RESEARCH ARTICLE

Catgut implantation at acupoints increases the expression of glutamate aspartate transporter and glial glutamate transporter-1 in the brain of rats with spasticity after stroke

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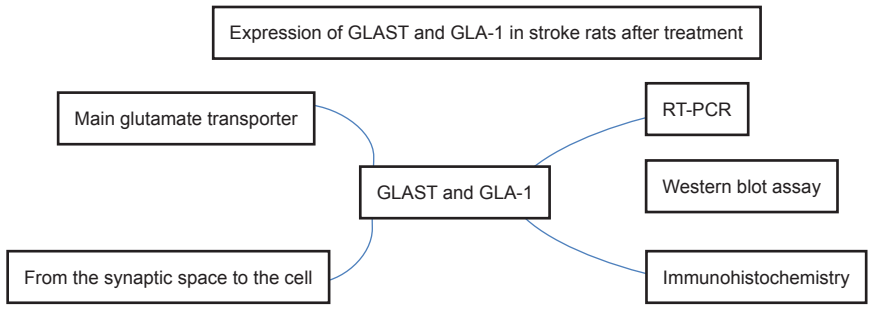
Graphical Abstract

Catgut implantation at acupoints can be used to treat middle cerebral artery occlusion rats by increasing the expression of GLAST and GLT-1 in the brain



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Abstract

Catgut implantation at acupoints has been shown to alleviate spasticity after stroke in rats. However, the underlying mechanisms are poorly understood. In this study, we used the rat middle cerebral artery occlusion model of stroke. Three days after surgery, absorbable surgical catgut sutures were implanted at *Dazhui* (GV14), *Jizhong* (GV6), *Houhui*, *Guanyuan* (CV4) and *Zhongwan* (CV12). The Zea Longa score was used to assess neurological function. The Modified Ashworth Scale was used to evaluate muscle tension. The 2,3,5-triphenyl-tetrazolium chloride assay was used to measure infarct volume. Immunohistochemical staining was performed for glutamate aspartate transporter (GLAST) and glial glutamate transporter-1 (GLT-1) expression. Western blot assay was used to analyze the expression of GLAST and GLT-1. Reverse transcription and polymerase chain reaction were carried out to assess the expression of GLAST and GLT-1 mRNAs. After catgut implantation at the acupoints, neurological function was substantially improved, muscle tension was decreased, and infarct volume was reduced in rats with spasticity after stroke. Furthermore, the expression of GLAST and GLT-1 mRNAs was increased on the injured (left) side. Our findings demonstrate that catgut implantation at acupoints alleviates spasticity after stroke, likely by increasing the expression of GLAST and GLT-1.

Key Words: *nerve regeneration; stroke; Dazhui (GV14); Jizhong (GV6); Houhui; Guanyuan (CV4); Zhongwan (CV12); catgut implantation at acupoints; limb spasm; glutamate transporter; neural regeneration*

Introduction

Stroke is a major cause of morbidity and mortality worldwide. The incidence of stroke is high in China, imparting a heavy burden on family and society (Shi, 2005). Numerous recent studies have focused on the diagnosis, treatment and pathogenesis of stroke, leading to a better understanding of the disease (Zhu et al., 2013; Qin et al., 2016).

Hemiplegic spasticity frequently occurs after stroke, and directly affects limb movement and reduces the quality of life of patients (Seo et al., 2013; Gunduz et al., 2014). Spasticity is a serious problem during recovery from hemiplegia (Zhang et al., 2009; Gao and Yang, 2014), and treating spasticity is a major challenge for rehabilitation medicine (Gong and Zhang, 2010). Gao and Yang (2014) showed that excessive excitatory transmission is the main cause of limb spasm after stroke. An important excitatory neurotransmitter, excessive glutamate increases the excitation of neurons, causing excitotoxicity (Hu and Chen, 2016), and thereby

inducing or aggravating limb spasm after stroke. Therefore, reducing glutamate content in the brain is an effective way to relieve limb spasm after stroke.

A previous study showed that catgut implantation at *Dazhui* (GV14), *Guanyuan* (CV4) and *Zhongwan* (CV12) alleviates upper limb spasticity after stroke in rats (Feng et al., 2013a). Additionally, catgut implantation at the acupoints can ameliorate upper limb spasm after stroke and enhance body functions and activities of daily living in paralytics (Feng et al., 2013a). Both the Modified Ashworth Scale and isolated muscle tone in the treatment group were dramatically decreased after treatment. Therefore, in the present study, we investigate the mechanisms underlying the therapeutic effectiveness of catgut implantation at the acupoints by examining the expression of glutamate transporters.

Materials and Methods

Animals

Healthy male specific-pathogen-free Sprague-Dawley rats (n = 125), aged 42 days and weighing 260 ± 20 g, were supplied by Beijing Vital River Laboratory Animal Technology, Beijing, China (license number: SCXK (Jing) 2016-0011). All rats were housed at 22 ± 1°C, with a humidity of 50 ± 5% and noise < 60 dB. The rats were kept in individually ventilated cages in the Central Laboratory of the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, and were given free access to food and water. The treatments conformed with the relevant ethical requirements, and the study was approved by the Animal Ethics Committee of the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, China (approval number 8150150901). The rats were randomly divided into ischemia/reperfusion (IR) (n = 50), treatment (n = 50) and sham (n = 25) groups.

Production of the stroke model

We generated the middle cerebral artery occlusion (MCAO) model of stroke (Longa et al., 1989; Jiang et al., 2011; Uluç et al., 2011). Rats were fasted from food and water 24 hours before surgery. Nylon sutures were prepared before surgery. The rats were intraperitoneally anesthetized with 2% pentobarbital sodium (45 mg/kg), secured onto the operation table, and the necks were shaved and disinfected with iodophor. In the midline of the neck, an incision was made to expose and separate the left common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA). After ligation of the CCA and ECA, the ICA was clipped with an arterial clamp. A small incision was made on the CCA near the bifurcation. The prepared nylon suture was inserted through the incision and into the ICA through the carotid bifurcation, and then stopped at the origin of the MCA. Subsequently, the nylon suture was bound to the CCA, and the incision was stitched. Reperfusion was performed 2 hours after surgery by removing the nylon suture.
Functional assessments with the Zea Longa neurological deficit score and Modified Ashworth Scale were performed by the same investigator. Rats with Zea Longa scores of 1–3 and Modified Ashworth Scale scores of 1–4 were included.

Zea Longa neurological deficit score

Neurological deficit was evaluated 3 days after MCAO surgery and 7 days after treatment. The Zea Longa neurological deficit score scale (Longa et al., 1989; Cao and Cheng, 2001) is as follows: 0, no neurologic deficit symptoms, activity is completely normal; 1, mild neurologic deficit, unable to fully extend the opposite front paw; 2, moderate neurologic deficit, turning to the opposite side when crawling; 3, severe neurologic deficit, tilt towards the opposite side when crawling; 4, loss of consciousness, inability to crawl; 5, death.

The Modified Ashworth Scale

The Modified Ashworth Scale was used to evaluate muscle tone (Chen, 2009; Yang et al., 2013) 3 days after MCAO and 7 days after therapy. The scale is as follows: 0, no increase in muscle tension, activity is completely normal; 1, mild increase in muscle tension, with catch and release during flexion or extension; 2, moderate increase in muscle tension, with limbs moving easily and mild ataxia; 3, severe increase in muscle tension, with passive/active difficulty and moderate ataxia; 4, limited flexion or extension, severe ataxia.

Catgut implantation at acupoints

The treatment group received catgut implantation at the acupoints 3 days after MCAO surgery. After disinfection, the catguts (PGA absorbable surgical suture; Shanghai Pudong Jinhuan Medical Products Co., Ltd., China; license No. 20000127) were cut into 0.5-cm segments and implanted at *Dazhui* (GV14), *Jizhong* (GV6), *Houhui*, *Guanyuan* (CV4) and *Zhongwan* (CV12) (Cui et al., 2010; Feng et al., 2013b) to a depth of 1 cm. *Dazhui* (GV14) is under the seventh cervical spine. *Jizhong* (GV6) is under the eleventh thoracic spine. *Houhui* is in the anterior medial side of the sixth lumbar transverse process. *Guanyuan* (CV4) is 25 mm below the umbilicus. *Zhongwan* (CV12) is 20 mm above the umbilicus. The catguts are absorbed over a 2-week period. Rats in the IR and sham groups received the same handling, but did not undergo implantation. Acupoint positioning was in accordance with a previous study (Li, 2007).

2,3,5-Triphenyl tetrazolium chloride (TTC) staining

After anesthesia, the rats were perfused with normal saline through the heart. The brain was quickly removed, placed on ice, and cut into 2-mm slices. The slices were placed in 2% TTC (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37°C in the dark for 20 minutes and photographed. The percent cerebral infarct volume was calculated using Image Pro Plus software (Cheng et al., 2015).

Immunohistochemical staining for glutamate aspartate transporter (GLAST) and glial glutamate transporter-1 (GLT-1)

After anesthesia, the rat was perfused through the heart with normal saline and 4% paraformaldehyde. The rat was decapitated and the brain was quickly removed and fixed in 4% paraformaldehyde for 48 hours. The tissue was then embedded in paraffin and sectioned into 5-μm-thick slices.

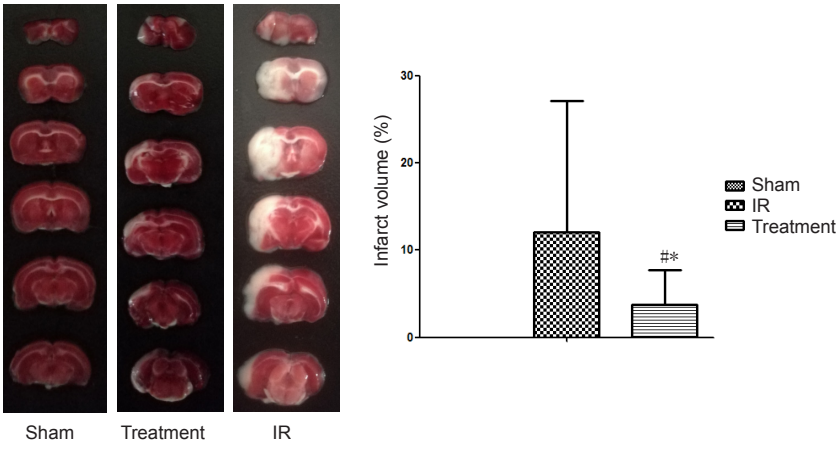

Figure 1 Effect of catgut implantation at acupoints on infarct volume in rats with spasticity after stroke.
(A) Catgut implantation at acupoints decreased infarct volume in rats with spasticity after stroke. (B) After treatment, infarct volume decreased in the treatment group. Data are expressed as the mean ± SD (sham group: $n = 10$; IR group: $n = 9$; treatment group: $n = 11$), and were analyzed with the independent-sample t-test. #$P < 0.05$, *vs.* IR group; *$P < 0.05$, *vs.* before treatment. IR: Ischemia/reperfusion.

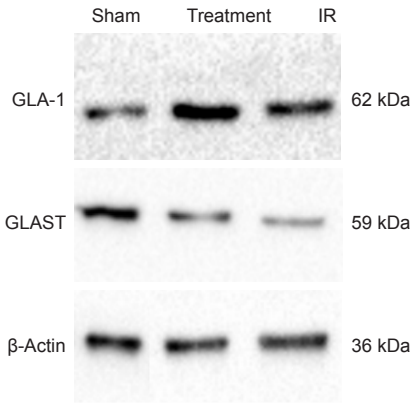

Figure 2 Effect of catgut implantation at acupoints on the protein expression levels of GLAST and GLA-1 in the injured brain of rats with spasticity after stroke, detected by western blot assay.
GLT-1: Glial glutamate transporter-1; GLAST: glutamate aspartate transporter; IR: ischemia/reperfusion.

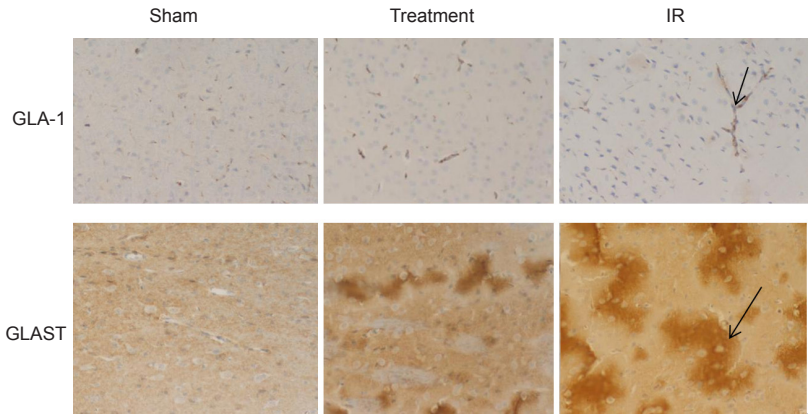

Figure 3 Effect of catgut implantation at acupoints on the expression of GLAST and GLA-1 in the injured brain of rats with spasticity after stroke (immunohistochemical staining).
Original magnification, 200×. Arrows indicate GLA-1 and GLAST immunoreactivity. GLT-1: Glial glutamate transporter-1; GLAST: glutamate aspartate transporter; IR: ischemia/reperfusion.

The slices were dehydrated and incubated with rabbit anti-EAAT1 polyclonal antibody (1:500; GeneTex, Irvine, CA, USA; No. GTX37432) or rabbit anti-EAAT2 polyclonal antibody (1:500; GeneTex; No. GTX20262) at 4°C overnight, and then with goat anti-rabbit IgG (1:200; MultiSciences Biotech Co., Ltd., Hangzhou, China) at 37°C for 1 hour. After conjugating the primary antibody to the secondary antibody, the slices were visualized with 3,3′-diaminobenzidine (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), counterstained with hematoxylin (Beijing Solarbio Science & Technology Co., Ltd.), and mounted. The slides were photographed with a light microscope (Olympus, Tokyo, Japan) and analyzed using Image Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) (Chang and Wang, 2003; Feng and Ji, 2017).

Western blot assay for GLAST and GLT-1

Protein concentrations were assessed with the bicinchoninic acid assay, and then, 20-μL aliquots of each sample, containing equal amounts of protein, were boiled for 5 minutes. After electrophoresis (120 V, 50 mA, 1.5 hours), the samples were transferred to a membrane, which was then blocked with 5% skim milk powder in TBST solution for approximately 1 hour, and incubated with rabbit anti-EAAT1 polyclonal antibody (1:200; GeneTex; No. GTX37432) or rabbit anti-EAAT2 polyclonal antibody (1:200; GeneTex; No. GTX20262) at 4°C overnight. The blot was then incubated with goat anti-rabbit IgG (H+L) conjugated to FITC (1:100) (Proteintech Group Inc., Chicago, USA) at 37°C for 1 hour. Developer solution was then added. Image Pro Plus software (version 6.0, Media Cybernetics) was used to analyze expression (Lai et al., 2012; Fu et al., 2014). The loading control was β-actin (β-actin antibody was from Proteintech Group, Inc., Wuhan, China). Expression was calculated as the ratio of the optical density value of the target protein to that of β-actin.

Reverse transcription-polymerase chain reaction (RT-PCR) for GLAST and GLT-1

Brain tissue was taken out from rats and immediately placed in liquid nitrogen and stored at −20°C. Tissue samples were homogenized in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The RNA was dissolved in DEPC-treated water, and the purity and concentration were assessed. Real-time quantitative

Table 1 Effect of catgut implantation at acupoints on the Zea Longa neurological deficit score in rats with spasticity after stroke

Group	*n*	Time	Score 0	Score 1	Score 2	Score 3	Score 4
Sham	25	Before treatment	25	0	0	0	0
		After treatment	25	0	0	0	0
IR	25	Before treatment	0	9	9	7	0
		After treatment	0	12	8	5	0
Treatment	31	Before treatment*	0	9	12	10	0
		After treatment#†	14	13	4	0	0

Data were analyzed using the Wilcoxon signed rank test. ∗P < 0.05, *vs.* sham group; #P < 0.05, *vs.* IR group; †P < 0.05, *vs.* before treatment. IR: Ischemia/reperfusion.

Table 2 Effect of catgut implantation at acupoints on the Modified Ashworth Scale in rats with spasticity after stroke

Group	*n*	Time	Level 0	Level 1	Level 2	Level 3	Level 4
Sham	25	Before treatment	25	0	0	0	0
		After treatment	25	0	0	0	0
IR	25	Before treatment	0	9	9	7	0
		After treatment	0	12	9	5	0
Treatment	31	Before treatment*	0	11	11	9	0
		After treatment#†	14	14	3	0	0

Data were analyzed with the Wilcoxon signed rank test. ∗P < 0.05, *vs.* sham group; #P < 0.05, *vs.* IR group; †P < 0.05, *vs.* before treatment. IR: Ischemia/reperfusion.

Table 3 Effect of catgut implantation at acupoints on the expression of GLAST and GLA-1 in the injured brain of rats with spasticity after stroke

	Sham group	IR group	Treatment group
mRNA expression (RT-PCR)			
GLT-1	1.46±0.61	1.98±0.50	5.05±2.25##
GLAST	1.31±0.60	1.78±0.90	3.39±1.42##
Protein expression (Western blot assay)			
GLT-1	0.32±0.13	0.45±0.19	2.28±0.74##
GLAST	0.50±0.15	0.63±0.17	2.79±0.49##
Immunopositivity (OD ratio to β-actin)			
GLT-1	1.27±0.66	1.83±0.64	3.09±1.21##
GLAST	0.25±0.09	0.39±0.18	0.68±0.24##

Data are expressed as the mean ± SD (sham group: n = 10; IR group: n = 9; treatment group: n = 11), and were analyzed with the independent-sample t-test. ##P < 0.01, *vs.* IR group. RT-PCR: Reverse transcription-polymerase chain reaction; OD: optical density; GLT-1: glial glutamate transporter-1; GLAST: glutamate aspartate transporter; IR: ischemia/reperfusion.

PCR (RT-PCR) was performed using a kit according to the manufacturer's instructions (Vazyme Biotech Co., Ltd., Nanjing, China), and relative mRNA expression was determined using the Ct value (Chen et al., 2008; Li et al., 2012).

Statistical analysis

All measurement data were expressed as the mean ± SD. Statistical analysis was performed with SPSS 17.0 software (IBM Corp., Armonk, NY, USA). The Wilcoxon signed rank test and independent-sample t-test were used. P < 0.05 was considered statistically significant.

Results

Quantitative analysis of experimental animals

No rats died in the sham group. Fifteen rats died, and 10 rats did not exhibit spasticity in the IR group. Eight rats died, and 11 rats did not exhibit spasticity in the treatment group. Rats in both the IR and treatment groups had Zea Longa neurological deficit scores of 1–3 and Modified Ashworth Scale scores of 1–4. Thus, the final analyses included 25 rats in the sham group, 25 in the IR group, and 31 in the treatment group. Ten rats each in the IR and treatment groups, and seven rats in the sham group were used for TTC staining. Six rats in the IR group, 10 rats in the treatment group and eight rats in the sham group were used for immunohistochemistry. Nine rats in the IR group, 11 rats in the treatment group, and 10 rats in the sham group were used for western blot assay and RT-PCR.

Catgut implantation at acupoints improved neurological function in rats with spasticity after stroke

The Zea Longa neurological deficit score was 0 in the sham group. Before treatment, the Zea Longa neurological deficit

scores in the IR and treatment groups were not significantly different ($P > 0.05$), while they were significantly higher compared with the sham group ($P < 0.05$). After treatment, the neurological deficit in the treatment group significantly improved, compared with the IR group ($P < 0.05$) and before treatment ($P < 0.05$; **Table 1**).

Catgut implantation at acupoints improved muscle tone in rats with spasticity after stroke

The Modified Ashworth Scale score was 0 in the sham group. Muscle tone levels were not significantly different between the IR and treatment groups before treatment ($P > 0.05$), but were significantly high compared with the sham group ($P < 0.05$). Muscle tone was significantly reduced in the treatment group compared with the IR group after treatment ($P < 0.05$) and before treatment ($P < 0.05$; **Table 2**).

Catgut implantation at acupoints decreased infarct volume in rats with spasticity after stroke

TTC staining showed that infarct volume was 0 in the sham group. Infarct volume was significantly decreased in the treatment group compared with the IR group after treatment ($P < 0.05$) and before treatment ($P < 0.05$; **Figure 1**).

Catgut implantation at acupoints elevated the expression of GLAST and GLA-1 in the injured brain of rats with spasticity after stroke

Immunohistochemical staining, western blot assay and RT-PCR results demonstrated that the expression of GLAST and GLA-1 was elevated in the IR group compared with the sham group ($P > 0.05$). The expression of GLAST and GLA-1 was significantly elevated in the treatment group compared with the IR group ($P < 0.05$; **Table 3**, **Figures 2** and **3**).

Discussion

Spasm refers to involuntary muscle contraction, which manifests as increased muscle tension and a specific pattern of abnormal muscle coordination. According to traditional Chinese medicine, the occurrence of spasm after stroke is caused by damage to the governor vessel and a deficiency of qi and blood. The acupoints for catgut implantation lie along the meridian, which is not only the channel of qi and blood, but also a link between the exterior and interior and the viscera of the body, and is a major component of the body's regulatory system. The governor vessel is in the middle of the spine, managing all the *Yang* meridians and entering into the brain. A damaged governor vessel leads to blood stasis in blood vessels, and the *Yang qi* does not disperse throughout the body, thereby resulting in spasm.

Catgut implantation at acupoints is an important part of traditional Chinese medicine and has a positive effect on physical dysfunction after stroke. Therefore, *Dazhui* (GV14), *Jizhong* (GV6) and *Houhui* located in governor vessels were selected to activate *Yang qi* and relieve spasm. The production of *qi* and blood by the spleen and stomach can be improved by *Guanyuan* (CV4) and *Zhongwan* (CV12) in the *Ren* channel, which can stimulate the *Yang qi* of the human body and relieve spasm (Jiang and Liu, 2009).

Limb spasm after stroke is caused by an impairment of the central inhibitory system and excitatory neurotransmitter release from central motor neurons, as well as increased excitability of α motor neurons, leading to stretch reflex hyperfunction (Yang et al., 2013). Zhang et al. (2009) showed that a reduction in glutamate in the brainstem can effectively relieve spasm. The glutamate transporter in the brainstem transports the accumulated glutamate to the cell, thereby terminating the excitation.

Previous studies have shown that cerebral ischemia and hypoxia-induced neuronal excitotoxicity results from impaired glutamate transporter activity and the accumulation of glutamate in the synaptic cleft (Barry et al., 2013; Sulkowski et al., 2014; Li et al., 2015). The removal of glutamate in the brainstem is mainly performed by GLAST and GLT-1 (Lagranha et al., 2014). GLAST is especially highly expressed in the cerebellum, with lower levels in the spinal cord and forebrain. GLT-1 is mainly expressed in the forebrain, hippocampus, cerebral cortex and striatum. Approximately 95% of glutamate is taken up by GLT-1 in the brain (Tani et al., 2014).

Because the accumulation of glutamate is a major reason for spasm after brain damage, we sought to investigate whether GLAST and GLT-1 expression are affected by catgut implantation at the acupoints. In our present study, infarct volume was reduced after suture implantation. GLAST and GLT-1 expression were increased by treatment following IR. GLAST and GLT-1 expression was higher in the IR group than in the sham group. Muscle tone was substantially reduced in the treatment group compared with the IR group. The muscle tone in the affected limb increased greatly 3 days after surgery, according to our preliminary experiments. Therefore, catgut implantation at the acupoints was carried out in the treatment group 3 days after MCAO, and GLAST and GLT-1 expression levels were assessed 7 days after treatment. GLAST and GLT-1 expression levels were not significantly different between the IR and sham groups, likely because the brain tissue was obtained 10 days after surgery, and therefore, the IR group had 10 days to recover from the operation. This amount of time was probably sufficient for GLAST and GLT-1 expression levels to recover to near-normal levels in the IR group.

In summary, limb spasm in rats after stroke was alleviated by catgut implantation at the acupoints, possibly because of the upregulation of GLAST and GLT-1. Our findings should be helpful for the development of clinical therapy for spasm after stroke. Further studies are needed to clarify the role of GLAST and GLA-1 in the pathogenesis of stroke.

Author contributions: *XDF designed the study. RQL, MYW, JS and HLW performed experiments. MYW wrote the paper. CML gave technical or material support. FLL, JH, RCL and LM analyzed data. All authors approved the final version of the paper.*
Conflicts of interest: *We declare that we have no conflicts of interest.*
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Institutional review board statement: *The study protocol was approved by the Animal Ethics Committee of First Affiliated Hospital of Henan University of Traditional Chinese Medicine of China (Approval number: CAP2016(A)0010). The experimental procedure followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1985).*

Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*

Plagiarism check: *Checked twice by iThenticate.*

Peer review: *Externally peer reviewed.*

Open peer review report:

Reviewer: *Ryan Hirschi, University of Utah School of Medicine, USA.*

Comments to authors: *This is a novel manuscript investigating the effect of catgut implantation at acupoints on the expression of GLAST and GLT-1 in the brain of rats with spasticity after stroke. It presents data supporting improved spasticity after stroke in a rat model, which is exciting. The paper has numerous figures and tables which are of good quality and add significantly to the paper.*

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