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Research paper

Clostridium butyricum pretreatment attenuates cerebral ischemia/reperfusion injury in mice via anti-oxidation and anti-apoptosis

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HIGHLIGHTS

- *C. butyricum* attenuates cerebral ischemia/reperfusion injury in the I/R mice.
- *C. butyricum* ameliorate neurological deficit in the I/R mice.
- *C. butyricum* exerts protective effects via anti-oxidative and anti-apoptosis.
- A butyrate might be involved in the neuroprotective effects of *C. butyricum*.

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ABSTRACT

Probiotics participate actively in the neuropsychiatric disorders. However, their roles on ischemic stroke remain unclear. This study aims to determine whether *Clostridium butyricum* (*C. butyricum*) could attenuate cerebral ischemia/reperfusion (I/R) injury and its possible mechanisms. Male ICR mice were intragastrically pretreated with *C. butyricum* for 2 successive weeks, and then subjected to cerebral I/R injury induced by the bilateral common carotid artery occlusion (BCCAO) for 20 min. After 24 h of the reperfusion, neurological deficit scores were evaluated. Histopathological changes of the hippocampus neurons were observed using Hematoxylin and eosin (H&E) and TUNEL staining. Malondialdehyde (MDA) contents and superoxide dismutase (SOD) activities in the brain were detected. The expression of Caspase-3, Bax and Bcl-2 were investigated by Western blot and immunohistochemistry analysis. The butyrate contents in the brain were determined. Our results showed that cerebral I/R injury led to neurological deficit, increased levels of Caspase-3 and Bax and decreased Bcl-2/Bax ratio. *C. butyricum* significantly improved neurological deficit, relieved histopathologic change, decreased MDA contents and increased SOD activities in the I/R injury mice. After *C. butyricum* pretreatment, the expression of Caspase-3 and Bax were significantly decreased, the Bcl-2/Bax ratio was significantly increased, and butyrate contents in the brain were significantly increased. These findings suggested that *C. butyricum* is able to exert neuroprotective effects against I/R injury mice through anti-oxidant and anti-apoptotic mechanisms, and reversing decrease of butyrate contents in the brain might be involved in its neuroprotection.

1. Introduction

Cerebral ischemia/reperfusion (I/R) is one of the major frequent causes of disability and death in the elderly and often causes irreversible brain damage [5]. It is characterized by the neuronal dysfunction and nerve cells damage and is caused by a sudden

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interruption of the cerebral blood supply [9]. However, the pathogenesis of I/R injury remains to be not fully understood. Oxidative stress and apoptosis of neurons are believed to be crucial mechanisms in ischemic stroke, which eventually lead to neuronal lesion and death [14]. The hippocampal CA1 region is well known as the most vulnerable region. Neuronal death in the hippocampal CA1 region occurs following transient ischemic insult. As for the oxidative stress, reactive oxygen species (ROS) involved in I/R injury is one of the primary factors resulting in neuronal loss. Accumulating evidence has demonstrated that excessive production of ROS may aggravate I/R injury and finally activate apoptotic cascades [4]. Endogenous anti-oxidant enzymes such as superoxide dismutase (SOD) and Glutathione peroxidase (GSH–PX) can clear the overproduction of ROS and ameliorate the various effects associated with I/R injury. On the other hand, apoptosis plays a leading role in the delayed neuronal death following cerebral I/R. The imbalance between the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 occurred in the progress of I/R injury [26,29]. Thus, inhibiting or blocking neuronal apoptosis could alleviate I/R injury. Taking into account the fact that I/R injury is associated with the oxidative and apoptotic mechanisms, some devote to exploring the neuroprotective agents that may attenuate the I/R injury.

Probiotics, live microorganisms, nutritional agent, and widely accepted by the public, have exhibited therapeutic effects on some gastrointestinal disorders [8,15]. Meanwhile, it has been shown that probiotics have exhibited potential therapeutic properties in neurologic disorders [3]. Particularly, probiotics have been proven to alleviate anxiety, prevent the chronic psychological stress, inhibit apoptosis in several brain regions and improve the cognitive function in mice [1,19,20]. *Clostridium butyricum*, producing high levels of butyrate in the gut lumen, is notably used as beneficial probiotics and plays a protective role against pathogenic bacteria and the intestinal injury [6]. Recent evidence showed that *C. butyricum* effectively could exert the therapeutic effects on some diseases, including antitumor effects by inducing apoptosis [22]. Moreover, a well-known property of *C. butyricum* is to produce butyrate and our previous study found that butyrate displayed neuroprotective effects against cerebral I/R injury in mice [24]. On the basis of these special functions, *C. butyricum* is seemed to have potential against cerebral I/R injury, but to our knowledge, the neuroprotective effects has not been investigated yet. Therefore, it is interesting to explore whether *C. butyricum* has positive effects on cerebral I/R injury.

In this study, we have evaluated that *C. butyricum* possesses protective effects against cerebral I/R injury induced by the bilateral common carotid artery occlusion (BCCAO) for 20 min in mice. Furthermore, we investigated the hypothesis that neuroprotective effect of *C. butyricum* pretreatment is associated with suppressing oxidative stress and apoptosis. Some possible protective mechanisms of *C. butyricum* are also proposed.

2. Material and methods

2.1. Animals

Male ICR mice (18–22 g, 6 weeks old) were purchased from the Experimental Animal Center of Wenzhou Medical University. Animals were housed in a specific room, and the treatment group was separated from each other to avoid cross contamination. Mice were provided with food and water ad libitum. All experiments were performed according to animal use guidelines and approved by the Animal Experimentation Ethics Committee of Wenzhou Medical University.

2.2. Pretreatment with C. butyricum

C. butyricum WZMC1018 (CGMCC 9830) provided by the China General Microbiological Culture Collection Center were harvested from the MRS broth for 48 h, centrifuged at 4.500 × *g* for 15 min, and resuspended in sterile saline. The mice were treated intragastrically with pretreatment *C. butyricum* (1×10^9 CFU) prior to BCCAO at doses of 200 μL once daily for successive 2 weeks.

2.3. Induction of cerebral I/R injury model

Cerebral I/R injury mouse model were performed according to previous method [25,27]. In brief, mice were anesthetized and fixed on an operating table. A midline incision was created in the ventral side of the neck to expose the right and left common carotid arteries. The cerebral I/R model were induced by BCCAO with vascular clips for 20 min. At the end of each occlusion, the clips were removed. The sham-operated controls (sham) underwent the same surgical procedure, but without carotid arteries occlusion. The sham control group and cerebral I/R model groups were treated with physiological saline at doses of 200 μL for successive 2 weeks prior to BCCAO.

2.4. Neurological deficit score

A neurological deficit score was performed at 24 h after the reperfusion according to Longa Score Scale [11]. (0) = no deficit; (1) failure to lift forepaw fully; (2) circling to the left; (3) falling to the left; (4) failure to walk spontaneously and/or depressed level of consciousness. The higher the neurological deficit score, the more severe injury.

2.5. Histopathology

After the neurological deficit score, mice were sacrificed and the brain were removed, fixed in 4% formaldehyde and embedded into paraffin. The brain tissues were sliced at 5 μm. The sections were then stained with either H&E or TUNEL (TUNEL, Roche Diagnostics, Germany) reagents using standardized protocols and analyzed, then examined under a light microscope. The numbers of karyopyknosis and apoptotic cells were counted, and the mean value was calculated.

2.6. SOD activity and MDA contents determination

The brain tissue was dissociated and stored at −80 °C until detection. The SOD activities and the MDA contents were measured using the biochemistry assay kit (Nianjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's manual.

2.7. Western blot analysis

The cerebral cortex samples were rapidly dissected, rinsed in 1 × phosphate buffered saline, and homogenized in an ice-cold homogenization buffer (Beyotime Institute of Biotechnology, Shanghai, China). The samples were then analyzed by Western blot, as described in our previous research [10,24]. The primary antibodies were anti-Bcl-2, anti-Bax, and anti-Caspase-3 (1:1000, Bioworld, USA). After incubation with primary antibodies; membranes were incubated with HRP conjugated secondary antibodies (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at room temperature. β-actin was performed as a loading control.