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RESEARCH ARTICLE

Laser Acupuncture at GV20 Improves Brain Damage and Oxidative Stress in Animal Model of Focal Ischemic Stroke

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Abstract
The burden of stroke is high and is continually increasing due to a dramatic growth in the world's elderly population. Novel therapeutic strategies are therefore required. The present study sought to determine the effect of laser acupuncture at GV20 on brain damage, oxidative-status markers in the cerebral cortex, and superoxide dismutase in the mitochondria of an animal model of focal ischemic stroke. Wistar rats, weighing 300–350 g, were divided into the following four groups: (1) control; (2) permanent occlusion of the right middle cerebral artery (Rt.MCAO) alone; (3) Rt.MCAO plus sham laser acupuncture; and (4) Rt.MCAO plus laser-acupuncture groups. Sham laser acupuncture or laser acupuncture was performed once daily at the GV20 (Baihui) acupoint for 14 days following Rt.MCAO. Half of the rats in each group were examined by 2,3,5-triphenyltetrazolium chloride staining to determine the brain infarct volume, while the other half were examined by biochemical assays to determine the malondialdehyde level, and the glutathione peroxidase, catalase, and superoxide-dismutase activities in the brain-cortex mitochondria. The results showed that laser acupuncture at GV20 significantly decreased the brain infarct volume and malondialdehyde level, and increased the catalase, glutathione peroxidase, and superoxide-dismutase activities in cerebral ischemic rats. In conclusion, laser acupuncture at GV20 decreases the brain infarct volume in cerebral ischemic rats, at least in part due to decreased oxidative stress. Further study is warranted to investigate other possible underlying mechanisms.

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1. Introduction

The burden of stroke is currently high and likely to increase in future decades as a result of demographic and epidemiological transitions in populations. Although stroke can occur as a result of either ischemia or hemorrhage, approximately 87% of strokes appear to occur due to ischemia [1]. It has been proposed that the brain damage caused by cerebral ischemia is due to decreased oxygen and glucose delivery to brain tissue [2]. As a result, cells produce less adenosine triphosphate (ATP), leading to less available energy and disturbance in the homeostasis of ionic concentration gradients across plasma membranes. While the primary function of the mitochondria is the production of energy in the form of ATP [3], they are also involved in apoptosis, which is the regulation of cellular metabolism and the generation of reactive oxygen species (ROS) [4]. Accumulating evidence suggests that the brain damage induced by cerebral ischemia, particularly during the period following reperfusion, is accompanied by the enhanced formation of oxygen free radicals in brain tissue. The excessive production of ROS, for example, superoxide anions, hydroxyl radicals, and hydrogen peroxide, is a known cause of oxidative stress and may result in cell injury [5]. In addition, increases in the capability of the endogenous antioxidant system, decreases in ROS production, decreases in neuroinflammation, and increases in ATP production are known to protect against brain damage from cerebral ischemic stroke [6,7]. Thus, targeting one or more of these mechanisms may provide neuroprotection of the brain following cerebral ischemia.

Low-level laser therapy (LLLT) has been clinically used for the treatment of various conditions, including spinal-cord injury, Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, brain injury, and stroke [8—10]. Moreover, it has a wide range of effects at molecular, cellular, and tissue levels, including the modulation of ROS and increasing ATP in the mitochondria [11]. Numerous studies have investigated the effect of transcranial laser therapy at a wavelength of 810 nm in animal models of stroke [12]. Laser therapy at a wavelength of 810 nm is highly effective in improving the neurological performance and in preventing cell death following traumatic brain injury [13]. Laser acupuncture is a form of alternative medicine that combines traditional acupuncture and LLLT. Giving that the traditional-acupuncture stimulation at Baihui (GV20) has been used to treat anxiety, headache, dizziness, and stroke [14], it has been proposed that laser acupuncture at GV20 may ameliorate cerebral ischemic stroke. In the present study, therefore, I investigated the effect of laser acupuncture at GV20 on brain damage, oxidative-status markers in cerebral cortex, and superoxide dismutase (SOD) in the mitochondria of an animal model, where focal ischemic stroke is induced by the permanent occlusion of the right middle cerebral artery (Rt.MCAO).

2. Materials and methods

2.1. Animals

Male Wistar rats (8 weeks old, weighing 300—350 g) were obtained from the National Laboratory Animal Center (Salaya, Nakhon Pathom). They were housed in groups of five per cage in standard metal cages at 22 ± 2 °C on 12:12 h of light—dark cycle. All animals were given free access to food and water *ad libitum*. All efforts were made to minimize animal suffering in accordance with the directives for the laboratory use and care of animals, issued by the Institutional Animal Care and Use Committee, Khon Kaen University, Thailand (record number AEKKU 28/2558).

2.2. Animal treatment

All animals were randomly divided into four groups, and each group comprised of 10 animals as follows:

(1) Group 1 (control group): Rats were given a sham operation and received no treatment.
(2) Group 2 (Rt.MCAO group): A focal cerebral ischemia was induced in rats by Rt.MCAO. No treatment was given.
(3) Group 3 (Rt.MCAO-plus-sham-laser-acupuncture group): A focal cerebral ischemia was induced in rats by Rt.MCAO, and laser acupuncture at a non-acupoint located 5 mm next to Baihui (GV20) was administered.
(4) Group IV (Rt.MCAO plus laser-acupuncture group): A focal cerebral ischemia was induced in rats by Rt.MCAO, and laser acupuncture at the Baihui (GV20) point was administered.

All animals were treated with the assigned intervention once daily for 14 days after the Rt.MCAO. Half of the rats in each group were examined by 2,3,5-triphenyltetrazolium chloride (TTC) staining to determine the brain infarct volume, while the other half were examined by biochemical assay to determine the malondialdehyde (MDA) level, and the glutathione peroxidase (GSH-Px), catalase (CAT), and SOD activities in the brain-cortex mitochondria.

2.3. Induction of focal cerebral ischemia

Before surgery, all experimental animals were fasted for 12 h, but they were allowed free access to water. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium at a dose of 40 mg/kg body weight. Focal cerebral ischemia was induced by permanent intraluminal Rt.MCAO [15]. In brief, the right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision and were proximally ligated. A silicone-coated round-tipped nylon monofilament (4-0) suture (The USS/DG[TM] division of United States Surgical; Tyco Healthcare Group LP, Norwalk, CT, USA) was inserted into the common carotid artery just below the carotid bifurcation, and advanced into the internal carotid artery approximately 19 mm distal to the carotid bifurcation until a slight resistance was felt. Occlusion of the origins of the anterior cerebral artery, the middle cerebral artery, and the posterior communicating artery was thereby achieved. The wound was sutured, and the incision sites were infiltrated with 10% povidone-iodine solution for antiseptic postoperative care. The rats were then returned to their cages with free access to food and water.

2.4. Laser acupuncture

The rats were anesthetized with pentobarbital sodium at a dose of 40 mg/kg body weight to minimize stress prior to the laser-acupuncture treatment. The animals were treated with laser acupuncture at the GV20 acupoint and non-acupoint once daily for 14 days. The laser beam (Weberneedle®, Lauenförde, Germany) was administered at a wavelength of 810 nm, laser-module output of 100 mW, as pulsed waves (50%), and laser spot diameter of 100 μm at acupoint and non-acupoint for 10 min at each point.

2.5. Determination of brain infarct volume

Under deep anesthesia, the rats were perfused with cold phosphate-buffered saline via the ascending aorta at the end of the experiment. Then, the brains were removed and cut coronally into 2-mm sections with a brain slicer. The sections were immersed in 2% TTC (Sigma–Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. The infarct areas were traced and evaluated in all sections of the brain with the aid of an Olympus light microscope model BH-2 (Olympus optical co. LTD, Tokyo, Japan) using Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA), and then were quantified by an image-analysis system [16].

2.6. Determination of oxidative-stress markers

The rats were perfused with cold phosphate-buffered saline to remove blood from the brain tissue. This was then rapidly removed and stored at −80 °C until used. The cerebral cortex was separated and homogenized in a buffer (10 mM sucrose, 10 mM Tris–HCl, and 0.1 mM Ethylenedinitrilo tetraacetic acid, EDTA (Sigma_Aldrich), and then adjusted to pH 7.4).

2.6.1. Protein determination

The method of Lowry et al [17] was used to determine the protein concentration in the aforementioned brain-homogenate samples, and bovine serum albumin (BSA) was used as a standard. The amount of proteins in the sample can be measured via reading the absorbance using a spectrophotometer at 650 nm.

2.6.2. MDA level

The method of Okhawa et al [18] was used to determine the MDA levels in the brain-homogenate samples via the thiobarbituric-acid reaction. The colored end product was read using a spectrophotometer at 540 nm. The results were expressed as nmoles MDA/mg protein.

2.6.3. CAT activity

The CAT activity was measured in the supernatant by recording the rate of decrease in H_2O_2 absorbance using a spectrophotometer at 240 nm [19]. The activity of CAT was expressed as units/mg protein.

2.6.4. GSH-Px level

GSH-Px was measured using a spectrophotometer recorded at 340 nm, and its activity was determined using *t*-butyl hydroperoxide as a substrate, and glutathione reductase (GR) and dihydronicotinamide-adenine dinucleotide phosphate (NADPH) as enzymatic and non-enzymatic indicators, respectively [20].

2.7. Isolation of mitochondria from the brain tissue for the determination of SOD activity

The cortex brain area was isolated and homogenized in mitochondrial isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% BSA, and 0.01% digitonin adjusted to pH 7.2 with potassium hydroxide (KOH)). The tissue homogenates were centrifuged at 1000 *g* for 2 min at 4 °C. The supernatant was placed aside, and the pellet was resuspended in 0.2 mL of the isolation buffer and centrifuged again at 1000 *g* for 2 min. The supernatants were combined and mixed with 0.07 mL of 80 vol% Percoll solution (1 M sucrose, 50 mM HEPES, and 10 mM EGTA adjusted to pH 7.0 with KOH). A volume of 0.7 mL of 10% Percoll solution was gently layered on top, and the mixture was centrifuged for 10 min at 18,500 *g*. The 10 vol% Percoll solution was prepared by diluting 80 vol% Percoll solution with the isolation buffer. The mitochondrial pellet was further purified by resuspending in the 0.7 mL of the washing buffer (5 mM HEPES, 250 mM sucrose, 0.1 mM EGTA, and 1 mg/mL of BSA adjusted to pH 7.2 with KOH) and centrifuging for 5 min at 10,000 *g*. Thereafter, the final mitochondrial pellet was suspended in 0.07 mL of the washing buffer and stored at −80 °C until used [21]. The SOD activity was determined using an SOD assay kit (Sigma–Aldrich) according to the manufacturer's instructions.

2.8. Statistical analysis

The data were expressed as mean value ± the standard error of the mean. The statistical significance of the data was determined by one-way analysis of variance followed by the *post hoc* least-significant-difference paired-comparison test. A $p < 0.05$ was considered statistically significant. All data were processed with SPSS statistical software (SPSS-IBM Inc., Chicago, IL, USA).

3. Results

3.1. Effect of laser acupuncture at GV20 on brain infarct volume

Previous research had demonstrated that brain impairments are related to the severity of the brain infarction. Therefore, the present study determined the brain infarct volume after treatment with laser acupuncture at GV20. The results in Fig. 1 show that the animals with focal ischemic stroke induced by permanent Rt.MCAO had a significantly increased infarct volume ($p < 0.05$) compared to the control group. In the laser-acupuncture-treated animals, this infarct volume was significantly decreased in both the cortical and subcortical areas ($p < 0.05$) compared to the Rt.MCAO-alone group and the Rt.MCAO-plus-sham-laser-acupuncture group.

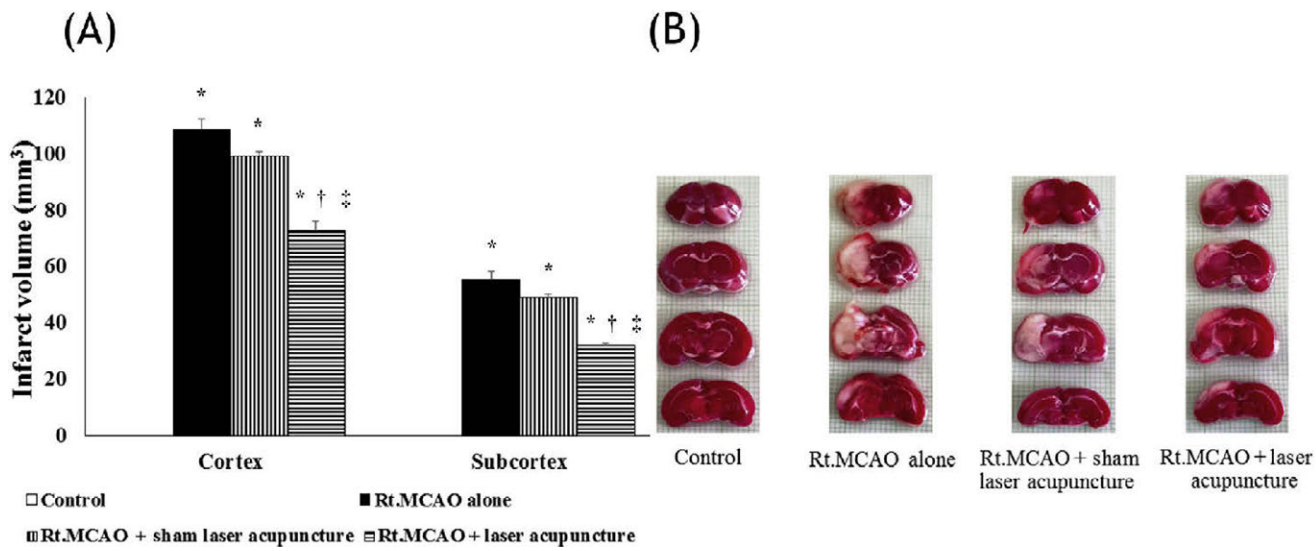

Figure 1 Effect of laser acupuncture at GV20 on brain infarct volume. (A) Representative sections of triphenyltetrazolium-chloride-stained brains from animals in each treatment group. Dark staining is indicative of viable tissue, and the absence of stain is indicative of an infarcted region. (B) Effect of laser acupuncture at GV20 on brain infarct volume in cerebral ischemic rats. Values given are the mean ± standard error of the mean ($N = 5$). *$p < 0.05$ when compared to the control. †$p < 0.05$ when compared to the Rt.MCAO-alone group. ‡$p < 0.05$ when compared to Rt.MCAO-plus-sham-laser-acupuncture group. Rt.MCAO = right middle cerebral artery occlusion.

3.2. Effect of laser acupuncture at GV20 on endogenous antioxidant enzymes

The endogenous antioxidant enzymes CAT and GSH-Px, as well as the oxidative-stress marker MDA, were also examined in this study. In the animals with permanent Rt.MCAO, the MDA level was significantly increased, and the CAT and GSH-Px activities were significantly decreased in the cerebral cortex ($p < 0.05$) compared to the control group. In the animals treated with laser acupuncture at GV20, the MDA level was significantly less elevated, and the CAT and GSH-Px activities were significantly less diminished ($p < 0.05$) compared to the Rt.MCAO-alone group and the Rt.MCAO-plus-sham-laser-acupuncture group (Figs. 2–4).

3.3. Effect of laser acupuncture at GV20 on the activity of SOD in the mitochondria

Rt.MCAO induced a significant decrease in the mitochondrial SOD activity compared to the control group ($p < 0.05$), as shown in Fig. 5. However, in the laser-acupuncture-treated rats, the mitochondrial SOD activity was significantly less diminished ($p < 0.05$) compared to the Rt.MCAO-alone group and the Rt.MCAO-plus-sham-laser-acupuncture group.

4. Discussion

The present study has demonstrated that focal ischemic stroke induced by the permanent Rt.MCAO is accompanied

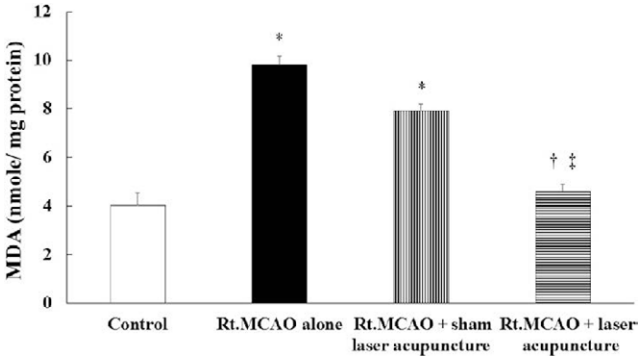

Figure 2 Effect of laser acupuncture at GV20 on MDA level in the cerebral cortex. The data are expressed as mean ± standard error of the mean ($N = 5$). *$p < 0.05$ when compared to the control. †$p < 0.05$ when compared to the Rt.MCAO-alone group. ‡$p < 0.05$ when compared to Rt.MCAO-plus-sham-laser-acupuncture group. MDA = malondialdehyde; Rt.MCAO = right middle cerebral artery occlusion.

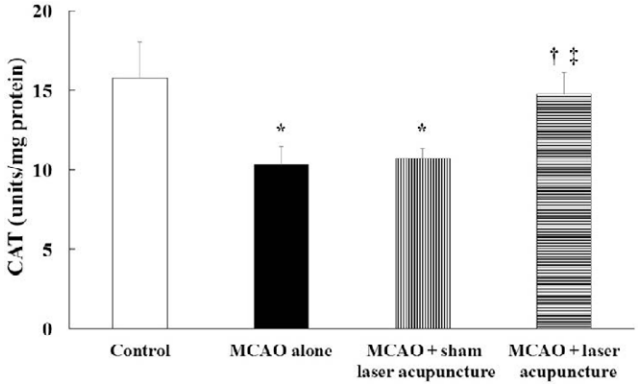

Figure 3 Effect of laser acupuncture at GV20 on CAT activity in the cerebral cortex. The data are expressed as mean ± standard error of the mean ($N = 5$). *$p < 0.05$ when compared to the control. †$p < 0.05$ when compared to the Rt.MCAO-alone group. ‡$p < 0.05$ when compared to the Rt.MCAO-plus-sham-laser-acupuncture group. CAT, catalase; Rt.MCAO = right middle cerebral artery occlusion.

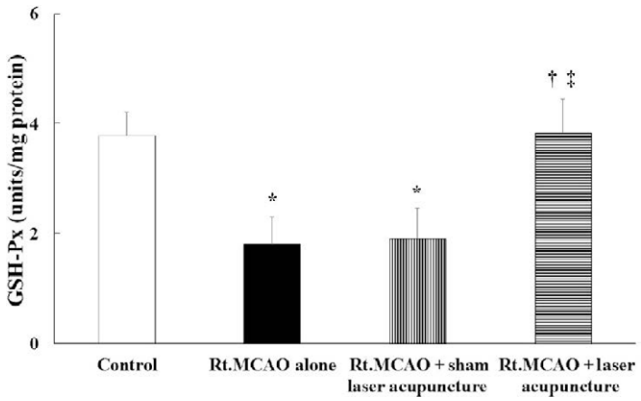

Figure 4 Effect of laser acupuncture at GV20 on GSH-Px activity in the cerebral cortex. The data are expressed as mean ± standard error of the mean ($N = 5$). *$p < 0.05$ when compared to the control. †$p < 0.05$ when compared to the Rt.MCAO-alone group. ‡$p < 0.05$ when compared to the Rt.MCAO-plus-sham-laser-acupuncture group. GSH-Px = glutathione peroxidase; Rt.MCAO = right middle cerebral artery occlusion.

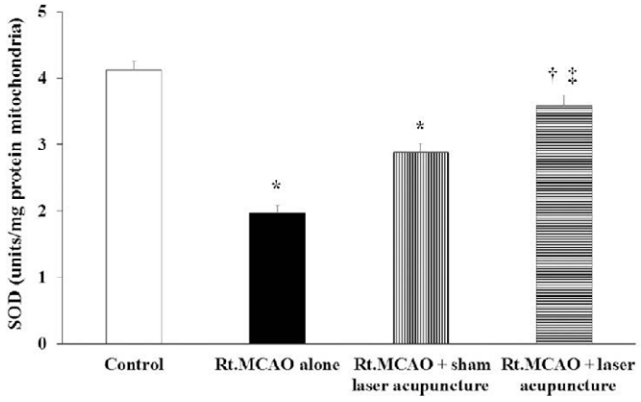

Figure 5 Effect of laser acupuncture at GV20 on the activity of SOD in mitochondria. The data are expressed as mean ± standard error of the mean ($N = 5$). *$p < 0.05$ when compared to the control. †$p < 0.05$ when compared to the Rt.MCAO-alone group. ‡$p < 0.05$ when compared to the Rt.MCAO-plus-sham-laser-acupuncture group. Rt.MCAO = right middle cerebral artery occlusion; SOD = superoxide dismutase.

by significantly increased infarct volume, but this infarct volume is significantly smaller in both the cortical and subcortical areas in the animals treated with laser acupuncture at GV20. Previous studies have reported that, in focal cerebral ischemia, there may be absolutely no blood flow in the very central core of the ischemia, and that this induces important changes, including energy failure. This in turn causes depolarization of the neuronal membrane, and excitatory neurotransmitters, such as glutamate, are released together. A marked influx of Ca^{2+} into neurons then occurs, followed by the occurrence of recessive free-radical production, which provokes the enzymatic process, leading to irreversible neuronal injury [22]. Evidence suggests that LLLT has the potential to stimulate tissue healing and repair after injury; increase cell proliferation; improve microcirculation to the brain; and reduce pain, inflammation, and swelling [23–25]. Laser therapy at a wavelength of 810 nm is highly effective in improving the neurological performance and in preventing cell death following traumatic brain injury [13]. In addition, there are several studies reporting that the traditional GV20 acupuncture treatment significantly increases cerebral blood flow to the brain [26,27]. The combination of acupuncture and laser therapy could therefore increase the flow of Qi and blood circulation to the brain.

In this experimental design, 5 mm next to Baihui (GV20) has been chosen for the sham acupoint, which is close to Sishencong (EX-HN1) [28]. The literature indicates that Sishencong (EX-HN1) also has some effects to treat stroke condition, but according to the traditional-Chinese-medicine theory, it has a weaker effect on ischemic-stroke treatment [29]. There are many acupoints located in the head, according to the atlas of the rat acupoint. Therefore, it is difficult to choose the sham acupoint that is far enough from the other acupoints. Liu et al used a fluorodeoxyglucose-based micro- positron emission tomography (PET) imaging technique to measure the glucose level in the brain, and used magnetic resonance imaging and TTC staining to measure the brain area injured after ischemic stroke. They found that real acupuncture had a positive effect in treating ischemic stroke more than the sham-acupoint stimulus [29]. In addition, our previous study also demonstrated that there was no significant difference between the blank control (MCAO with no treatment) and the non-acupoint stimulus [30].

It is important to note the methodological limitation of this study. The author used the laser beam (Weberneedle laser) at a wavelength of 810 nm, laser-module output of 100 mW, as pulsed waves (50%), and laser spot diameter of 100 μm at acupoint and non-acupoint for 10 min at each point, resulting in an energy density of about 30 J. Literature reviews insist that the laser-acupuncture application to stimulate acupoints on the body in chronic stroke patients was given at 51 J/cm^2, and deeper points were treated at 103 J/cm^2 [31]. Moreover, the red laser stimulation of the Baihui acupoint in an energy density of about 20 J/cm^2 decreased the heart rate in human participants [32], and laser beam at a wavelength of 405 nm, an output power of 100 mW, and a spot diameter of 500 μm for 10 min applied at HT7 point could reduce the cognitive impairment in the animal models of Alzheimer's disease [33]. On the other hand, laser acupuncture uses red or near-infrared light with a wavelength between 600 and 1000 nm, and power between 5 and 500 mW, or LLLT with 1–4 J/cm^2 per acupoint might have a photobiostimulation effect [34]. Therefore, an energy density of about 30 J from this study might more than low-level laser-acupuncture application. Further studies, the optimal energy density or instensity for laser acupuncture must be concerned on each point, must make sure that the power energy is not too high.

Given the crucial role of free radicals in the pathophysiology of cerebral ischemia, the present study also determined the effect of laser acupuncture at GV20 on the lipid peroxidation (LPO) and the activity of scavenger enzymes. It was found that laser acupuncture at GV20 could decrease the LPO product. Recent evidence supports the involvement of

oxidative stress in brain injury mediated by cerebral ischemia and stroke [35]. The roles of ROS and LPO have been proposed to be important factors in the reduction of cerebral blood flow and reperfusion injury [36,37]. Neurodegeneration has also been postulated to be associated with ROS, which reacted with cellular macromolecules, such as lipids, proteins, and nucleic acids, leading to neuron damage [38]. Therefore, the endogenous-antioxidant-enzyme activity of the ischemic brain was particularly important, and the measurement of antioxidant enzymes could be used as a tool to assess the vulnerability of the ischemic-brain areas [39]. The present study showed that the permanent Rt.MCAO significantly decreased the CAT, GSH-Px, and mitochondrial SOD activities in the mitochondria of the cerebral cortex, and increased the LPO product as mentioned earlier. Under these conditions, the administration of an 810-nm laser at GV20 significantly improved the activity of scavenger enzymes and diminished the elevated MDA level. Numerous studies have investigated the effect of transcranial laser therapy at a wavelength of 810 nm in animal models of stroke [12]. The molecular mechanisms of LLLT have been reported to include increasing mitochondrial function and ATP production [11], and the modulation of ROS and intracellular calcium, leading to antioxidant, anti-apoptotic, pro-proliferation, and anti-inflammatory activities [40,41]. Moreover, the stimulation of the Baihui point improves oxygenation and attenuates the oxidative stress in the brain of cerebral ischemic rats [42].

In conclusion, the current study clearly demonstrates that laser acupuncture at GV20 can integrate the beneficial effects of the aforementioned two treatments, and is related to antioxidant action. Thus, laser acupuncture is a novel and potentially useful therapeutic option for focal stroke. Further research is warranted to determine if additional mechanism(s) contribute to the beneficial therapeutic effects observed in this study.

Disclosure statement

I have read the submitted manuscript that includes my name as an author and vouch for its accuracy. I certify that I have participated sufficiently in the conception, design, analysis of the data of this work and the writing of this manuscript. I really believe my work represents valid and honest work. The author declare no conflict of interest.

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