Roles of electro-acupuncture in glucose metabolism as assessed by \(^{18}\text{F-FDG/PET}\) imaging and AMPK\(\alpha\) phosphorylation in rats with ischemic stroke

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Received December 31, 2016; Accepted June 27, 2017

DOI: 10.3892/ijmm.2017.3057

Abstract. Targeted energy metabolism balance contributes to neural survival during ischemic stroke. Herein, we tested the hypothesis that electro-acupuncture (EA) can enhance cerebral glucose metabolism assessed by \(^{18}\text{F-fluorodeoxyglucose/positron emission tomography (\(^{18}\text{F-FDG/PET}\))}\) imaging to prevent propagation of tissue damage and improve neurological outcome in rats subjected to ischemia and reperfusion injury. Rats underwent middle cerebral artery occlusion (MCAO) and received EA treatment at the LI11 and ST36 acupoints or non-acupoint treatment once a day for 7 days. After EA treatment, a significant reduction in the infarct volume was determined by T2-weighted imaging, accompanied by the functional recovery in CatWalk and Rota-rod performance. Moreover, EA promoted higher glucose metabolism in the caudate putamen (CPu), motor cortex (MCTX), somatosensory cortex (SCTX) regions as assessed by animal \(^{18}\text{F-FDG/PET}\) imaging, suggesting that three-brain regional neural activity was enhanced by EA. In addition, the AMP-activated protein kinase \(\alpha\) (AMPK\(\alpha\)) in the CPu, MCTX and SCTX regions was phosphorylated at threonine 172 (Thr172) after ischemic injury; however, phosphorylation of AMPK was further increased by EA. These results indicate that EA could promote AMPK\(\alpha\) phosphorylation of the CPu, MCTX and SCTX regions to enhance neural activity and motor functional recovery after ischemic stroke.

Introduction

Approximately 800,000 individuals suffer a stroke each year worldwide, and 70% of stroke survivors suffer from motor impairments, resulting in a reduction in the quality of life and a large socioeconomic burden (1,2). In addition to standard rehabilitation therapies, acupuncture is currently being investigated as a potential treatment for improving motor function in stroke (3).

Acupuncture has broad clinical applications and involves the insertion of needles along specific pathways (meridians) as described in traditional Chinese medicine (TCM). Electrical stimulation added to traditional acupuncture is a technique called electro-acupuncture (EA), which intensifies the inserting dose and enhances treatment efficiency (4). An expanding literature and clinical studies have substantiated that EA at selected acupoints can improve the scores of motor assessment scales in post-stroke hemiplegia patients (5,6). In animal studies, EA was found to ameliorate motor deficits via reduction in cerebral infarct volume and histopathological damages in rats that suffered an induced stroke (7-9). Therefore, EA is a safe and effective therapy for improving motor function in stroke; however, its functional mechanism has not been fully elucidated.

Within the core of the ischemic stroke, where cerebral blood flow is most severely restricted, oxygen and glucose deprivation of neural cells occurs within minutes. In prolonged ischemia, an imbalance in energy supply and demand results in surrounding neural hypoxia and dysfunction, which induces delayed damage, including production of reactive oxygen species, excitotoxicity, intracellular calcium overload, endoplasmic reticulum stress and inflammatory injury (10). Conversely, restoration of energy metabolism balance in the surrounding cells following acute ischemic stroke may reduce ischemic injury. Studies have demonstrated that EA improves energy metabolism in normal rats (11,12). Therefore, these findings indicate that EA could increase glucose metabolism to protect against neural injury under ischemic stroke.

Small animal positron emission tomography scanner (microPET) is a non-invasive technique which produces high quality images that provide information concerning relative
access to food and water. All experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (FUTCM).

Methods and materials

Animals and statement of ethics. Adult male Sprague-Dawley (SD) rats weighing 200-250 g were provided by Shanghai Laboratory Animal Co., Ltd. (SLAC), Shanghai, China (license no. SCXK 2014-007) for this study. All rats were maintained in standard conditions under constant temperature (23±2°C), humidity (60-70%) and 12-h light/dark cycle with ad libitum access to food and water. All experiments were strictly conducted in accordance with the guidelines of the National Institutes of Health (NIH), and the care and use of the rats were carried out following protocols approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (FUTCM).

Ischemic model and grouping. Rats were randomly divided into the sham operation group (n=16) and the ischemia group (n=54). Rats were anesthetized with 1.5% isoflurane in 68.5% N₂O and 30% O₂. The model of cerebral ischemia was performed by middle cerebral artery occlusion (MCAO) on animals in the ischemia group, as described previously (22). Briefly, a 2-cm midline incision was made on the neck, and the left common, external and internal carotid arteries were carefully exposed and identified. The external and common carotid arteries were ligated, and then 4-0 nylon filament (Jia Ling Biological Technology Ltd., Guangzhou, China) with a heat-blunted tip was introduced into the internal carotid artery from the common carotid artery to block the origin of the MCA for 2 h. Animals in the sham group had the same surgical procedures but received no occlusion on the artery. The rats were reperfused by withdrawing the filament. The MCAO surgery and sham-operation were carried out and the body temperature of the sham-operated or operated rats was maintained at 37a±0.5°C during the surgical procedure (23,24). The Zea-Longa score conducted in a blinded manner at 24 h was made to exclude the rats of failed ischemia and death (25). Using a random number table, the rest of the rats in the ischemia group were randomly assigned into the MCAO group (MCAO), the EA treatment group (EA) and the non-acupoint EA treatment group (non-EA) (n=16 each group).

EA treatment. The groups received EA or non-EA treatment at 24 h after reperfusion and continued treatment for a consecutive 7 days by using an electrical stimulator (Model G6805; SMIF, Shanghai, China). Meanwhile, the Sham and MCAO rats received the same holding but were not subjected to EA or non-EA. A stainless steel needle (0.3 mm in diameter and 30 mm in length) was inserted into the right unilateral paralyzed limb of Zusani (ST36, located on the anterior lateral side of the leg close to the anterior crest of the tibia) and Quchi (LI11, located at the midpoint between the lateral end of the transverse cubical crease) at a depth of 5 mm. Additionally, the non-acupoint EA treatments were performed by stimulating the distal non-acupoints. One of the non-acupoints is ~3 cm distal from the ST36 acupoint toward the tail and opposite to the knee joint. It is located over the semitendinosus muscle at 5 mm from the tail base. The other one is ~3 cm distal from the LI11 acupoint toward the shoulder joint, located over the biceps brachii inside of the elbow. The parameters of the EA stimulation consisted of a dense disperse wave of 2/20 Hz, 30 min each time, once a day (26).

Neurological function assessments. Neurological function assessments based on a 5-point scale (25) and modified neurological severity scores (mNSS) were performed to evaluate the degree of neurological deficits at 24 h and day 7 after surgery. Behavioral test was performed by the Zea-Longa 5-point scale. To assess neurologic function, mNSS is recognized as a crucial mean, which focuses on overall performance of motion, sensation, balance function and nerve reflex (27,28).

Magnetic resonance imaging. The infarct volumes were detected by T2-weighted imaging (T2WI) at day 7 after reperfusion. Before the scans, animals were anesthetized with 3% isoflurane for 5 min. Rats were placed into a holder compatible with MRI acquisition systems and body temperature was maintained at 37±0.5°C. Assessments were performed by 7.0 T MRI (Pharmascan, microMRI; Bruker, Karlsruhe, Germany), and T2WI spin-echo sequence was used with the parameters: echo time/repetition time, 35/4200 msec; field of view, 32x32 mm; slice thickness, 0.8 mm; slices, 24; matrix, 256x256; average, 4.

Magnetic resonance imaging image analysis. Infarct regions were defined on the region of increased signal in the ipsilateral hemisphere by using ImageJ software (NIH, Bethesda, MD, 2010).
Metabolic SUV right/metabolic SUV left x 100%.

was expressed as the percentage obtained through the formula:

\[
\text{Percentage} = \left( \frac{\text{SUV right}}{\text{SUV left}} \right) \times 100\%.
\]

The extent of metabolism was calculated by multiplying the total region of the delimited region by the slice thickness.

CatWalk gait analysis. CatWalk XT9 (Noldus Information Technology, Wageningen, The Netherlands), a real-time and quantitative gait analysis system (29,30) was used for this study in which animals cross a 7-cm wide glass walkway illuminated by green light from the side in a darkened room. When the paws contact the glass floor, the light is scattered and captured by a digital high-speed camera which is then recorded and measured with CatWalk software 10.0. For each animal, we performed analysis of a package of footprint parameters included base of support, stride length, step sequence, print width, swing speed, cadence, average speed and duration (31). The rats were trained for one week, 6 times a day (32). The continual training without any stoppers for the entire distance and 3 valid result recordings are a basic requirement (33). Animals received gait assessment at day 7 after EA treatment.

Rota‑rod test. Motor function was also evaluated by Rota‑rod test (Ugo Basile, Varese, Italy) which is widely used in ischemic animal research (34-36). The animals had 3 sessions of training at 4 rpm for 3 days in the pre‑training period. After EA treatment, the time to fall on the accelerating rod at 4-40 rpm over a period of 4 min was recorded (37). Each trial was repeated three times.

Micro PET/CT. 18F-fluorodeoxyglucose (FDG)‑PET (MILabs BV, Utrecht, The Netherlands) examination was conducted at day 7 after EA treatment to measure glucose metabolic activity of the brain. The rats were sent to the small animal PET/CT laboratory after 12 h of fasting with only water, and were then subjected to the following procedure. i) The rats received gas (3.5% isoflurane, 400 ml/min) anesthesia in the anesthesia induction chamber. ii) 18F-FDG (1 mCi; PET‑CT Center of Nuclear Medicine of Fujian Province Hospital, China) was injected via the tail vein. iii) The rats were allowed to rest for 30 min in a dark room without noise disturbance. iv) Thirty minutes later, the rats again were gas anesthetized, and were subjected to a PET scan until fully anesthetized. v) Rats underwent PET scanning on a VECT or +CT scanner with constant anesthesia (2.0% isoflurane, 250 ml/min). After setting the scanning parameters (55 kV voltage, 615 μA intensity), spiral CT detection was performed for the first 10 min, and PET scanning was then conducted for 15 min. Any signs of distress include the breathing of the rats were monitored while the image was acquired.

Wild‑type animals were euthanized by dissention into the left caudate putamen (CPu), motor cortex (MCTX), somatosensory cortex (SCTX) tissue and frozen in liquid nitrogen. The brain samples were stored at -80°C. Samples were homogenized in lysis buffer (RIPA); after centrifugation, a BCA (Thermo Fisher Scientific, Waltham, MA, USA) assay was used to determine protein concentrations. An equal amount of protein was subjected to electrophoresis on a 5-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio‑Rad, Hercules, CA, USA) and subsequently transferred to polyvinylidene difluoride membranes (0.45 μm; Millipore, Billerica, MA, USA). Total AMPKα (t‑AMPKα, 1:1,000; no. orb14583; Biorbyt, San Francisco, CA, USA), phosphorylated AMPKα (p‑AMPKα, Thr172, 1:1,000; no. 2531; Cell Signaling Technology, Danvers, MA, USA) were the primary antibodies, and β-actin (1:8,000; Sigma, St. Louis, MO, USA) was used as the loading control. Blots were maintained at room temperature for 1 h in 5% BSA and primary antibody incubation was carried out overnight at 4°C. Incubation with the secondary antibody (1:5,000, goat anti-rabbit IgG; Chemicon, Temecula, CA, USA) was carried out for 2 h, and the ECL detection kit was used for signal detection.

Statistical analysis. All data are expressed as the mean ± SEM or mean ± SD in each group. Multiple comparisons were carried out with one-way ANOVA, and comparison of two groups was carried out with the LSD test or Mann-Whitney U test. P-value of <0.05 or <0.01 was indicative of statistical significance.

Results

EA treatment improves neurological deficits in MCAO rats. As shown in Fig. 1, compared with the Sham group, the rats with reperfusion had significantly higher scores in terms of the neurological deficit according to the 5-point scale and the mNSS score (P<0.01), whereas no significant differences were noted among the MCAO group, the EA group and the non-EA group (P>0.05). However, the neurological deficits were improved after EA treatment at day 7 (Fig. 1) (P<0.05).

EA treatment decreases the infarct volumes in MCAO rats. As shown in Fig. 2, the cerebral infarct volume was significantly reduced in the EA group compared with that in the MCAO group and non-EA group at day 7 after EA treatment (P<0.05). These results suggest that EA treatment attenuated the cerebral infarct lesion.

Western blotting. After EA treatment, all rats were euthanized. Then the brains were rapidly removed from the skull, followed by dissention into the left caudate putamen (CPu), motor cortex (MCTX), somatosensory cortex (SCTX) tissue and frozen in liquid nitrogen. The brain samples were stored at -80°C. Samples were homogenized in lysis buffer (RIPA); after centrifugation, a BCA (Thermo Fisher Scientific, Waltham, MA, USA) assay was used to determine protein concentrations. An equal amount of protein was subjected to electrophoresis on a 5-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio‑Rad, Hercules, CA, USA) and subsequently transferred to polyvinylidene difluoride membranes (0.45 μm; Millipore, Billerica, MA, USA). Total AMPKα (t‑AMPKα, 1:1,000; no. orb14583; Biorbyt, San Francisco, CA, USA), phosphorylated AMPKα (p‑AMPKα, Thr172, 1:1,000; no. 2531; Cell Signaling Technology, Danvers, MA, USA) were the primary antibodies, and β-actin (1:8,000; Sigma, St. Louis, MO, USA) was used as the loading control. Blots were maintained at room temperature for 1 h in 5% BSA and primary antibody incubation was carried out overnight at 4°C. Incubation with the secondary antibody (1:5,000, goat anti-rabbit IgG; Chemicon, Temecula, CA, USA) was carried out for 2 h, and the ECL detection kit was used for signal detection.

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an overall significant increase in these parameters in the EA group (P<0.05), which was not observed in the non-EA group (P>0.05). In regards to the rat crawling speed, the EA group showed a significant higher score than the MCAO group and the non-EA group (P>0.05).

Rota-rod test. In the model MCAO group, the animal motor performance was significantly decreased as compared with the Sham group (P<0.01). However, the rats in the EA treatment group also had improved post-ischemic motor outcome as shown in Fig. 4 both at the fixed and the accelerated modes, as assessed by the Rota-rod test, compared to the MCAO group and the non-EA group (P>0.05).

EA treatment enhances the glucose metabolism in MCAO rats. $^{18}$F-FDG/PET imaging is a sensitive and reliable means of assessing cerebral ischemic metabolism. In normal rats, there was no asymmetric metabolism between the left and right hemispheres (Fig. 5A). There was a significantly metabolic activity reduction in the left hemisphere compared with the right in rats after ischemia and reperfusion. Compared with the Sham group, the MCAO group exhibited a lower level of glucose metabolism in the CPu, MCTX and SCTX regions (P<0.01) (Fig. 5B). Compared with the MCAO and non-EA groups, the EA group rats exhibited a higher level of glucose metabolism in the CPu, MCTX and SCTX regions (P<0.05 or P<0.01) (Fig. 5B). There was a significant difference in the CPu and SCTX regions between the MCAO group and the non-EA group (P<0.01 or P<0.05) (Fig. 5B).

EA treatment increases the levels of p-AMPKα in the CPu, MCTX and SCTX regions of MCAO rats. AMPKα, a key sensor of cellular energy levels, is activated under conditions of pathological stress, including hypoxia, oxidative stress, glucose deprivation or heat shock (38). Activation of AMPKα, as assessed by phosphorylation status, can provide a short-term energy supply through astrocytes for ischemic neurons (39,40). Therefore, to determine whether AMPKα is activated, western blot analysis was conducted to test the levels of t-AMPKα and p-AMPKα in the CPu, MCTX and SCTX regions. As shown in Fig. 6, the expression of t-AMPKα showed no difference among the Sham, MCAO, EA and non-EA groups. However, the ratio of p-AMPKα/t-AMPKα showed no difference among the Sham, MCAO, EA and non-EA groups. However, the ratio of p-AMPKα/t-AMPKα in the CPu, MCTX and SCTX regions was increased in the MCAO group compared with the ratio in the Sham group (P<0.01 or P<0.05), whereas the level of p-AMPKα/t-AMPKα was further increased in the EA group (P<0.01 or P<0.05). A significant
difference was noted between the EA and the non-EA group (P<0.01 or P<0.05). These results suggest that EA treatment could stimulate AMPK α phosphorylation in the CPu, MCTX and SCTX regions.

Discussion

In the present study, we demonstrated that EA at the LI11 and ST36 acupoints could improve the neurological deficit and subsequent motor functional impairment after ischemia reperfusion injury in rats. Furthermore, the beneficial effects of EA were mediated by enhancing glucose utilization in the regions of the motor control system including CPu, MCTX and SCTX as assessed by 18F-FDG/PET imaging. We further explored the mechanism that EA could enhance neural activity-related AMPKα phosphorylation.

Clinical studies have shown that EA at the LI11 and ST36 acupoints promotes limb functional recovery after stroke (41,42). A retrospective study showed that EA could reduce vasospasm and improve Rankin scale and subsequent functional recovery in patients with cerebral vascular disease (43). Similarly, animal experiments also found that EA treatment could improve neurological function in cerebral ischemic injury rats (44,45). Our previous studies confirmed that EA at the LI11 and ST36 acupoints could ameliorate neuroethology (46‑48) and neurological deficits (49) in MCAO rats. The present study found that treatment with EA at the LI11 and ST36 acupoints for 7 days could improve the neurological deficits and reduce infarct volumes assessed by T2WI following MCAO in rats. Furthermore, to address the EA effect on motor function, we used CatWalk gait analysis and Rota-rod test which are sensitive to ischemic insult. Cerebral ischemia led to a reduction in swing speed and the number of steps per second, which was consistent with a previous study (50). The variation in parameters (decrease of stride length and swing speed, increase in swing time and single foot standing time) was associated with the symptoms (walking instability, walking speed slowdown and limb swing reduction) of stroke patients, which reflect the motor dysfunction due to ischemic insult (51). Consistent with previous observations (52,53), we
found that limb motor dysfunction caused by MCAO was significantly improved by EA treatment, in regards to the running average speed, step length and limb swing speed, as well as an increase in retention time on the accelerating rod.

Glucose metabolism is the dominant cerebral energy provider, which is closely related to the degree of neural activity. Increased level of energy metabolism has been proposed as a mechanism of functional recovery after cerebral ischemia (54). In the study of brain function, PET imaging can quantitatively show the utilization of glucose in the brain, which reflects the brain function region of different activities through the PET scan of the brain with $^{18}$F-FDG as a tracer (55). Many researchers have observed the variation of glucose utilization in different brain regions of rats with ischemic stroke by small animal PET, and suggested the reduction of metabolism in the ischemic region (18,19,56). In the present study, we found that glucose metabolism of the CPu, MCTX and SCTX regions was decreased. As known, cerebral CPu, MCTX and SCTX regions are closely associated with limb motor function and are involved in the preparation and implementation of the exercise process (57-59). Subcortical motor control center is mainly involved in regulating muscle tension to maintain coordination and stability of muscle movement. However, EA at the LI11 and ST36 acupoints for 7 days improved the glucose utilization in the CPu, MCTX and SCTX regions of the rats. This suggests that EA at the LI11 and ST36 acupoints improved motor function by regulating brain function of the CPu, MCTX and SCTX regions in a rat model of ischemic stroke.
AMPKα is an important regulator of energy switch in the cell, when the body is under a condition of hypoxia and ischemia (40.60.61). In cerebral ischemia reperfusion injury, AMPKα is activated followed by an increase in AMPKα phosphorylation, which improves the survival of neurons (62). In the present study, we found that phosphorylation of AMPKα was upregulated in a compensatory manner to restore metabolism after cerebral ischemia. AMPKα activation can promote decomposition and inhibit synthesis, leading to increased ATP production to maintain the energy metabolism in cells, which has attracted attention as a potential therapeutic target (63). The results of this study are similar to Ran et al (64) who demonstrated that EA treatment markedly increased phosphorylation of AMPKα in the CPu, MCTX and SCTX regions to maintain energy metabolism in neurons, suggested that the treatment of EA at the LI11 and ST36 acupoints could improve neural activity of motor-related brain regions through the promotion of AMPKα phosphorylation after ischemic stroke.

In conclusion, the study demonstrated that treatment with EA at the LI11 and ST36 acupoints enhanced glucose metabolism of the CPu, MCTX and SCTX regions as assessed by 18F-FDG/PET imaging, and enhanced the phosphorylation of AMPKα after ischemic stroke.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 81373778) and the Natural Science Foundation of Fujian Province (no. 2016J01382) and the Foundation of Collaborative Innovation Center of Rehabilitation Technology (Fujian Province) (no. 755010017-collaborative).

References


