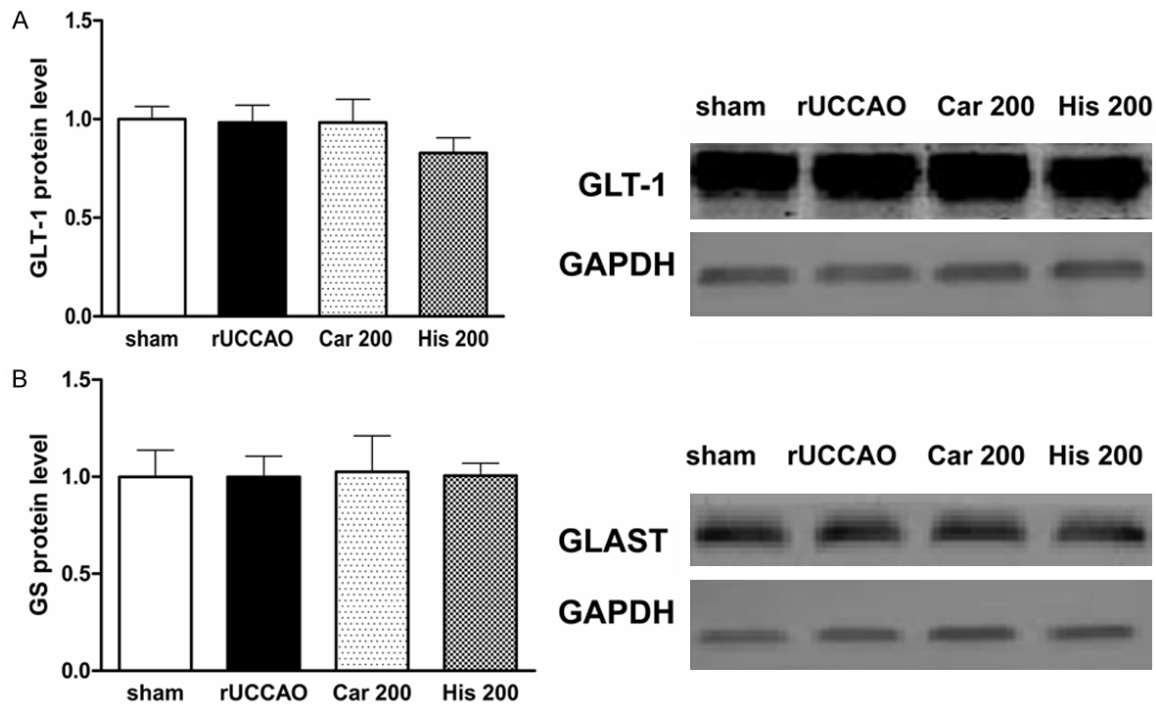
Figure 5. Effects of carnosine on the expression of GLT-1 and GLAST. Western blot analysis of GLT-1 (A) and GLAST (B) protein levels in the WT mice after SIVD. Values show means ± SEM.
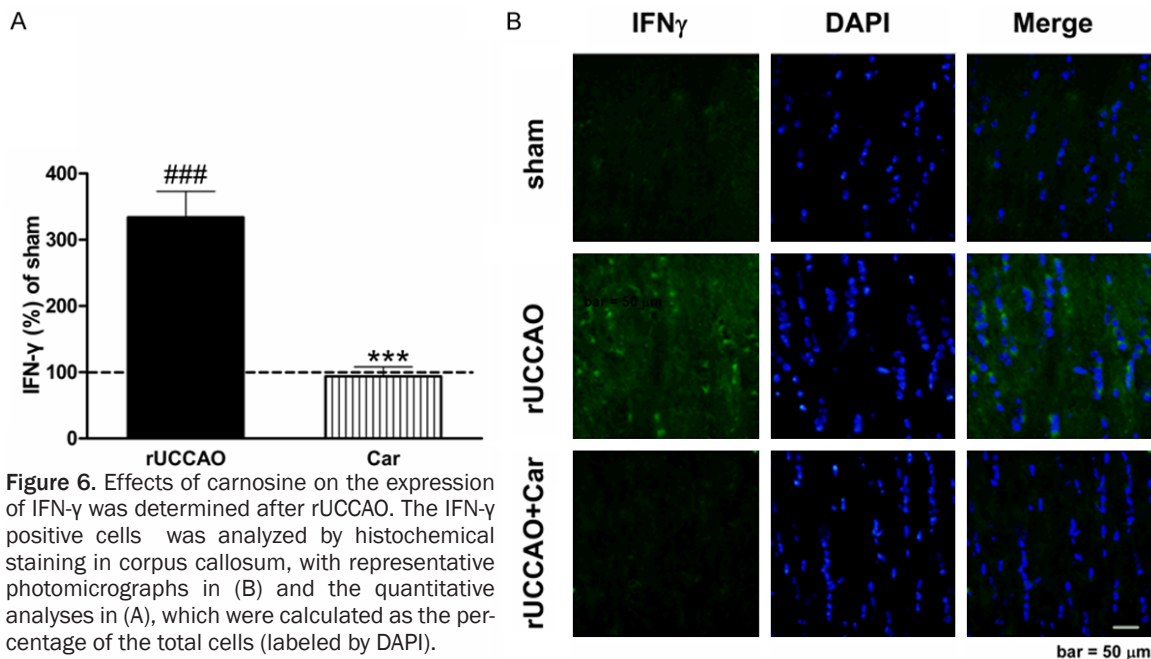

Figure 6. Effects of carnosine on the expression of IFN-γ was determined after rUCCAO. The IFN-γ positive cells was analyzed by histochemical staining in corpus callosum, with representative photomicrographs in (B) and the quantitative analyses in (A), which were calculated as the percentage of the total cells (labeled by DAPI).

vated the time in center ($P < 0.05$). In fear conditioning test, rUCCAO induced a significant reduction in context memory, which was completely reversed by treatment of carnosine (**Figure 1B**, $P < 0.001$). However, in cue memory of fear conditioning test, rUCCAO did not induce change of percentage freezing time (data not shown).

The protective effect of carnosine on white matter damage

White matter damage in the truncus corporis callosi was examined by Klüver-Barrera staining. Fiber density in the truncus corporis callosi was greatly lower than the sham group at day 32 after rUCCAO (**Figure 2**, $P < 0.001$). On day 32 after rUCCAO, carnosine markedly recovered the fiber density to the level in the sham group ($P < 0.01$).

The protective effect of carnosine on astrocyte activation

In the truncus corporis callosi, the mean fluorescent intensity of GFAP immunopositive astrocytes greatly increased at day 32 after rUCCAO, indicating the activation of astrocyte (**Figure 3**, $P < 0.001$). Carnosine (200 mg/kg) inhibited the activation of astrocyte as attenuating the elevation of GFAP fluorescent intensities.

The protective effect of carnosine on astrocyte activation is independent of the histaminergic pathway

Histidine decarboxylase (HDC) synthesizes histamine from histidine in mammals, and the HDC-KO mice show a histamine deficiency and lack histamine-synthesizing activity from histidine or carnosine. So, the effects of histidine and carnosine on astrocyte activation after rUCCAO were evaluated in WT or HDC-KO mice. Histidine (200, 500 mg/kg) did not change the astrocyte activation after rUCCAO (**Figure 4A** and **4B**). HDC-KO sham group showed a significant higher level of GFAP fluorescent intensities in the astrocyte activation than the WT sham group. Although HDC-KO mice showed no difference in astrocyte activation after rUCCAO (**Figure 4C** and **4D**), carnosine 200 mg/kg greatly reversed the activation of astrocyte.

The effect of carnosine on the expression of GLT-1 and GLAST

To assess GLT-1 and GLAST after SIVD, we isolated the corpus callosum after rUCCAO in WT mice. Western blot analysis showed that there was no difference between rUCCAO and sham group in the expression of GLT-1 and GLAST, and carnosine did not affect the expression of GLT-1 and GLAST after SIVD (**Figure 5**).

The protective effect of carnosine on the inhibition of proinflammatory cytokine

Activated astrocyte is the major sources of proinflammatory cytokines after cerebral ischemia. Here we determined the effects of carnosine on the proinflammatory cytokine levels in the corpus callosum 32 day after rUCCAO in WT mice and found that proinflammatory cytokine, IFN-γ was lower in the carnosine-treated group than in the saline group (**Figure 6**).

Discussion

In this study, we found that under chronic cerebral hypoperfusion induced by rUCCAO, carnosine ameliorated astrocyte activation both in HDC-KO mice, which are lack of histamine, and WT mice almost to the same extent. Histidine did not show the same effect. We found that hypoperfusion and carnosine treatment both did not affect the expression of GLT-1 and GLAST after SIVD. Carnosine significantly attenuated the increase of IFN-γ to the level in the sham group at days 32 after rUCCAO. It is likely that the neuroprotective effect of carnosine on astrocyte activation in SIVD is neither dependent on the histaminergic pathway nor the regulation of GLT-1 and GLAST, but may due to the inhibition of proinflammatory cytokine IFN-γ.

Although the neuroprotection of carnosine on white matter lesion in SIVD has been addressed in our present study, we only observed the white matter lesion in genu of corpus callosum [15]. Here we investigated the white matter lesion in truncus corporis callosi which indicated that chronic cerebral hypoperfusion induced diffuse white matter lesion in corpus callosum. Meanwhile carnosine has extensive neuroprotective effect on white matter damage and astrocyte activation in corpus callosum after SIVD. It was proposed that carnosine may protect astrocyte function through the histaminergic pathway, because carnosine serves as a non-mast-cell reservoir for histidine, utilized for the synthesis of histamine [20]. However, we found that carnosine significantly inhibited the astrocyte activation after rUCCAO in both HDC-KO and WT mice almost to the same extent, which indicates that carnosine has the same protective effect on astrocyte activation in SIVD whether or not mice lack histamine. Meanwhile, histidine, which will be transformed to histamine, did not show the protective action on

astrocyte activation in WT mice after rUCCAO, although histamine is considered to participate in astrocyte activation [21], indicated by the increase of GFAP fluorescent intensities in HDC-KO mice without rUCCAO. These data revealed that the protective effect of carnosine on astrocyte activation in SIVD may not involve the carnosine-histidine-histamine metabolic pathway.

Because astrocytes are known to play an important role in glutamate uptake by GLT-1 and GLAST to limit excitotoxic injury of oligodendrocyte [22], we further determined the effects of hypoperfusion and carnosine on the expression of GLT-1 and GLAST. In our previous study, we only observed the level of glutamate was not different between the rUCCAO and sham group in HPLC analysis. We can not rule out the changes of transport and recycle of glutamate. In this study, as shown in **Figure 5**, we found the expression of GLT-1 and GLAST in corpus callosum were not changed at day 32 after rUCCAO and carnosine did not affect the expression of GLT-1 and GLAST after SIVD. These data revealed that the protective effect of carnosine on astrocyte activation in SIVD may not involve the regulation of GLT-1 and GLAST. Moreover, our data suggest that histamine may not be involved in astrocyte activation in SIVD, since there is no significant difference between WT and HDC-KO mice after rUCCAO, and histidine has no protection against astrocyte activation after rUCCAO, but histamine is found to participate in astrocyte activation in acute cerebral ischemia from a lot of previous studies [23, 24]. So, our data further confirm that pathological mechanism of SIVD is different from the acute cerebral ischemia which causes excitotoxicity due to extracellular accumulation of glutamine and involves histamine

Activated astrocyte is the major sources of proinflammatory cytokines and white matter lesions have frequently been suggested to have the relationship with glia activation [25]. We found that carnosine markedly inhibited the activation of astrocyte as attenuating the elevation of GFAP fluorescent intensities. Meanwhile, we found that IFN-γ was significant increased in corpus collosum after rUCCAO and carnosine markedly attenuated the increase of IFN-γ at days 32 after rUCCAO. The pro-inflam-

matory cytokine IFN-γ is present during episodes of neuroinflammation, and is considered to play a role in the demyelination that occurs in white matter damage [26]. IFN-γ initiates lesion formation by amplifying the activation of astrocyte and microglia and myelin phagocytosis [27, 28]. Some research found that treated with IFN-γ (10 ng/ml) decreased the oligodendrocyte progenitor's self-renewal [29]. The death of oligodendrocyte could be induced by IFN-γ [30, 31]. IFN-γ also has been reported to have the inhibitory effect on oligodendroglial lineage cell proliferation and differentiation in vitro [32, 33]. Carnosine is considered to have an anti-inflammatory effect [34] and has been reported to have the neuroprotective effect in astrocytes exposed to LPS- and IFN-γ-induced nitrosative stress [35]. Therefore, our results suggest that the protection of carnosine against white matter damage after rUCCAO may be through inhibiting the release of IFN-γ from astrocytes.

In conclusion, we found that carnosine had a protective effect on astrocyte activation in SIVD induced by rUCCAO, which neither involve the carnosine-histidine-histamine metabolic pathway nor the regulation of GLT-1 and GLAST, but may be due to an inhibition of pro-inflammatory cytokine IFN-γ. These data suggest that carnosine may have anti-inflammatory value for the therapeutic treatment in SIVD.

Disclosure of conflict of interest

None.

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