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Anti - Inflammatory Effects of Tianma Gouteng Decoction Granule on Cerebral Ischemic Injury in Rats

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Abstract: ***Stroke is one of the leading causes of long - term disability and death around the world. Although enormous efforts have been made to investigate the pathogenesis of stroke and explore new therapeutic strategy, improving the prognosis of patients with stroke remains an unmet medical need. The beneficial effects of traditional Chinese medicine (TCM) as an add - on to thrombolytic therapy are getting more attention. Here, we describe the therapeutic effects of a granule product of a classic TCM, Tianma Gouteng decoction (TM granule), in a transient cerebral ischemia rat model. Middle cerebral artery occlusion (MCAO) for 90 min followed by reperfusion for 24 h (ischemia/reperfusion; I/R) were performed in adult male Sprague - Dawley rats. Post - stroke treatment with TM granule ameliorated I/R - induced cerebral infarct and restored stroke - affected neurobehavioral defects. Immunohistochemical analysis showed that the expression of proinflammatory cytokine, interleukin - 6 (IL - 6) was increased in the cerebral cortex of I/R rats but the effect was reduced by TM granule treatment. Enzyme - linked immunosorbent assay further revealed that the upregulation of proinflammatory cytokine, IL - 6 and tumor necrosis factor - α (TNF - α), in I/R rats was attenuated by TM granule administrations and the treatment significantly increased the level of an anti - inflammatory cytokine, transforming growth factor - β (TGF - β) in a dose - dependent manner. These results provide an evidence that TM granule represents an add - on treatment for stroke treatment and the neuroprotective effects are associated with its anti - inflammatory actions.***

Keywords: Stroke, ischemic/reperfusion, traditional Chinese medicine, Tianma Gouteng decoction, inflammation

1. Introduction

Stroke leads to acute serious injuries of the central nervous system and is a leading cause of death and disability around the world [1]. Stroke can be divided into two major types: ischemic stroke and intracerebral hemorrhage [2]. According to epidemiological investigation, ischemic type is the most common form of stroke, which is accounted for about 87% of all cases [2]. Although enormous efforts have been made to explore new therapeutic strategy, improving the prognosis of patients with ischemic stroke remains an unmet medical need. Except for thrombolysis, there is currently no specific treatment for ischemic stroke [3].

The pathogenesis of ischemic stroke is complicated, involving heterogeneous pathogenic factors such as inflammation, mitochondrial dysfunction, and oxidative stress [2, 4]. In addition to the detrimental signaling pathways induced by ischemia itself, reperfusion following administration of thrombolytic drug tissue plasminogen activator (t - PA) could induce deteriorative inflammatory responses and further brain injury [5]. At initial stage after stroke, resident resting microglia become activated microglia morphologically and functionally. Among the activated phenotypes, M2 microglia can clear the cell debris and release interleukin - 10 (IL - 10) and transforming growth factor - β (TGF - β) topromote cell repairedin the core of the infarct [6 - 10]. On the other hand, the other group of microglia (i. e., M1) appears in the peri - infarct area and then in the core of the infarct. The production of proinflammatory cytokines including interleukin - 1β (IL - 1β), interleukin - 6 (IL - 6), and tumor necrosis factor - α

(TNF - α) by M1 microglia is detrimental for stroke outcome [6 - 10]. Except microglia, neutrophils and monocytes invade the brain parenchyma from peripheral blood and involve in the modulation of neuroinflammation during the course of stroke [6, 7]. Although the role of inflammatory mechanisms in stroke is not fully understood, modulation of inflammation is considered as a potential target of therapeutic intervention in stroke.

Traditional Chinese medicines (TCMs) have been clinically used in the treatment of brain disorders for centuries. Based on empirical experience, a classic formula of TCMs, Tianma Gouteng decoction (Tianma Gouteng Yin; TM), is widely used to treat cerebrovascular diseases including cerebral ischemia and hypertension that is a key risk factor behind ischemic stroke [11 - 13]. In addition, TM is reported to have neuroprotective effects in experimental animal models [14]. TM is composed of the following eleven different herbs: Tianma (*Rhizoma Gastrodiae*), Gouteng (*Ramulus Uncariae Cum Unicis*), Shijueming (*Concha Haliotidis*), Zhizi (*Fructus Gardeniae*), Huanqin (*Radix Scutellariae*), Chuanniuxi (*Radix Cyathulae*), Duzhong (*Cortex Eucommiae*), Yimucao (*Herba Leonuri*), Sangjisheng (*Herba Taxilli*), Yejiaoteng (*Cauls Polugoni Multiflori*), and Fushen (*Poria*). Recent study showed that water extract of a combination of Tianma (*Rhizoma Gastrodiae*) and Gouteng (*Ramulus Uncariae Cum Unicis*) has anti - oxidative and anti - apoptotic properties in oxygen - glucose - deprived neuronal cell model and middle cerebral artery (MCA) occlusion (MCAO) rat model [13]. The detailed mechanisms underlying the neuroprotective effects of TM such as modulation of inflammation in stroke model are yet to be elucidated.

Traditionally, the raw herbs in TM are made as a decoction before oral administration. For convenient use by patients, TM has been processed as a granule product that is commonly prescribed in clinics. However, the therapeutic efficacy of TM granule after ischemic stroke and reperfusion has never been evaluated. This study was aimed to investigate the therapeutic effects of TM granule on neurobehavioral functions and its anti - inflammatory mechanisms in a rat model of cerebral ischemic stroke.

2. Materials and Methods

2.1 Experimental animals

Adult male Sprague - Dawley rats weighting 250 ~ 300g were purchased from BioLasco Co., Ltd. (Taipei, Taiwan). Rats were allowed to acclimatize for 7 days prior to any experiments. Rats were housed in cages with a 12 hours light - dark cycle and with ad libitum access to chow and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Taipei Tzu Chi Hospital (approval no.107 - IACUC - 002).

2.2 MCAO model

MCAO was performed according to a published protocol in rats[10]. In brief, rats were anesthetized by a Zoletil 50 (50 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Body temperature of animals was carefully maintained at 37°C with a warm pad and a heating lamp throughout the surgical procedure. After reducing the brain blood flow by a ligation of right carotid artery, rats were fixed on a stereotaxic instrument and the right MCA was ligated by a 10/0 nylon thread. After the occlusion for 90 minutes, the nylon thread was withdrawn to allow blood flow through the MCA for 24 h. At initiation of occlusion and reperfusion, the blood flow in the MCA was verified undera microscope. In control group, the sham surgery was performed but the MCA was not ligated. After the surgery, each rat was kept in individual cage to prevent further wound damage.

2.3 Experimental grouping and treatments

Rats were randomly divided into four groups: sham control group, ischemia/reperfusion (I/R) group, I/R group treated with low - dose TM (I/R + L - TM), and I/R group treated with high - dose TM (I/R + H - TM). After sham or MCAO operation, the rats in the sham group and I/R group were received normal saline orally; the rats in the treatment groups were administered with 0.5 g/kg or 2.5 g/kg of TM by oral gavage twice daily for 7 consecutive days. TM granule (the powdered product of TM) was obtained from Sun - Ten Pharmaceutical Co. Ltd. (New Taipei City, Taiwan) and manufactured under PIC/S good manufacturing practice (GMP) guidelines. The components of the TMgranule were listed in Table 1. Rats were sacrificed for histological and biochemical analyses at 24 h after the last administration of saline or TM.

2.4 Neurobehavioral assessments

At day 1, day 7, and day 14 after MCAO operation, rats were subjected to neurobehavioral assessments. Neurological deficit was evaluated using a modified neurological severity score (mNSS). The mNSS included motor, sensory, beam balance, and reflexes absent and abnormal movement was performed according to previously research[10]. The corner test that can assess sensorimotor and postural asymmetries was performed as described previously[11].

2.5 Measurement of cerebral infarct volume

The procedure of infarct size analysis was performed as described previously[10]. In brief, rats with sham or MCAO operation were sacrificed at 24 h after the last administration of saline or TM. Rats were anesthetized andthe brain was carefully collected and sliced to a thickness of 2mm. The brain sections were soaked in 2, 3, 5 - triphenyltetrazolium chloride (TTC) solution (2%) for 30 min at 37°C and then put into 10% formalin overnight. The areas of cerebral infarction were examined by Image - J (v1.50i, National Institutes of Health, Bethesda, MD, USA). The percentage area of cerebral infarction was calculated as (infarct area/area of whole brain sections) × 100%.

2.6 Hematoxylin and eosin (H&E) staining

At 24 h after the last administration of saline or TM, rats were perfused with normal saline via left ventricle before brain collection and fixation. For evaluation of histological changes, H&E staining was performed on formalin - fixed, paraffin - embedded sections. After deparaffinization with xylene and rehydration through a series of ethanol and water, the brain sections were applied with hematoxylin and subsequently with eosin. The sections were then dehydrated in upstream ethanol and xylene. The H&E staining was used to observe the degree of tissue necrosis, connection, and infiltration under an optical microscopeat 40× and 200× magnification.

2.7 Immunohistochemistry

The brain sections were prepared as described above. After being washed with phosphate buffer saline (PBS), the brain sections were treated in citrate buffer (pH6.0) for heat - induced antigen retrieval and then immersed in 3% H_2O_2 for 15 min to block endogenous peroxidase. These sections were blocked with normal goat serum and then incubated with primary antibody for one hour at room temperature and overnight at 4°C. The primary antibodies included rabbit polyclonal anti - IL - 6 (1: 500; Abcam, Cambridge, UK) and IL - 10 (1: 500; Abcam, Cambridge, UK). After being washed with PBS, the sections were incubated with horseradish peroxidase (HRP) - conjugated secondary antibodies for 30 min at room temperature. The secondary antibodies were reacted with standard ABC kit reagent (Vector Laboratories, Burlingame, CA, USA) and the immunostaining signaling was developed with 0.01% 3, 3 - diaminobenzidine tetrahydrochloride (DAB) (Sigma - Aldrich, St. Louis, MO, USA) at room temperature. The immuno - positive areas were examined under a light microscope and the average integral optical density were calculated with image J.

2.8 Enzyme - linked immunosorbent assay (ELISA)

The concentrations of TNF - α, IL - 6, IL - 10, and TGF - β, and malondialdehyde (MDA) in fresh brain tissues were measured by using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN, USA). The standard ELISA procedure was performed according to manufacturer's instructions. Optical intensities of samples after reaction were read at 450 nm on an ELISA reader.

2.9 Statistical analysis

Data were expressed as the mean ± standard deviation (SD). All statistical analyses were performed using SPSS 20.0 software (Chicago, IL, USA). The statistical differences between groups were analyzed using one - way ANOVA, followed by Least Significant Difference (LSD) test. The significance was determined on a criterion of $P < 0.05$.

3. Result

Post - stroke treatment with TM granule ameliorated I/R - induced neurobehaviorl deficits in rats

To test the neuroprotective effects of oral administration of TM granule against I/R - induced injury, we first performed the corner test at Day 1, Day 7, and Day 14 after reperfusion. The results of the corner test were shown in Figure 1A. Sham - operated rats showed no neurobehavioral impairment throughout the test, suggesting no functional damage due to surgical procedure. The score of corner test in I/R group was significantly higher than that in the sham group. However, treatments with TM granule resulted in improved performance in the corner test that reached a statistical significance on Day 7 and Day 14 after reperfusion. Notably, treatment with high dose TM granule in I/R rats led to a further improvement in the corner test compared with low dose TM treatment group.

To further evaluate the effects of I/R and TM granule on neurological deficits, including motor, sensory, balance, and reflexes, another neurobehavioral assay, mNSS test, was also carried out on Day 1, Day 7, and Day 14 after reperfusion. As shown in Figure 1B, similar changes were observed in the mNSS test. Together, these results demonstrate that post - stroke oral administration of TM granule can ameliorate the neurobehavioral deficits induced by I/R and the beneficial effects of TM granule on neuronal functions are dose - dependent.

Post - stroke treatment with TM granule reduced I/R - induced cerebral infarction in rats

To test whether treatment with TM granule after stroke is protective against the formation of cerebral infarctinduced by ischemia and reperfusion, we used TTC staining to examine the infarct area in the brain of rats with sham - operated surgery, I/R, and I/R treated with TM. Consistent with the neurobehavioral functions described above, sham - operated rats did not exhibit any infarct area. As expected, MCAO and reperfusion produced an obvious infarct in the cerebral cortex of rats in I/R group (approximately 5% of total area). I/R rats orally treated with TM granule for 7 days displayed significantly reduced cortical infarct areas. While the infarct area in low dose TM group was reduced to about 3% of total area, that in high dose TM group was about 2% (Figure 2). These results show that post - stroke treatment with TM can attenuate I/R - induced cerebral injury, supporting the findings in neuro - functional improvement.

Post - stroke treatment with TM granule protected against the histological changes induced by I/R in rats

H&E staining was employed to examine the histological alterations in groups of sham - operated rats, I/R rats, and I/R rats treated with TM granule. Compared with sham group, there were many shrunken and ruptured neurons and infiltrated inflammatory cells in the cerebral cortex of MCAO hemisphere in I/R group. Moreover, microvascular destruction and cerebral hemorrhage were also observed in the I/R group. However, treatment with TM granule after I/R can significantly improve neuronal damage and cell infiltration in I/R rats (Figure 3).

Post - stroke treatment with TM granule normalized I/R - induced upregulation of IL - 6 in the cerebral cortex of rats

Inflammatory responses are implicated in the pathogenesis and prognosis of ischemic stroke. To explore if the neuroprotective effects of TM were associated with the modulation of inflammation in stroke condition, we first examined the cortical levels of a proinflammatory cytokine IL - 6 and an anti - inflammatory cytokine IL - 10 obtained from sham - operated and I/R rats treated with or without TM granule. The immunohistochemical staining showed that the expression of IL - 6 was induced in the cerebral cortex of I/R rats, compared with the sham group. Treatment with TM after I/R normalized the upregulation of IL - 6 (Figure 4A). On the other hand, neither I/R nor administration of TM granule significantly changed the expression of IL - 10 in the cortical slices (Figure 4B).

Post - stroke treatment with TM granule effectively reduced the levels of proinflammatory cytokines IL - 6 and TNF - α and increased the levels of an anti - inflammatory cytokine TGF - β in the cerebral cortex of I/R rats

The above findings suggested that oral treatment with TM granule may be efficacious in stroke model by modulating inflammatory status. ELISA was further performed to quantitatively examine the levels of several inflammatory cytokines in cortical lysates obtained from sham - operated and I/R rats treatment with or without TM granule. Consistent with the results of immunohistochemistry, treatment with TM significantly reversed upregulation of IL - 6 in the cortex of I/R rats (Figure 5A). In addition, TM treatment also attenuated I/R - induced increase in the level of TNF - α (Figure 5B). In terms of anti - inflammatory cytokines, matching with the results of immunohistochemistry, the levels of IL - 10 were comparable among all four groups (Figure 6A). Interestingly, treatment with high dose, but not low dose, of TM strongly increased the level of TGF - β in the cortex of I/R rats (Figure 6B). Collectively, these results demonstrate that post - stroke treatment with TM pushes immune responses towards a more anti - inflammatory phenotype in the injured cortex.

Post - stroke treatment with TM granule reduced the level of lipid peroxidation in the cerebral cortex of I/R rats

To further investigate whether the anti - oxidant activity ofTM is associated with the protection against I/R - induced brain injury, ELISA was used to examine the level of MDA, a biomarker of lipid peroxidation, in the cortical lysates of sham rats, I/R rats treated with or without TM granule. The results showed that oral treatment with TM significantly reduced I/R - induced increase in cortical MDA levels, whereas the beneficial effects of low dose and high dose TM on lipid peroxidation were comparable (Figure 7).

4. Discussion

Enormous efforts have been made to investigate the pathogenesis and new treatment strategy for ischemic stroke, it is still one of the top causes of death and disability throughout the world. In recent years, more attention has focused on the therapeutic potential of TCMs on stroke. Although the water extract of TM is used for indications including stroke for centuries, the evidence - based study demonstrating the therapeutic effects and its mode of action remains largely lacking. In addition, to improve patient compliance and reduce health burden, powdered products of TM are increasingly prescribed in clinical practice, despite without efficacy confirmation. The present study showed that, in condition of ischemia and reperfusion, post - stroke treatment with TM granule can evoke an anti - inflammatory immune responses that is associated with its therapeutic effects.

Human recombinant t - PA is currently the only Food and Drug Administration (FDA) approved thromolytic drug for ischemic stroke treatment. However, the 4.5 - h narrow therapeutic window and potential side effects after reperfusion largely limit its clinical use [4, 6]. Our data showed that the powdered product of TM had promising beneficial effects on stroke, in terms of rescuing the deficits in neurobehavioral performance by two different functioal assays. The therapeutic effects were in parallel with the reduction of infarct area in the cerebral cortex. Given that we used a animal model with both ischemia and reperfusion injury, the present findings supported the idea that TM granule can serve as an add - on therapy to current thrombolytic approaches.

Inflammation is one of the most important contributors to the pathophysiology of ischemic stroke. Under ischemic condition, pro - inflammatory responses may trigger oxidative stress, lipid peroxidation, mitochondrial impairment, subsequently leading to neuronal death and functional deficits [15 - 17]. Therefore, modulation of proinflammatory microenvironment is expected to be protective in stroke [18, 19]. Our present data showed that ischemia and reperfusion injury significantly induced the cortical levels of IL - 6 and TNF - α. Post - stroke administration of TM granule can reverse the elevation of these proinflammatory cytokines in the brain of I/R rats. In patients with stroke, these proinflammatory cytokines are associated with early neurological deterioration, larger infarct size and poor long - term prognosis [10, 20 - 22], supproting the efficacy potential of TM granule in human stroke. With respect to anti - inflammatory cytokines, oral administration of TM granule was found to dramatically increase the level of TGF - β in the cerebral cortex of I/R rats. Previous study showed thatanti - inflammatory cytokines including TGF - β can modulate Th1 and Th2 responses and promotes T regulatory cell development [23], resulting in neuroprotection in experimental stroke [24]. More importantly, direct injection of recombinant TGF - β or adenoviral - mediated TGF - β expression can rescue inflammatory responses and neuronal injury in ischemic animal models [25, 26]. Thus, upregulation of TGF - β may be also involved in the neuroprotective effects of TM granule in I/R rats. However, unlike Bu Yang Huan Wu decoction,

another classic TCM formula [12], TM treatment did not have effects on the regulation of IL - 10.

Although the present study has demonstrated the therapeutic and anti - inflammatory effects of TM granule on ischemic stroke, there are still unsolved questions regarding the underlying molecular mechanisms. Our data showed that treatment with TM granule attenuated inflammation in the central nervous system, whereas the effects of TM treatment on cerebrovascular system and peripheral immune system required further investigation. Previous studies showed that TM formulations were efficacious for the treatment of hypertension [27, 28]. Given that high blood pressure is a major risk factor for ischemic stroke, whether the beneficial effects of TM on systemic and cardiac vascular regulation are also involved in the neuroprotection is worth an attention. Future study is needed to investigate this possibility in an ischemic model concomitant with hypertension.

In conclusion, this study for the first time demonstrates that post - stroke administration of TM granule orally has beneficial therapeutic effects on an ischemia and reperfusion stroke model. The underlying mechanisms of action are associated with its anti - inflammatory effects. These findings provide an evidence - based insight of using TM granule for stroke patients receiving thrombolytic therapy.

5. Conclusion

This study for the first time demonstrates that post - stroke administration of TM granule orally has beneficial therapeutic effects on an ischemia and reperfusion stroke model. The underlying mechanisms of action are associated with its anti - inflammatory effects. These findings provide an evidence - based insight of using TM granule for stroke patients receiving thrombolytic therapy.

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Data Availability
The data used to support the findings of this study are presented within the article. All raw data are available by request to corresponding author.

Competing interests
The authors declare no conflict of interest.

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Author Profile

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Table 1: The component of the TM granule used in this study

The following herbs yield an amount of extract		4.0 g
Tianma (*Rhizoma Gastrodiae*)	1.2 g	
Gouteng (*Ramulus Uncariae Cum Unicis*)	2.0 g	
Shijueming (*Concha Haliotidis*)	4.0 g	
Zhizi (*Fructus Gardeniae*)	1.2 g	
Huanqin (*Radix Scutellariae*)	1.2 g	
Chuanniuxi (*Radix Cyathulae*)	1.6 g	
Duzhong (*Cortex Eucommiae*)	2.0 g	
Yimucao (*Herba Leonuri*)	2.0 g	
Sangjisheng (*Herba Taxilli*)	3.2 g	
Yejiaoteng (*Cauls Polugoni Multiflori*)	4.0 g	
Fushen (*Poria*)	2.0 g	
Starch		2.0 g

Each 6 g of the TM granule contains the above components

Figure Legend

Figure 1: Post - stroke treatment with TM granule ameliorated I/R - induced neurobehaviorl deficits in rats. (A and B) The corner test (A) and modified neurological severity scores (mNSS) (B) of sham - operated rats and MCAO/reperfusion (I/R)

rats receiving vehicle, low - dose (L) or high - dose (H) TM granule at 1, 7, and 14 days after sham operation and I/R. Data were presented as mean ± SD (n = 3 in each group). ***, P < 0.001 compared with sham - operated group; +, P < 0.05, ++, P < 0.01 and +++, P < 0.001 compared with I/R group; #, P < 0.05, ###, P < 0.001 between I/R groups treated with low - dose and high - dose TM granule.

Figure 2: Post - stroke treatment with TM granule reduced I/R - induced cerebral infarction in rats. (A) Representative cortical sections from sham - operated rats and MCAO/reperfusion (I/R) rats receiving vehicle, low - dose (L) or high - dose (H) TM granule stained with 2, 3, 5 - triphenyltetrazolium chloride (TTC). (B) Higher magnification images of the fourth sections of all groups are presented. (C) The area of infarcted brain tissue at 7 days after sham operation and I/R treated with or without TM granule was expressed as a percentage of the whole section area. Data were presented as mean ± SD (n = 3 in each group). ***, P < 0.001 compared with sham - operated group; +, P < 0.05, ++, P < 0.01 compared with I/R group.

Figure 3: Post - stroke treatment with TM granule protected against the histological changes induced by I/R in rats. (A) Representative brain sections from sham - operated rats and MCAO/reperfusion (I/R) rats receiving vehicle, low - dose (L) or high - dose (H) TM granule stained with hematoxylin and eosin (H & E) (B) Higher magnification of the H & E staining in the cerebral cortex. Scale bars represent 100 μm in (A); 50 μm in (B).

Figure 4: Post - stroke treatment with TM granule normalized I/R - induced upregulation of IL - 6 in the cerebral cortex of rats (A and B) Representative cortical sections from sham - operated rats and MCAO/reperfusion (I/R) rats receiving vehicle, low - dose (L) or high - dose (H) TM granule stained for IL - 6 (A) and IL - 10 (B). Scale bars represent 50 μm.

Figure 5: Post - stroke treatment with TM granule reduced the levels of proinflammatory cytokines IL - 6 and TNF - αin the cerebral cortex of I/R rats (A and B) The protein levels of IL - 6 (A) and TNF - α (B) measured by ELISA in the brains obtained from sham - operated rats and MCAO/reperfusion (I/R) rats receiving vehicle, low - dose (L) or high - dose (H) TM granule at 7 days after sham operation or I/R. Data were presented as mean ± SD (n = 4 in each group). ***, P < 0.001 compared with sham - operated group; +++, P < 0.001 compared with I/R group; #, P < 0.05, ###, P < 0.001 between I/R groups treated with low - dose and high - dose TM granule.

Figure 6: Post - stroke treatment with TM granule increased the levels of anti - inflammatory cytokines TGF - β in the cerebral cortex of I/R rats (A and B) The protein levels of TGF - β (A) and IL - 10 (B) measured by ELISA in the brains obtained from sham - operated rats and MCAO/reperfusion (I/R) rats receiving vehicle, low - dose (L) or high - dose (H) TM granule at 7 days after sham operation or I/R. Data were presented as mean ± SD (n = 4 in each group). **, P < 0.001 compared with sham - operated group; +, P < 0.05, +++, P < 0.001 compared with I/R group; ###, P < 0.001 between I/R groups treated with low - dose and high - dose TM granule.

Figure 7: Post - stroke treatment with TM granule reduced the level of lipid peroxidation in the cerebral cortex of I/R rats. The level of malondialdehyde (MDA) measured by ELISA in the brains obtained from sham - operated rats and I/R rats receiving vehicle, low - dose (L) or high - dose (H) TM granule at 7 days after sham operation or I/R. Data were presented as mean ± SD (n = 4 in each group). ***, P < 0.001 compared with sham - operated group; ++, P < 0.01, +++, P < 0.001 compared with I/R group.

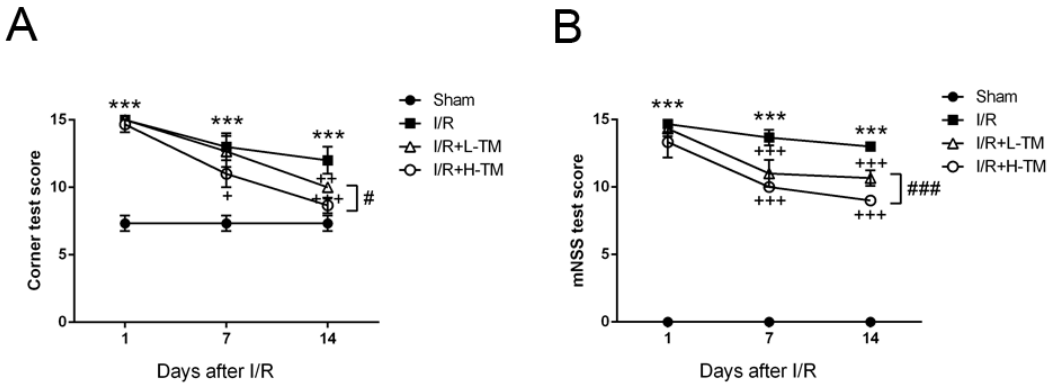

Figure 1

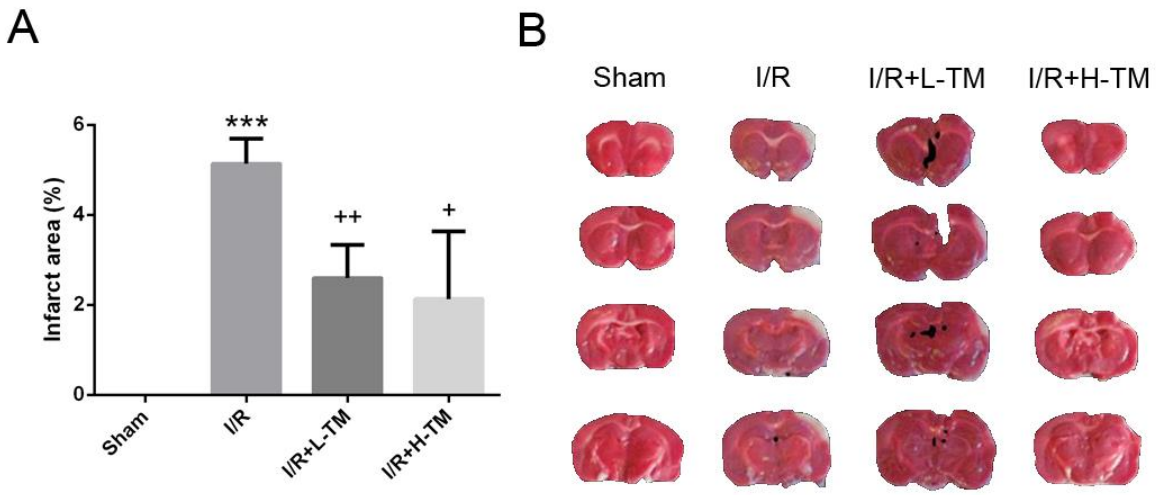

Figure 2

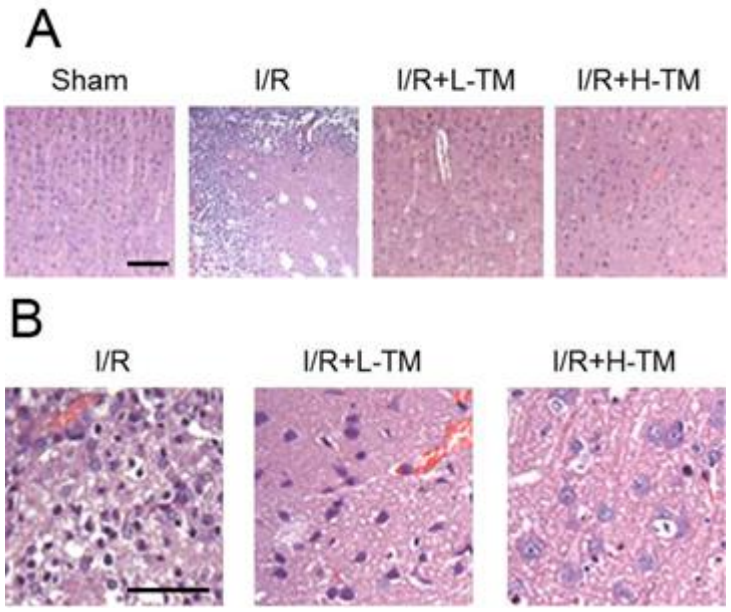

Figure 3

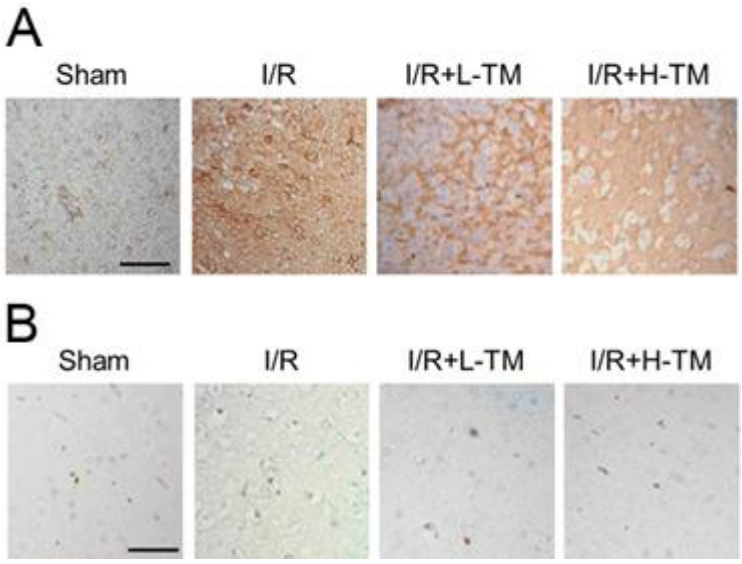

Figure 4

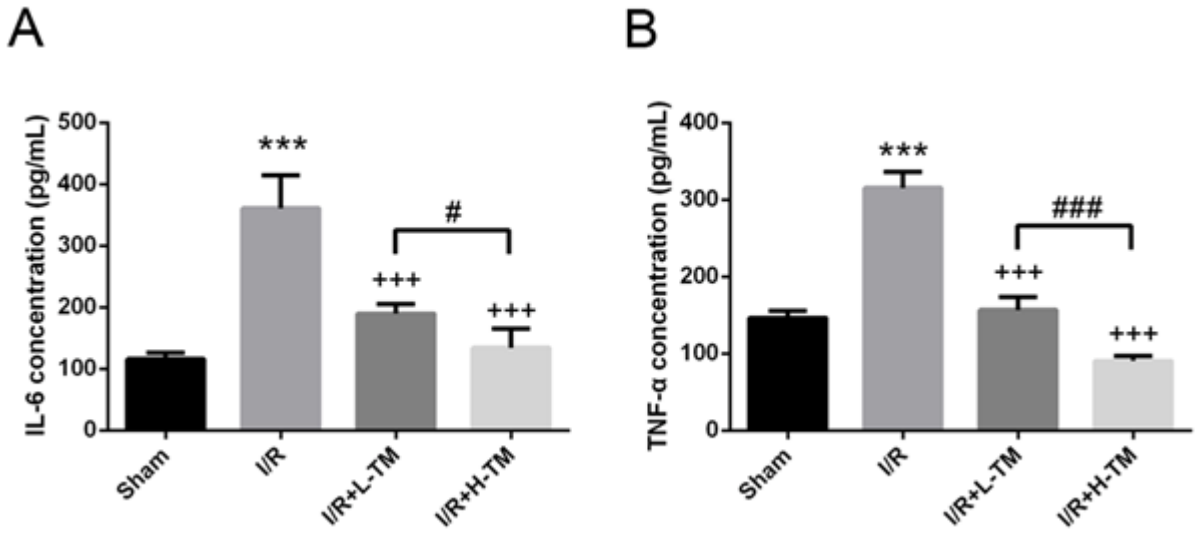

Figure 5

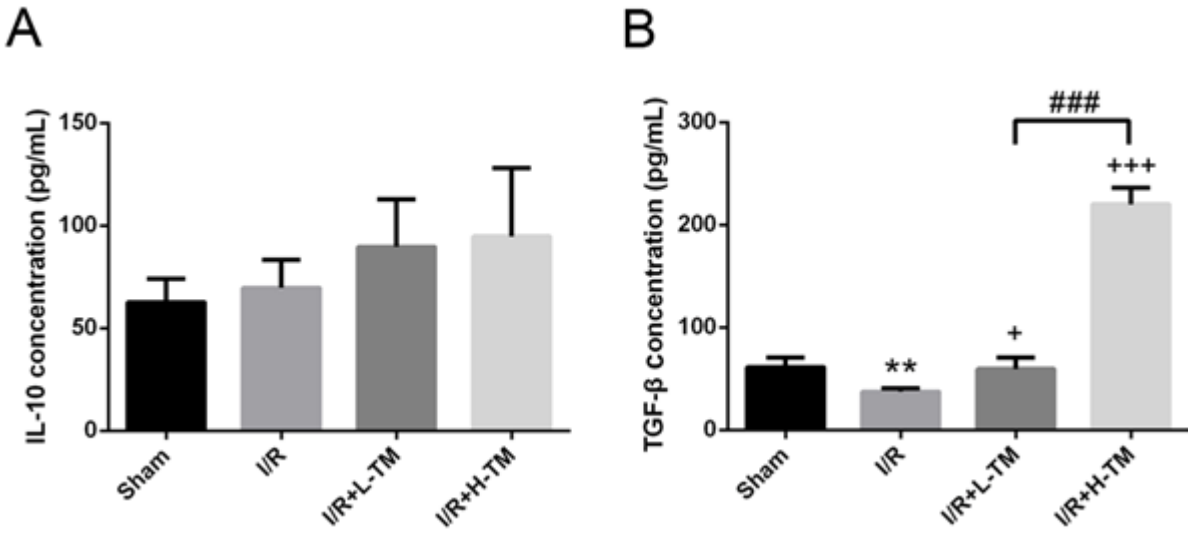

Figure 6

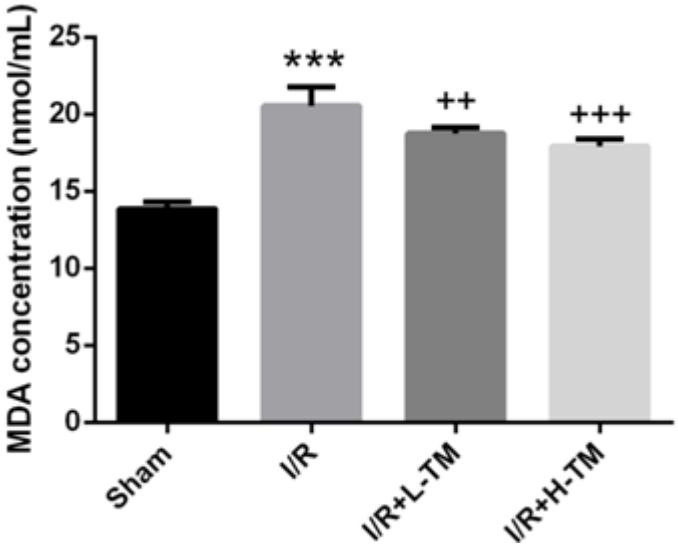

Figure 7