Title

Protective Effect of Tanshinone IIA Against Infarct Size and Increased HMGB1, NFκB, GFAP and Apoptosis Consequent to Transient Middle Cerebral Artery Occlusion

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Abstract: Acute inflammation plays an important role in brain damage following cerebral ischemia and reperfusion (I/R) injury. The present study employed a rat model of middle cerebral artery occlusion (MCAO) to explore the neuroprotective effects of tanshinone IIA (TSN), which is widely used in China for promoting circulation. Rats were randomly divided into sham-operated group, and I/R transiently occluded then reperfused groups. Two out of each of these three groups received a different dose of TSN. After 15 days, triphenyl tetrazolium chloride and hematoxylin and eosin staining revealed fewer lesions in the TSN -treated I/R group relative to I/R alone. TSN treatment dramatically improved infarct sizes and reduced content of HMGB1 in the I/R model group. Nuclear translocation of NFκB was also attenuated in I/R animals subsequently receiving TSN. TUNEL staining revealed more apoptosis in the I/R model group and this was reduced in the I/R animals treated with TSN for 15d. Thus TSN mitigates the severity of damage effected by I/R.

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August 13, 2012

Dear Sirs,

We are submitting this manuscript entitled "Protective effect of tanshinone IIA against infarct size and increased HMGB1, NFκB, GFAP and apoptosis consequent to transient middle cerebral artery occlusion" for consideration as a publication in **Neurochemistry International**

This article is original work and has not been previously submitted to any other journal.

There is no conflict of interest between any of the authors and the substance of this article.

Thank you for your consideration of this study.

Sincerely yours,

Stephen Bondy, Ph. D.
Professor

***Highlights (for review)**

- Tanshinone IIA mitigates changes in brain after ischemia-reperfusion injury.
- Tanshinone IIA after ischemia-reperfusion insult, reduces indices of inflammation.
- The nuclear migration of NF-κB after ischemic injury is lowered by tanshinone IIA.
- Astroglial activation after ischemia- reperfusion, is reduced by tanshinone IIA.
- Tanshinone IIA may have clinical utility, as it can act *after* the neural injury.

Protective effect of tanshinone IIA against infarct size and increased HMGB1, NFκB, GFAP and apoptosis consequent to transient middle cerebral artery occlusion

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Abstract

Acute inflammation plays an important role in brain damage following cerebral ischemia and reperfusion (I/R) injury. The present study employed a rat model of middle cerebral artery occlusion (MCAO) to explore the neuroprotective effects of tanshinone IIA (TSN), which is widely used in China for treating cerebrovascular and cardiovascular diseases. Rats were divided into a sham-operated group, and I/R transiently occluded then reperfused groups. Some of the I/R animals were treated daily for 7d or 15d with two different doses of TSN. After 15 days, triphenyl tetrazolium chloride and hematoxylin and eosin staining revealed fewer lesions in the TSN -treated I/R group relative to the untreated corresponding I/R group. TSN treatment dramatically improved infarct sizes and reduced content of HMGB1 following I/R. Nuclear translocation of NFκB was also attenuated in I/R animals subsequently receiving TSN. TUNEL staining revealed more apoptosis in the I/R model group and this was reduced in the I/R animals treated with TSN for 15d. Thus TSN mitigates the severity of damage effected by I/R.

Keywords: Apoptosis; inflammation; middle cerebral artery occlusion; Tanshinone IIA

1. Introduction

As worldwide populations age, the incidence of human cerebrovascular diseases continues to increase, and ischemic cerebrovascular diseases accounts for 80% of these (Feigin et al., 2003). The high incidence and significant mortality rates of ischemic cerebrovascular disease are risks to human health (Pendlebury and Rothwell, 2009). Consequently, much attention has been given to understanding pathological mechanisms of focal cerebral ischemia and on finding effective treatments (Durukan and Tatlisumak, 2007, Froehler and Ovbiagele, 2010). The traditional Chinese medicine *Salvia miltiorrhiza* is the dry root and rhizome of *S. miltiorrhiza Bunge*. The lipid soluble constituent of this root, tanshinone IIA, (TSN) is the most abundant active component in this root from which it can be extracted (Xia et al., 2005). It has widespread pharmacological effects and has recently become a subject of much research (Niu et al., 2000, Zhou et al., 2011). TSN effectively inhibits cell lipid peroxidation, prevents apoptosis and methyl guanine transferase activity (Gao et al., 2008, and can reduce cerebral infarction area in I/R rats (Yu et al., 2010). Tanshinone has been to protect against ischemic/reperfusion-induced brain damage and has been used to treat focal cerebral ischemia, as well as a range of cardiovascular diseases (Lam et al., 2003, Xia et al., 2005,Xu et al., 2009, Dong et al., 2009, Tang et al., 2010, Wang et al., 2010). This protective effect may be mediated by down-regulation of high mobility

group box-1 (HMGB1) receptor for advanced glycation endproducts (RAGE), toll-like receptor 4 (TLR4), and nuclear factor-κB (NFκB), (Wang et al., 2010), and with upregulation of BDNF expression (Lui et al.,2010). HMGB1, which was originally characterized as a nuclear DNA-binding protein with a highly conserved structure in several species, participates in nucleosome formation and regulates gene transcription (Thomas and Travers, 2001). When cells are injured or become necrotic, HMGB1, which normally resides in nuclei, translocates to the cytoplasm and/or extracellular space (Wang et al., 1999, Scaffidi et al., 2002). HMGB1 is a key cytokine that plays an extracellular role in cellular activation and proinflammatory responses (Yang et al., 2007). The presence of extracellular HMGB1 has been proven in necrotic tissue damaged by trauma or ischemia, and HMGB1 has been identified in the active secretions of many types of cells, including activated immune cells (Orlova et al., 2007). HMGB1 been implicated in several inflammatory diseases (Andersson et al., 2000, Scaffidi et al., 2002, Muhammed et al., 2008, Qui et al., 2008) and is released from ischemic brain tissue, and is elevated in serum from stroke patients (Goldstein et al., 2006, Luan et al., 2010). HMGB1 exerts its biological effects through interaction with its receptor, TLR-4. Activation of TLR-4 can then lead to activation of NFκB (Park et al., 2006, Tian et al., 2007, Qiu et al., 2008) which will further increase expression of leukocyte adhesion molecules and the production of proinflammatory mediators by hematopoietic and endothelial cells, thereby promoting inflammation and causing organ and cell damage (Treutiger and Mullins, 2003). This is in part mediated by

furthering of the maturation and migration of immune cells leading to increased reactivity of TLR (Palumbo et al., 2007, Tsung et al., 2007). NF-κB is an important transcription factor, and persistent activation of NF-κB makes neurons vulnerable to ischemic insult (Clemens et al., 1997, Zhang et al., 2005). In the non-activated state, NF-κB and IκB are found as a covalently bound trimer in the cytoplasm. When cells are stimulated by physical or chemical factors, IκB phosphorylation and degradation occurs, which results in movement of activated NFκB p65 from the cytoplasm to the nucleus, where it acts as a transcription factor to promote the production of inflammatory factors and thereby expand the inflammatory response. IKK interacts with IκB-α and specifically phosphorylates IκB-α on site that trigger its degradation. The functional IKK complex contains three subunits, designated IKKα, IKKβ, and IKKγ; each making an essential contribution to IκB phosphorylation. Elevated HMGB1 and NF-κB expression are involved in ischemia-evoked neuronal injury and death via a pro-inflammatory mechanism (Wang et al., 2010). Collectively, the evidence suggests that HMGB1 associated with NFκB activation pathway plays an important role in injury processes incurred after stroke (Ridder et al., 2009). In view of the reported neuroprotective effect of TSN, we have evaluated the effect of ischemic followed by reperfusion on phosphorylation and nuclear translocation of HMGB1 and NFκB, and the potential of TSN to mitigate any changes observed. The relationship between NFκB and IKKBα, p-IκBα, IKKα, p-IKKα, was also explored. We have investigated the neuroprotective mechanism of TSN in order to provide a theoretical foundation for

future research. The effects of TSN on rats with cerebral I/R injury following middle coronary artery occlusion have been studied by assessing the extent of phosphorylation and nuclear translocation of the HMGB1/NFκB signaling pathway.

GFAP is an important cytoskeletal component of astrocytes, which are the most abundant population of glial cells in the central nervous system microenvironment. They are intimately associated with brain homeostasis and the maintenance of brain function. Astrocytes not only provide metabolic and trophic support for neurons, they also modulate levels of neurotransmitters and synthesize and release neurotrophic factors, thus playing an important role in neurogenesis (Lee et al., 2012). However when excessively activated, they are also capable of initiating inflammatory events. Shortly after brain ischemia injury, a series of imbalances occurs that leads to an increased number of reactive astrocytes (Choi et al., 2010). In ischemia-reperfusion injury, delayed neuronal death is associated with apoptosis especially in the ischemic penumbra (Cho and Toledo-Pereyra, 2008). Therefore, in this work, we also evaluated astroglial activation using the GFAP assay and cell death using the TUNEL assay.

2. Materials and methods

2.1. Animal treatment

80 healthy adult male Sprague Dawley rats weighing 220-240 g were provided by the medical experimental animal center of Xiangya Medical School,

Central South University. Humidity levels were maintained at 55 ± 5%, and the rats were kept under a 12-h light–dark cycle at 22 ± 2 °C. All animal procedures were performed in accordance with the Guidelines of the Chinese Society for Laboratory Animals Science. The rats were randomly divided into eight experimental groups (n= 8 each). In the sham groups, animals were subjected to the same daily treatment with saline and the same surgical procedures as the I/R group but without filament fixation. In the MCAO groups, the animals received a single I/R injury at the beginning of the study in addition to an intraperitoneal (i.p.) injection of vehicle (phosphate-buffered saline, PBS). In the TSN IIA groups, the animals were treated as the I/R group but also received TSN IIA (5 mg/kg or 10 mg/kg dissolved in saline and administered i.p.) immediately after surgery. Animals were sacrificed 7 or 15 days subsequently, and the brains were removed for histology, immunohistochemistry, and western blotting.

2.2. Ischemia-reperfusion surgery

Induction of ischemia followed by reperfusion (I/R) was carried out as previously described (Longa et al., 1989). Briefly, rats were anesthetized with 10% chloral hydrate (350 ml/kg), and an incision was made in the midline of the neck. Then, the right common carotid artery and branches of the external carotid artery were ligated with a 4–0 filament (Beijing Shadong Biology Company, Beijing, China). The diameter of the filament is 0.26 mm, but the diameter of the tip is 0.34 mm to create a globular stopper that was introduced into the internal

carotid artery and inserted until it meets resistance. The filament was then fixed and removed after 2 h. Rats in the sham group underwent the same surgical procedure without the introduction of the filament into the internal carotid artery. Rectal temperatures were recorded and maintained at 37 °C throughout the procedure. The standard of judging successful I/R is that upon recovery from anesthesia, the rats appear to have left-sided paralysis, difficulty standing, and circle to one side when held by the tail. Neural function was scored using Longa and Bederson's 5-point scale within 24 h after recovery from anesthetic. The scoring is performed as follows: 0, no nerve damage symptoms; 1, cannot fully extend front paws on opposite side; 2, resistance to the opposite side drops; 3, circles to the opposite side; 4, cannot spontaneously walk, loses consciousness.

2.3. Brain sample preparation

At 7 or 15 days after surgery, rats were deeply anesthetized with 10% chloral hydrate (350 ml/kg), and the heart was exposed to perfuse PBS through the left ventricle. Next, PBS was changed to 4% paraformaldehyde (PFA) and perfusion continued at 10 ml/min. Brain tissue was then collected and fixed in 4% PFA at 4 °C overnight, then transferred into 0.02% sodium azide at 4 °C and stored until use. Some tissue was rinsed with PBS, dehydrated in a graded ethanol series, embedded in paraffin, and coronally sectioned at 5 μm. For 2,3,5-triphenyltetrazolium chloride (TTC) staining (Wexler et al., 2002), fresh brain slices were cut and placed in 2% TTC solution for 30 min. Infarcted areas

appeared as unstained tissue while normal brain tissues stained red.

2.4. Immunohistochemistry

Dewaxed sections were immersed in antigen retrieval solution (Vector Lab, Burlingame, CA, USA) for 20min (Zhou et al., 2008) and endogenous peroxidase was blocked by incubation in 3% H_2O_2, and nonspecific background staining was blocked by incubating in 2% bovine serum albumin (BSA). Sections were then incubated overnight with primary antibodies raised against HMGB1 (1:500; Abcam, Cambridge, UK) and NFκB (1:200; Cell Signaling Technology, Beverly, MA, USA). The sections were then incubated with secondary antibodies and ABC reagent (Vector Laboratories, Burlinghame, CA, USA Finally, the sections were incubated for approximately 2~5 min with 3,3'-diaminobenzidine (Vector), dehydrated in a series of graded ethanols and xylene, then coverslipped with DePeX (Biomedical Specialties, Santa Monica, CA, USA).

2.5.Western blotting

Tissue was homogenized in 10 volumes of Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4 and 150 mM NaCl) containing a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) (Zhou et al., 2008). Samples were electrophoretically separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred to polyvinylidene fluoride membranes, which were subsequently blocked in 5% skim milk and then incubated primary in

antibodies (HMGB1, from Abcam; NFκB-p65, p-NFκB from Cell Signaling Technology); IκB-α, p-IKB-α, IKKα/β, p-IKKα/β from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and glial fibrillary acidic protein (GFAP) from Dako (Carpenteria, CA, USA) overnight at 4°C. The next day, blots were incubated with the corresponding horseradish peroxidase-labeled secondary antibodies (1:2000). Labeling was detected using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA). The blots were subsequently re-probed with antibodies to assess equal loading (actin from Sigma and β-tubulin from Santa Cruz) following the same procedures as above. Bands were analyzed using densitometric software (Scion Corp., Frederick, MD, USA) and statistically analyzed with one-way analysis of variance (ANOVA). Analysis of these bands was from at least three separate experiments performed on different days.

2.6. TUNEL staining

After the sections were treated with proteinase K (Sigma, St. Louis, MO, USA), they were subsequently incubated in dUTP-digoxin mixture followed by anti-DIG-anti-alkaline phosphatase solution. Sections were next treated in nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche, Nutley, NJ, USA) for color development. Finally, the slides were mounted with water-soluble mounting medium (Vector).

2.7. Image acquisition.

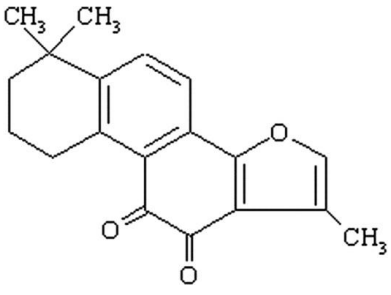
Immunostaining was quantitated using a Nikon Eclipse 80i microscope (Tokyo, Japan), and images were taken of the cortex and hippocampus with a high-resolution Nikon DS digital color camera.

2.8. Statistical analysis

The percentage of immunopositive area (immunopositive area/total image area × 100) was determined for all of the markers by averaging 4-5 images per section that covered most of the regions using NIS-Elements AR 3.0 software (Nikon). Statistical significance was determined by one-way ANOVA. In all cases, differences were considered significant if $P < 0.05$ (two-tailed).

2.9. Reagents

Tanshinone (1,6,6-Trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-b]furan-10,11-dione), TSN was purchased from Jiangsu Carefree Pharmaceutical Co., Ltd (Jiangsu, China). The chemical structure of TSN is shown:



A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit used for detection of apoptosis was from Roche (Indianapolis, IN, USA). All other chemicals and reagents were of analytical or high-performance liquid chromatography grade.

3. Results

3.1. Tanshinone IIA, TSN reduces brain infarction area in I/R

The control groups had normal brain tissue structure, whereas the I/R groups revealed ischemic changes in the infarcted areas as judged by an increase in unstained areas (Fig1a). In animals treated with TSN, there were different degrees of improvement in brain infarction area. Quantitation of these results showed the most pathology in the I/R group receiving no TSN (Fig. 1b). Pathological changes were partly but not completely reversed by TSN treatment, the reduction being greatest and significant only in the high-dosage group treated for 15d.

3.2. TSN suppresses the increase in HMGB1 protein occurring after ischemic injury

HMGB1 was measured in brain tissue by immunohistochemistry and quantitated by Western blotting. I/R treatment markedly increased levels of this protein which however remained above control values (Fig. 2a). Only the 15d high-dose TSN significantly reduced this elevation of HMGB1. Quantitation of the corresponding Western blots revealed that this reduction effected by TSN was apparent in both the corresponding cytoplasmic and nuclear fractions (Fig. 2b).

3.3. TSN IIA suppresses the increase in NFκB occurring after ischemic injury

NFκB was quantitated by immunohistochemistry (Fig. 3a, b) and by analysis

of Western blots (Fig. 3c). Both assays showed I/R to enhance NFκB levels. This was reduced by TSN only in the nuclear fraction and only at the longest duration of exposure and highest dose used. This suggests that the migration of active NFκB into the nucleus was impaired by TSN.

Western blotting of cytoplasmic and nuclear fractions allowed distinction between NFκB and its activated phosphorylated form, pNFκB. Compared to I/R surgery alone, the longer period (15d) and higher dose of TSN dosing led to elevation of the proportion of NFκB present in the phosphorylated form in cytoplasm, but to depression of pNFκB in the nucleus (Fig. 3d).

3.4. I/R elevates cytoplasmic IκB and IKKα/β and TSN can enhance cytoplasmic p-IκB and p-IKKα/β

I/R treatment led to a significant increase in cytoplasmic IκBα and -IKKα/β at 7d or 15d post-surgery, but there changes attributable to subsequent TSN treatment were limited to partial reduction of IKKα/β after the shorter period of TSN treatment (Fig. 4a). I/R did not alter cytoplasmic content of the de-activated phosphorylated inhibitors of NFκB, p-IκB and p-IKKα/β. However both of these were greatly increased by the higher dose of TSN (Figs. 4b). These changes were not however reflected in the corresponding nuclear levels of either native NFκB inhibitors or their phosphorylated derivatives all of which were unaltered by any treatment (data not shown).

3.5. TSN IIA attenuates GFAP expression after I/R

GFAP expression was raised several fold in I/R animals after 7d and 15d, and treatment with the higher dose of TSN reduced this significantly (Fig. 5). This further suggests that the neuroprotective effects of TSH may primarily be based on its anti-inflammatory properties.

3.6. TSN reduces I/R induced apoptosis in the cortex and hippocampus

TUNEL staining revealed apoptotic nuclei in brain tissue from all groups except the sham operated controls (Fig. 6). The number of apoptotic cells was increased in the ischemic penumbrae and focal ischemic areas in all experimental groups. The intensity of TUNEL staining decreased over time. The extent of apoptosis was significantly further decreased after 15d of treatment with the higher dose of TSN. In this case, both the size of the infarction area and number of apoptotic cells were strongly reduced. These findings imply that TSN treatment effectively attenuated I/R-induced apoptosis.

4. Discussion

The overall value of TSN treatment was indicated by its ability to lead to a decreased infarct area after I/R (Fig. 1). Down-regulation of HMGB1-induced NFκB activation might be a potential mechanism accounting for TSN's neuroprotective effects following cerebral ischemia. TSN also significantly reduced the effect of I/R in elevating GFAP levels, and this inhibition of astroglial

activation may also be related to the protective effects of TSN in the ischemic region (Fig. 5).

Apoptosis occurred in frontal cortex and hippocampus of I/R treated groups. Apoptosis occurred as a time-dependent dynamic process, the number of apoptotic cells decreasing in 15d I/R treated rats. The higher dose of TSN applied for 15d further reduced the extent of apoptosis (Fig. 6).

The primary mechanism underlying the cerebral protective effect of TSN IIA has not yet been clearly pinpointed. In addition to the pathways suggested by the current study, inhibition of calcium influx, antioxidant effects, and improved Na+, K+-ATPase function have all been suggested as primary mechanisms (Xie et al., 2011). However, the current results suggest that an upstream event inhibited by TSN, is the inhibition of the injury-provoked translocation of HMGB1 to the nucleus (Fig. 2). This may lead to the observed inhibition of nuclear migration of both NFκB and pNFκB (Fig. 3), and initiate the anti-inflammatory properties of TSN. The increased phosphorylation of nuclear IκBα, and IKKα effected by TSN reflects inactivation of NFκB inhibitors, (Fig.4) is difficult to account for. It may be that this apparently pro-inflammatory change is overwhelmed by the multiple anti-inflammatory effects of TSN.

In conclusion, TSN reduced brain infarct area in the ischemic hemisphere. TSN effectively attenuates the inflammatory response and apoptosis following I/R injury in rats. Reducing nuclear HMGB1, NFkB by retarding their translocation from cytoplasm may inhibit inflammatory gene activation and

consequently reduce the production of inflammatory cytokines. Collectively, these results illustrate the value of this traditional Chinese medicine approach for treating cerebral I/R injury. The fact that TSN can significantly reduce pathological changes following I/R has implications for the clinical treatment of cerebral injury following stroke.

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Figure Legends

Figure 1. Effects of TSN on H&E staining

a) Staining reaction of cortex (×200). Infarcted brain tissue is filled with unstained tissue.

b) Quantitation of the histochemical data. **A** = Control 7d, **B** = I/R 7d, **C** = I/R + TSN (5mg/kg) 7d, **D** =I/R + TSN (10mg/kg) 7d, **E** = Control 15d, **F**= I/R 15d, **G** = I/R + TSN (5mg/kg) 15d, **H** = I/R 15d + TSN (10mg/kg). I/R = subjected to ischemia/reperfusion, TSN = treated with 5 or 10 mg/kg tanshinone IIA daily for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding I/R group.

Figure 2. HMGB1 levels assayed by immunohistochemistry and Western blotting.

a) Staining reaction of cortex (×200). b) Quantitation of the histochemical data. c) Quantitation of Western blotting from cytoplasmic and nuclear fractions.

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSN (5mg/kg) 7d, **D** =I/R + TSN (10mg/kg) 7d, **E** = Control 15d, **F**= I/R 15d, **G** = I/R + TSN (5mg/kg) 15d, **H** = I/R 15d + TSN (10mg/kg). I/R = subjected to ischemia/reperfusion, TSN = treated with 5 or 10 mg/kg tanshinone IIA daily for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding I/R group.

Figure 3. NFκB expression assessed by immunohistochemistry and Western blot

a) Staining reaction of NFκB in cortex (×200). b) Quantitation of the histochemical data for NFκB. c) Quantitation of Western blotting of NFκB from cytoplasmic and nuclear fractions. d) Quantitation of Western blotting of pNFκB from cytoplasmic and nuclear fractions.

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSN (5mg/kg) 7d, **D** =I/R + TSN (10mg/kg) 7d, **F**= I/R 15d, **G** = I/R + TSN (5mg/kg) 15d, **H** = I/R 15d + TSN (10mg/kg). I/R = subjected to ischemia/reperfusion, TSN = treated with 5 or 10 mg/kg tanshinone IIA daily for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding I/R group. n = 5-6.

Figure 4. Cytoplasmic levels of modulators of NFκB activity, IKBα , p-IKBα ,IKKα/β and p- IKKα/β quantitated by Western blot

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSN (5mg/kg) 7d, **D** =I/R + TSN (10mg/kg) 7d, **F**= I/R 15d, **G** = I/R + TSN (5mg/kg) 15d, **H** = I/R 15d + TSN (10mg/kg). I/R = subjected to ischemia/reperfusion, TSN = treated with 5 or 10 mg/kg tanshinone IIA daily for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding I/R group. n = 5-7.

Figure 5. Effect of TSN treatment upon GFAP content after I/R quantitated by Western blot

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSN (5mg/kg) 7d, **D** =I/R + TSN (10mg/kg) 7d, **F**= I/R 15d, **G** = I/R + TSN (5mg/kg) 15d, **H** = I/R 15d + TSN (10mg/kg). I/R = subjected to ischemia/reperfusion, TSN = treated with 5 or 10 mg/kg tanshinone IIA daily for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding I/R group. n = 5-7.

Figure 6. Apoptotic cells in the cortex and hippocampus assessed by TUNEL staining.

a) Cortical section, b) Hippocampal section, c) Quantitation of the histochemical data derived from both cortex and hippocampus.

A = Control 7d, **B** = I/R 7d, **C** = I/R + TSN (5mg/kg) 7d, **D** =I/R + TSN (10mg/kg) 7d, **F**= I/R 15d, **G** = I/R + TSN (5mg/kg) 15d, **H** = I/R 15d + TSN (10mg/kg). I/R = subjected to ischemia/reperfusion, TSN = treated with 5 or 10 mg/kg tanshinone IIA daily for 7 or 15 days. *: $P < 0.05$ differs from corresponding control group; #: $P < 0.05$ also differs from corresponding I/R group. n = 5-7.

Figure 1

Fig. 1a

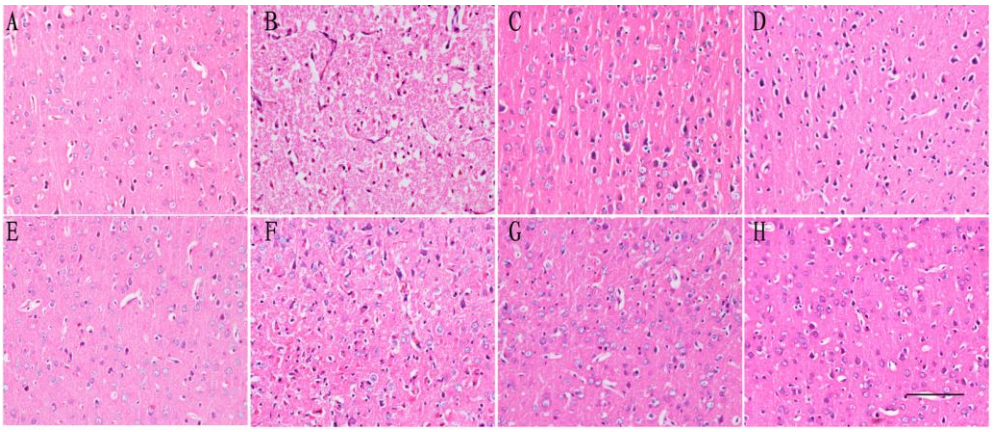


Fig. 1b

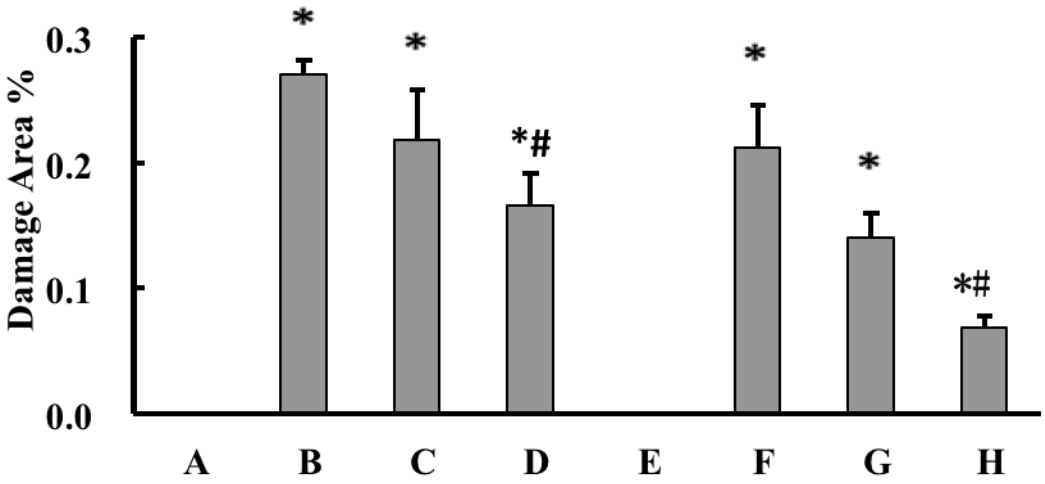
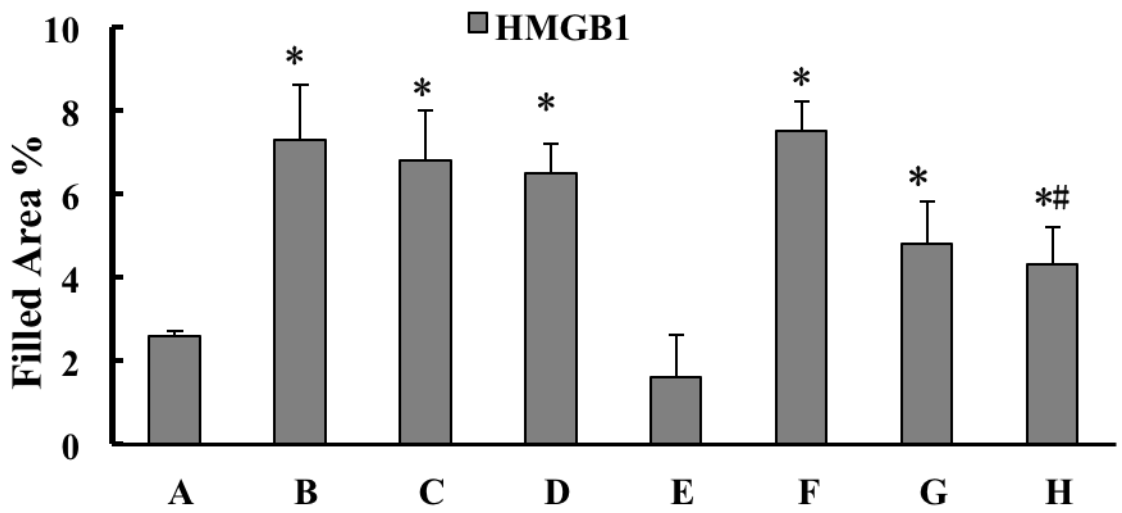


Figure 2

Fig. 2A



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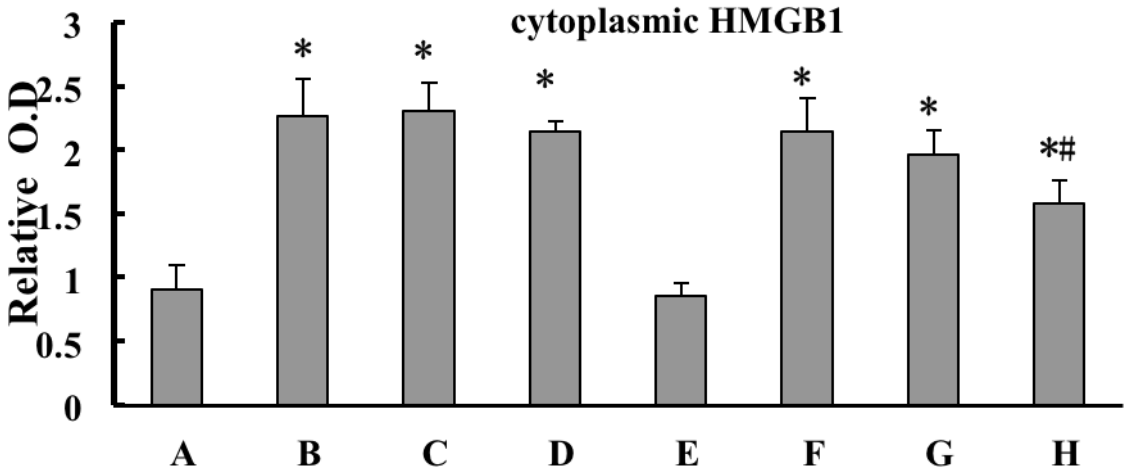

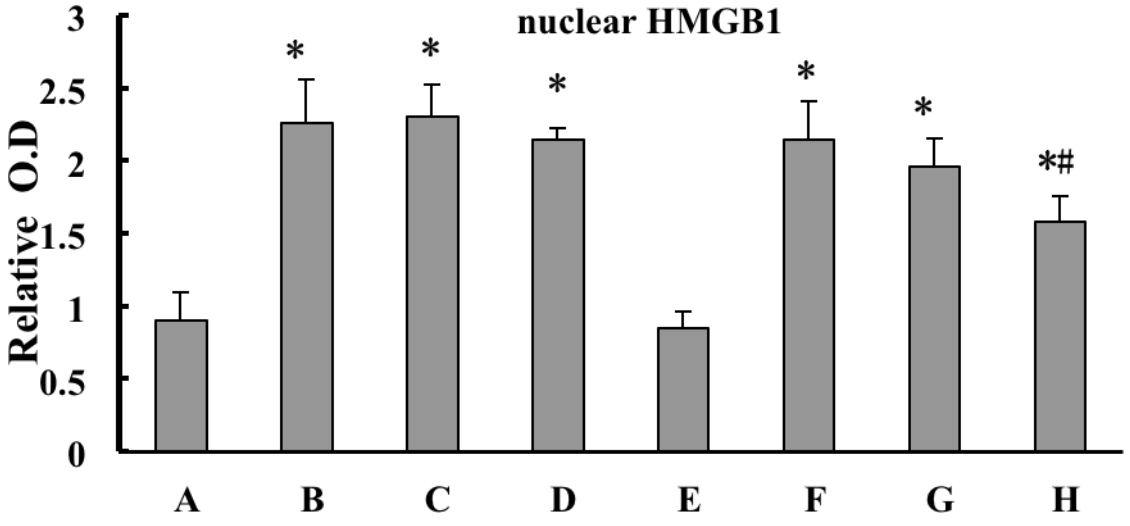
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Relative O.D
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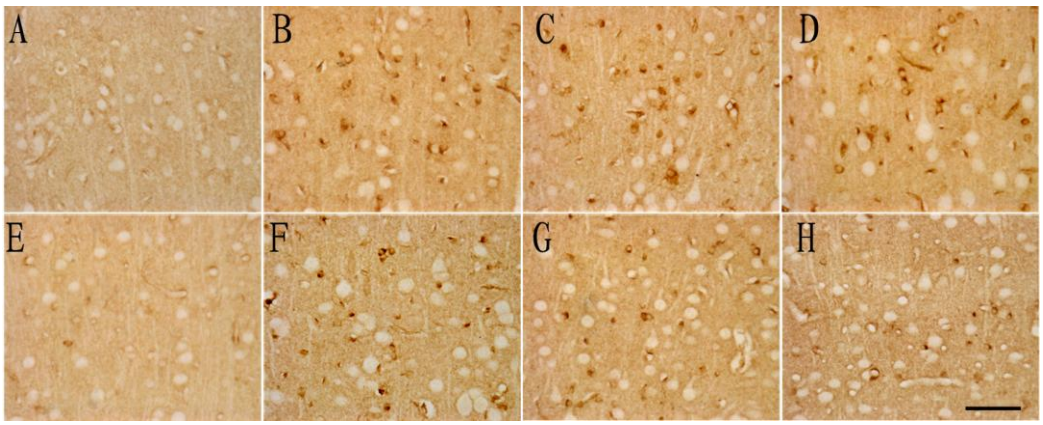
2.5
2
1.5
1
0.5
0
*
*
*
*
*
*#
A
B
C
D
E
F
G
H

Figure 3

A



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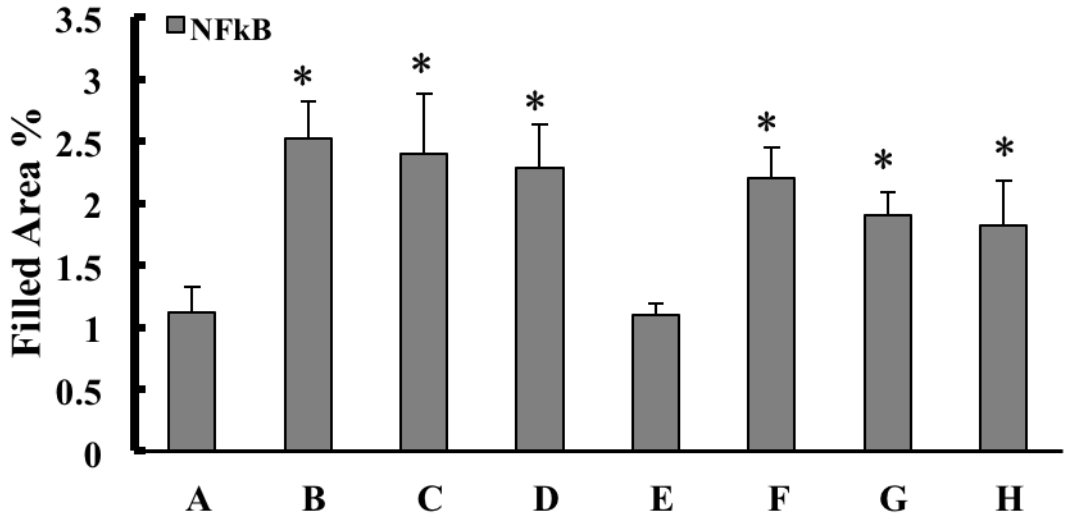

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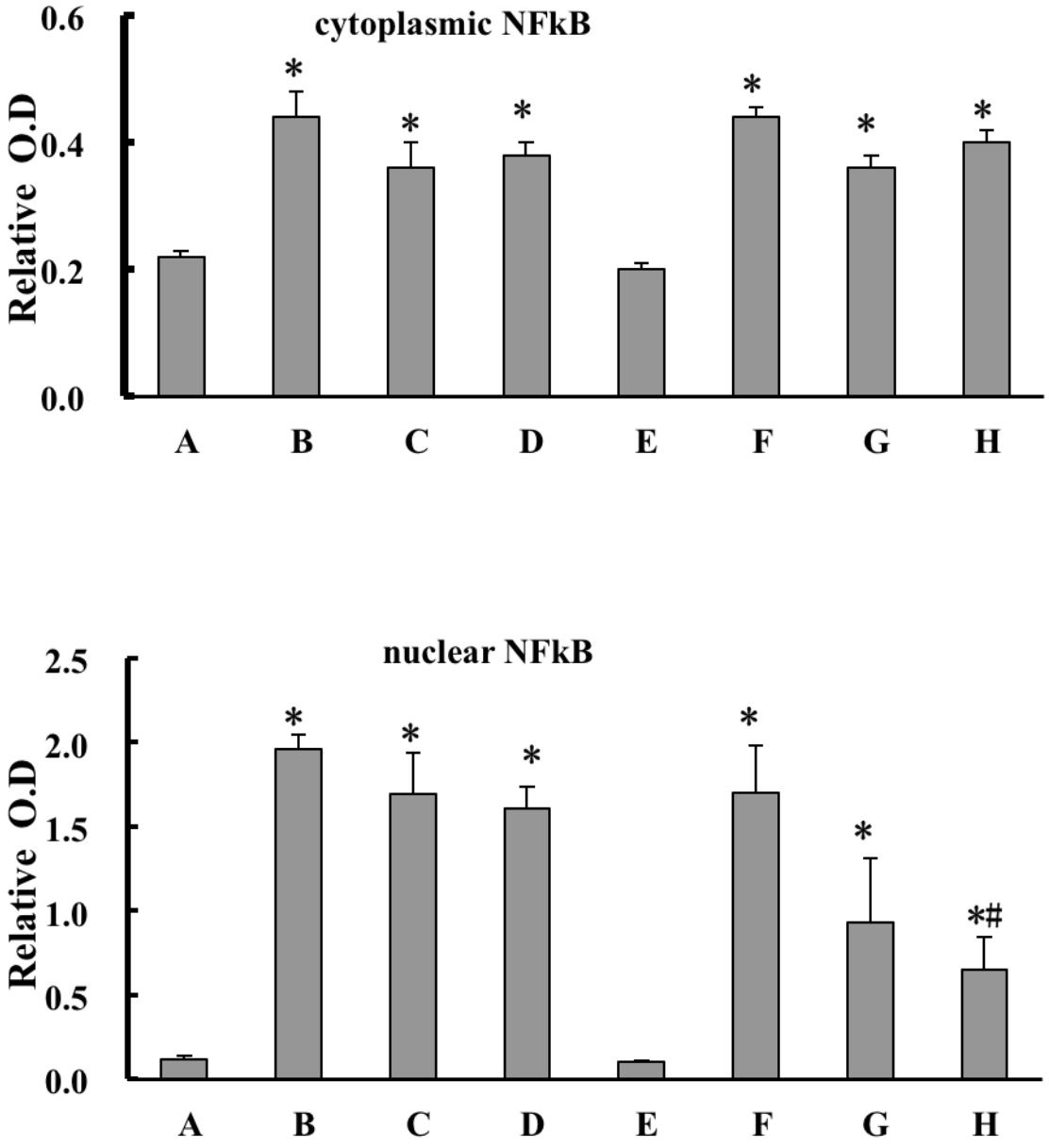
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A
B
C
D
E
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H
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Relative O.D
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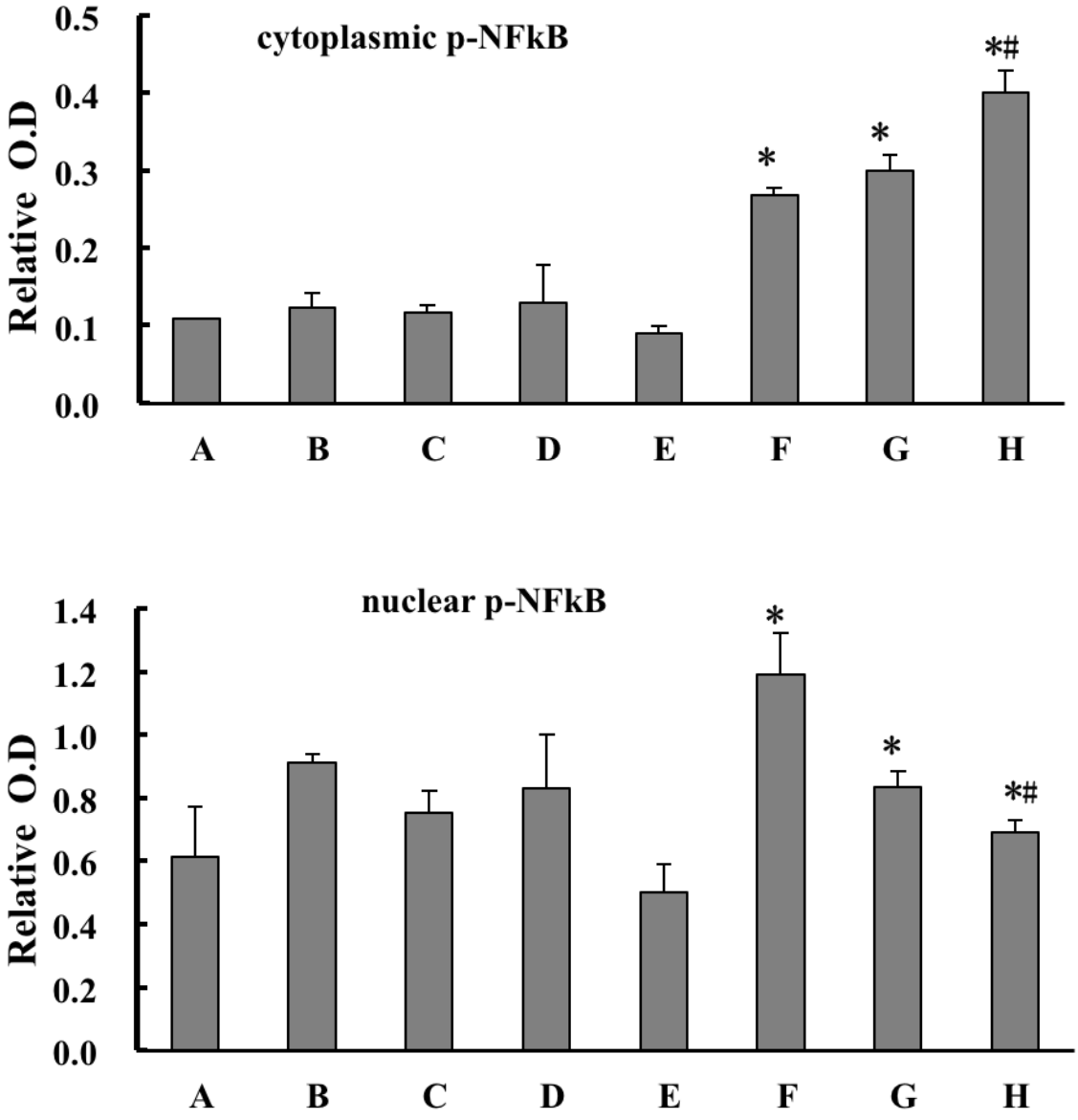
2.0
1.5
1.0
0.5
0.0
*#

D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*
*#
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B
C
D
E
F
G
H
nuclear p-NFkB
Relative O.D
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
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A
B
C
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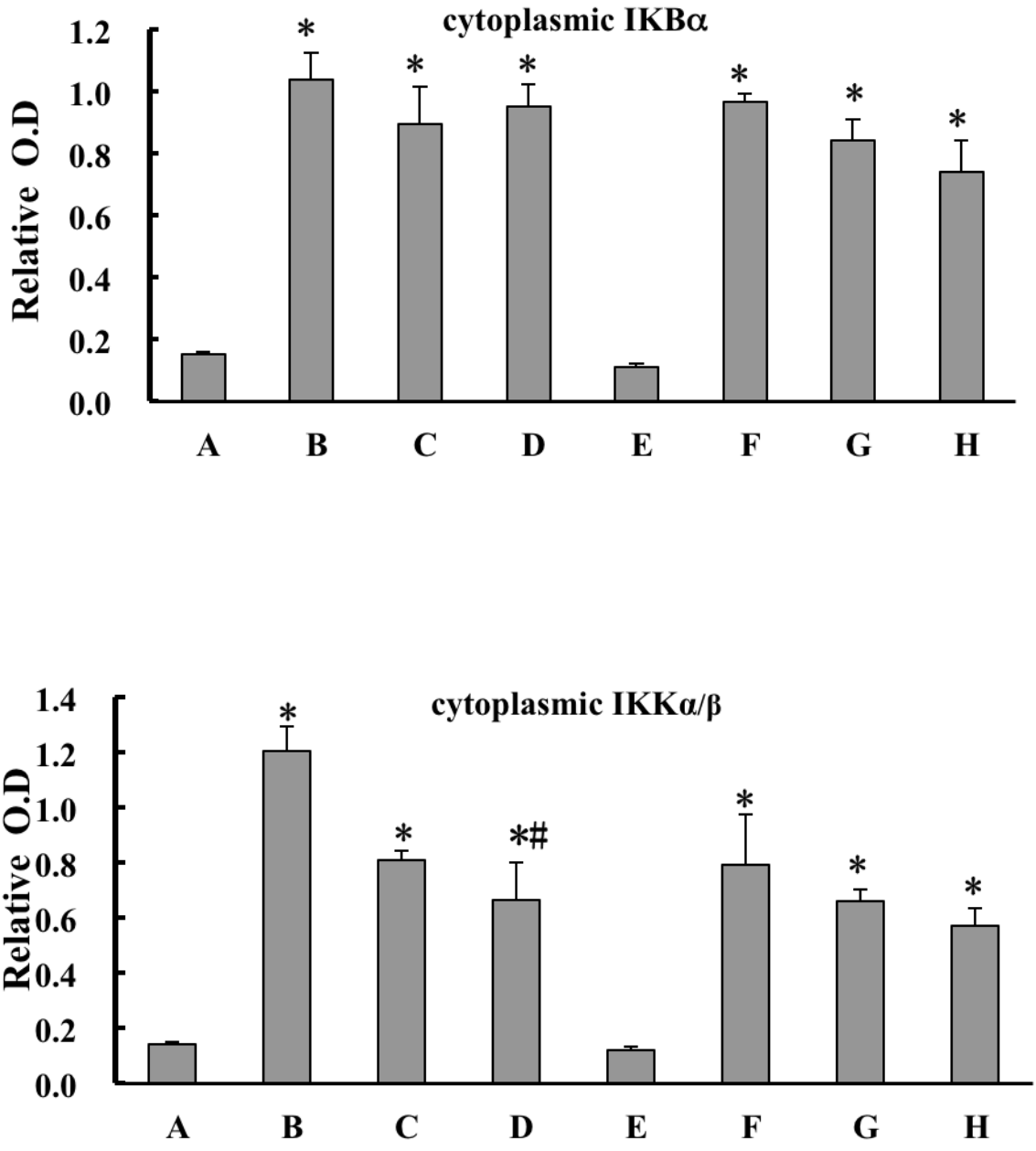
Figure 4

A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Relative O.D
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0.8
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0.4
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B
C
D
E
F
G
H
cytoplasmic IKKα/β
Relative O.D
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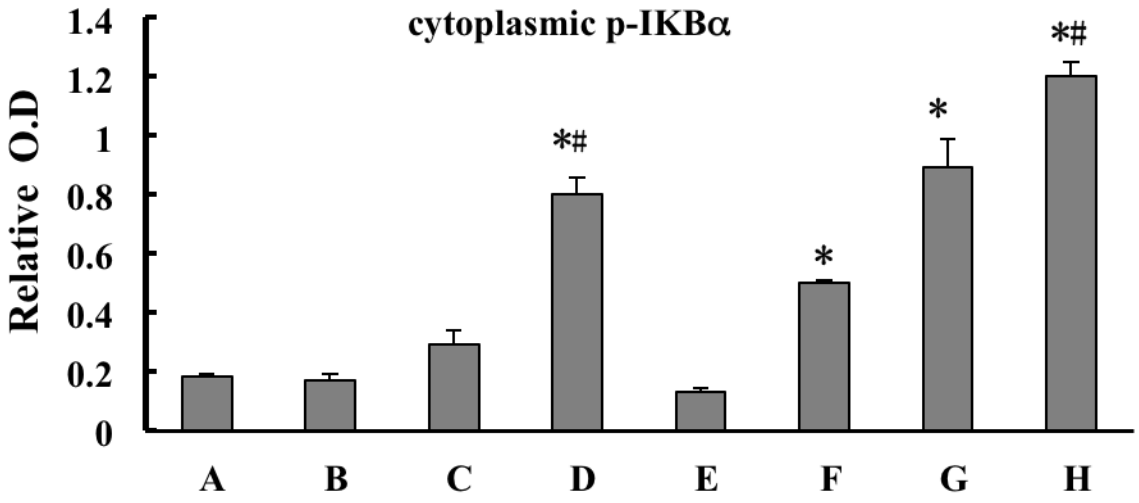
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*#

B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Relative O.D
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1.2
1
0.8
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*#
A
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G
H

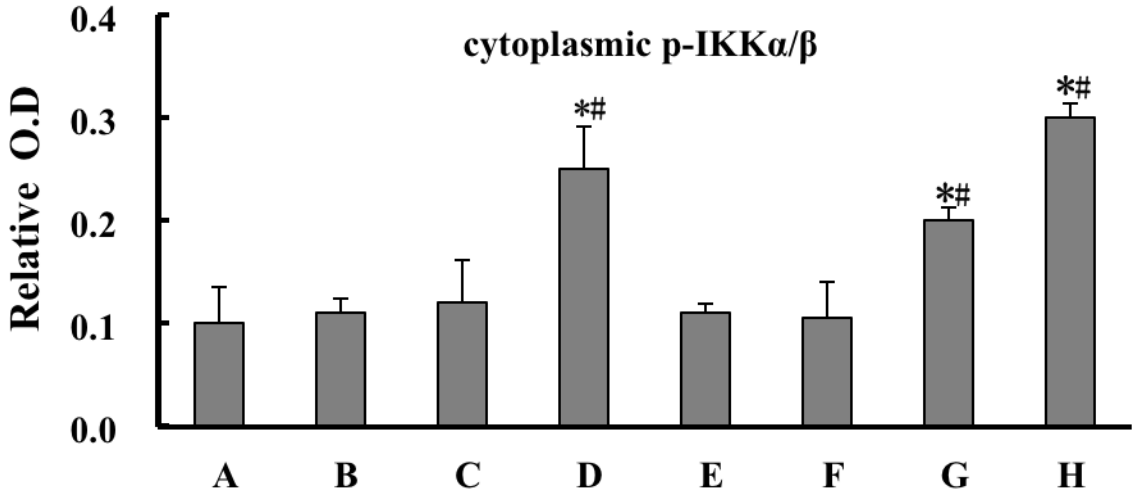
cytoplasmic p-IKKα/β
Relative O.D
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Figure 5

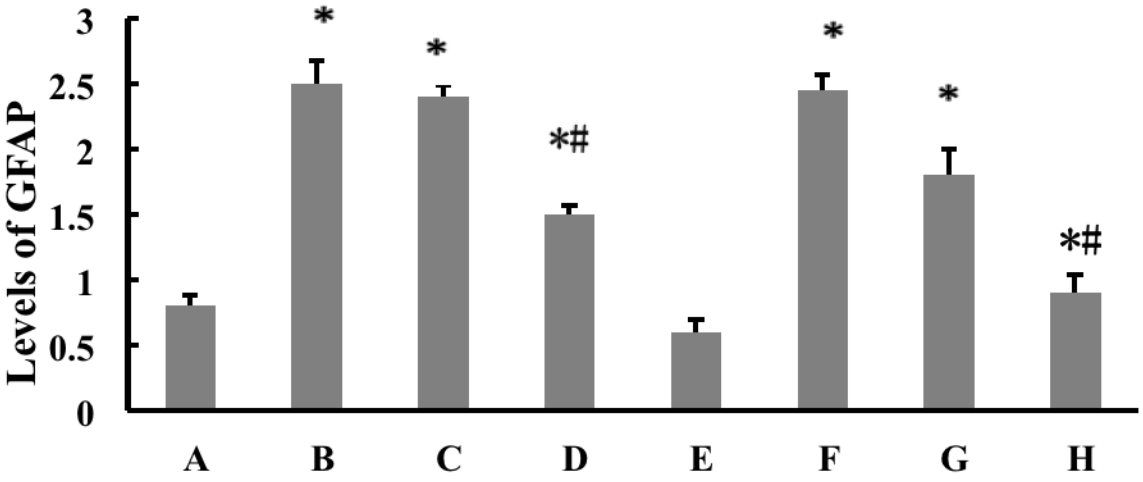
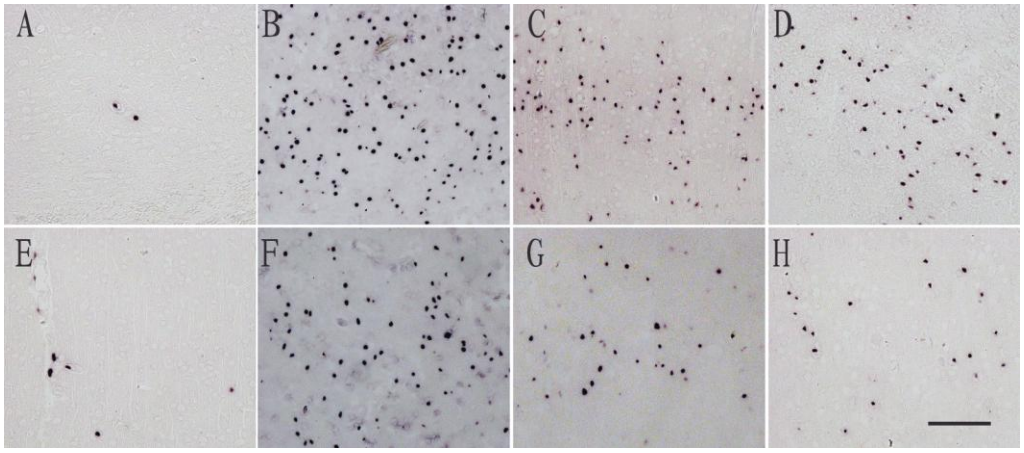
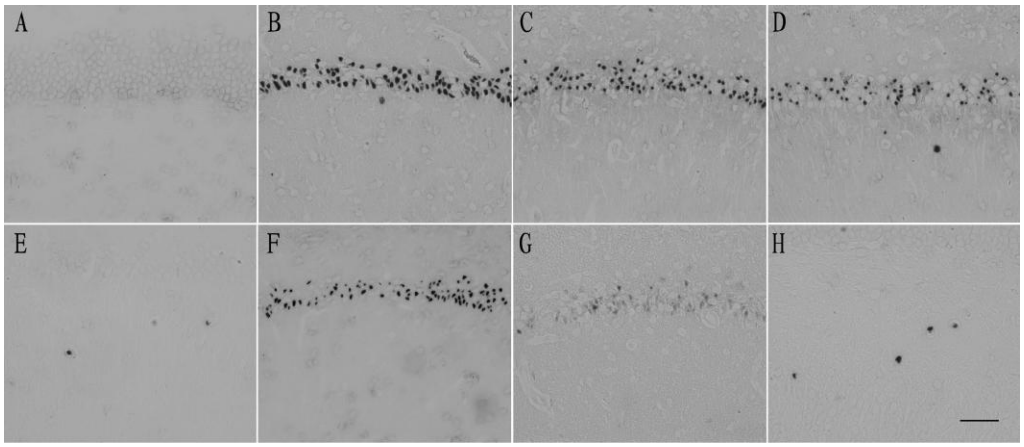


Figure 6

a) Cortex



b) Hippocampus



c)