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Original Article

Acamprosate protects rat brain against middle-cerebral-artery-occlusion-induced ischemic injuries through anti-oxidative activities

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Abstract: It is has been reported that acamprosate significantly reduces neurological deficits following transient hemispheric ischemia, which may be related to its antiglutamatergic effect. However, it is unclear whether acamprosate may provide anti-oxidative neuroprotection in cerebral ischemic condition. To investigate the anti-oxidative neuroprotective effects of acamprosate during focal cerebral ischemia, rats were treated with acamprosate through sublingual vein injection 30 min after the onset of MCAO-induced cerebral ischemia. The behavioral tests were performed to evaluate neurological performance. 2,3,5-triphenyltetrazolium chloride (TTC) staining was used to evaluate the infarct area of brain tissue. Spectrophotometric assay was used to determine the activities of superoxide dismutase (SOD) and glutathione-peroxidase (GSH-Px), and contents of malondialdehyde (MDA) and adenosine triphosphate (ATP). Furthermore, the respiratory control ratio (RCR = State 3/State 4) was calculated to determine the brain mitochondria activities. Acamprosate at doses of 30 and 100 mg/kg, but not 10 mg/kg, exhibited significant neuroprotective effects against focal cerebral ischemic injury by markedly attenuating MCAO-induced neurological deficit, and reducing the infarct area. Meanwhile, acamprosate significantly improved mitochondrial energy metabolism, and countered the reduction of SOD and GSH-Px activities and elevation of MDA level induced by cerebral ischemia. Acamprosate might provide neuroprotection against the cerebral ischemia-induced injury through anti-oxidative actions in the brain.

Keywords: Acamprosate, cerebral ischemia, energy metabolism, free radical, neuroprotection, mitochondrial

Introduction

Ischemic stroke, accounting for 87% of all stroke cases, is a devastating cause of death and the most causative factor for long term disability worldwide [1]. During cerebral ischemia, there is an over-activation of postsynaptic glutamate receptors in response to the excessive release of presynaptic glutamate, which leads to massive excitation then death of the brain cells [2]. The reduced oxygen and glucose supply to the brain during cerebral ischemia is also accompanied by reduced formation of adenosine triphosphate (ATP) [3]. The brain is vulnerable to free radical-mediated insult during the cerebral ischemia, which is due to its characteristic aerobic metabolism, limited energy reserves, and lack of ample free radical scavengers, but large amount of polyunsaturated fatty acid as a major component of the neuronal cell membrane that subject to free radical damage [3].

Acamprosate, first used to prevent relapse in alcohol dependence, is believed to modulate the NMDA-receptor activity through blocking the metabotropic glutamate receptor subtype 5 [4]. It has been reported that acamprosate significantly reduces neurological deficits following transient hemispheric ischemia, which may be related to its antiglutamatergic effect with consecutive reduction of transmembraneous Ca^{2+} flux through NMDA-activated ion channels [5]. Furthermore, glutamine receptor antagonist inhibits NMDA-receptor-mediated intracellular Ca^{2+} accumulation in rat primary cortical neurons, reduces the brain infarct volume in rats that undergo middle cerebral artery occlusion

(MCAO), and abates the lipid peroxidation as well as the elevation of reactive oxygen species (ROS) [6]. Recently, Doeppner *et al*. [7] found that acamprosate can also reduce the effects of MCAO, but it is unclear whether acamprosate may provide anti-oxidative neuroprotection in cerebral ischemic condition.

Using the typical animal model with MCAO-induced cerebral ischemia, we investigated if acamprosate is neuroprotective against cerebral ischemic damage and explored if the neuroprotection involves anti-oxidative activities in the brain by examining the modulation of energy metabolism in the brain. Edaravone, the neuroprotective agent known to have anti-oxidative effect against ischemic brain injuries, was used in this study for positive comparison.

Materials and methods

Animals

All experiments were approved by the Animal Ethics Committee of Zhejiang University School of Medicine. Ninety-six adult male Sprague-Dawley rats (250-300 g) were obtained from the Experimental Animal Center of Zhejiang University School of Medicine. The rats were housed in cages in a room with controlled temperature (25°C) and 12:12 h light-dark cycle. All rats were randomly assigned into six groups (n = 16 for each group) which consisted of sham, control, three acamprosate (Sigma-Aldrich, MO, USA)-treatment groups (with dosages at 10, 30 and 100 mg/kg, respectively), and edaravone (Yuanye Biotech, Shanghai, China) group (10 mg/kg). All treatments were administered 30 min after the onset of ischemia. The animals of sham or the control group were injected with the same volume of saline as that for acamprosate.

Induction of cerebral ischemia by middle cerebral artery occlusion

All rats, except for sham groups, were subjected to left total MCAO using an intraluminal technique [8]. The rats were anesthetized with a mixture of xylazine (1.5 mL of 100 mg/mL) and ketamine (100 mg/mL, 0.1 mL/100 g of body weight) by intraperitoneal injection. In brief, the left external carotid artery (ECA), common carotid artery (CCA) and internal carotid artery (ICA) were separated, and the ECA was ligated. A microvascular clip was applied at the left ICA and an arteriotomy was made at the CCA, 5 mm from the bifurcation of ECA and ICA. A 4-0 nylon monofilament with a blunted tip was inserted into the left ICA via the opening of the left CCA until a mild resistance was felt [8]. At this point, the intraluminal suture totally blocked the origin of the middle cerebral artery for 60 minutes and then withdrawn to allow reperfusion for 24-hours.

Evaluation of neurological deficit

After 24 hours reperfusion, an evaluator blinded to the drug treatment was assigned to assess the animals' neurological performance. 5-point scale was used to rate the neurological deficit: [9] 0, no deficit; 1, difficulty in extending the right paw; 2, circling to the right; 3, difficulty in maintaining balance while falling to the right side; 4, lower level of consciousness without spontaneous walk movement.

Sloping board experiment

After 24 hours reperfusion, the rats underwent sloping board test, as previously described with minor changes [10]. Briefly, the animals were placed on the slanted board at 75°. The time duration (monitored for up to 3 min) in which animals remain on the slope board was recorded.

2, 3, 5-Triphenyltetrazolium chloride staining for infarct volume evaluation

After 24 hours reperfusion, the animals (n = 8 in each group) were sacrificed and the brains were rapidly removed. The brain tissue was cut at 1.5 mm, and then stained with 2,3,5-triphenyltetrazolium chloride (TTC). The images of stained brain slices were captured and then processed on a Compix system computer (C imaging 1280 system; Compix Inc. Image Systems) to determine the infarct area and the total hemispheric area of each brain slice. The infarct volume for all seven slices was calculated [11] and presented as total infarct volume (in mm^3).

Preparation of brain homogenate and mitochondria isolate for evaluation of antioxidant activity

After 24 hours reperfusion, the rats (n = 8 in each group) were sacrificed, and the brains

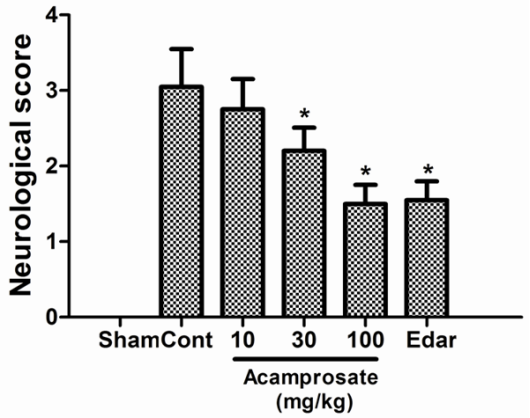

Figure 1. Effects of acamprosate on neurological deficit at 24 hours after reperfusion. Data were represented as mean ± S.E.M. *P < 0.05, compared with control, n = 8. Cont, control; Edar, edaravone.

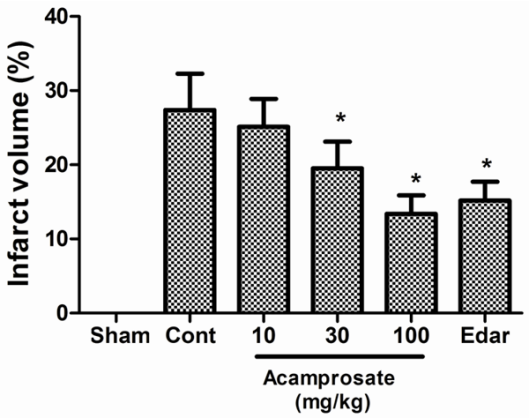

Figure 2. Effects of acamprosate on size of brain infarct area at 24 hours after reperfusion in rats. Data were represented as means ± S.E.M. *P < 0.05, compared with control, n = 8. Cont, control; Edar, edaravone.

were rapidly removed and dissected. The right parietal lobes adjacent to the contusion foci were minced and then homogenized in mitochondria isolation buffer (10 mmol/L Tris-HCl, pH 7.4, 250 mmol/L sucrose, 0.5 mmol/L EDTA, and 0.5% bovine serum albumin, ice cold). The homogenate of brain tissue was centrifuged at 750 g for 10 min. 2 ml of the supernatant was extracted and frozen -80 C for later use, with the remainder centrifuged again at 10,000 g for 10 min. The mitochondrial pellets were rinsed twice in isolation buffer and then re-suspended. The protein content of the mitochondria was measured using Coomassie blue protein-binding technique, with bovine serum albumin serving as standard. Antioxidant activi-

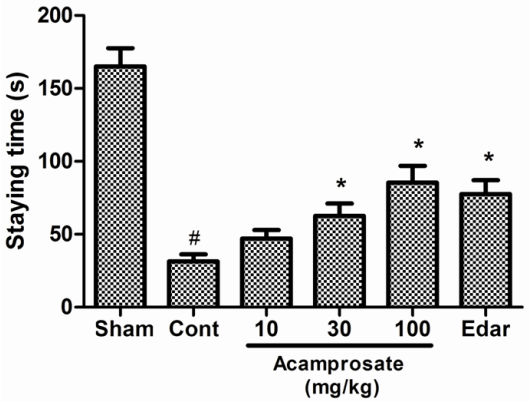

Figure 3. Effects of acamprosate on duration of staying time on slope board at 24 hours after reperfusion in rats. Data were represented as mean ± S.E.M. *P < 0.05, compared with control; #P < 0.05, compared with sham group, n = 8. Cont, control; Edar, edaravone.

ties of the homogenized tissue were then evaluated. The MDA content in the homogenate was determined by the TBA kits (Jiancheng Bioengineering Ltd, Nanjing, China).

Determination of ATP levels in isolated mitochondria

Mitochondria pellet was re-suspended in a buffer (100 mmol/L KCl, 75 mmol/L mannitol, 25 mmol/L sucrose, 10 μmol/L EGTA, and 5 mmol/L potassium phosphate, pH 7.4). Mitochondria (0.5 mg protein per milliliter) were treated with glutamate/malate (2.5 mmol/L) plus ADP (25 mmol/L), aspirin (0.03 to 0.3 mmol/L), and sodium salicylate (0.03 to 0.3 mmol/L) for 7 minutes. ATP levels of mitochondria were then assessed. The respiratory control ratio (RCR) of mitochondria was evaluated as previously described [12]. Briefly, RCR was calculated as the ratio of State 3 to State 4. The 1 ml reaction mixture contains sucrose (150 mmol/L), Tris-HCl (25 mmol/L), phosphate buffer (10 mmol/L, pH 7.4), and mitochondrial protein (1.0 mg), and the assay was performed at 30°C.

Statistical analysis

SPSS 16.0 (SPSS Inc, Chicago, IL, USA) was used for data analysis. The data were presented as mean ± S.E.M. One-way analysis of variance (ANOVA) and post hoc Dunnett's test were used to determine statistical significance. P < 0.05 was considered statistically significant.

Table 1. Effects of acamprosate on brain ATP content and mitochondrial respiratory function in rats with MCAO-induced ischemia

Groups	Dose (mg kg^{-1})	R3 (nM $min^{-1}mg^{-1}$)	R4 (nM $min^{-1}mg^{-1}$)	RCR	ATP (μM mg^{-1})
Sham	Saline	82.3 ± 8.7	23.1 ± 3.3	3.3 ± 0.5	2.9 ± 0.6
Control	Saline	38.2 ±4.5**	31.3 ± 4.1**	1.4 ± 0.3**	1.7 ±0.3**
Acamprosate	10	44.3 ± 5.2	29.4 ± 3.3	1.6 ± 0.6	2.6 ± 0.5
	30	57.2 ± 6.5#	24.2 ± 2.5#	2.4 ± 0.5#	2.1 ± 0.4#
	100	68.5 ±7.8##	21.5 ± 2.6##	3.3 ± 0.4##	1.9 ± 0.3##
Edaravone	10	67.7 ± 7.4##	22.4 ± 2.3##	3.2 ± 0.5##	1.9 ± 0.4##

Values are expressed as the mean ± S.E.M. **P < 0.01, compared with sham group; #P < 0.05, ##P < 0.01, compared with control group, n = 8.

Table 2. Effect of acamprosate on SOD/GSH-Px activities, and MDA content of brain mitochondria in rats with MCAO-induced ischemia

Group	Dose mg kg^{-1}	SOD (u mg^{-1} Pro)	GSH-Px (u mg^{-1} Pro)	MDA nM mg^{-1} Pro
Sham	Saline	162.1 ± 18.6	16.4 ± 3.2	8.4 ± 1.9
Control	Saline	101.1 ± 12.6**	7.8 ± 2.2**	13.6 ± 2.4**
Acamprosate	10	115.4 ± 14.3	9.5 ± 2.3	10.7 ± 2.3
	30	135.2 ± 17.3#	12.4 ± 2.7#	9.6 ± 2.2#
	100	147.5 ± 14.8##	14.4 ± 2.4##	8.7 ± 1.8##
Edaravone	10	142.3 ± 13.4##	13.6 ± 2.7##	8.8 ± 1.9##

Values are expressed as the mean ± S.E.M. **P < 0.01, compared with sham group; # P < 0.05, ##P < 0.01, compared with control group, n = 8.

Results

Neurological deficits

The control group demonstrated the most neurological deficit (determined by neurological score) compared with other groups, which suggested that MCAO-induced brain ischemia caused motor and somatosensory dysfunctions. These dysfunctions may be the result of ischemic injuries to the caudoputamen and frontoparietal cortex, as both areas receive their blood supply from middle cerebral artery and are important in motor, perceptual, cognitive skill and spatial task. There was significant reduction of neurological deficit in acamprosate (30 and 100 mg/kg) -treated and edaravone (10 mg/kg) treated groups (**Figure 1**).

Infarct volume

There was significant infarction in the rat brain at 24 hours after reperfusion, compared with the sham group. It's understood that the infarction is due to MCAO-induced cerebral ischemia. For the acamprosate treatment group, the MCAO-induced infarct volume was significantly reduced by 28.8% (with the drug dosage at 30 mg/kg) and 51.1% (with dosage at 100 mg/kg). Between the acamprosate and edaravone treatment groups, it was found that acamprosate at higher dosage (100 mg/kg) had higher potency than edaravone at 10 mg/kg (**Figure 2**).

Sloping board experiment

The rats were subjected to slope board experiment at 24 hours after reperfusion. It was found that the rats with MCAO-induced cerebral ischemia had a significantly shorter duration of maintaining on the slope board, compared with the sham group. The rats in drug treatment groups (acamprosate groups with dosage at 30 and 100 mg/kg; and edaravone group with dosage at 10 mg/kg), however, showed longer duration on the slope board than that of the MCAO control group (**Figure 3**).

Energy metabolism

To investigate the effect of acamprosate on brain energy metabolism during MCAO-induced cerebral ischemia in rats, we quantified the ATP content in the brain tissue and calculated the brain mitochondrial RCR as well. The data indicated that the rat brains from the MCAO control group had lower levels of ATP and reduced mitochondrial RCR compared with those of sham group, but treatments with edaravone (10 mg/kg) or acamprosate (30 and 100 mg/kg, but not 10 mg/kg) significantly reversed the MCAO-induced reduction of ATP level and mitochondrial RCR in the brain (**Table 1**).

Anti-oxidative activity of brain tissue

The changes in activities of endogenous antioxidant enzymes and MDA contents in rat

brains were evaluated 24 hours after reperfusion. As indicated in **Table 2**, the MCAO group had significantly lower anti-oxidative activities compared with shame group, which were manifested by the reduced activities of SOD and GSH-Px, while edaravone (10 mg/kg) and acamprosate (30 and 100 mg/kg, but not 10 mg/kg) treatment significantly attenuated the ischemia-induced reduction of SOD and GSH-Px activities. Meanwhile, edaravone and acamprosate at above mentioned doses inhibited the MCAO-induced increase of MDA contents in brain tissue. The data suggest that edaravone and acamprosate can significantly abate the cerebral ischemia-induced free radical generation in the brain.

Discussion

Our results indicated that in protecting against MCAO-induced ischemic brain injuries, acamprosate significantly attenuated the ischemia-induced neurological deficits, decreased the brain infarction, ameliorated the brain mitochondrial energy metabolism during MCAO-induced brain ischemia, and abated the decrease of SOD and GSH-Px activities and the elevation of in MDA level in the brain tissue.

While acamprosate protected rat brains against focal cerebral ischemic injuries in a dose-dependent manner, there was no significant efficacy difference between acamprosate (100 mg/kg) and edaravone (10 mg/kg) treatment. The potential mechanism underlying the drug's neuroprotection against brain ischemia is likely due to the anti-oxidative properties, as it was reported that edaravone worked against the cellular oxidative activities during brain ischemia [13]. Since cell oxidation is associated with cell mortality, the net effect of this inhibition improves cell survival during ischemic episodes [14]. Acamprosate modulates NMDA-R responses by inhibiting mGluR5 [4], and it has been found that NMDA-R antagonist has anti-oxidant actions in cortical neurons [6]. Thus, we investigated whether the neuronal protection by acamprosate is mediated by its anti-oxidative actions in the brain.

Our study found that, with the treatment of acamprosate (at doses of 30 and 100 mg/kg, but not 10 mg/kg) in rats subjected to MCAO-induced brain ischemia, the improvements in motor function correlated with the reduction of brain infarct volumes. We then investigated the anti-oxidative activities of acamprosate in the brain, which included the use of the TTC staining technique. TTC may provide morphological evidence of ischemia-caused cell death, but in addition to this, it is also a sensitive histochemical indicator of mitochondrial respiratory enzyme function [15]. Our findings indicated that acamprosate can protect the mitochondrial activities against the cerebral ischemia-induced oxidative stress, which improves the neuronal survival and reduces the brain infarct volume during ischemic insult. ATP in the brain is crucial for various neuronal functions including protein synthesis, neurotransmitter transport, and maintaining neuronal network structure. During cerebral ischemia, the decreased oxygen and glucose supplies result in decreased ATP level [16]. In consistency with the results on anti-oxidative activities by acamprosate, our study also indicated that acamprosate treatment reversed the MCAO-induced reduction of mitochondrial RCR and decreased level of brain ATP, and thus ameliorated the brain energy metabolism during cerebral ischemia.

As previous report indicated, the anti-oxidative activities of the drug treatment during cerebral focal ischemia can be manifested by the substantial elevation of antioxidant enzyme activities and reduction of lipid peroxide contents in the cortex [17]. Previous study showed that the cerebral focal ischemia induces the generation of ROS in the brain, which is involved in the pathogenesis of brain diseases, such as stroke [18]. Our data indicated that acamprosate inhibites the MCAO-induced cerebral ROS formation, and reverses the reduction of brain mitochondrial SOD and GSH-Px activities, and hinders the brain MDA production during the cerebral ischemia.

Thus, this study demonstrated that acamprosate is neuroprotective against MCAO-induced cerebral ischemia, and its neuroprotection is attributable to the enhanced brain mitochondrial activities, improved brain energy metabolism and other anti-oxidative actions, including the inhibition of ROS formation and lipid peroxidation, and boost of endogenous free radical scavenging enzyme activities. Taken together, these findings suggested that acamprosate may serve as a potential therapeutic and preventative agent for cerebral ischemia-induced brain injuries.

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Disclosure of conflict of interest

None.

Authors' contribution

Conceived and designed the experiments: HD. Performed the experiments: QL. Analyzed the data: LX. Wrote the paper: HD.

Abbreviations

ATP, adenosine triphosphate; mGluR5, metabotropic glutamate receptor subtype 5; MCAO, middle cerebral artery occlusion; ROS, reactive oxygen species; ECA, external carotid artery; CCA, common carotid artery; ICA, internal carotid artery.

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