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Full Length Article

Protective Effect of the Total Flavonoids from *Carya cathayensis Sarg.* Leaves on Myocardial Ischemia-reperfusion Injury through Anti-oxidative and Anti-apoptotic Activities

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Abstract

Many flavonoids have been reported to have myocardial protective effects against ischemia/reperfusion injury. Our previous studies have demonstrated that the total flavonoids (TFs) from *Carya cathayensis* Sarg. leaves contains various pharmacological activities. The present study is aimed to investigate the TFs' protective effects on myocardial ischemia/reperfusion (I/R) induced injury and its underlying mechanism. Model of I/R injury were established in rats and H9C2 cells. The results revealed that pretreatment of TFs significantly protected cardiac functions and attenuated heart infarct area size induced by myocardial I/R injury in rats. The results from in vitro experiments also showed that TFs protected H9C2 cells against H/R injury. Moreover, TFs also could protect the heart and cells by increasing the levels of LDH and SOD, and decreasing MDA level. Furthermore, our results found that TFs pretreatment significantly increase the contents of Bcl-2, decrease the level of Bax. Simultaneously, the expressions of caspase-3 were significantly decreased. It can be concluded that TFs pretreatment attenuates myocardium I/R-induced injury by decreasing oxidative stress and anti-apoptotic activities. © 2018 Friends Science Publishers

Keywords: Total flavonoids; *Carya cathayensis* Sarg.; Myocardial ischemia/reperfusion injury; Anti-oxidative; Anti-apoptotic

Introduction

Acute myocardial infarction is a majority of mortality and disability in the world (Finegold *et al*., 2013). Reperfusion strategies are the standard therapy for myocardial infarction. However, reperfusion may lead to cardiomyocyte dysfunction and worsens the tissue damage (Slone and Fleming, 2014). Studies have shown that various factors were involved in the myocardial ischemic/reperfusion (I/R) induced injury; apoptosis, inflammation and oxidative stress play a major role in this process (Song *et al*., 2014). Oxidative stress is a well-known cause of myocardial damage associated with I/R injury (Dhalla *et al*., 2000; Bartel, 2009). Moreover, data in mounting numbers revealed that apoptosis is one of the most significant mechanisms of I/R injury (Patrice *et al*., 2013).

Recent research finds that flavonoids may be important in treating cardiovascular disease with their protective effects against ischemia injury, improve coronary function, and inhibit oxidative stress and inflammation (Kim *et al*., 2009; Lee *et al*., 2011; Wang *et al*., 2012; Song *et al*., 2014; Xi *et al*., 2014; Adegbola *et al*., 2017). *Carya cathayensis Sarg*. is a Chinese medicinal herb and it has been widely used to combat various inflammatory diseases. Our previous works have found that total flavonoids (TFs) from *Carya cathayensis Sarg*. leaves (LCC) contains five main components (Cao *et al*., 2012). A few reports have yet found that the flavonoids isolated from LCC have perfective effects such as anti-tumor and anti-oxidation (Cao *et al*., 2012; Shen *et al*., 2014; Tian *et al*., 2014; Jin and Ding, 2017). Cardamonin, for instance, one of flavonoids in TFs, could alleviate cardiac remodeling and dysfunction induced by pressure overload through inhibition of oxidative stress. Furthermore, Cardamonin could significantly abrogate the expression of Bax and enhanced the level of Bcl-2 and Bcl-xl (Li *et al*., 2016). Pinostrobin was revealed to have many pharmacological activities, such as antioxidant and anti-inflammatory activities (Xu *et al*., 2013; Patel *et al*., 2016). Pinocembrin was reported to have cardioprotective benefits during I/R through its antiapoptotic and anti-oxidative stress activities (Lungkaphin *et al*., 2015). Wogonin also showed cardioprotective effects in vivo with its antioxidant capacity and anti-inflammatory effects, by improved the anti-oxidases activities including SOD and CAT, decreased the

level of ROS and MDA production (Khan *et al.*, 2016). Chrysin has antioxidant, anti-apoptotic, anti-inflammatory and anti-autophagic effects (Kandemir *et al.*, 2017). We also found that TFs and these pure flavonoids present angiogenic effects (Tian *et al*., 2014). Overall, given the collective anti-oxidative properties and anti-apoptosis effects of these main components, we estimate that TFs has the potential cardio-protective activities against myocardial I/R induced injury. Thus, this study we aim to clarify weather TFs prevents against myocardial I/R injury in rats and H9C2 cells, and to further explore the underlying mechanisms.

Materials and Methods

Sample Preparation

The TFs with approximately 80% flavonoid content were extracted from the leaves of *Carya cathayensis* Sarg. as described previously (Cao *et al.*, 2012). Dantonic® (The capsule of Compound Danshen dripping pills, DSP, lot number: 20140629) was provided by Tasly Pharmaceutical Co. Ltd. (Tianjin, China). All the reagents in the study were got from commercial suppliers and were of analytical grade.

Animals and Grouping

A total of 90 wistar rats (200 ± 20 g, male, 3-month-old) were provided by Zhejiang Chinese Medical University. The animals were housed at standard conditions (24±2°C under 12/12 h light/dark cycle) with free access to food and water. The rats were allocated into 6 groups randomly: sham group, myocardial I/R group (I/R group), I/R plus treatments groups, including compound Danshen dripping pills (I/R+DSP group, 80 mg/kg), TFs low dose (I/R+TFs 50 mg/kg group), TFs middle dose (I/R+TFs 100 mg/kg group), and TFs high dose (I/R+TFs 200 mg/kg group).

All animal experiments were approved by the Animal Research Center of Zhejiang Chinese Medical University and performed according to animal ethical guidelines of the Chinese National Health and Medical Research Council.

Myocardial Ischemia-reperfusion Injury Model

All rats were fasted for 12 h prior to myocardial I/R injury and anesthetized with 10% choral hydrate (3 mg/kg) before endotracheal intubation. During I/R operation, Electrocardiogram (ECG) in rats was detected. The rats were fixed and the chest was open to expose the left anterior descending coronary artery (LAD), a 7-0 nylon suture was tied around the LAD for 30 min. Then the slipknot was released, allowing the reperfusion for 120 min. The same surgical procedure was performed on rats in the sham group except for no occlusion for 30 min.

Heart Hemodynamic Parameters Measurement

The hemodynamic parameters were detected continuously by a Biopac research workstation (Biopac, USA). The following functional parameters were measured: including Left ventricular systolic pressure (LVSP), left ventricular enddiastolic pressure (LVEDP), maximum rise/down velocity of left intraventricular pressure ($\pm dp/dt_{max}$).

Infarct Size Measurement

The myocardial infarct area was measured by Evans Blue/triphenyltetrazolium chloride (TTC, Sigma) double-staining. After reperfusion treatment, 1 mL of 1% Evans Blue dye was injected into the tail vein. The heart was excised quickly and cleaned. Subsequently, the hearts were frozen on dry ice and sliced transversally into 2 mm thick sections. The slices were incubated in 1.5% TTC for 30 min at 37°C, and then fixed with 4% formaldehyde for 30 min. The infarction size was measured as the percentage of the infarct volume to the total volume. Staining area were quantitated by Image-Pro plus software.

Cell culture and Hypoxia/Reoxygenation treatment

The H9C2 cells used in the present study was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco). Cells were maintained in a humidified incubator supplied with 5% CO_2 at 37°C.

To mimic the hypoxia conditions, cell was cultured in serum-free medium and placed into an anaerobic chamber (Thermo Forma) equilibrated with 2% O2, 5%CO2 and 93% N2 at 37°C for 24 h. reoxygenation was carried out with standard growth media in 95% air, 5% CO_2 for 12 h before they were used for further analysis. In the control cultures, Cells under normal condition throughout the experiments were included.

The H9C2 cells were assigned to five groups randomly: (1) the control group; (2) H/R group; (3) TFs-treated H/R group, the cells were pretreated with TFs at indicated concentrations (2.5, 5, or 10 μg/mL) for 24 h before subjected to H/R.

Cell Viability Analysis

Cell viability was examined by a modified MTT method. The cells (5×10^3 cells/well) were seeded into a 96-well plate. After the aforementioned treatments, 20 μL of 5 mg/mL MTT solution were added to each well, and the cells were incubated at 37°C for 4 h. Then, the supernatant was removed, 150 μL DMSO was added into each hole to dissolve the formazan crystals. The absorbance was read at 490 nm by using a microplate reader.

Measurement of Enzymes Activities

After treatments, the serum of rats and the cell culture medium were collected and homogenized. Levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), and

superoxide dismutase (SOD) were detected by using respective kits following the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China).

Hoechst 33324 Binding Assay

Hoechst 33324 staining was used to detect nuclear fragmentation in situ. After treatment, cells were harvested and washed with cold PBS, then incubated with Hoechst 33324 (1 μg/mL) and PI (1 μg/mL) for 30 min at room temperature in the dark. After being washed with PBS, cells were visualized under a fluorescence microscopy (Nikon Ti-S, Japan).

Annexin V-FITC/PI Doulbe Staining Assay

Cell apoptosis analysis was measured by using Annexin V and PI fluorescein staining kit (KeyGEN BioTECH, Nanjing, China). After drug treatment, the cells were harvested, washed 3 times with cold PBS, and then incubated with PI, annexin V-FITC for 15 min at room temperature in the dark. Then, the stained cells were harvested and cellular fluorescence was analyzed by a flow cytometer (Gavua insyte 5, Millipore).

Western Blot Analysis

Total protein was isolated from heart tissue or treated cells using RIPA lysis buffer (Beyotime, Shanghai China). The protein concentrations were examined by a BCA protein assay kit. Bovine serum albumin was used as the standard. Protein samples (30 μg) were separated by denaturing 10% SDS-polyacrylamide gels and then electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). After the membranes were blocked by 5% milk for 2h, the primary antibodies of Anti-Bax antibody (dilution 1:1000; ImmunoWay), anti-Bcl-2 antibody (dilution 1:1000; ImmunoWay), anti-caspase-3 antibody (dilution 1:1000; ImmunoWay) were incubated overnight respectively. The horseradish peroxidase conjugated secondary antibodies were used to incubated for 2 h at 4˚C. β-actin was used as loading reference control.

Statistical Analysis

All quantitative data were expressed as the mean ± SD from three separate experiments at least. Statistical comparisons between groups were analyzed by one-way ANOVA. All data analyses were performed by GraphPad Prism 5 software. A value of $P<0.05$ was considered statistically significant.

Results

TFs improved the Cardiac Function Recovery after I/R injury

As shown in Table 1, the hemodynamic of left ventricle, including LVEDP, LVSP and ±dp/dtmax in I/R group became worse, while compared with sham-operated group ($p<0.01$). On the contrary, compared with I/R group, pretreatment with TFs (100 mg/kg) could significantly increase the LVSP and ±dp/dtmax, and decreased the LVEDP ($p<0.05$, $p<0.01$). These results indicated that TFs could improve cardiac function.

TFs Pretreatment Reduces Infarct Size

As shown in Fig. 1, significant myocardial infarction size was induced by I/R. However, pretreatment with TFs (200 mg/kg) could markedly reduce the percentage infarct size following myocardial I/R injury.

Effects of TFs on Cells Survial in H/R Treated H9C2 Cells

Cell exposure to H/R caused to a reduction of cell viability compared to control group (Fig. 2) MTT assay results disclosed that pretreatment with TFs was able to restore the decreased cell viabilities. TFs could protect H9C2 cells in concentration-dependent against H/R injury.

Effects of TFs on Release of LDH in Vivo and vitro

As shown in Fig. 3, LDH is a well-known indicator of cell damage. The release of LDH was higher in I/R induced group than that in control group both in vitro and in vivo. While compared with I/R and H/R group, The LDH release was remarkably lowered by pretreatment with TFs. These results demonstrated that TFs protected myocardial injury induced by I/R in vitro and in vivo.

Effect of TFs on Myocardial Histology

Changes in the morphology of myocardial tissue by HE staining are shown in Fig. 4. the myocardial structure in I/R group showed morphological damage, such as cardiac structural disorder, myocardial cell denaturation and necrosis, and fibrous scar tissue formation. However, pretreatment with 200 mg/kg TFs significantly weakened morphological disorders induced by myocardial I/R injury.

Effects of TFs on Myocardium Antioxidant Enzymes in vivo and vitro

The I/R or H/R treatment group showed increased MDA content when compared with the sham group in rats and H9C2 cells, Moreover, antioxidant enzyme activities SOD was significantly decreased ($P<0.01$). However, TFs pretreatment obviously reduced the increased MDA content induced by I/R or H/R. Furthermore, the level of SOD was all restored by the TFs pretreatment. These results revealed that TFs could attenuate myocardium oxidative stress in vivo and vitro.

Table 1: Effects of TFs on hemodynamic changes in I/R-treated rats

Treatment	LVSP (mmHg)	LVEDP (mmHg)	+dp/dtmax (mmHg/ms)	-dp/dtmax (mmHg/ms)
Sham	121.24 ± 14.72	1.74 ± 1.14	4.92 ± 0.87	3.97 ± 1.06
I/R	91.68± 10.70**	8.56 ± 3.68**	3.01 ± 1.57**	2.20 ± 1.22**
DSP (80 mg/kg)	105.51± 20.06	5.15 ± 2.63$^{\#}$	3.66 ± 1.18	3.32 ± 0.74$^{\#}$
TFs (50 mg/kg)	102.24± 12.50	6.87 ± 1.09	3.25 ± 1.05	2.71 ± 0.57
TFs (100 mg/kg)	112.59± 13.04$^{\#}$	2.25 ± 2.31$^{\#\#}$	4.03 ± 0.56$^{\#}$	3.08 ± 0.43$^{\#}$
TFs (200 mg/kg)	106.64± 12.35	5.54 ± 3.37	3.90± 0.78	2.64 ± 0.68

Values are expressed by mean ± SD(n=10). **P<0.01 vs sham; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs I/R

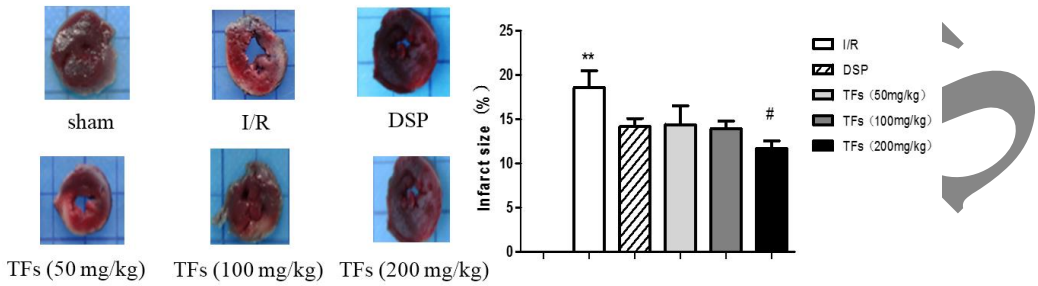

Fig. 1: TFs reduced the myocardial infarction area induced by I/R. The pictures of heart slice are representatives TTC staining of 10 individual animals for each group. Values are expressed by mean±SD, n = 10. **P < 0.01 vs sham; #P < 0.05 vs I/R)

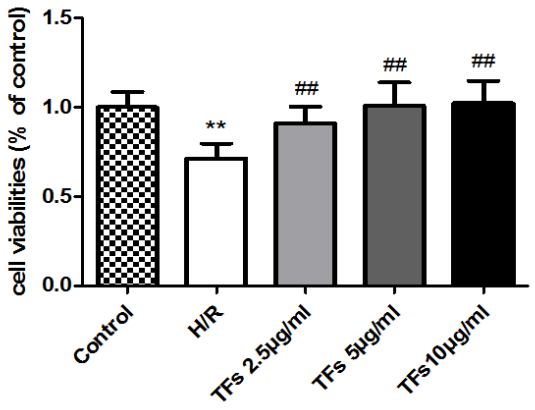

Fig. 2: Effect of TFs on cell viability from H/R injury. Values are expressed by the mean ± SD, **P < 0.01 vs sham; $^{\#\#}$P < 0.01 vs H/R. n = 6

Effects of TFs Suppress Apoptosis Induced by H/R in vitro

To further understanding the function of TFs in I/R-induced cardiac injury, we examined cardiomyocyte apoptosis in H/R-treated H9C2 cells. As showed in Fig. 6, Microscopy analyses with fluorescence staining of Hoechst 33342 and PI also demonstrated identical morphological changes of H9C2 cells. We detected nuclear modifications of fragmentation and chromatin condensation in cells after H/R injury, pretreat with TFs could markedly reduce this phenomenon. Annexin V and PI binding assay revealed that TFs markedly suppressed the H/R-induced apoptosis rate as compared with control.

Apoptosis Inhibition by TFs is Related to Bcl-2 Family

To investigate the protective effect of TFs on the role of decrease myocardial apoptosis, the expressions of the apoptosis regulatory proteins including caspase-3, Bcl-2 and Bax was examined by Western blot. The results showed in Fig. 7, compare with model group (I/R in rats and H/R in H9C2 cells), TFs reversed the alteration of expression of apoptosis associated proteins induced by I/R or H/R in rats' heart tissues and H9C2 cells. The results indicated that TFs suppressed apoptosis by inhibit the pro-apoptotic indexes expression level, such as Bax and Caspase3; and promoted the expression of anti-apoptotic index of Bcl-2.

Discussion

Heart dysfunction is an important etiological factor in I/R-induced injury (Jiang *et al.*, 2014). Significant cardiac dysfunction of serial changes in hemodynamic parameters, including LVDP, HR, CF and ±dp/dtmax could be observed after I/R treatment (Marchelak *et al.*, 2017). The increase of infarct size is documented to be a reliable index of myocardial I/R injury (Adegbola *et al.*, 2017). LDH serves as an important metabolic enzyme in cardiomyocytes and could be leaked from injured cardiomyocytes. Hence, LDH level in the culture medium is a primary index to evaluate cell damage (Xia *et al.*, 2014). In this study, we found that TFs reduced the myocardial infarction area, and improved recovery of altered hemodynamic parameters by I/R

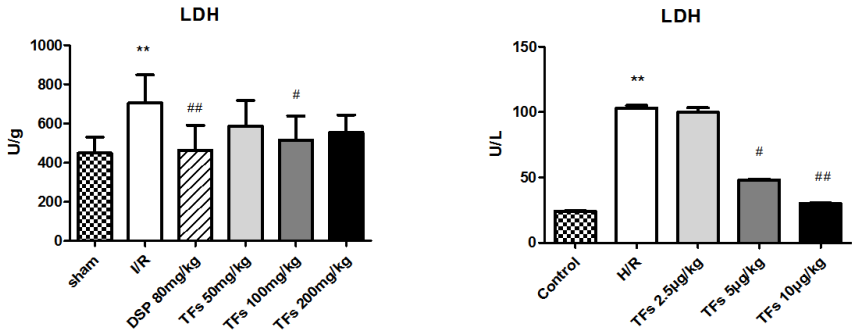

Fig. 3: TFs decreased the level of LDH in heart tissues and H9C2 cells. Values are mean±SD, n = 3-10. $^{**}P < 0.01$ vs sham or control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs I/R or H/R)

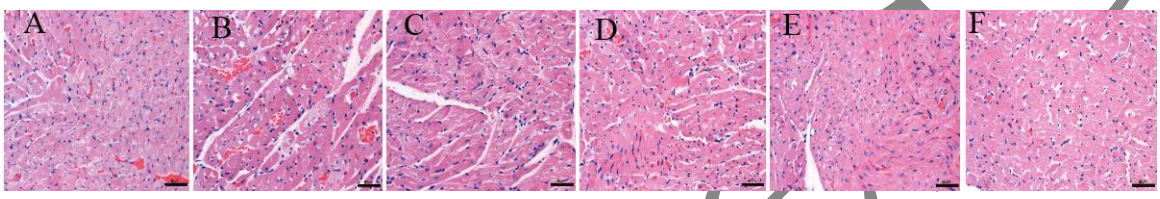

Fig. 4: Effects of TFs on cell morphology (×200) (n = 8). Scar bar: 50 μM A: Sham; B: I/R; C: DSP (80 mg/kg); D: TFs (50 mg/kg); E: TFs (50 mg/kg); F: TFs (200 mg/kg)

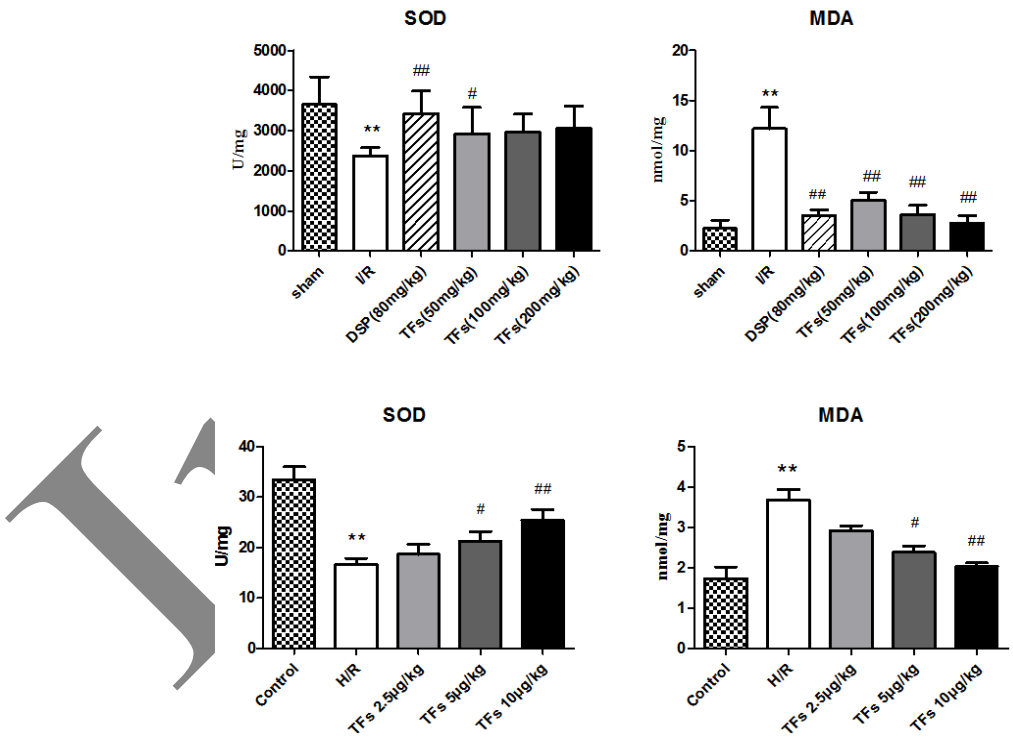

Fig. 5: TFs enhanced the antioxidant enzymes activities in rat heart tissue and H9C2 cells. Values are mean±SD. $^{**}P<0.01$ vs sham or control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs I/R or H/R (n=3-10)

(LVEDP, LVSP and ±dp/dtmax), inhibited I/R-induced enzyme LDH release in vivo and vitro. Our results suggest that TFs pretreatment improves the recovery of hemodynamic performance after I/R-treat.

Myocardial I/R injury can induce oxidative stress, which is characterized by Reactive oxygen species (ROS) generation (Huang *et al.*, 2014). In the normal condition, the ROS can be suppressed by antioxidant systems,

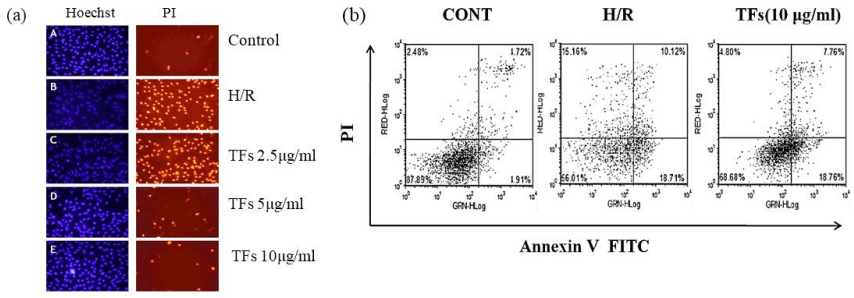

Fig. 6: (a) Representative images of fluorescence microscopy analysis showing the effects of treatment with TFs on nuclei apoptosis and cell necrosis in H9C2 cells. Cells were stained with Hoechst and PI, and images were taken under magnification of ×20. **(b)**. H9C2 cells were double stained by annexin V/PI, and determined with flow cytometry. Percentage of the bottom right quadrant and the top right quadrant showing the representative events of early and late apoptosis, respectively

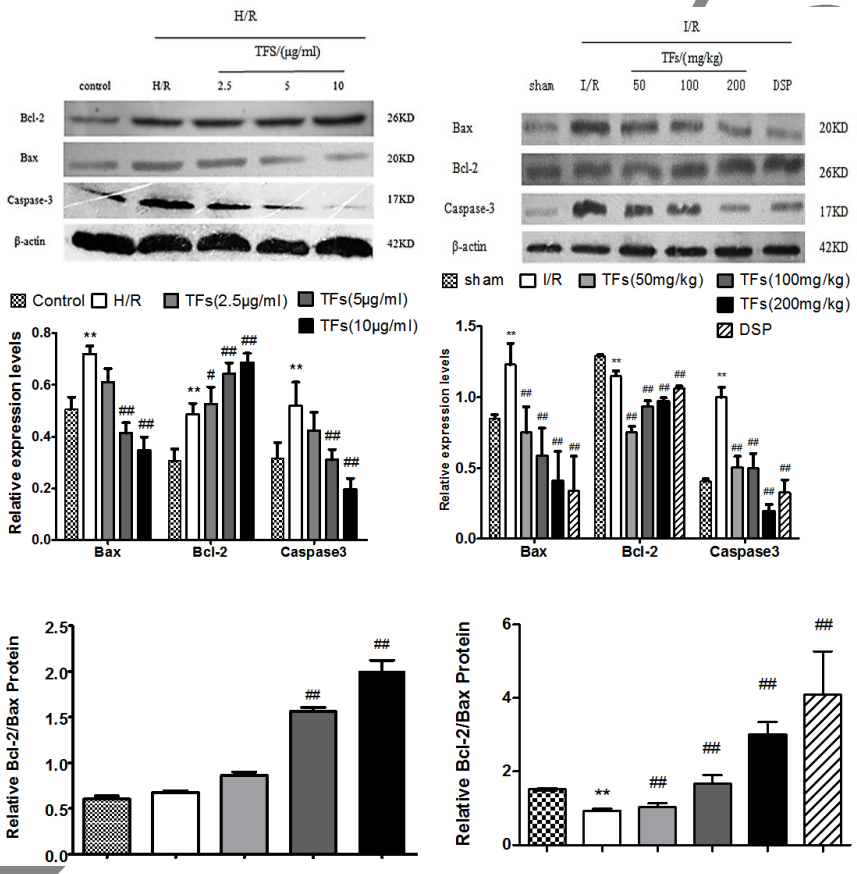

Fig. 7: Effects of TFs on apoptosis related proteins expression examined by western blot analysis. Gel pictures are representatives of Western blot results. Bar figures are statistical summaries. Values are mean ± SD, $*P < 0.05$ and $**P < 0.01$ vs control or sham; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs H/R or I/R. n = 3

Such as speroxide dismutase (SOD) and (glutathione peroxidase) GSH (Zhou *et al.*, 2015). The imbalance between ROS production and antioxidant defenses leads to oxidative stress. I/R injury can produce numerous of oxygen free radicals in cardiomyocytes, and subsequently causes lipid peroxidation and leads to cell damage (Mahmoudabady *et al.*, 2017; Xu *et al.*, 2017). Thus, lipid peroxidation is one of mechanisms of cellular damage. MDA, a product of lipid peroxidation, was applied to assess oxygen-free radicals-induced myocardial I/R injury. MDA is the end product after reactive oxygen species attack unsaturated fat in cell membrane system, thus its content

reflects lipid peroxidation in cells. So, MDA reflects ROS-induced damage and used as a key marker for evaluating oxidative injury of cell. SOD is antioxidant enzymes which can catalyzes reduction of the superoxide (O^{2-}) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Wu *et al*., 2017). Our study revealed that SOD activity was significantly decreased, but MDA level was markedly increased in model group (H/R in vitro and I/R in vivo), which demonstrated that severe oxidative stress was induced by H/R or I/R injury. Pretreatment of TFs significantly decreased the contents of MDA, promoted the activities of SOD in serum of rats and cell culture medium, which indicated that the cardio protection of TFs against I/R injury was at least associated with its antioxidant property.

Apoptosis is an important change induced by ischemia after I/R injury. Oxidative stress is known to induce activation of the apoptotic cascade (Li *et al.*, 2017). During this procedure, caspase-3 activation was essential for the execution step in programmed cell death and was a crucial mediator of ischemic injury. Caspase-3 and Bcl-2 family plays important roles in apoptosis of myocardial cells (Grünenfelder *et al*., 2001; Kim *et al.*, 2014). Bcl-2 is a proto-oncogene and can suppress apoptosis. The Bcl-2 gene can also help in the survival of the cells by anti-apoptotic action. This is also reported that overexpression of Bcl-2 gene may decrease the cardiac apoptosis after reperfusion and cause protection of heart tissues against IR injury (George *et al.*, 2017). Bcl-2 can block programmed cell apoptosis through preventing Bax-mediated mitochondrial apoptosis, whereas the increase of Bax promotes cell apoptosis; so the Bcl-2/Bax ratio was important for determine the cells apoptotic activity (Sun *et al*., 2015). We found TFs pretreatment inhibited the expression of Bcl-2, which accompany with the increased expressions of Bax after reperfusion both in H9C2 cells and rat. The increase of the Bcl-2/Bax ratio was beneficial to anti-apoptosis. Bcl-2 suppresses cell apoptosis by reducing the release of mitochondrial cytochrome C in order to inhibit the activation of caspase-3. To date, multiple studies have clarified that caspase-3 is a major effector in the process of apoptosis, and that its activation marks the irreversible stage of apoptosis (Ran *et al*., 2015). Our results showed that TFs effectively decreased caspase-3 expression. Therefore, All the data above demonstrated that TFs has protective function in myocardial cells against I/R induced injury in vivo and in vitro, by suppressing apoptosis, and its mechanism may be included with suppressing the activities of Bcl-2/Bax/caspase-3 signaling pathway.

In summary, the major findings in the present study are: (1) TFs exerted a protective effection on reducing I/R injury in rat. (2): TFs pretreatment alleviates I/R-induced injury by suppressing the MDA level and increasing SOD activity. Thus, the anti-oxidative property might be one of the mechanisms in TFs protected myocardial I/R-induced injury. (3) The cardioprotective effect of TFs inhibits apoptosis was shown to be associated with Bcl-2/Bax/caspase-3 signaling pathway, which are closely related with the mitochondria pathway of apoptosis. However, future studies are required to further clarify and confirm its potential mechanisms.

Conclusion

In total, the present study provided evidence that the TFs from *Carya cathayensis Sarg.* Leaves has protective function against I/R injury in vivo and in vitro by attenuating oxidative stress and inhibiting apoptosis. Above all, TFs may have potential for use as one new therapeutic agent for protection against myocardial I/R injury in the future.

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