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The brain protection effects of tea polyphenols against cells death resulting from JNK activation

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Cardiac arrest is a major health problem, affecting approximately 15–20% of all deaths. Despite advancements in prevention and treatment, neurofunction intact survival rates remains low worldwide, more than 80% subjects are unable to recover to an independent way of life because of neurological deficits. Tea polyphenols (TP) has been proven to protect against neuronal diseases. The present study aimed to evaluate the effects of TP on the brain cells of rat subjected to cardiac arrest (CA) /cardiopulmonary resuscitation (CPR). Thirty rats were divided randomly into 3 groups: sham, CA, TP. Except sham group, rats were subjected to CA for 5 min followed by CPR operation. The resuscitation rats were then injected with saline and TP (10 mg/kg) in CA and TP groups respectively. The brains were collected to measure the levels of p-JNK/JNK, caspase-3, Bax, Bcl-2, TUNEL-positive cells and double fluorescent staining of p-JNK/TUNEL. Our results showed that TP significantly reduced the percentage of apoptotic neurons. The pJNK/JNK, caspase-3, Bax levels in the TP-treated group were lower than in the CA group. TP significantly increased Bcl-2 of reperfusion as compared to CA group. Our results demonstrated that TP has natural neuroprotective effect for the treatment of cerebral ischemia induced by CA/CPR. Our findings suggest that TP inhibits neuronal apoptosis through the inhibition of JNK signaling pathway as well as Bax and activation of Bcl2.

Keywords: Tea polyphenols, JNK signaling pathway, cardiac arrest, cerebral ischemia, apoptosis

INTRODUCTION

Cardiac arrest (CA) is one of the leading cause of death over the world, affecting more than 400000 individuals in the United States [Makary and Daniel.(2016)] and 500000 individuals in China annually [Zhang, S.(2015)]. After successful cardiopulmonary resuscitation, cerebral hypoperfusion may lead to decrease cerebral oxygenation and cause the death of vulnerable neurons with further deterioration of cerebral region [Iordanova *et a*l(2017), Geocadin *et al* (2008)]. In patients who achieved return of spontaneous circulation (ROSC), more than 80% of cardiac arrest survivors remain in coma for variable period of time due to neurological deficits [Puttgen *et al* (2009)]. The new approach to cure cardiac arrest is not successful, because the mechanism of injured brain was less understood [Patil *et al* (2015)]. It is suggested that the cerebral ischemia reperfusion response results in the production of reactive oxygen species (ROS) and oxidative stress, being one of the main factor responsible for cerebral damage after ischemic reperfusion[Sun *et al* (2018), Granger and Kvietys (2015).]. ROS activation is associated with phosphorylation of MAPK pathway, which initiates a cascade of events results in neuronal damage [Zhang *et al* (2016), Mao *et al* (2008) , Drigotas *et al* (2013)]. Our previous study showed that oxidative stress results in cerebral injury, because it promotes ERK activation in vascular cells and cortical neurons and inhibition of ERK has positive effect on brain after CA[Nguyen Thi *et al* (2016)]. Moreover, JNK signaling is one of the major stress response pathway, which often triggers apoptosis [Shklover et al (2015),Igaki (2009) , Dhanasekaran and Reddy (2017)]. Indeed, activation of JNK pathway has been involved in the development of apoptosis of neurons such as PC12 cells[Shepelev *et al* (2011)].

Tea polyphenols (TP) contains four main catechin derivatives including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) and their structures are shown in Figure 1. Moreover, TP has been investigated as a mean to reduce apoptosis against ischemic stroke with varying degrees of success [Gundimeda *et al* (2012), Liu *et al* (2018) ,He *et al* (2015)]. Our previous study indicated that TP improved survival rate and neurologic deficit scores, and reduced cerebral ROS generation in rats induced CA/CPR[Zhuo *et al* (2016)]. Treatment with Green TP is beneficial for preventing ischemic stroke against cell death induced by oxygen-glucose deprivation/reoxygenation [Gundimeda *et al* (2012)]. Green TP

can protect blood brain barriers through the regulation of TJ and PKCα signaling [Liu *et al* (2018)]. Recent study showed that EGCG in tea exerts neuroprotection in intracerebral hemorrhage by suppressing c-Jun-N-terminal kinase (JNK) phosphorylation and caspase-3 activation [He *et al* (2015)]. Molecular mechanisms underlying cerebral injury are not completely understood, but a role of JNK activation has been suggested. Thus, the aim of the present study was to evaluate the protective role of TP which effects on JNK pathway in brain rat subjected CA/CRR .

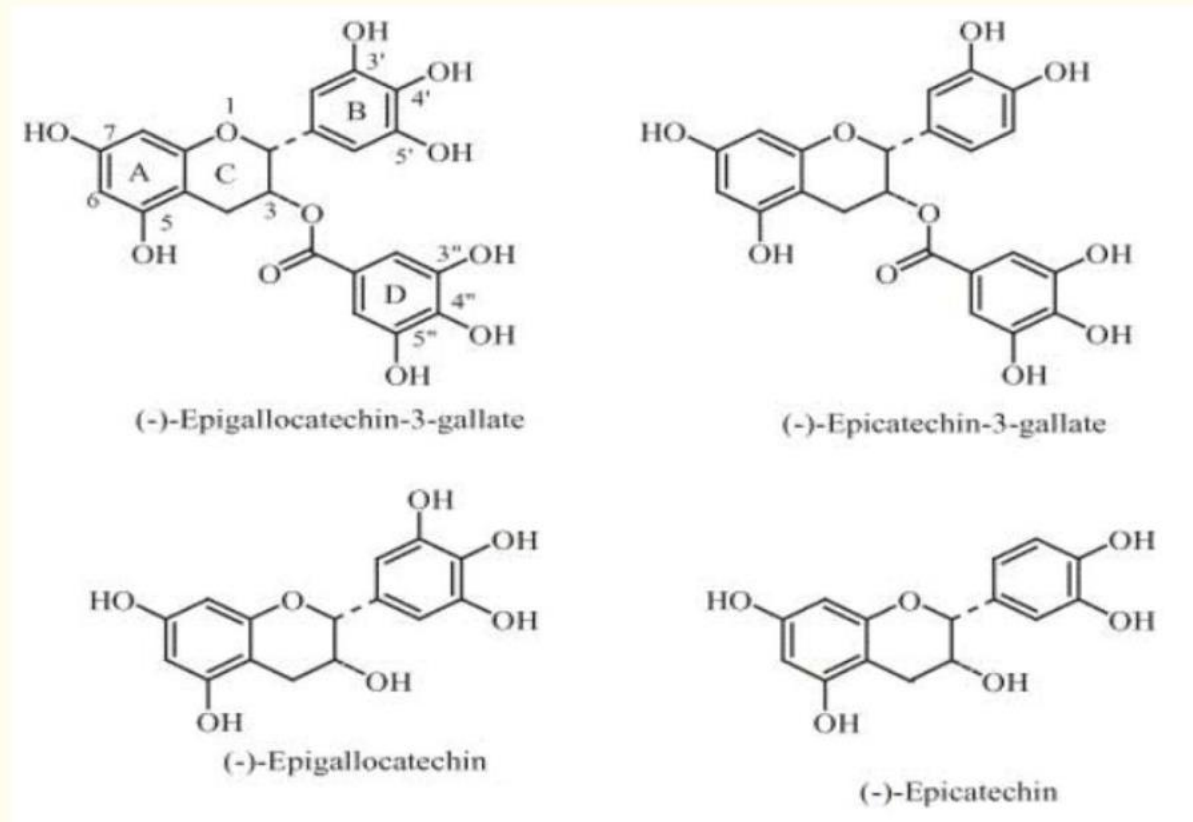

Figure 1. Structures of the major tea polyphenols

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following commercial sources: Tea polyphenol (Sigma, CAS Number 84650-60-2); complete protease inhibitor cocktail tablets (Sigma, CAS Number 4693116001)
primary antibodies, SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), Bcl2, Bax (Cell Signaling Technology, Danvers, MA); secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-rat IgG, In Situ Cell Death Detection Kit, Fluorescein (Sigma, CAS Number 11684795910)

Animal preparation

The experimental protocol was in accordance with the Regulations of Laboratory Animal Care. Thirty adult male rats were randomized into 3 group: sham, CA, TP. The rats except sham group were subjected to CA for 5 min followed by CPR operation following CA/ CPR model that was established by Chen *et al* [Chen *et al* (2007)]. After Restoration of spontaneous circulation (ROSC), the rats were immediately injected with TP 10 mg/kg (TP group) or saline (CA group). Animals were sacrificed at 12h and 72h after reperfusion and the brain was collected.

Western blot

Western blotting was performed using standard method [Zhao *et al* (2015)]. The primary antibodies were as follows: Primary antibodies against phospho-SAPK/JNK, SAPK/JNK, GAPDH, Caspase-3, Bax, Bcl-2.

Cell apoptosis assay

To detect apoptotic cells, paraffin blocks were cut into 5 μm thickness coronal sections, TUNEL staining was

then performed with an In Situ Cell Death Detection Kit (POD, ROCHE, cat: 11684817910) according to the manufacturer's protocol.

Double fluorescent staining

To clarify the spatial relationship between phospho-JNK expression and DNA fragmentation, phospho-JNK and TUNEL (terminal deoxynucleotidyl transferase-mediated uridine 5´-triphosphate-biotin nick end labeling) were used as double staining agents and monitored with fluorescence microscopy. Some samples were single stained with TUNEL for quantification of TUNEL positive cells.

Statistical analysis

All data are expressed as means ± standard deviation (SD). Statistical analysis of the data between groups was performed using independent samples t-tests, and analysis of variance was used to analyze the data within each group. P<0.05 was considered to be statistically significant.

RESULTS

pJNK levels were decreased in TP group

Western Blot of pJNK and JNK are shown in Figure 2A. The pJNK/JNK levels in CA group were higher than that in sham group (p<0.05; Figure 2B). However, treatment with TP significant decreased pJNK/JNK at 12h and 72h (p<0.05; Figure 2B).

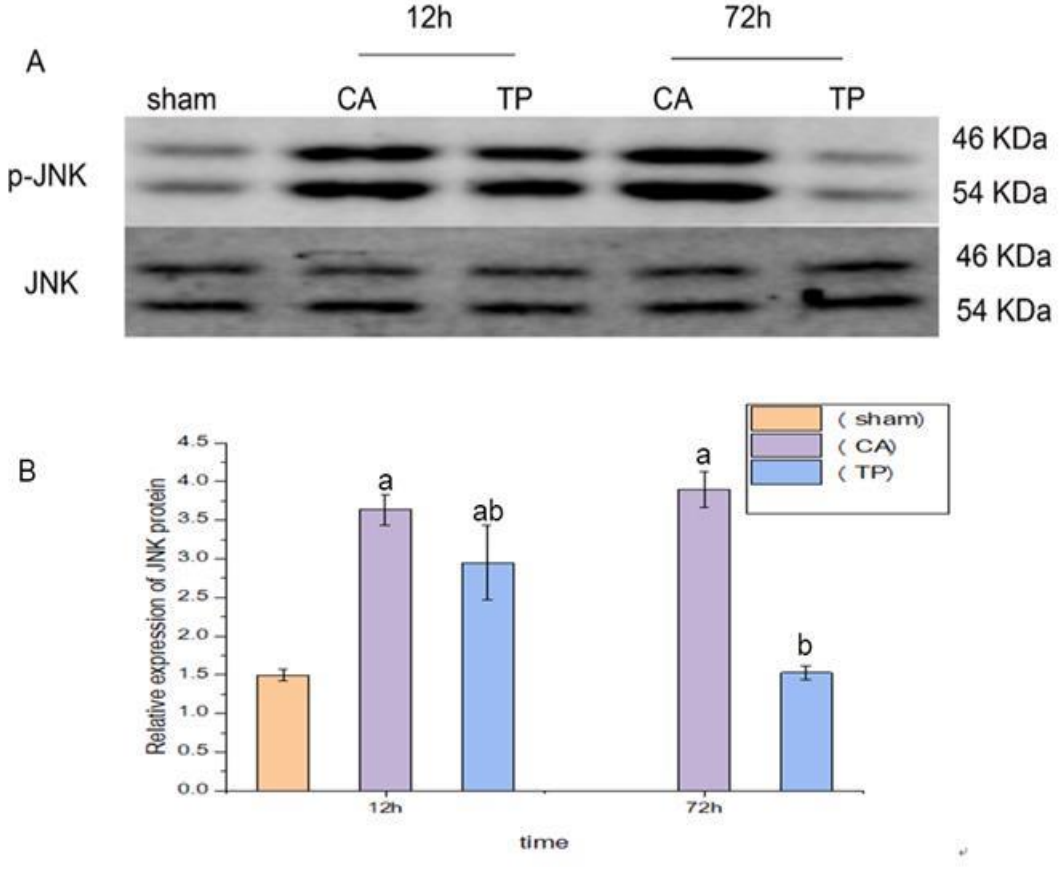

Figure 2. p- JNK levels in brain (A) Western blot. (B) JNK ratios (phosphorylated vs total protein); mean ± standard deviation. n = 6 for each group (a = P < 0.05 vs the sham group; b = P < 0.05 vs the CA group).

TP decreases TUNEL positive neurons in cortex

TUNEL-positive cells in CA group and TP group were higher than that in the sham group at 72 h after CA/CPR (p<0.01; Figure 3 a-b; Figure .3 c-d, Figure .3 e-f). However, as compared to CA group the number of TUNEL-positive cells was significantly lower in TP group (p<0.01). These results suggest that TP effectively inhibited the expansion of apoptotic cell death in CA/CPR model.

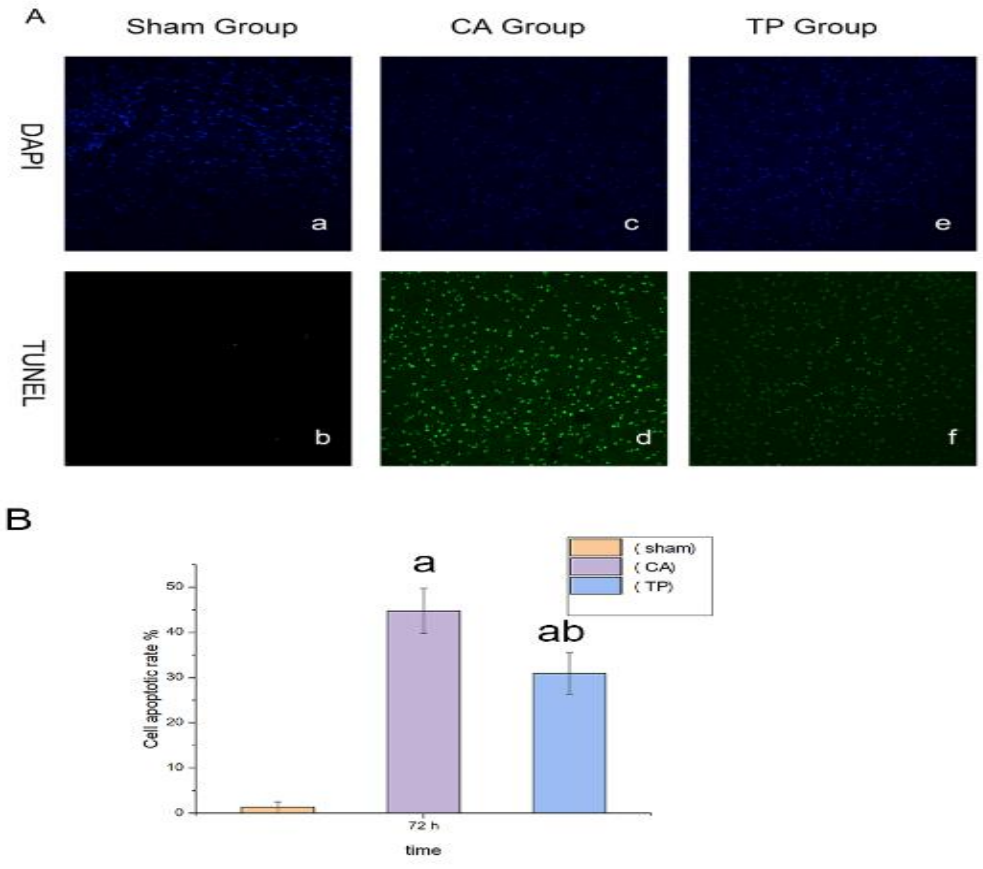

Figure 3. TP decrease neuronal apoptosis in the cortex. (A) The images of TUNEL positive cells in sham, CA, and TP group. (B) The percentage of TUNEL positive cell in sham, CA, and TP group, mean ± standard deviation, n= 6 for each group. (a = $p < 0.01$ vs the sham group; b = $p < 0.01$ vs the CA group).

p-JNK/TUNEL double staining

The fluorescence signal of p-JNK/TUNEL double staining in cortex at 72h after ROSC, showed that the average p-JNK/TUNEL co-positive cells in CA group (Figure 4 A-B-C) was higher than that in TP group (Figure 3 D-E-F) (32.7±3.05%versus 5.3±0.85 % respectively($p<0.01$).

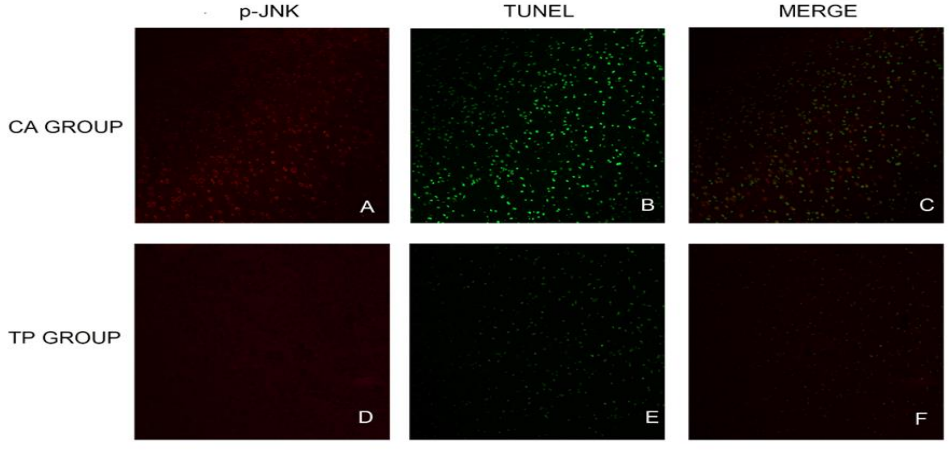

Figure 4. Immunofluorescence staining for p-JNK and TUNEL. TUNEL (green) and p-JNK (red) in rat at 72h (A-B-C) The images of immunofluorescence staining in CA group; (D-E-F) in TP group, magnification x200; mean ± standard deviation; n=6.

TP decreases cleaved caspase-3

The western blot images of cleaved caspase-3 expression were showed in Figure 5A. The level of cleaved caspase 3 in CA group was significantly higher than those in sham and TP group at 12h and 72h (Figure 5B; $p<0.05$).

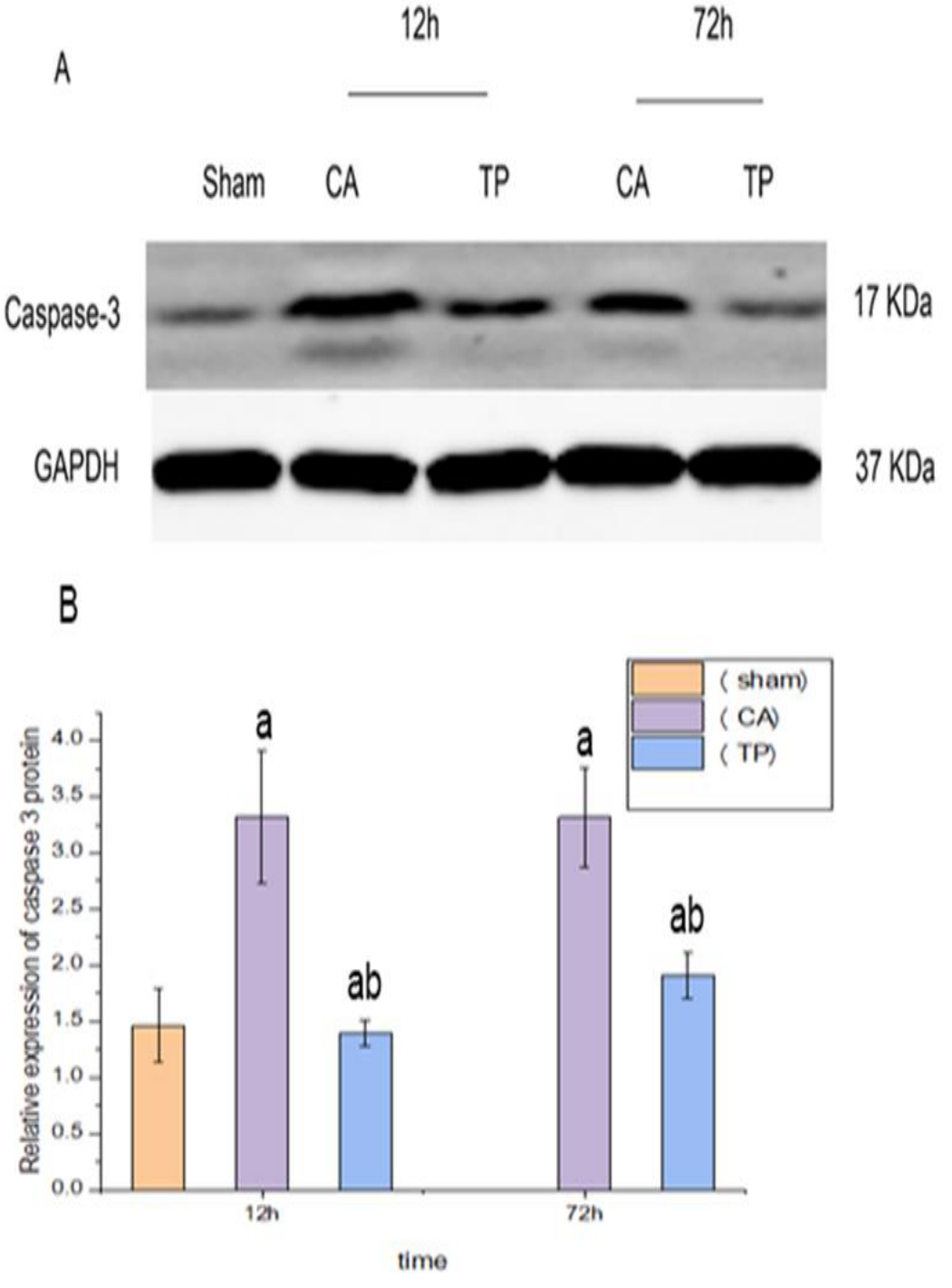

Figure 5. TP down regulated cleaved caspase-3. (A) Western blot of cleaved- caspase-3 and GAPDH. (B) The level Caspase-3 in group sham, CA and TP; mean ± standard deviation. n = 6 for each group (a = $p < 0.05$ vs the sham group; b = $p < 0.05$ vs the CA group).

TP regulates Bax and Bcl-2 expression

TP upregulates the Bax level in CA group as compared to sham and TP group ($p<0.05$; Figure 6B). By contrast, Bcl-2 protein of CA group markedly down-regulated than that in sham group and TP group $p<0.05$; Figure 6C).

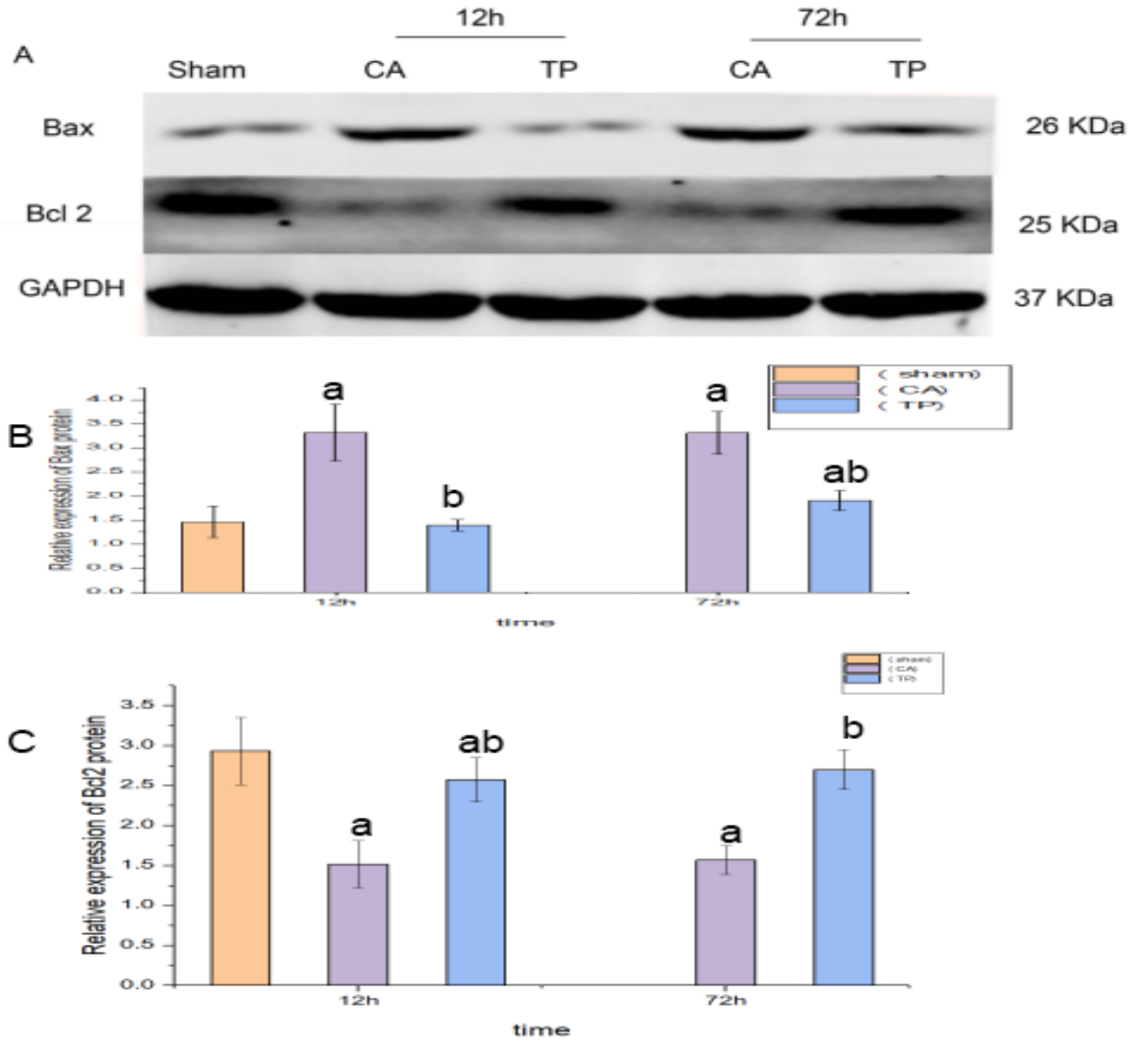

Figure 6. Effect of TP on the expression of Bax and Bcl-2. (A) Western blot images of Bax and Bcl-2 (B) The levels of Bax and (C) the levels of Bcl-2 ; mean ± standard deviation; n = 6 for each group (a = $p < 0.05$ vs the sham group; b = $p < 0.05$ vs the CA group).

DISCUSSION

In the present study we demonstrated neuroprotective role of TP against cerebral ischemia in CA/CPR rat model. Treatment with TP reduced apoptotic neuronal cells and JNK phosphorylation. More over TP could decrease caspase-3, Bax and increase Bcl-2 in cortical region of brain subjected to CA/CPR.

Activation of JNK is associated with development of inflammatory responses, blood-brain barrier breakdown, apoptotic neurodegeneration, and synaptic protein loss, regulated apoptotic processes and impaired motor function [Rehman *et al* (2018) , Zhao and Herdegen (2009)]. The mechanism of JNK in neuronal apoptosis may be associated with phosphorylation of Bcl2 member [Guan *et al* (2006),Dhanasekaran and Reddy (2008) , Lei and Davis(2003).]. The Bcl-2 family of proteins, comprise both pro-apoptotic and anti-apoptotic members, are vital to the intrinsic apoptotic pathway [Hata *et al* (2015)]. As a pro-apoptotic member of Bcl-2 family, under physiological condition, Bax is inactivated in cytosol by interactions of several proteins. When cells are exposed to various oxidative stress stimuli, JNK phosphorylation can cause Bax translocation to outer mitochondrial membrane and release cytochrome c to cytosol [Lei and Davis(2003)], subsequently activate caspase-3 [Gogvadze *et al* (2006)]. The activation of caspase-3 is well known key player in apoptosis process which promotes fragmentation of DNA, and lead to neuronal cells death[Cao *et al* (2001)]. By contrast, Bcl-2 as an anti-apoptotic protein, it is essential for maintaining mitochondrial membrane integrity, preventing cytochrome c release and inhibiting oligomerization of Bax [Akhtar *et al* (2004)] . Thus, decrease in Bcl-2 protein expression may activate caspase-3 lead to cell death. Moreover, JNK can promote neuronal death by reducing the anti–

apoptotic function of Bcl-2 in transient brain -ischemia/reperfusion [Kuan *et al* (2003)]. In present study, the down-regulation of bax, caspase-3 and up-regulated expression of Bcl-2 in TP treated rats, validates the anti-apoptotic effect of TP, which is consistent with the previous reports[He *et al* (2015)].

In conclusion, our study shows that treatment with TP significantly reduced the percentage of apoptotic neurons. The mechanisms underlying the neuroprotective effects of TP may be associated with the inhibition of JNK pathway and regulation of apoptotic proteins by targeting caspase-3, Bax and Bcl-2 Thus, TP exerts natural neuroprotection for the treatment of cerebral ischemia induced by CA.

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