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Publication Date

2015-03-01

DOI

10.1016/j.neuroscience.2014.12.028

Peer reviewed

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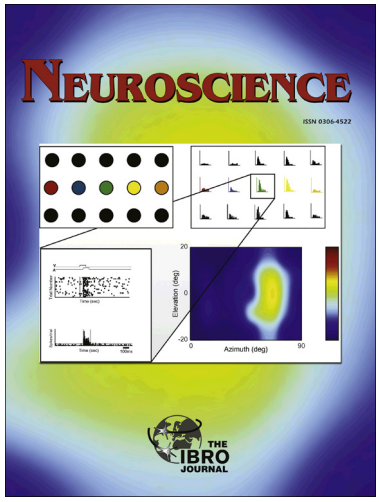

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PII: S0306-4522(14)01077-X
DOI: http://dx.doi.org/10.1016/j.neuroscience.2014.12.028
Reference: NSC 15925

To appear in: *Neuroscience*

Accepted Date: 17 December 2014

Please cite this article as: L. Zhou, S.C. Bondy, L. Jian, P-y. Wen, F-z. Yang, H. Luo, W-w. Li, J. Zhou, Tanshinone IIA attenuates the cerebral ischemic injury-induced increase in levels of GFAP and of caspases-3 and -8, *Neuroscience* (2014), doi: http://dx.doi.org/10.1016/j.neuroscience.2014.12.028

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Tanshinone IIA attenuates the cerebral ischemic injury-induced increase in levels of GFAP and of caspases-3 and -8

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Abstract

Tanshinone IIA (TSA) is a lipid soluble agent derived from the root of *Salvia miltiorrhiza* (Danshen). This plant is a traditional Chinese herb, which has been used widely in China especially for enhancing circulation. However the mechanisms underlying its efficacy remain poorly understood. The present study was designed to illuminate the events that may underlie the apparently neuroprotective effects of TSA following ischemic insult. Adult Sprague Dawley rats were subjected to transient focal cerebral ischemia by use of a middle cerebral artery occlusion model. They were then randomly divided into a sham-operated control group, and cerebral ischemia/reperfusion groups receiving a two-hour occlusion. Further subsets of groups received the same durations of occlusion or were sham-operated but then received daily i.p. injections of high or low doses of TSA, for 7 or 15 days. Hematoxylin and eosin staining revealed lesions in the entorhinal cortex of both the rats subject to ischemia and to a lesser extent to those receiving TSA after surgery. Levels of glial fibrillary acidic protein (GFAP), caspase-3 and caspase-8, were quantified by both immunohistochemistry and Western blotting. TSA treatment after middle cerebral artery occlusion, markedly reduced infarct size, and reduced the expression of caspase-3, and caspase-8. These changes were considered protective and were generally proportional to the dose of TSA used. These results suggest that TSA may effect neuroprotection by way of reduction of the extent of cell inflammation and death within affected regions.

Keywords

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Tanshinone IIA; cerebral ischemia; caspase; glial fibrillary acidic protein

Abbreviations

GFAP, glial fibrillary acidic protein; I/R, cerebral ischemia/reperfusion; TSA, Tanshinone IIA;

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1. Introduction

Stroke is one of the leading causes of death worldwide and can also be a source of permanent disability survivors. Cerebral ischemic stroke accounts for approximately 80% of all strokes (Chen et al., 2012；Donnan et al., 2008；Feigin et al., 2003). The most common cause of ischemic stroke is the occlusion of a blood vessel by a thrombus, resulting in an immediate loss of the normal supply of oxygen and glucose to cerebral tissue (Genoverse et al., 2011). A variety of therapeutic strategies are currently being considered as a means of minimizing the neuronal damage resulting from ischemia, leading to increasing study of the pathological mechanisms underlying stroke.

The diterpenoid tanshinone IIA (TSA) is an important component of Chinese medicine, made from the dried rhizome of danshen, *Salvia miltiorrhiza*. This herb is traditionally used for the treatment of cardiovascular and cerebrovascular diseases, including ischemic stroke (Yang et al., 2008; Xu et al., 2009). TSA is a derivative of phenanthrenequinone;1,6,6-trimethyl-8,9-dihydro-7H-naphtho[8,7-g][1]benzoxole-10, 11-dione (Fig. 1). Many plant-derived terpenoids have been found to possess anti-inflammatory and anti-neoplastic properties, and TSA has been reported to modulate many kinds of biological activities, including the quenching of reactive oxygen species, and antagonism of calcium-promoted events. Such molecular changes may account for the ability of TSA to promote microcirculatory function, inhibit neutrophil migration, and exert anti-tumor effects (Han et al., 2008). TSA is able to traverse the blood brain barrier although the brain content is limited to 30% of

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the plasma concentration (Chen et al., 2007), and this could relate to its effects in reducing the volume of cerebral infarction and maintenance of neuronal function (Lam et al., 2003). TSA has been shown to have protective effects against focal cerebral ischemia/reperfusion (I/R) injury in animal models (Tang et al., 2010; Liu et al., 2010) and has been proposed as a potential therapeutic agent in heart disease, liver disease and cancer treatment (Dai et al., 2012).

The present study was intended to evaluate the potentially protective effects of TSA in a stroke model and illuminate on the mechanisms that could underlie this. A suture-occlusion method was used to block the middle cerebral artery, followed by release of the block thus initiating reperfusion. Levels of the pro-inflammatory indicators glial fibrillary acidic protein (GFAP) were increased after I/R together with levels of two caspases associated with apoptosis; caspase-3 and capsase-8. Treatment with TSA after the surgical procedure partially reversed all of the changes caused by I/R. This provides a mechanistic basis accounting for the utility of TSA on the treatment of stroke.

2. Experimental procedures

2.1. *Materials*

TSA was purchased from Jiangsu Carefree Pharmaceutical Co., Ltd (Jiangsu, China). Male Sprague-Dawley rats weighing 220~280g were obtained from the Department of Medical Experimental Animal Center of Xiang-ya School of Medicine, Central South University. Rats were housed in groups of eight each, with food and

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water supplied *ad libitum,* and were maintained on a 12-h light/dark cycle. The rats were kept under standardized temperature, humidity and light conditions with free access to food and water. All experimental protocols and animal handling procedures were approved in advance by the Animal Care and Use Committee of Xiang-ya School of Medicine (Central South University) and were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. *The Middle Cerebral Artery Occlusion Model*

Focal cerebral ischemia was induced by transient middle cerebral artery occlusion in the right hemisphere (Genoverse et al., 2011). Rats were anesthetized with 10% chloral hydrate (350ml/kg, administered i.p. in 0.3 ml saline). The surgery to occlude the medial carotid artery consisted of the insertion of a 4–0 nylon filament (Beijing Shadong Biology Company, Beijing, China; diameter 0.26mm) precoated with a hardener with diameter 0.34 mm, via the external carotid artery into the internal carotid artery to block the inflow of blood into the medial carotid artery according to the procedure originally described by (Longa et al., 1989) and modified by (Melani et al., 1999). After 2 hours of ischemia, the nylon filament was carefully pulled out to establish reperfusion. Sham-operated (control) rats underwent the same surgical procedures without occlusion of the common carotid arteries. Rectal temperature was recorded and maintained at 37℃ throughout the whole surgical procedure. The operation of was carried out with all efforts to minimize animal suffering.

This transient medial carotid artery occlusion caused measurable neurological deficits. We used Longa and Bederson's 5-point scale within 24 h after recovery from

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anesthetic to judge whether the surgery was successful. The scoring system was as follows: 0, no deficit; 1, flexion of the contralateral torso and forelimb when held by tail; 2, circling to affected side; 3, leaning to affected side; 4, no spontaneous locomotor activity or barrel rolling. Any animal without deficit in the intra-ischemic period was excluded from the study (Li et al., 2010).

2.3. *Study design in animal models*

Sixty-four rats were randomized into eight groups (n=8 for each group): Sham (control) animals were subjected to surgical procedures without artery occlusion shock and treated with a daily i.p. injection of physiological saline for 7 or15 days.. I/R operated rats were subjected to artery occlusion for 2 hours followed by reperfusion. They then received daily i.p. injections of saline for 7 or 15d respectively. I/R operated rats subsequently treated with a low dose (4 mg/kg/day i.p.) or a high dose of TSA (8mg/kg/day).

2.4. *Brain sample preparations*

After the experiments were completed, rats were deeply anesthetized with 10% chloral hydrate (350 ml/kg), and the heart was exposed to perfuse transcardially with cold 0.1M phosphate-buffered saline PH 7.4 through the left ventricle. After dissection one half of the brain was immediately frozen in -80 °C refrigerator (for Western blotting) and the other half fixed in 4% paraformaldehyde overnight (for immunohistochemistry and hematoxylin-eosin staining). Thereafter, fixed tissue was transferred into PBS/0.02% Na azide at 4°C until use. Afterward, the fixed brain tissues were further processed by dehydration in graded ethanol prior to paraffin

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embedding. Finally, the blocks were coronally cut in serial 5-μm-thick sections and stored at 4 °C before being stained.

2.5. *Hematoxylin-eosin Staining and Immunohistochemistry*

After deparaffinization, sections were washed in double distilled water for ten m, and were incubated in hematoxylin, then treated with with 0.2% hydrochloric acid. Subsequently, eosin red was used in a 2 m incubation, and this was followed by fixation in 95% alcohol.

Immunohistochemistry was performed on paraffin embedded sections. After deparaffinization, sections were permeabilized with in antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 20 min with 80°C. Endogenous peroxidase in issues was quenched with hydrogen peroxide for 5 min. Non-specific adsorption was minimized by incubating the sections in 2% BSA with 0.3% Triton X-100 for 40~60 min. Then sections were incubated with anti-GFAP rabbit polyclonal antibody 1:500 in PBS (DAKO Troy, MI, USA), anti-caspase 3 rabbit polyclonal antibody (Abcam, Cambridge, MA, USA, 1:500 in PBS), anti-caspase 8 rabbit polyclonal antibody1: 50 in PBS (Abcam,) overnight at 4 °C. Sections were then immersed in PBS with 0.1% Triton-X and incubated with secondary antibody (1:400, Vector Laboratories, Burlinghame, CA, USA) for 2 h. In order to amplify the sensitivity and specificity of biological response, an avidin-biotin complex marked with enzyme (ABC), was used as a secondary marker antibody. Reaction product was detected with 3, 3'-diaminobenzidine-tetrahydrochloride, DAB (Vector). Sections were processed by dehydration in graded ethanol, cleared with xylene, and then

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coverslipped with DPX (Biomedical Specialties, Miami Lakes, FL, USA). Some sections were incubated with the primary antibody alone or with the secondary antibody alone. In this manner the immunoreaction in all experiments was confirmed to be specific to the target protein.

2.6. *Western Blotting*

Samples of cortex prepared from fresh frozen brain tissue were homogenized in 10 volumes extraction ice-cold tris-buffered saline (20 mM Tris-HCL buffer PH 7.4, 150 mM NaCl) (0.150g tissue/2 ml buffer) with 0.5 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail from Sigma. Homogenates were centrifuged at 12000×*g* (10 min, 4°C) so that the supernatant was collected, and then the protein concentration measured with BCA protein assay (Novagen Inc. Madison, WI, USA). Samples (30–60μg protein/well) were separated in 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (Amersham Biosciences, Piscataway, NJ, USA) membrane (300 mA for 1.5 h). The membrane was incubated with 5% non-fat dried milk (PM) for 40~60 min and subsequently probed with GFAP (1:3000, DAKO Troy, MI, USA), caspase-3, 1:1000 (Abcam, Cambridge, MA, USA), caspase-8 (1:200, Abcam), at 4°C overnight. After washing, blots were incubated with the corresponding HRP-labeled secondary antibodies 1:3000 dilution (Jackson Labs, Bar Harbor, ME, USA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were incubated in the presence of the antibody against β-actin 1:1000 (Santa Cruz Biotechnology). Chemiluminescence was detected with ECL Plus substrate kit (Beyotime, China) by Image Quant 350 (GE

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Healthcare, Little Chalfont, UK).

2.7. *Image and Statistical Analysis*

Immunostaining was quantified using a Nikon Eclipse 80i microscope (Nikon, Japan) and images acquired with a Nikon DS high-resolution digital color camera (1280 × 1024 pixel) using NIS-Elements AR 3.0 software. Digital images were analyzed using Ver.3.00 analysis program (Nikon). The percentage of immunostained area (field area of immunostaining/total image area ×100) was determined for all the markers studied by averaging several images per section that cover all or most of the region of study. Western blots bands were analyzed by Image J software. All experiments were repeated at least twice, with n=6–8 animals per group per marker. All quantitative comparisons were performed on sections processed at the same time. Single ANOVA statistical analysis was used to assess the significance of the differences in anti-GFAP, anti-caspase-3, anti-caspase-8, among the animal groups. A two-tailed *P*-value of less than 0.05 was considered significant. All the statistical analysis was carried out without knowledge of the treatments (Impellizzeri et al., 2011).

3. Results

The consequence of ischemic injury on a variety of relevant parameters was studied together with the outcome of daily treatment with TSA following surgery for 7 or 15 days. It was found that all values obtained after TSA treatment following sham surgery, did not differ significantly from the corresponding values following sham

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surgery alone. Thus, in order to simplify the figures these results are not shown.

3.1. *Hematoxylin-eosin staining*

The cerebral infarction cause by I/R was examined in brain slices stained with hematoxylin-eosin (Fig. 2). Sections from the control group had normally staining brain tissue (Fig. 2A,E) , whereas the I/R treatment groups had a paler less stained region reflecting the area of ischemic damage in the infarcted area (Fig. 2B,F).

Treatment with TSA resulted in an incomplete reversal of the loss of staining in the infarcted area. The infarct volume (white area) was attenuated in these groups. This was marginal in the group receiving the lower dose of TSA for 7d (Fig. 2C), and especially pronounced in the group receiving the higher dose of TSA for 15d (Fig. 2H) suggesting a dose- and time- response restorative relationship.

3.2. *Levels of GFAP in the cortex*

Immunohistochemical staining for GFAP revealed that I/R treatment led to a significant and sustained increase in the GFAP levels in the injured cortex relative to controls (Fig. 3A). GFAP and CD11b levels were quantitatively confirmed by densitometric analysis (Fig. 3B) and confirmed by Western blotting of homogenates (Figs. 3C, D, E, F). The effect of repeated treatment with TSA after surgery led to a pronounced attenuation of GFAP content. There was a clear dose-response relationship in that the effect of the higher dose of TSA was greater than that of the low dose.

3.3. *Caspase content*

I/R groups exhibited higher expression of caspase-3 and caspase-8 than sham

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groups as judged by immunohistochemistry (Figs. 4 A, B, and 5 A, B) and this was confirmed by parallel quantification made with Western blotting (Figs. 4 C, D and 5 C, D). The increase in caspase levels caused by I/R was around 10-fold. The effect of TSA treatment on the content of caspases-3 and -8 was to partially reverse the increase of both caspases caused by I/R. This reduction was around 50% and was apparent using either of the quantitative caspase assays. A clear dose-response relation between high and low amounts of TSA was only observed for caspase-3 in the group treated with TSA for 15 days.

4. Discussion

Middle cerebral artery occlusion is a generally accepted model of cerebral ischemia (Hoffman et al., 2006). The findings presented suggest that indices of apoptosis in rat brain induced by I/R can be considerably reduced (but not totally prevented) by TSA.

The reduction of GFAP content after TSA treatment, further indicated that TSA can mitigate the inflammation and the impairments in metabolism induced by I/R. Astrocytes, the most abundant population of glial cells that constitute the microenvironment of the central nervous system (Liu et al., 2012), are intimately associated with the brain homeostasis and maintenance of brain function. While astrocytes are significant in effects repair processes in the CNS, excessive prolongation of their activation is known to lead to undesirable inflammatory outcomes (Sun et al., 2012). Since GFAP is a marker of astrocytic reactivity our

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findings suggest that TSA may owe some of its neuroprotective effects in ischemia regions by restoring the resting condition of astrocytes.

A substantial body of evidence suggests that apoptosis after cerebral I/R injury is a major pathway leading to cell death (Faruk et al., 2010). After transient occlusion of the middle cerebral artery followed by reperfusion, the number of apoptotic cells in the striatum and cortex of rats was directly proportional to the duration of ischemia (Raff et al., 1993). The mechanism of apoptosis involves a signal transduction process by which intracellular protein is degraded by a family of cysteine-dependent aspartate-directed proteases (caspases). The caspases, central regulators of apoptosis, have been demonstrated to play a key role in ischemia-induced cytotoxicity in vivo and in vitro (Bi et al., 2008; Lee et al., 2007; Shimmyo et al., 2008). Based on their function, caspases are classified into two groups, initiator caspases such as caspase-8, and effector caspases such as caspase-3. Generally, the initiator caspases activate downstream effector caspases by a proteolytic cascade, resulting in the cleavage of a variety of cellular substrates involved in apoptosis (Liu et al. 2011a). Caspase-3 can be activated by upstream initiator caspases such as caspase-8 through two distinct pathways, i.e., the death receptor-mediated extrinsic caspase-8 pathway or the mitochondria dependent-cytochrome c/caspase-9 intrinsic pathway, respectively (Hu et al., 2003). Caspase-3 has been shown to be up-regulated and activated in ischemic brain tissue (Rami et al., 2003), and genetic deletion or pharmacologic inhibition of caspase-3 leads to reduced neuronal death in the ischemic brain (Le et al., 2002). The protective effect of neuronal deletion of caspase-8 has also been shown

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following impact injury to the brain. Such deletion led to reductions in lesion size, the extent of neuronal cell death and less activation of caspase-3. Behaviorally impacted animals with deletion of the caspase-8 gene had a lower neuropathological score, better learning performance, superior retention of spatial memory and improved sensorimotor function (Krajewska et al., 2011). Caspase-3 activity increased when myocardial cells were treated with angiotensin II and TSA greatly inhibited this elevation (Hong et al., 2010). Apoptosis of cultured myocardial cells has also been reported to be blocked by TSA and this was attributed to inhibition of caspase-3 activity (Jin et al., 2013). Our finding parallels these reports in that the levels of caspases in brain tissue were significantly increased when rats were subjected to I/R, and that TSA treatment greatly reduced the magnitude of this increase. The results presented here show that TSA inhibited ischemia-induced cell death in the entorhinal cortex. This finding parallels that of a study using prostatic cancer cells, where TSA also had an anti-apoptic effect (Won et al., 2010). Since many neurodegenerative diseases involve cell death and inappropriately elevated levels of immune activity, it is likely that an agent such as TSA with anti-inflammatory and anti-apoptic properties, will also find application in the treatment of a broader range of age-related diseases of the nervous system. For example, a protective effect of TSA has been reported in animal models of Alzheimer's disease (Yin et al., 2008, Jiang et al., 2014).

In animals subjected to I/R and not treated with TSA, the indices of damage were generally lower 15 days after surgery than after 7 days. Thus the effect of TSA may be to improve or accelerate any intrinsic recovery possible after a serious I/R insult.

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Conclusions

The present study found that TSA protects nerve cells from I/R-induced apoptosis. This neuroprotective effect appears to involve several processes, including suppression of excess activation of glial cells, and inhibiting the activity of caspase-3 and caspase-8. The initial cellular site of TSA action, which can lead to a sequence of protective events, remains to be determined. Furthermore other properties of TSA such as its ability to inhibit platelet aggregation (Liu et al., 2011b) may also contribute to its reputed effectiveness in stroke treatment.

It is noteworthy that, since TSA was effective when administered after the induced lesion, this allows its consideration as a therapeutic drug. TSA seems to be an appropriate candidate for the treatment of stroke or other apoptosis-related brain diseases. Further studies are needed in order to form a strong basis for human clinical trials.

Acknowledgement

The financial support provided by Hunan Provincial Social Science Foundation of China under the contract No. 2011WK3047 is greatly appreciated here.

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Figure Legends

Fig. 1. Structure of tanshinone IIA (from PubChem open Chemistry Database); 1,6,6-trimethyl-8,9-dihydro-7H-naphtho[8,7-g][1]benzoxole-10, 11-dione.

Fig. 2 Representative coronal sections of cortex (×200, H&E)

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSA (4mg/kg) 7d, **D** =I/R + TSA (8mg/kg) 7d, E = Control 15d, **F** = I/R 15d, **G** = I/R + TSA (8mg/kg) 15d, **H** = I/R 15d + TSA (8mg/kg). I/R = subjected to ischemia/reperfusion, TSA: daily injections for for 7 or 15 days.

Fig. 3. Effect of TSA on GFAP levels

A) GFAP immunostaining reaction of cortex (×200). **(B)** Densitometric analysis of cortex. Data are expressed as % total tissue area. **(C)** Representative Western blot **(D)** Western blot quantitation normalized relative to β-actin.

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSA (4mg/kg) 7d, **D** = I/R + TSA (8mg/kg) 7d, **E** = Control 15d, **F** = I/R 15d, **G** = I/R + TSA (8mg/kg) 15d, **H** = I/R 15d + TSA (8mg/kg). I/R: subjected to ischemia/reperfusion, TSA: daily injections for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding high-dose TSA-treated group.

Fig. 4. Effect of TSA on caspase-3 levels.

A) Caspase-3 immunostaining reaction of cortex (×200). **(B)** Densitometric analysis of cortex. Data are expressed as % total tissue area. **(C)** Representative Western blot

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(D) Western blot quantitation normalized relative to β-actin.

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSA (4mg/kg) 7d, **D** = I/R + TSA (8mg/kg) 7d, **E** = Control 15d, **F** = I/R 15d, **G** = I/R + TSA (8mg/kg) 15d, **H** = I/R 15d + TSA (8mg/kg). I/R: subjected to ischemia/reperfusion, TSA: daily injections for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding high-dose TSA-treated group.

Fig. 5. Effect of TSA on capsase-8 levels

A) Caspase-8 immunostaining reaction of cortex (×200). **(B)** Densitometric analysis of cortex. Data are expressed as % total tissue area. **(C)** Representative Western blot **(D)** Western blot quantitation normalized relative to β-actin.

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSA (4mg/kg) 7d, **D** = I/R + TSA (8mg/kg) 7d, **E** = Control 15d, **F** = I/R 15d, **G** = I/R + TSA (8mg/kg) 15d, **H** = I/R 15d + TSA (8mg/kg). I/R: subjected to ischemia/reperfusion, TSA: daily injections for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding high-dose TSA-treated group.

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Fig. 1

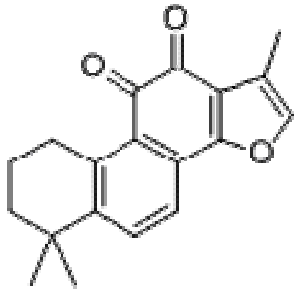

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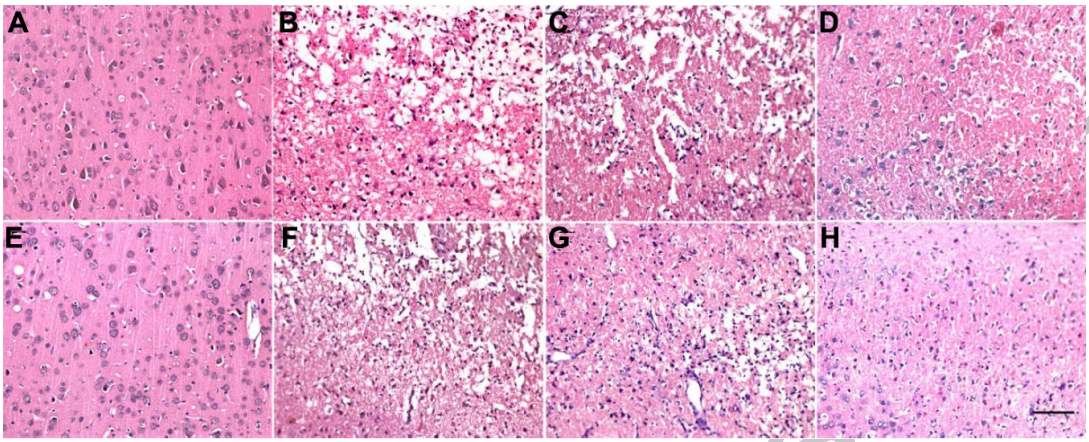
Fig. 2



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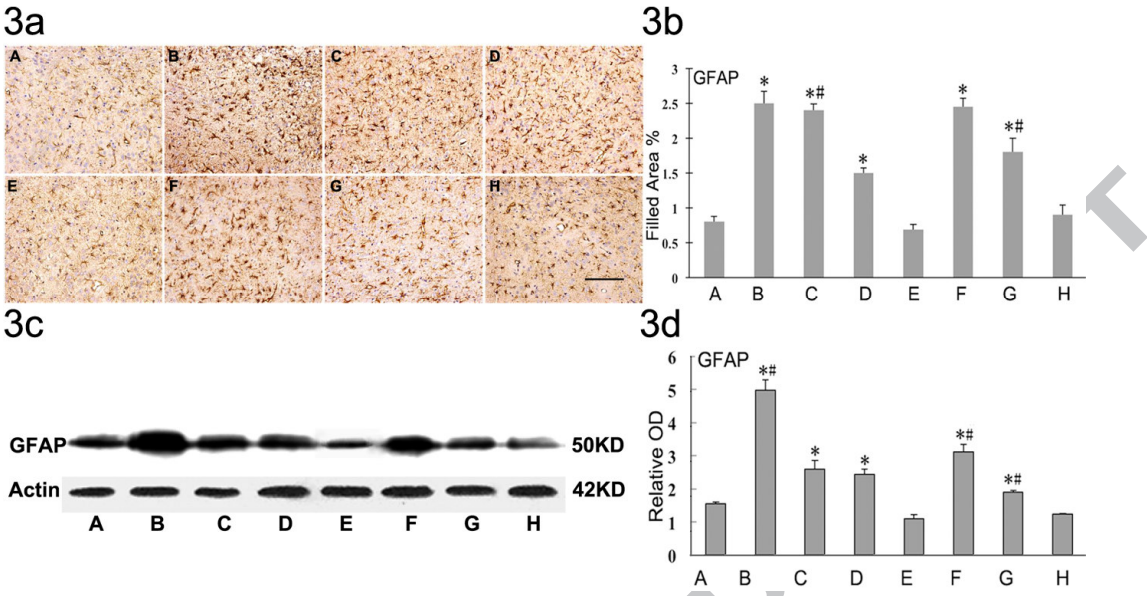

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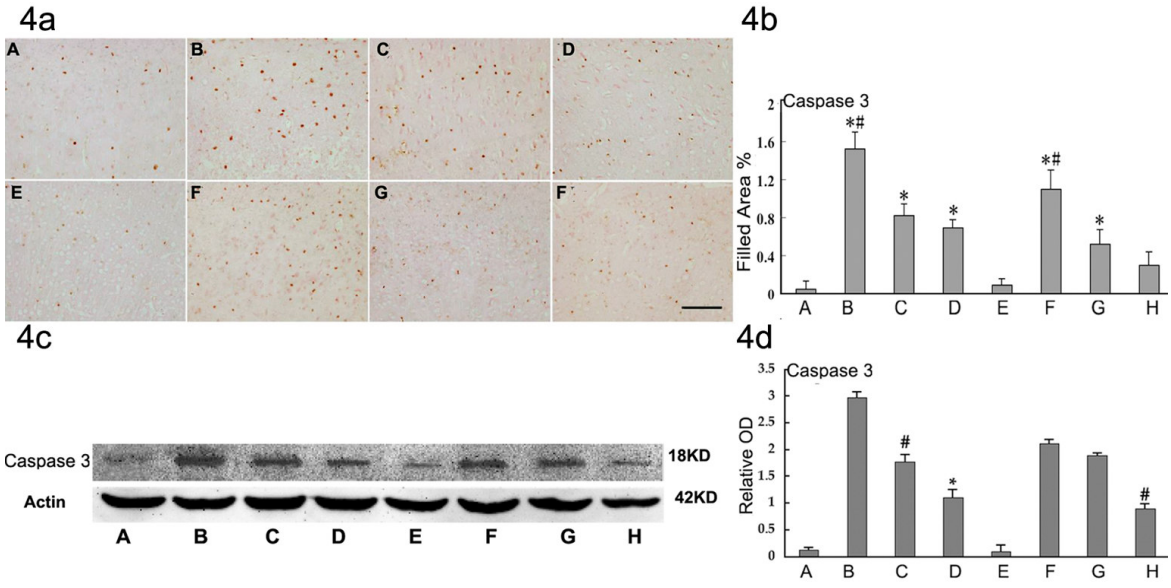

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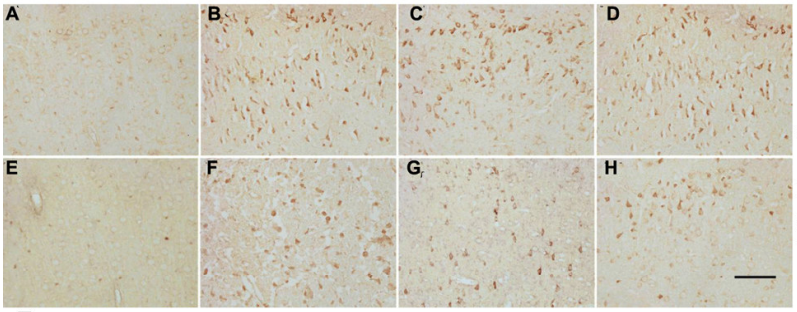

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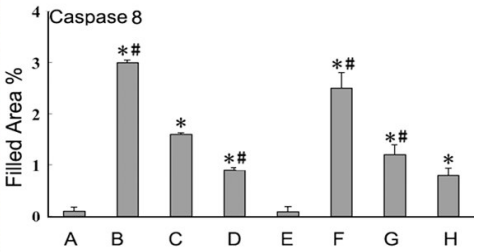

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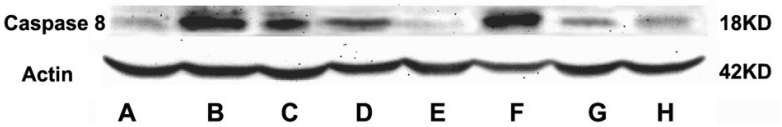

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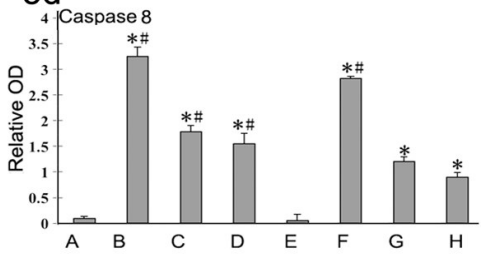

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Highlights

- Two hours of ischemia, increased levels of caspases -3 and -8, and GFAP, in cortex
- Tanshinone IIA reduced indices of inflammation and cell death.
- Tanshinone IIA treatment reduced infarct size after middle cerebral artery occlusion.
- Tanshinone IIA may effect neuroprotection by reduction of inflammation and cell death

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