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Combination of curcumin and *vagus* nerve stimulation attenuates cerebral ischemia/reperfusion injury-induced behavioral deficits

Jian Xu[a], Xinjuan Kong[b], Hong Xiu[c], Yihe Dou[a], Zeyu Wu[a], Peng Sun[a,*]

[a] Department of Neurosurgery, The Affiliated Hospital of Qingdao University, Qingdao 266000, China
[b] Department of Gastroenterology, The Affiliated Hospital of Qingdao University, Qingdao 266000, China
[c] Department of Emergency, The Affiliated Hospital of Qingdao University, Qingdao 266000, China

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ABSTRACT

Aim: Previous studies indicated that cerebral ischemia/reperfusion injury (CI/RI) could induce behavioral deficits. Single treatment of *vagus* nerve stimulation (VNS) or curcumin is reported to restore CI/RI-induced behavioral deficits. However, the synergic effect remains unclear.

Materials and methods: Rats were divided into 6 groups: sham, CI/RI, VNS, CI/RI + VNS, VNS + curcumin and CI/RI + VNS + curcumin groups. Each group was further divided into three or four subgroups for further assessments. In specific, Morris water maze task and shuttle box test were used to evaluate cognitive capacity. Rota-rod test, neurological deficits scores, 2,3,5-triphenyltetrazolium chloride staining, TUNEL staining were performed to estimate motor capacity, neurological deficits, the size of infarct volume and neural apoptosis, respectively. Finally, the expressions of apoptosis-associated proteins and key kinases in the AKT/extracellular signal-regulated kinase-2 (ERK2) pathway were measured by Western blot analysis.

Results: Combination of curcumin and VNS significantly restored the CI/RI-induced cognitive and motor impairments compared with the CI/RI + VNS group ($P < 0.05$ and $P < 0.01$). Moreover, combination of curcumin and VNS significantly lowered CI/RI-induced neurological deficits, infract volume, neural apoptosis (all $P < 0.05$) and inflammatory cytokines release ($P < 0.05$ and $P < 0.01$) when compared to the CI/RI + VNS group. Additionally, the phosphorylation levels of AKT and ERK2 were both increased by combination of curcumin and VNS compared with the CI/RI + VNS group.

Conclusion: Combination of curcumin and VNS restored CI/RI-induced behavioral deficits by inhibiting apoptosis and inflammatory response. Besides, the AKT/ERK2 pathway might be implicated.

1. Introduction

Cerebral ischemia/reperfusion injury (CI/RI) is a common complication caused by various events, such as craniocerebral and thrombolytic trauma and surgical operations [1]. During the process of CI/RI, the brain suffers sever damage twice. One is due to interruption of blood flow and the other one is the reestablishment of blood supply, which even causes more deleterious damage than cerebral occlusion itself [2,3]. Accumulating evidence proposed that even a short duration of CI/RI could lead to multiple pathophysiological processes, including necrosis, apoptosis, mitochondrial dysfunction, inflammation, excitotoxicity and oxidative stress, resulting in cognitive deficiency [3–5]. Despite the advances on treatment in animals, effective strategies for clinical therapy are very few [6]. Hence, it is urgent to explore more effective adjuvant treatment.

Vagus nerve stimulation (VNS), which is approved by the Food and Drug Administration for treatment of refractory epilepsy, is a safe and effective therapeutic strategy for neurological disease, pain and obesity [7–10]. Previous studies have attempted to use VNS to induce a precisely timed release of neuromodulators and enhance neural plasticity, and thereby improve stroke recovery [11,12]. Furthermore, VNS is reported to attenuate CI/RI through endogenous cholinergic pathway in rat [13]. A previous study showed that VNS could modulate neuro-inflammatory response and exert anti-inflammatory effect on CI/RI rats [14]. Another study also well reported the effect of VNS on recovery of cognitive functioning, suggesting the potential therapeutic action of VNS [15].

Curcumin is a natural polyphenol extracted from rhizome of *Curcuma longa* [16]. In ancient Ayurveda, curcumin is used to treat different pathologies including allergy, anorexia, sinusitis, and so on [17]. With the fast development of modern medicine, curcumin is reported to possess substantial medicinal benefits, such as anti-oxidant,

* Corresponding author at: Department of Neurosurgery, The Affiliated Hospital of Qingdao University, No.16, Jiangsu Road, Qingdao 266000, China.
E-mail address: sunpeng1616@126.com (P. Sun).

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anti-inflammatory and anti-carcinogenic properties [18–20]. More recently, curcumin is proved to exhibit protective influence during neurological disorders, such as neurodegenerative diseases, CI/RI, and depression [21]. However, the effect of curcumin on learning and motor capacity remains unclear.

Both VNS-induced neuroprotection and curcumin-induced protective influence are possibly associated with anti-inflammatory and anti-apoptotic mechanisms. Therefore, we hypothesized that the combination of curcumin and VNS treatment could act as a potential avenue for restoration of CI/RI-induced learning and motor capacity disorders. In our study, we attempted to evaluate whether the synergistic effect of VNS and curcumin treatments could enhance learning and motor capacity of rats suffered CI/RI. The neural cell apoptosis and possibly involved signaling pathway were also investigated.

2. Materials and methods

2.1. Animals

A total of 220 adult Sprague-Dawley rats (male, 250–300 g) were procured from Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were maintained in cages with standard rat chow and water *ad libitum* at 22–24 °C, along with a 12 h light/dark cycle (lights on at 7:00 a.m). Experimental protocols were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. We tried our best to minimize the number and the pain of rats in our study.

2.2. CI/RI and electrical VNS

CI/RI model was constructed by right middle cerebral artery occlusion (MCAO)/reperfusion as described previously [14]. In brief, rats were deeply anesthetized with pentobarbital intraperitoneally (40 mg/kg), along with normothermia maintaining in a heating plate and continuous monitoring of blood pressure and heart rate with a SAII Monitoring System (Small Animal Instruments, Inc. Stony Brook, NY, USA). Then, a silicon-coated nylon filament was used to induce transient focal cerebral ischemia. Monitored by continuous laser Doppler flowmetry (Perimed, North Royalton, OH, USA), the CI/RI model was successfully constructed when the decrease in cerebral blood flow was 70–80% during the first 30 min and the flow recovery within the initial 10 min of reperfusion was more than 70%.

After transient MCAO for 30 min, right VNS was performed using a Grass Model S48 stimulator according to a previous study [22]. Electrodes, composed of two curved silver wires, were sutured to the sternocleidomastoid muscle and wrapped around the right cervical nerve with a microscope (Olympus, Tokyo, Japan). VNS was delivered as a 30 ms train of 0.5 ms square pulses (0.5 mA, 20 Hz), which were repeated every 5 min for 60 min. Two hours after surgery, reperfusion was performed. The rats were under anesthesia during the surgery and were sent to cages after recovery from anesthesia.

2.3. Experimental protocol

For each test, rats were randomly assigned into 6 groups (n = 30 for each group): (1) sham group, (2) CI/RI group, (3) VNS group, (4) CI/RI + VNS group, (5) VNS + curcumin group, and (6) CI/RI + VNS + curcumin group. Each group was further divided into three subgroups (n = 10 for each subgroup) for Morris water maze (MWM) task, shuttle box test and rota-rod test, respectively. Then, the neurological deficits of rats in sham group, CI/RI group, CI/RI + VNS group and CI/RI + VNS + curcumin group were scored and then all the rats were sacrificed, followed by studies of infarct area, neural apoptosis, inflammatory secretion and signaling pathway. Curcumin (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in normal saline with 1% dimethyl sulfoxide (DMSO, Sigma-Aldrich). Rats received daily intraperitoneal injection of curcumin (50 mg/kg) for five consecutive days before MCAO. Rats in sham group received same surgical procedures without occlusion using filament, along with the same frequency of the intraperitoneal injection of normal saline containing 1% DMSO.

2.4. MWM task

MWM task was performed to examine spatial learning and memory test of rats as described previously [23]. In brief, the task was consisted of navigation test and probe trial. Specifically, the navigation test was performed three times per day for 5 consecutive days before surgery, and then the probe trials were conducted on 1 d, 3 d, 7 d, 10 d, 14 d and 21 d after surgery. In each test or trial, rats (n = 10 for each group) were originally put into one of the three quadrants without the hidden platform. If the rats failed to find the submerged platform within 1 min, the rats were gently placed on the platform to avoid being exhausted. At the end of each swim, rats were dried and rested for 30 min. Performances of rats were videotaped by a video camera, and time taken to find the hidden platform (latency, s) and velocity (cm/s) were both analyzed using image tracking software (2020 Plus Tracking System; HVS Image, Buckingham, UK). The spatial learning and memory of rats were evaluated based on the latency.

2.5. Shuttle box test

As described previously, an automated shuttle box was employed for active avoidance test [24]. Briefly, there were two compartments in the box, and the floors were equipped with independently electrified stainless steel bars. Before surgery, rats (n = 10 for each group) received 5 consecutive days' training of conditioned (tone) and unconditioned (foot shock) stimulus. In specific, rats were allowed to habituate to the test environment for 2 min before trials. During the trials, the rats needed to response to a tone (80 dB, 2.4 kHz, 10 s), presenting as a quick movement to the other side of the box. If the rats were failed to response, an electrical foot shock (1.5 mA) was given for 10 s. The trials were repeated ten times per day with an interval of 60 s. The number of trials and active avoidance responses for each test were recorded on 3 d and 1 d before surgery as well as 1 d, 3 d, 7 d, 10 d, 14 d and 21 d after surgery.

2.6. Rota-rod test

Rota-rod test was used to evaluate motor coordination and motor learning ability. Before surgery, rats (n = 10 for each group) received 5 consecutive days' training with three trials per day. In each trial, rats were placed on a rota-rod apparatus (diameter 6.0 cm, Ugo Basile, Milano, Italy) with a constant speed at 20 rotations per minute (rpm) for no longer than 300 s. Rats were rested for 15 min between two trials. The latency to fall off the rotarod was recorded on 3 d and 1 d before surgery as well as 1 d, 3 d, 7 d, 10 d, 14 d and 21 d after surgery.

2.7. Neurological deficits scores and evaluation of cerebral infarct volume

Neurological scores of rats (n = 30 for each group) were assessed after behavioral test by a blinded assessor based on a 18-point scale [25]. Rats received a score depending on the severity of the symptom. Higher score means the rat condition is worse. Subsequently, rats were all sacrificed with sevoflurane overdose. Then, brains of rats (n = 10 for each group) were quickly removed and frozen, followed by coronal section resulting as five 2 mm intervals. The slices were then stained with 2,3,5-triphenyltetrazolium chloride (TTC, 2% in phosphate-buffered saline, PBS) at 37 °C for 15 min, and fixed with 10% formalin in PBS. Afterwards, the infarct volume, calculated by multiplying infarct area by slice thickness, was evaluated according to the analysis of stained slices.