

Total flavone of *Abelmoschus manihot* L. medic exhibits protective effect against hind-limb ischemia in rat model

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Abstract: Total flavones of *Abelmoschus manihot* L. Medic (TFA) is the major active component isolated from Chinese herb *Abelmoschus manihot* L. Medic. TFA has shown neuroprotective effect against cerebral ischemia injury in rats and rabbits. However, the effects of TFA on hind-limb ischemia and the underlying mechanisms remain unclear. Therefore, in the present study, we used a rat hind-limb ischemia model to investigate protective effect of TFA against limb ischemia injury. The rat model of hind-limb ischemia was established. Treatment groups received TFA at two different doses (160 and 40mg/kg) daily for 10 days. Sham operated control group and model group received saline. At the end the rats were sacrificed, hindlimb tissues were stained with Haematoxylin-Eosin and Masson's trichrome. RNA and protein were extracted from tissues for PCR and Western blot analysis. The results showed that TFA reduced lower limb ischemic injury, recovered tissue volume and diminished fibrosis and muscle degeneration. Mechanistically, we showed that TFA increased the expression of anti-apoptotic factor such as Bcl-2 and survivin, decreased the expression of pro-apoptotic factor such as Caspase 3, Bax and Bak and inhibited the activation of caspase 3 and 9. In summary, this study proves new evidence that TFA protects hind-limb against ischemia injury by inhibiting apoptosis and could be a promising therapeutic agent for acute lower extremity ischemia.

Keywords: TFA, hind-limb ischemia, apoptosis, Bcl-2, Caspase.

INTRODUCTION

Acute lower extremity ischemia is a significant clinical problem with a mortality rate of 9-42% in elderly patients and an amputation rate of 20% in survivors (Olson and Glasgow 2005; Rutherford 2009). Currently, the administration of fluid resuscitation, thrombolysis and anticoagulant therapy remain as the main treatment methods for acute lower extremity ischemia (Crowther and Warkentin, 2008). Thrombolysis decreases the mortality associated with limb reperfusion, but the mortality rate remains high and thrombolysis is associated with increased incidence of bleeding complications (Doyle *et al.*, 2009). Therefore, there is an urgent need to understand the pathogenesis of acute lower extremity ischemia.

Bcl-2 is a well-known anti-apoptotic protein. Apoptotic activity coincides with reduced Bcl-2 activity in endothelial cells during the late limb ischemia phase (Kumar *et al.*, 2004). Bcl-2 plays an important role in ischemia reperfusion injury in the hind-limb ischemia model (Iwata *et al.*, 2010).

Abelmoschus manihot L. Medic is a plant rich in flavones such as quercetin, hyperin and rutin. Total flavone of *Abelmoschus manihot* L. Medic (TFA) is the major active component of this plant and has been used as a traditional Chinese medicine for the treatment of vascular injury (Liu *et al.*, 2009). TFA has shown neuroprotective effect

against cerebral ischemia injury in rats and rabbits (Yan *et al.*, 2005). However, the effects of TFA on hind-limb ischemia and the underlying mechanisms remain unclear. Therefore, in the present study, we used a hind-limb ischemia model in the rat to investigate vascular protection effects of TFA and explore the mode of action of TFA.

MATERIALS AND METHODS

Reagents

TFA (content of flavone glycosides over 99%) was extracted from Flos *A. manihot* L. Medic by the Department of Chinese Materia Medica, Nanjing University of Chinese Medicine (Nanjing, China). All of the seven flavone glycosides in TFA were characterized by high-performance liquid chromatography, with the following composition: hyperoside (43.2%), hibifolin (27.1%), isoquercetin (13.7%), quercetin-3'-O-glucoside (8.8%), quercetin-3-O-robinobioside (3.8%), myricetin (3.2%), and quercetin (0.2%). The hyperoside contents in different preparations were measured for quality control. TFA was suspended in 1% carboxymethyl cellulose solution at different concentrations for oral administration.

Animals

Rats were purchased from Shanghai Experimental Animal Center. All animals were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996) and the animal experiments

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were performed under approved protocols of the Animal Care and Use Committee of Jiangsu Province Hospital of Traditional Chinese Medicine. The rat model of hind-limb ischemia was established as described previously (Wang *et al.*, 2010). After surgery, the rats were injected with penicillin for 3 days and got normal feeding. 45 model rats were randomly divided into three groups as follows: Model group, low dose group and high dose group (n=15). Low and high dose group was administrated with TFA of 40 or 160mg/kg/day, respectively. The sham operation group and model group were given the same volume of saline. All rats were administrated for 10 days.

Histological analysis

At the end of the experiment period, rat lower limb tissues were removed and rapidly fixed in 10% formaldehyde and embedded in paraffin. Sections of 5 μ m were stained with Haematoxylin-Eosin (H&E) and Masson's trichrome, and the stained sections were mounted for observation under light microscope.

PCR analysis

Total RNA was extracted from lower limb tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μ g RNA was reversely transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit (Fermentas). PCR was performed using 2X Maxima SYBR Green/ROX qPCR Master Mix kit with ABI7500. The program was initially 2min at 95 $^{\circ}$ C, followed by 40 cycles of 0.5min at 95 $^{\circ}$ C and 1min at 60 $^{\circ}$ C. Data were analyzed by the $\Delta\Delta$ Ct relative quantification analysis.

Western blot analysis

Total protein was extracted from lower limb tissues, separated by 8% SDS-PAGE and transferred to PVDF membrane (Millipore, USA). Next, the membrane was incubated with specific antibody for Bcl-2, survivin, Caspase 3, activated Caspase 3 and activated Caspase 9 or β -actin (Abcam, USA) at 4 $^{\circ}$ C overnight. The membrane was washed with TBST for 3 times, then incubated with HRP-conjugated secondary antibody (Cell Signaling, USA) for 1h at room temperature. The membrane was developed using Enhanced Chemiluminescence (Cell Signaling, USA) according to the manufacturer's protocol.

STATISTICAL ANALYSIS

For statistical analysis, one-way analysis of variance (ANOVA) was used. The significance was set at $p < 0.05$.

RESULTS

TFA improves the tissue volume of lower limbs in ischemia model

Compared to control group, lower limb tissue volume was significantly reduced in ischemia model group. However, administration of TFA could increase lower limb tissue volume in model group in a dose dependent manner (fig. 1). These results indicate that the TFA may reduce lower limb ischemic injury and recover tissue volume.

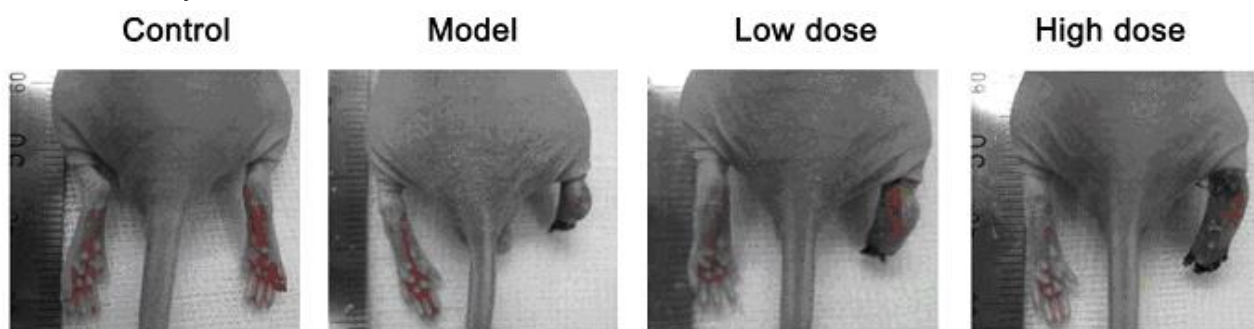


Fig. 1: The morphology of lower limbs in different groups after 10 days of administration of saline or TFA.

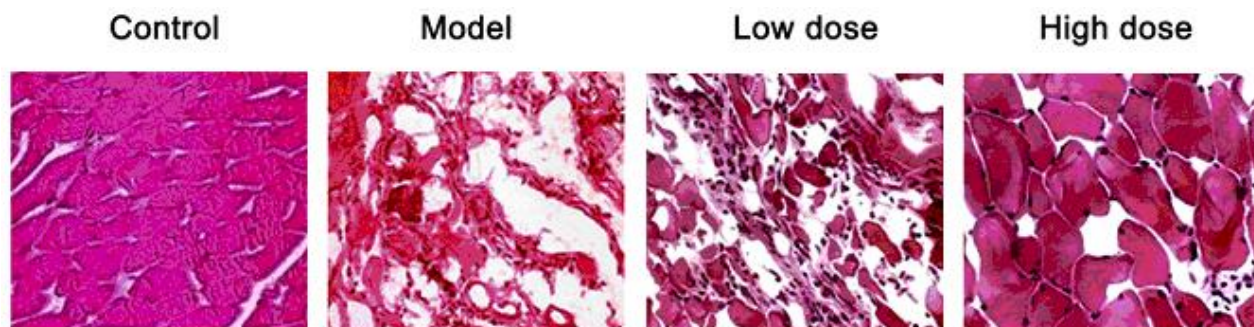


Fig. 2: H&E staining of lower limbs in different groups. Magnification: x 200.

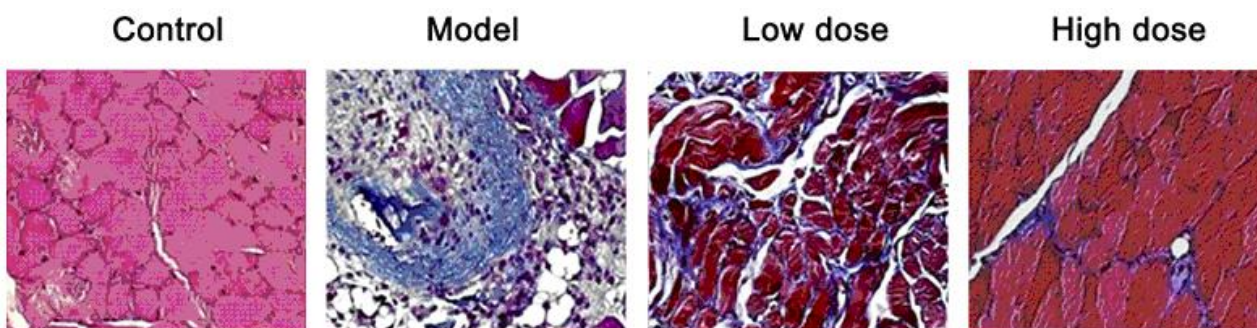


Fig. 3: Masson's staining of lower limbs in different groups. Magnification: x 200.

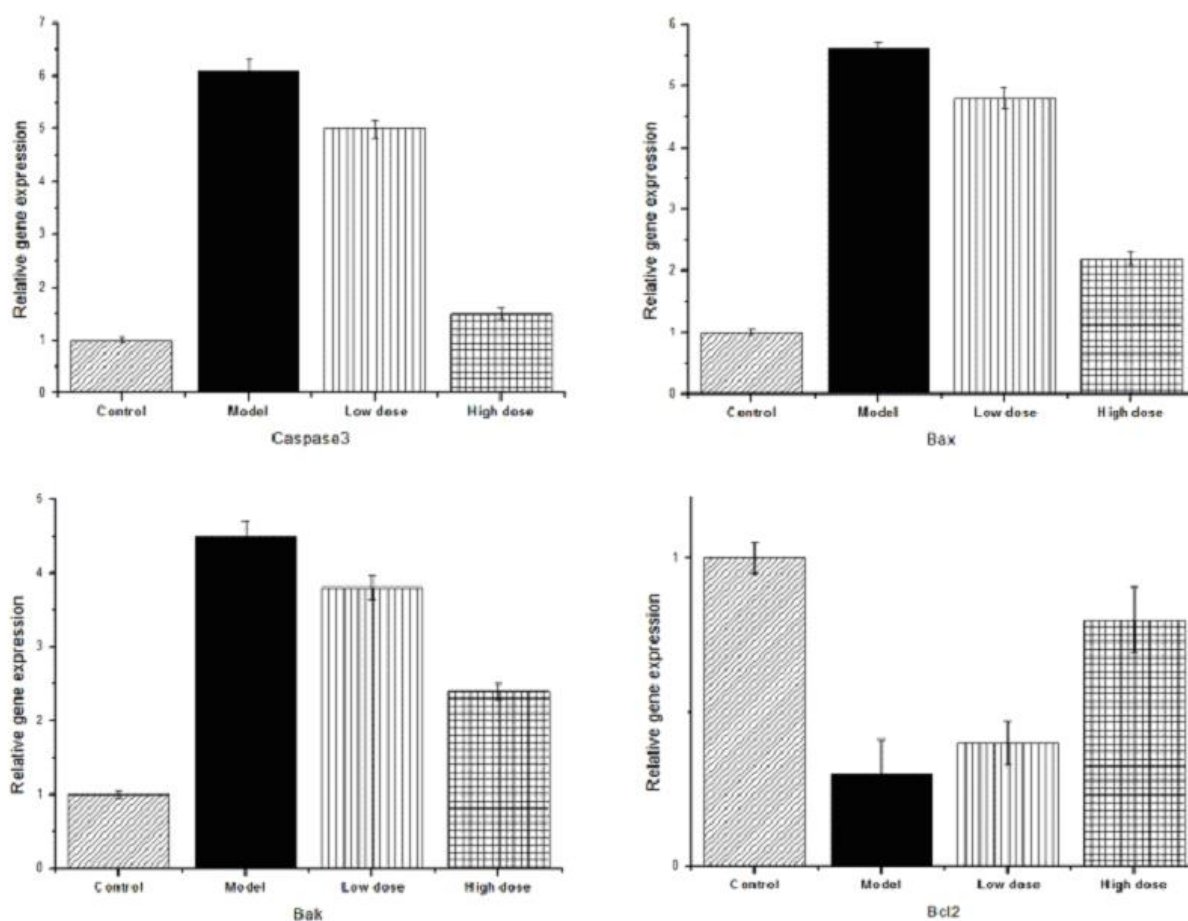


Fig. 4: Quantitative PCR analysis of mRNA levels of Bcl-2, Bak, caspase-3 and Bax in lower limbs in different groups (n=5).

TFA improves the histomorphology of lower limbs in ischemia model

Next we examined histological and fibrotic aspects of lower limbs subjected to ischemia. H&E staining showed that in control group, lower limb tissues were rich of veins, and smooth muscle cells had no obvious abnormalities. In ischemia model group, we observed a gland cavity-like structure, and epithelial cells on the cavity surface underwent apoptosis. After the administration of TFA, gland cavity shrunk inward,

epithelial cells on the cavity surface showed improved morphology, especially in group administrated with high dose of TFA (fig. 2).

In addition, by Masson's trichrome staining we observed fibrosis and fat vacuolation in lower limb tissues in ischemia control group, compared to control group. However, after the administration of TFA, we observed diminished fibrosis and muscle degeneration, especially in group administrated with high dose of TFA (fig. 3).

TFA regulates the expression of apoptosis-related proteins in lower limbs in ischemia model

To explore the molecular mechanisms underlying the beneficial effects of TFA, we evaluated the expression of apoptosis-related proteins. Quantitative PCR analysis showed that TFA reduced the expression of pro-apoptotic factors such as Caspase 3, Bax and Bak, while increased the expression of anti-apoptotic factor Bcl-2, in a dose dependent manner (fig. 4).

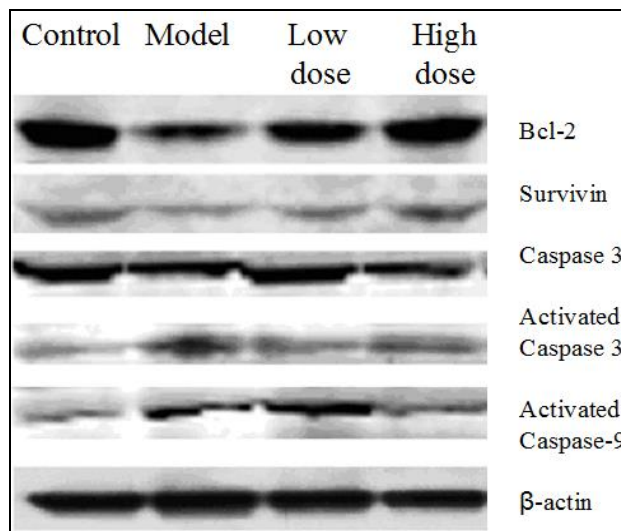


Fig. 5: Western blot analysis of Bcl-2, survivin, caspase 3, activated caspase 3, and activated caspase 9 in lower limbs in different groups. Shown were representative blots from three independent experiments with similar results. β -actin was used as loading control.

Furthermore, we performed Western blot analysis and found that TFA reduced protein levels of pro-apoptotic factors such as Caspase 3, activated Caspase 3 and activated Caspase 9, while increased protein levels of anti-apoptotic factors Bcl-2 and survivin, in a dose dependent manner (fig. 5). Taken together, these results confirm that TFA inhibits cell apoptosis in lower limb tissues subjected to ischemia.

DISCUSSION

Apoptosis is a physiologic process that is involved in the growth and regression of normal tissues (Rai *et al.*, 2005). Apoptosis can be induced by extrinsic factors such as chemotherapeutic drugs and radiation. It has been shown previously that the process of apoptosis is involved in the cyclic growth of normal vein (Persengiev and Green, 2003).

TFA is a traditional Chinese medicine for the treatment of vascular injury in the past thousands years. However, as a Chinese herbal compound, the composition of TFA is extremely complex and its therapeutic mechanism has not

been elucidated. In recent years, with the application of modern biotechniques in the field of traditional Chinese medicine research, the molecular mechanisms of TFA action have been gradually revealed. A study showed that TFA significantly reduced ROS level through anti-oxidation pathway (Yan *et al.*, 2005). However, the role of TFA in apoptosis remains elusive.

Hypoxia is known as an important factor that induces the apoptosis in the tissues subjected to ischemia (Pan and Zhou, 2012). In this study we used rat lower limb ischemia model to demonstrate that a physiological dose of TFA could inhibit apoptosis of endothelial cells in lower limb ischemic tissues. In the model group, hematoxylin-eosin staining and Masson's trichrome staining showed obvious ischemic damage of the tissues, compared to control group. Interestingly, the pathological changes of ischemic tissues were reversed when TFA was administered. These data indicate that TFA protects hind-limb tissue against ischemia through inhibiting apoptosis.

To elucidate how TFA exerts anti-apoptotic effects, we focused on the expression of apoptosis-related factors. PCR and Western blot analysis demonstrated that TFA upregulated the expression of anti-apoptotic factor such as Bcl-2 and survivin, while downregulated the expression of pro-apoptotic factor such as Caspase 3, Bax and Bak. Moreover, we found that TFA inhibited the activation of caspase 3 and 9, both of which are the members of the cysteineaspartic acid protease family and are crucial mediators of apoptosis (Hensley *et al.*, 2013). These results suggest that TFA regulates the process of apoptosis.

CONCLUSION

This study proves new evidence that TFA is a potent inhibitor of ischemia induced apoptosis and could be a promising therapeutic agent for acute lower extremity ischemia.

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