Neuroprotective effect of the traditional Chinese herbal formula *Tongxinluo*: a PET imaging study in rats

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Abstract

Tongxinluo has been widely used in China for the treatment of acute stroke and for neuroprotection. However, there are few positron emission tomography (PET) studies on the neuroprotective effect of *Tongxinluo* on cerebral ischemia/reperfusion in small animals. In the present study, *Tongxinluo* superfine powder suspension or its vehicle was administered intragastrically to rats for 5 successive days before middle cerebral artery occlusion. ^{18}F-fluorodeoxyglucose (FDG) small animal PET imaging showed that at 1 and 2 weeks after cerebral ischemia/reperfusion, glucose metabolism in the ischemic area was greater in rats that had received *Tongxinluo* than in those that had received the vehicle. Nissl staining showed that 2 weeks after cerebral ischemia/reperfusion, there was less neuronal loss in the prefrontal cortex in *Tongxinluo*-treated rats than in controls. In addition, *Tongxinluo*-treated animals showed better neurologic function and lower cerebral infarct volume than rats that received the vehicle. These findings suggest that *Tongxinluo* exhibits neuroprotective effects in cerebral ischemia/reperfusion injury and demonstrates that ^{18}F-FDG small animal PET imaging is a useful tool with which to study the molecular pharmacology of traditional Chinese medicine.

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Introduction

Ischemic stroke remains a leading cause of death and long-term disability globally (Heiss and Kidwell, 2014). Restoration of blood flow to the ischemic brain as early as possible is the only way to rescue patients exposed to cerebral ischemia. However, reperfusion itself has the potential to produce additional injuries in the ischemic brain and the pathogenesis of cerebral ischemia/reperfusion (I/R) injury is not yet completely understood. Tissue plasminogen activator (tPA) is the only FDA approved agent for acute ischemic stroke. However, only 2% of patients benefit from tPA treatment. Because of the high morbidity and mortality of ischemic stroke, and the extent of disability that it can cause, there is a critical need for novel therapeutic strategies in stroke management. For many decades, researchers have struggled to treat ischemia by neuroprotection and have aimed to develop target molecules with anti-apoptotic, anti-calcium, anti-inflammatory, and anti-oxidative properties (Schmidt and Minnerup, 2014).

Traditional Chinese medicine is gaining increasing attention from scientists and patients because of its mild nature, emphasis on maintaining balance in individuals, and effect on multiple targets (Lao et al., 2014). However, little remains known about the interaction between traditional Chinese medicine and the organism, and evaluating the holistic efficacy of traditional Chinese medicine remains a difficult task because of the lack of information on the active compounds and synergistic actions of multiple components. Metabonomics is an ideal tool for bridging traditional Chinese medicine and molecular pharmacology. Currently, positron emission tomography (PET) is one of the preferred methods by which to evaluate cerebral energy metabolism in clinical practice, providing excellent sensitivity to small molecular changes (on the nanogram scale, compared to milligrams or micrograms for magnetic resonance imaging (MRI) or computed tomography (CT)), and can provide important information regarding changes in brain metabolism after cerebral I/R injury. There are many advantages of using small animal PET for drug research. First, the results of the study *in vitro* cannot be directly applied to human studies, while small animal PET imaging methods and results can be extrapolated to humans, thus bridging animal and human studies (Malaney and Nicosia, 2014). Moreover, with PET imaging, experimental procedures can be repeated in the same animals
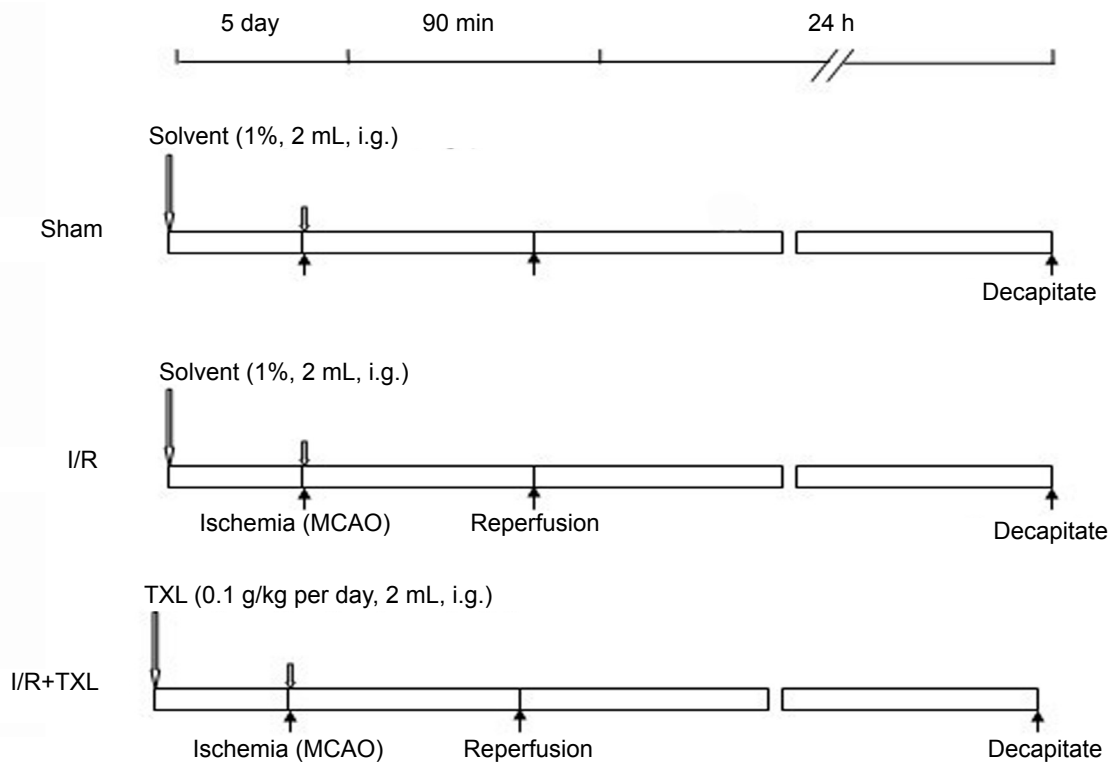
Figure 1 Experimental groups and protocols for the investigation of cerebral protection by *Tongxinluo* pretreatment in rats with middle cerebral artery occlusion-induced 90 minute ischemia followed by 24 hour reperfusion.
TXL: *Tongxinluo*; I/R: ischemia-reperfusion injury; MCAO: middle cerebral artery occlusion.

without damage, thus reducing the use of laboratory animals, improving ethical acceptability and saving experimental costs (Toyohara and Ishiwata, 2011). The biggest scientific advantage of small animal PET is its use in studying the same animals at different time points, which can eliminate interindividual differences. In addition, compared to many other methods of animal research in which the majority of observations are carried out postmortem, small animal PET requires less time to obtain data and contributes to greater experimental continuity. Small animal PET imaging is the dynamic scanning of experimental animals (Tsukada, 2011) and has been used in a longitudinal study to investigate the therapeutic efficacy of a drug (Funk et al., 2004). PET with ^{18}F-fluorodeoxyglucose (FDG) is a powerful tool with which to study the etiopathogenesis and progression of neurologic diseases, and has recently been used to detect subtle changes in glucose metabolism *in vivo* after therapy in various neurologic disease models, including cerebral ischemia and reperfusion injury (Heiss, 2011), traumatic brain injury (Stocker et al., 2014), Parkinson's disease (Kiessling, 2014; Stoessl et al., 2014; Wood, 2014), and Huntington disease (Ahmad et al., 2014). For non-invasion imaging, a positron-emitting isotope such as ^{18}F can be attached to FDG resulting in [^{18}F]-FDG that accumulates in brain tissue in proportion to glucose uptake and phosphorylation, and is quantifiable using PET imaging (Reivich et al., 1979). In the present study, we used FDG-PET to measure alterations in cerebral glucose

metabolism non-invasively after I/R injury, to investigate the therapeutic efficacy of *Tongxinluo*.

With the progression of modern technology, an increasing number of herbal compound extracts are being authenticated, standardized, and administered successfully in clinical practice. *Tongxinluo* has properties that might be effective in acute stroke (Editor's note, 2002). *Tongxinluo* capsules are composed of *Radi Ginseng*, *Scorpio*, *Hirudo*, *Eupolyphaga seu Steleophage*, *Scolopendra*, *Periostracum Cicadae*, *Radix Paeoniae Rubra*, and *Borneolum Syntheticum* (Wu et al., 2001; Editor's note, 2002; Wang and Xiong, 2014). These materials are powdered and prepared as 0.38 g capsules (Wu et al., 2001). *Tongxinluo* capsules have been widely used in the treatment of cardiovascular and cerebrovascular diseases in China (Chen et al., 2009; Zhang et al., 2009). This herbal compound has a protective role in the blood-brain barrier after I/R injury (Liu et al., 2013). Metabonomic studies have suggested that *Tongxinluo* prevents endothelial dysfunction by regulating multiple metabolic pathways (Dai et al., 2011). A previous study has shown that *Tongxinluo* reduces myocardial no-reflow and alleviates I/R injury by stimulating the phosphorylation of endothelial nitric oxide synthase *via* the PKA pathway (Li et al., 2010). However, it remains unclear whether *Tongxinluo* can provide neuroprotection against I/R injury. Therefore, in the present study, we used rat models of focal cerebral I/R injury induced by middle cerebral artery occlusion (MCAO) to identify whether a low dose of *Tongxinluo*
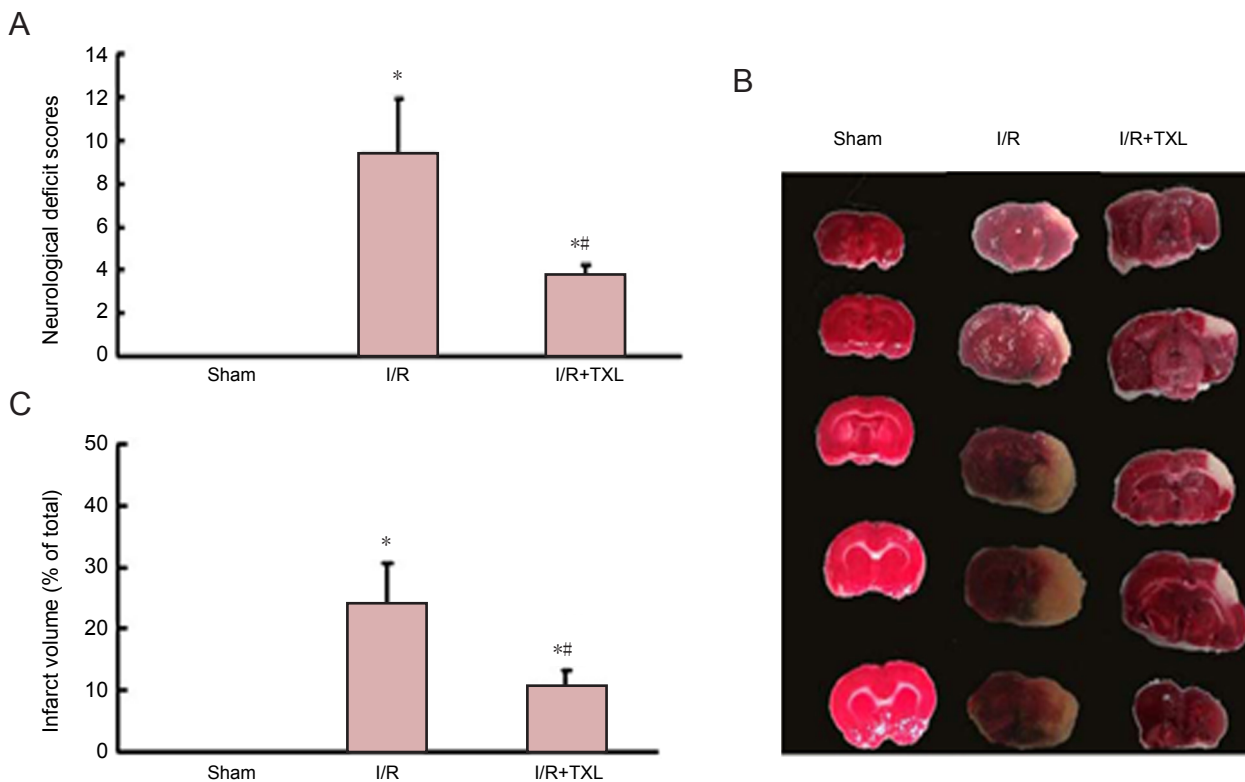
Figure 2 Effects of TXL on neurological deficit score and infarction volume after cerebral I/R in rats.
(A) Neurological deficit scores of rats measured after 24 hours of reperfusion. (B) Representative photograph of 2,3,5-triphenyltetrazoliumchloride (TTC) staining of coronal brain sections (1 mm thick) from rats with cerebral I/R. Non-ischemic region is in red and the infarct region appears in white. (C) Quantification of infarct volume after 24 hours of reperfusion in rats with cerebral I/R. Data are expressed as mean ± SD of six rats in each group. *$P < 0.01$, *vs.* sham group; #$P < 0.01$, *vs.* I/R group (one-way analysis of variance and Tukey-Kramer multiple comparison tests). TXL: *Tongxinluo* capsule; I/R: ischemia/reperfusion.

administered for 5 successive days before ischemia can enhance glucose metabolic activity and improve neurological outcome after focal cerebral I/R injury in rats. Metabolic recovery of the cerebral infarction area was evaluated by ^{18}F-FDG small animal PET, histological analyses, and tests of neurologic function.

Materials and Methods

Animals
A total of 186 adult male Sprague-Dawley rats, weighing 240–270 g, aged 180–220 days, were obtained from the Laboratory Animal Center of Sun Yat-sen University, China (license No. SCXK (Yue) 2011-0029). All rats had free access to water and food. All surgical procedures were conducted aseptically in accordance with the Chinese National Health and Medical Research Council (NHMRC) animal ethics guidelines and approved by the Sun Yat-sen University Animal Experimentation Ethics Committee.

Establishment of rat models of I/R
The right middle cerebral artery was occluded according to previously described methods (Chen et al., 2001; Zhang and Chen, 2014), with some modifications. Briefly, animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg), and a midline ventral incision was made in

the neck to expose the right and left common carotid arteries. The right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were exposed and carefully isolated. A nylon monofilament (40 mm in length and 0.26 mm in diameter) was inserted from the lumen of the ECA to that of the right ICA to occlude the origin of the right middle cerebral artery (MCA). The right MCA was occluded for 90 minutes, and thereafter the brain was re-perfused with blood by withdrawing the nylon monofilament. A heat pad was used to maintain the body temperature of the rats at 37 ± 0.5°C throughout surgery and the postoperative period until the animals recovered fully from anesthesia. The rats were killed at the designated reperfusion times. The sham-operated group underwent identical manipulations as the reperfusion groups, except for the occlusion of the CCA.

Drug administration and protocols
Tongxinluo powder was provided by Shijiazhuang Yiling Pharmaceutical Factory (Shijiazhuang, Hebei Province, China). The herbal drugs were authenticated and standardized against marker compounds according to the Chinese Pharmacopoeia 2005 (Wang et al., 2014). The 12 medicinal components of *Tongxinluo* were ground to superfine (≤ 10 μm) powder by a micronizer and prepared as capsules. To reduce the dose variability of *Tongxinluo* capsules between different
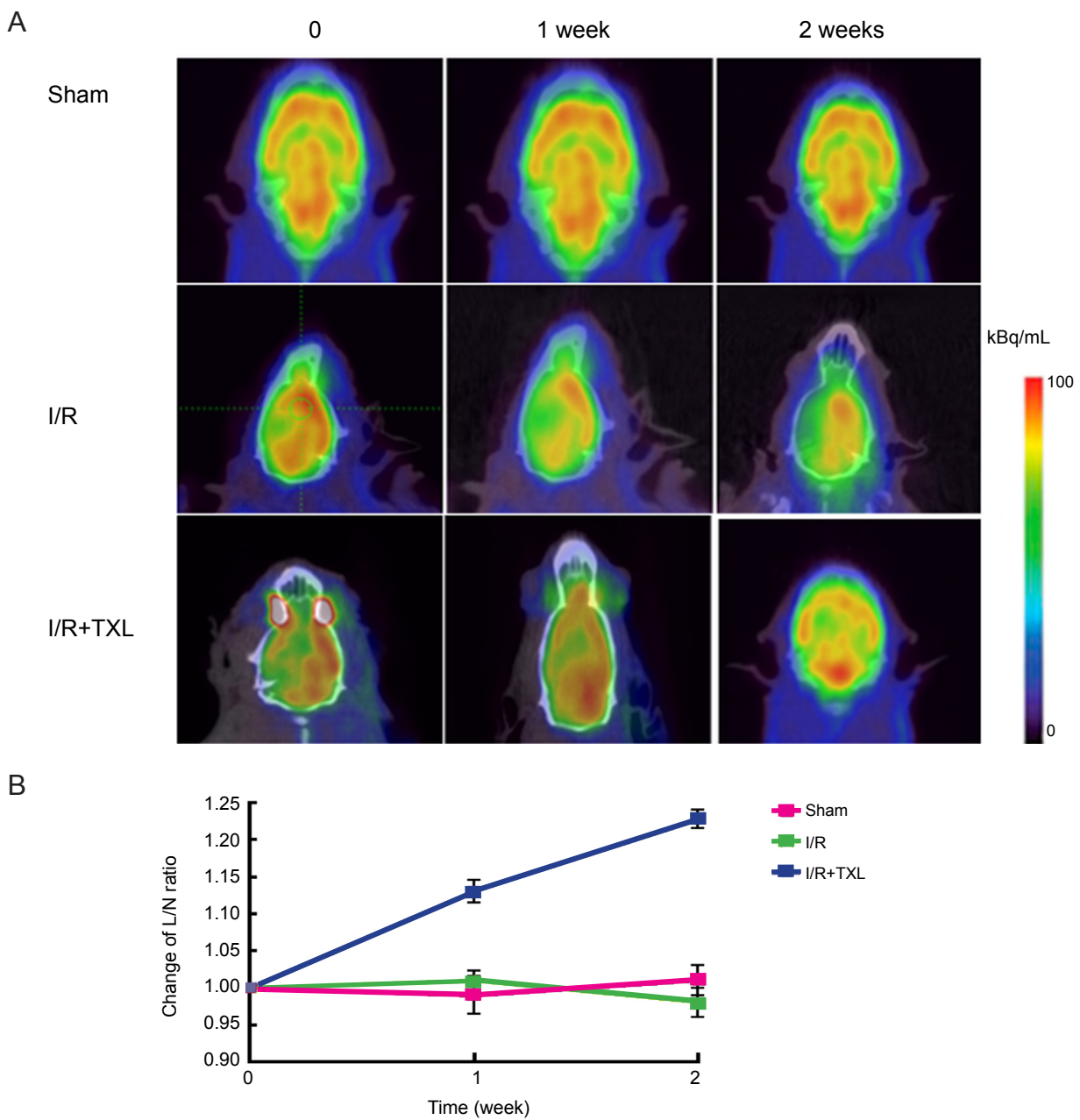
Figure 3 Effect of TXL on ^{18}F-FDG PET evaluation in rats subjected to cerebral I/R injury.
(A) Serial PET images demonstrate metabolism recovery after *Tongxinluo* pretreatment for cerebral I/R injury in rats. Images are shown in axial view. Scale was set according to signal intensity. (B) Semi-quantitative analysis of variance of glucose metabolism after cerebral I/R in each group (shown as change of L/N ratio at each time point after treatment relative to L/N ratio before treatment). L/N ratios were both significantly greater in the TXL + I/R group after 1 and 2 weeks of reperfusion compared to the I/R group ($P < 0.05$). Data are expressed as mean $\pm$ SD of six rats in each group at each time point. One-way analysis of variance was used to analyze the differences among groups followed by Tukey-Kramer multiple comparison tests. TXL: *Tongxinluo* capsule; ^{18}F-FDG PET: ^{18}F-fluorodeoxyglucose positron emission tomography; I/R: ischemia/reperfusion; L/N ratio: counts per pixel of lesion region of interest/mean counts per pixel of contralateral normal area.

batches, the species, origin, harvest time, medicinal parts, and preparation methods for each component were strictly standardized. Moreover, high performance liquid chromatography, high performance capillary electrophoresis, and gas chromatography were used to quantitate the components of the *Tongxinluo* capsule.

The rats were divided randomly into three groups (**Fig-ure 1**): sham group; I/R group (MCAO for 90 minutes then reperfusion for 24 hours); and I/R + *Tongxinluo* group (100 mg/kg *Tongxinluo* suspension administered intragastrically daily for 5 successive days before I/R surgery). *Tongxinluo* suspension was prepared by dissolving superfine *Tongxinluo* powder in 1% sodium carboxymethylcellulose (Li et al., 2010). The sham and I/R groups received equivalent vol-
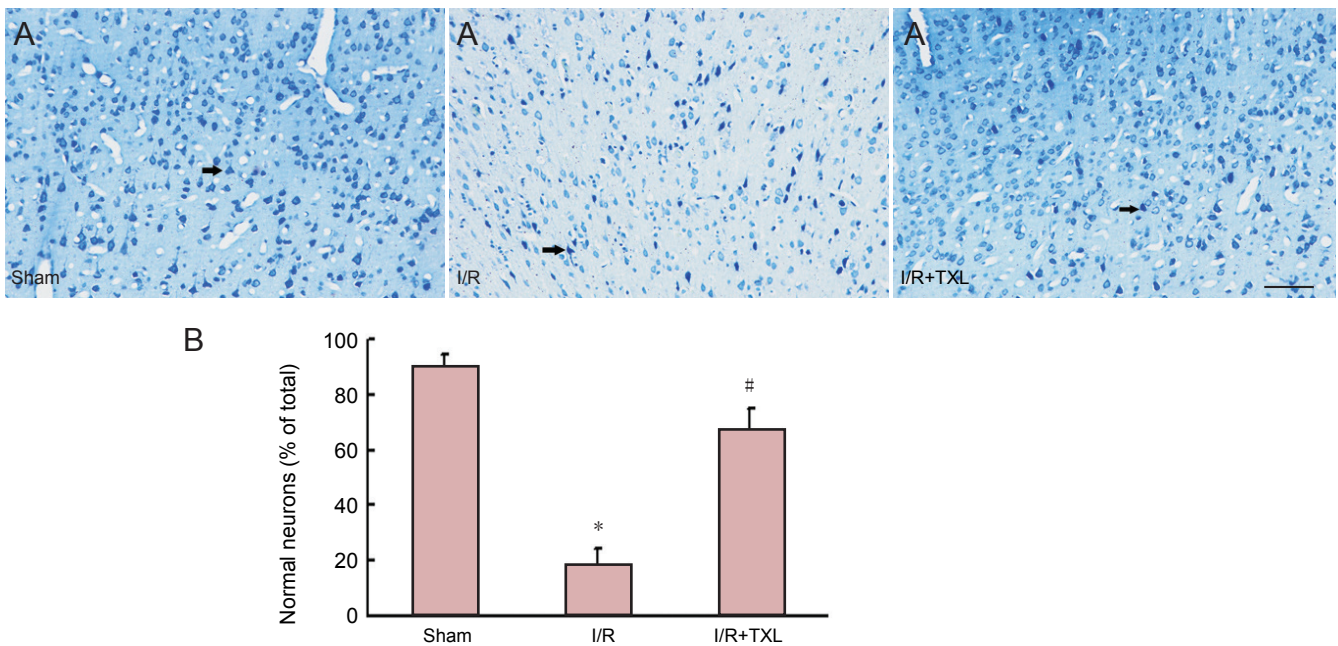
Figure 4 Effect of TXL on neuron survival in the prefrontal cortex after 2 weeks of reperfusion (Nissl staining).
(A) Morphological evaluation in the prefrontal cortex 24 hours after middle cerebral artery occlusion in the sham, I/R, and I/R + TXL groups. Scale bar: 100 μm. (B) Percentage of normal neurons among total neurons in the sham, I/R, and I/R + TXL groups. Data are expressed as mean ± standard deviation of six rats in each group. $*P < 0.01$, *vs.* sham group; $\#P < 0.01$, *vs.* I/R group. One-way analysis of variance was used to analyze the differences among groups followed by Tukey-Kramer multiple comparison tests. TXL: *Tongxinluo* capsule; I/R: cerebral ischemia/reperfusion.

umes of 1% sodium carboxymethylcellulose as the *Tongxin-luo*-treated rats, by gavage. Neurological deficit score and cerebral infarct volume were determined 24 hours after I/R injury. Rats were detected for ^{18}F-FDG PET analysis at 0 days, 1 week and 2 weeks of reperfusion ($n = 6$).

Neurological deficit score
Testing of the modified Neurologic Severity Score (mNSS) was performed 24 hours after MCAO by a single investigator blinded to the experimental groups, as described previously (Chen et al., 2001). mNSS score is the integrated score for movement, sensation, reflection and balance functions. The maximum score is 18, with a higher score indicating more severe damage (mild injury: 1–6; moderate injury: 7–12; severe injury: 13–18).

Determination of cerebral infarct volume
Cerebral infarct volume was determined as described previously (Liang and Luan, 2014; Tan and Li, 2014). Rats were decapitated 24 hours after MCAO, and the brains were removed quickly. Each brain was frozen immediately at −20°C and 2 mm slices were cut coronally. The slices were stained by incubation with 2% 2,3,5-triphenyltetrazoliumchloride (TTC, pH 7.4) at 37°C for 30 minutes, and fixed in 10% formalin. The size of the infarct area (unstained) was quantified with Image-Pro Plus 6.0 (Media Cybernetics Company, Commerce, GA, USA). The total infarct volume (sum of the infarct areas in the five sections) was expressed as a percentage of the volume of the contralateral hemisphere (Alessan-

drini et al., 2001).

***In vivo* PET study and image analysis (^{18}F-FDG PET/CT)**
^{18}F-FDG PET/CT was performed as described previously (Wang and Chao, 2013; Wang and Song, 2014). All rats were fasted for 12 hours and had no access to water for 4 hours before the experiments. After 0 day, 1 week and 2 weeks of reperfusion, rats were anesthetized with isoflurane (2%) and injected with approximately 18.5 MBq (500 mCi) of ^{18}F-FDG *via* the tail vein. At 40 minutes after ^{18}F-FDG injection, the rats were anesthetized again with isoflurane (2%) and positioned prone in the microPET scanner (Siemens Inveon Micro-CT/PET, Siemens Medical Solutions, Erlangen, Germany) for a 10 minute static acquisition with the mid skull in the center of the field of view. The images were reconstructed using a modified back-projection algorithm. ^{18}F-FDG uptake was calculated as the percentage injected dose per gram of tissue using Inveon Research Workplace 4.1 software (Siemens Preclinical Solutions, Knoxville, TN, USA). In each image plane, three-dimensional regions of interest 2 mm in diameter were drawn over the infarct area of the cerebral cortex and the contralateral normal tissue, and the mean percentage injected dose per gram of tissue was calculated. The scan parameters of this experiment were as follows: 64 LSO crystals, PET axial field of view of 12.7 cm, axial resolution < 1 cm from the center 1.7 ram, time resolution < 1.5 ns, acquisition field of view 12.37 cm × 12.66 cm, CT tube voltage range 30–80 kV, tube current 50–500 μA, detector pixel size 32 μm, maximum effective pixel area 4,096 × 4,032, scan field 10 cm × 9.7 cm. The

lesion-to-normal homologous contralateral (L/N) ratio was used for semiquantitative analysis and calculated using the following formula: mean counts per pixel of lesioned region of interest/mean counts per pixel of contralateral normal area. The average radioactivity concentration within the infarct area was obtained from the mean pixel values, normalized to that of non-ischemic cortex, and expressed as a percentage. Because there were no differences among the three groups after 0 days of reperfusion, L/N ratio at this time point was taken as the baseline.

Nissl staining and neuron counts

Nissl staining was carried out according to a protocol described in our previous study (Zhou et al., 2013; Meng and Wang, 2014). Rats were decapitated after 2 weeks of reperfusion and brains were removed quickly. Each brain was frozen immediately at 20°C and sliced into coronal sections (15 mm) at 150 mm intervals (from olfactory bulb to cerebellum) and stained. The images were viewed under a light microscope (Olympus BX60). Six sections from each animal were selected for Nissl staining of the prefrontal cortex.

Statistical analysis

Data analysis was performed with SPSS version 16.0 (SPSS, Chicago, IL, USA). All variables were expressed as mean ± standard deviation. One-way analysis of variance was used to analyze the differences among groups followed by Tukey-Kramer multiple comparison tests. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of *Tongxinluo* pretreatment on neurological deficit score and cerebral infarct volume in rats subjected to focal cerebral ischemia/reperfusion

The neuroprotective effect of *Tongxinluo* on rats subjected to focal cerebral I/R was evaluated by neurological deficit score and infarct volume. The neurological deficit score was 9.3 in the I/R group, which was significantly greater than that in the sham group ($P < 0.01$), and 3.7 in the I/R + *Tongxinluo* group, which was significantly lower than that in the I/R group ($P < 0.01$) (**Figure 2A**). Correspondingly, the infarct volume was 23.9% in the I/R group, significantly greater than that in the sham group ($P < 0.01$), and 10.3% in the I/R + *Tongxinluo* group, which was significantly lower than the I/R group ($P < 0.01$) (**Figure 2B, C**).

Effect of *Tongxinluo* pretreatment on glucose metabolic activity in cerebral ischemic areas of rats with I/R injury

^{18}F-FDG PET imaging showed that pretreatment with *Tongxinluo* enhanced glucose metabolic activity in cerebral I/R injury model rats. The ^{18}F-FDG PET scans allowed the visualization and quantification of glucose metabolism throughout the brain at each time point (**Figure 3A**). Semi-quantitative measurement of ^{18}F-FDG radioactivity in the ischemic area revealed no significant difference in the L/N ratio between the I/R and I/R + *Tongxinluo* groups after

0 days of reperfusion (**Figure 3B**). However, the L/N ratio was significantly greater in the I/R + *Tongxinluo* group than in the I/R group at 1 and 2 weeks of reperfusion ($P < 0.05$). In addition, there was no focal abnormal increase in glucose metabolism in the cerebral ischemic area, indicating no tumor or teratoma formation after *Tongxinluo* treatment.

Effect of *Tongxinluo* pretreatment on neuron survival in rats with cerebral I/R injury

Nissl staining was performed to show neuronal damage in the prefrontal cortex region after 2 weeks of reperfusion. The normal neurons in the prefrontal cortex were packed tightly and in an orderly manner, with clear nuclei, while the injured neurons showed shrunken cell bodies accompanied by shrunken and pyknotic nuclei (**Figure 4A**). The number of normal neurons was 18.2 ± 5.7% in the prefrontal cortex in the I/R group ($P < 0.01$ compared with the sham group; Figure 4B), and 66.7 ± 8.3% in the I/R + *Tongxinluo* group ($P < 0.01$ compared with I/R group; **Figure 4B**). Neuronal loss occurred in the rat prefrontal cortex after I/R injury, which was inhibited by *Tongxinluo* pretreatment (**Figure 4**). However, survival of neurons in the I/R + *Tongxinluo* group was still markedly lower than that in the sham group ($P < 0.05$).

Discussion

In the present study, rat models of I/R injury were established using MCAO to investigate the therapeutic effect of *Tongxinluo* capsule pretreatment by means of neurological deficit score, cerebral infarct volume assay, small animal PET (^{18}F-FDG PET/CT) imaging and Nissl staining. Our results showed that rats that had been pretreated with *Tongxinluo* not only had significantly lower neurological deficit scores and cerebral infarct volumes, but also promoted the survival of neurons. Moreover, ^{18}F-FDG PET evaluation revealed that the L/N ratios were significantly increased in the I/R + *Tongxinluo* group after 1 and 2 weeks of reperfusion, which demonstrated that *Tongxinluo* could effectively promote glucose metabolism and alleviate I/R injury. According to the present results, *Tongxinluo* has neuroprotective effects on cerebral I/R injury, which is supported by evidence from a number of studies. For instance, Li et al. (2010) investigated the neuroprotective role of *Tongxinluo* on blood-brain barrier disruption after I/R injury and found that administration of *Tongxinluo* before or after I/R effectively protected the brain from blood-brain barrier disruption via the inflammatory response. Furthermore, continuous administration of *Tongxinluo* before and throughout the ischemia period is recommended because of the multiple functions of *Tongxinluo*. To our knowledge, we are the first to use ^{18}F-FDG PET imaging to measure alterations in cerebral glucose metabolism after I/R injury in the investigation of the therapeutic effect of *Tongxinluo*. Using these methods, we show that functional recovery occurs after *Tongxinluo* pretreatment in a rat model of cerebral I/R injury by PET/CT imaging, and demonstrate that small animal PET imaging is an ideal tool with which to bridge traditional Chinese medicine and mo-

lecular pharmacology.

PET imaging of ^{18}F-FDG is widely used to quantify changes in glucose metabolism because of the greater sensitivity and specificity offered by metabolic information than anatomic imaging alone (Moore et al., 2000). Small animal PET (microPET) scanning with ^{18}F-FDG has become a useful tool for the monitoring of cerebral metabolic patterns in rat brains owing to its improved resolution (Shimoji et al., 2004). The resolution of PET (1 to 2 mm) is still lower than MRI and CT, meaning it can only provide limited anatomical information, but it is highly sensitive and inherently quantitative enough to detect functional changes (Riemann et al., 2008; Franc et al., 2008). Furthermore, quantitative PET methods provide detailed information about the activity of measured tissue and the amount of radiotracer delivered to the tissue during the PET scan period, which means that the metabolic situation can be clearly monitored and quantified over time (Yang et al., 2014).

PET scans use positron-emitting isotopes, which are usually produced in a cyclotron and have short half-lives (e.g. [18]F has a half-life of 110 minutes), allowing repetition of experiments within short time periods (Haubner et al., 2001). In the present study, we found that the results detected by PET were similar to those obtained using classical approaches such as ethology and immunohistochemistry, demonstrating that microPET is a viable technique for exploring the therapeutic effect of drugs, in particular those indicated for cardiovascular and cerebrovascular diseases.

A large number of studies have revealed that *Tongxinluo* has multiple functions, and the mechanism underlying its therapeutic effects is complicated. As described above, the different functions of *Tongxinluo* arise from the actions of its various components, including dilation of blood vessels and inhibition of platelet adhesion, leading to its effectiveness across many diseases (Zhuo et al., 2008). Here, we found that *Tongxinluo* was neuroprotective. The precise mechanism, however, is still unclear, because of the complex mixture of components and action at multiple targets in the organism, and further studies are needed to determine the therapeutic mechanism of this useful drug.

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