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Tanshinone II A Relieves Adriamycin-induced Myocardial Injury in Rat Model

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Abstract

As an effective antineoplastic agent, adriamycin (ADR) remains a use for the treatment of cancer. However, it is limited by the serous cardiotoxicity. Tanshinone II A is the main effective component of Salvia miltiorrhiza Bunge which has been used for treatment of cardiovascular diseases. The purpose of this study is to evaluate the protective effect of Tanshinone II A on adriamycin-induced myocardial injury in rat and explore the mechanism of this effect. Male Wistar rats (200 ± 20 g) were divided into three groups, control (CON) group, adriamycin (ADR) group and ADR + Tanshinone II A (TRA) group. At the end of the 4 week treatment period, cardiac function was evaluated by transthoracic echocardiography. Molecular and cellular measurements were performed in atrial muscle to examine histopathological changes, the formation of fibrosis, Inflammation and apoptosis. Cardiac dysfunction was induced by adriamycin, as indicated by significant decreases in ventricular fractional shortening and ejection fraction. This adriamycin-induced cardiac dysfunction was prevented by the treatment of Tanshinone II A. Adriamycin induced pathological changes and fibrosis, activated apoptosis (increased TUNEL index, apoptotic DNA fragmentation,and caspase-3 activity and decreased Bcl-2/Bax ratio), inflammation and suppressed phosphorylation status (eIF2α and PERK) in atrial. All these molecular and cellular alterations induced by ADR were not found in the rats treated with Tanshinone II A. These findings demonstrate clearly that Tanshinone II A protects the cardiomyocytes against the ADR-induced cardiomyopathy by preventing the activation of cardiac fibrosis and apoptosis, and the effects are probably mediated through ERS pathway.

Keywords: tanshinone II A, adriamycin, cardiomyopathy, apoptosis

1. Introduction

As an anthracycline antibiotic, adriamycin (ADR) is widely used for treatment of cancer (Blum et al., 1974). After the crossing of the cellular membrane, adriamycin inhibits the synthesis of RNA and DNA, killing various growth cycle of tumor cells (Shi et al., 1993). As same as other anticancer drugs, adriamycin has a series of side effects, such as nausea and vomiting which disappear after stopping medication (Huang et al., 2004). However, high concentrations of ADR tend to cause severe toxicity to normal tissues, including cardiotoxicity, limiting the application of adriamycin (Lefrak et al., 1973).

As traditional Chines medicine, the root of Salvia miltiorrhiza Bunge has been used for treatment of cardiovascular diseases for a long time (Bristow et al., 1978). Tanshinone II A is one of the major active components of Salvia miltiorrhiza Bunge. It has been reported that Tanshinone II A inhibit atherosclerotic calcification (Futian et al., 2007). It also relieves the apoptosis of cardiomyocytes (Lin et al., 2015). Additionally, Tanshinone II A has been found to anti-inflammation (Gao et al., 2008).

Some researchers have reported Tanshinone II A protect against cardiotoxicity induced by adriamycin. Jiang et al provided evidence on the potential of Tanshinone II A as a proteictive agent against adriamycin induced cardiotoxicity (Zhi-yuan et al., 2008). Tanshinone II A significantly inhibits adriamycin-induced cardiomyocyte apoptosis in a

dose-dependent manner also has been reported (Baohong et al., 2009), and Akt-signaling pathways related with this phenomenon (Hong-Jye et al., 2012). Some studies have implicated the role of ER stress in myocardial pathology (Xin et al., 2007;Toth et al., 2007). ER stress is related with the development of ischemic heart disease in mice (Azfer et al., 2006). Whether Tanshinone II A protect against cardiotoxicity induced by adriamycin through ERS or other activity. Therefore, this study determined to examine the effect of Tanshinone II A on adriamycin-induced cardiomyocythy. We test Tanshinone II A whether protect against myocardial fibrosis and apoptosis induced by adriamycin. To further examine whether the effects of Tanshinone II A are mediated by ERS pathway. Last, we test the effects of Tanshinone II A on adriamycin-induced inflammation related factors.

2. Materials and Methods

2.1 Materials

Adriamycin was purchased from the medicine of WanDong (ShenZhen, China). Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay kit was purchased from Beyotime Biotechnology (ShangHai, China). Tanshinone II A was provided from SHANGHAI NO.1 Biochemical and Pharmaceutical. Co., Ltd. The structure of Tanshinone II A is shown in figure 1.

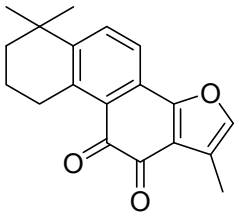

Figure 1. the chemical structure of Tanshinone II A

2.2 Experimental Animals and Rat Model of Adriamycin Induced

Male Wistar rats (200 ± 20 g) were obtained from YiSi Laboratory Animal Technology Co., Ltd (ChangChun). All of rats were housed under standard conditions. All experiments in this research were performed in accordance with the Guidelines of Animal Experiments form the Committee of Medical Ethics, National Health Department of China (1998). These rats were divided into three groups, control (CON) group, adriamycin (ADR) group and ADR + Tanshinone II A (TRA) group. CON group intraperitoneal injected saline at the indicated time; ADR group were intraperitoneal injected 2mg/kg/week adriamycin for 3 weeks and ADR + TRA group intraperitoneal injected 2mg/kg/week adriamycin and 2mg/kg/week Tanshinone II A for 3 weeks. All the rats were maintained on a 12 : 12 hour light-dark cycle, at the temperature of 20 ± 2 ℃ and were allowed free access to food and tap water. After 4 weeks, the rats were sacrificed.

2.3 High Frequency Echocardiography Analysis

The Color Ultragraphy was used for the echocardiography of rats. Echocardiography was performed according to a previously described protocol (Xiao et al., 2014). Animals in each group were anaesthetised with sodium pentobarbital (100 mg/kg). Hair from chests was removed with an electrical clipper and hair removal gel prior to the examination. Two-dimensional grayscale ultrasound scanning was performed to assess the cardiac structures in the parasternal short-axis view at the midpapillary level. The grayscale echocardiographic view was used to position the M-mode echocardiographic line. Left ventricle (LV) internal dimensions and anterior and posterior wall thickness were then measured. LV end-diastolic (LVEDD) and end-systolic dimensions (LVESD) were assessed from the M-mode tracing. Fractional shortening (FS), the percent change in LV cavity dimension, was calculated using the equation FS (%)=[(LVEDD-LVESD)/LVEDD]×100. Ejection fraction (EF) represents stroke volume as a percentage of end-diastolic LV volume and was derived as EF (%)=Y+[(100-Y)×0.15], where Y=[(LVEDD2-LVESD2)/LVEDD2]×100. All measurements were averaged over three consecutive cardiac cycles.

2.4 Enzyme-linked Immunosorbent Assay

Heart tissue levels of TNF-α and TGF-β1 were determined using the rat TNF-α and TGF-β1 ELISA kit (NEOBIOSCIENCE, China), according to the manufacturer's instructions.

2.5 HE staining, Masson Staining and VG Staining

Harvested hearts were fixed in 4 % formalin and embedded in paraffin for hematoxylin and HE, masson or VG staining. For observation of fibrosis, HE staining, VG staining and masson staining were used to stain collagen fibers. The results were detected by microscopy.

2.6 Transmission Electron Microscopy Analysis

The hearts in each group were immersed in a 4 % formalin. After dehydration in a graded concentration of alcohol, ultrathin sections of tissues were mounted on formvar-coated slot grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 electron microscope (JEOL Co., Tokyo, Japan).

2.7 TUNEL Staining

DNA fragmentation of apoptotic cells was detected by TUNEL staining following the manufacturer's instructions. After the cardiomyocytes were cultured for 24 h, the cells were fixed by 4% paraformaldehyde solution for 30 min. then 0.3% H_2O_2 was used for block endogenous peroxidase activity in cells. Last TUNEL reaction was used to incubate cells. The results were detected by microscopy.

2.8 Western Blot Analysis

Western blot was performed as described previously (Wang et al., 2014). The primary antibodies contain anti-CX40 (1 : 500), anti-PERK (1 : 500), anti-p-PERK (1 : 500), anti-capase-3 (1 : 500) (Bioss), anti-eIF2α, (1 : 1000), anti-p-eIF2α,(1 : 1000), anti-Bcl-2 (1 : 1000) (Wanleibio), anti-Bax (1 : 400), anti-ATF-4 (1 : 400) (Boster).

2.9 Statistical Analysis

All values were expressed as mean ± SE. Multi-group comparisons of the means were carried out by matched t-test using SPSS 11.5.

3. Results

3.1 Effect of Tanshinone II A on Adriamycin-induced Myocardial Injury

Echocardiographic functional data are physiologically important to confirm the cardioprotective role. In our results, all rats in ADR group showed progressive thickening of left ventricular end-diastolic dimension (LVEDd) and left ventricular end-systolic dimension (LVEDs), ejection fraction (EF) was decreased compared with control rats (Table 1). Tanshinone II A treatment significantly reversed the changing of heart function induced by ADR (figure 2).

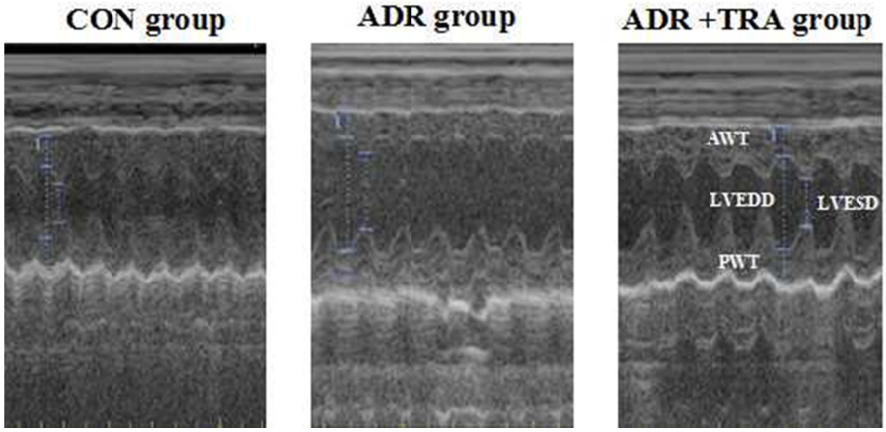

Figure 2. The postintervention M-mode echocardiographic image

LVESD, left ventricular end-systolic dimension; LVEDD, left ventricular end-diastolic dimension; AWT, anterior wall thickness; PWT, posterior wall thickness.

Table 1. Echocardiographic parameter

	CON (n=8)	ADR (n=7)	ADR+TRA (n=8)
IVSd	2.04 ± 0.02mm	1.8 ± 0.01 mm	2.12 ± 0.008 mm
LVIDd	4.86 ± 0.11 mm	5.8 ± 0.009 mm	5.33 ± 0.001 mm
LVPWd	1.96 ± 0.01 mm	1.65 ± 0.02 mm	1.88 ± 0.21 mm
LVIDs	2.66 ± 0.05 mm	3.68 ± 0.01 mm	2.74 ± 0.06 mm
EF	82 ± 1.6 %	72 ± 1.9 %	85 ± 2.1%
%FS	45 ± 1.7 %	36 ± 2.0 %	49 ± 1.4 %

IVSd: InterventricularSeptal Thickness at Diastole; LVIDd: left ventricular end-diastolic dimension; LVPWd: Left ventricular posterior wall thickness; LVIDs: left ventricular end-systolic dimension; FS, fractional shortening; EF, ejection fraction.

3.2 HistopathologicalObservation by HEStaining

HE staining showed that no pathological changes were present in the CON group. While the heart form ADR treated rats revealed the formation of cytoplasmic vacuole and myofibrillar loss. Tanshinone II A significantly decreased myocardial lesions (figure 3).

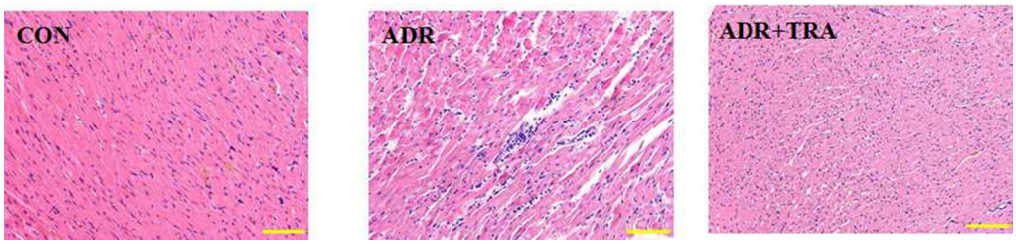

Figure 3.The effects of Tanshinone II A on histological features of heart from different treatment (H&E staining).

Myocardial fibrosis significantly increased in heart of rats form ADR group compared with CON group (figure 4). However, Tanshinone II A treatment reduced myocardial fibrosis induced by ADR.

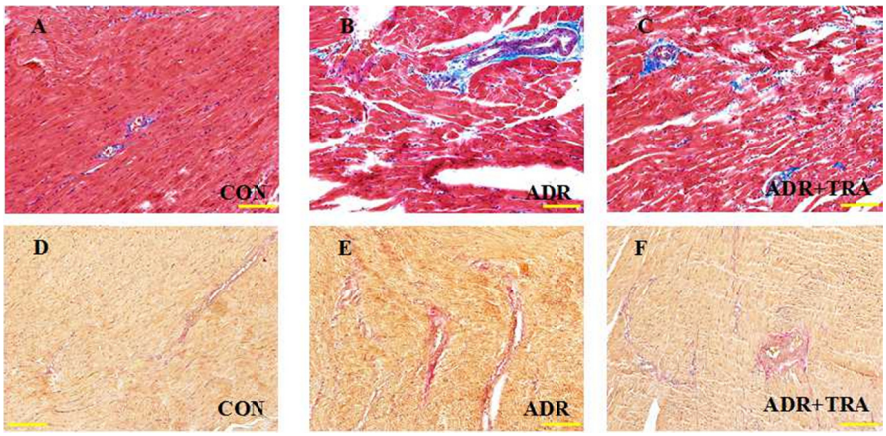

Figure 4.The effect of Tanshinone II A on heart fibrosis (Masson and VG staining).

Representative photomicrographs of rat cross sections stained with Masson (A, B and C), VG (D, E and F).

3.3 Heart Tissue Levels of TNF-α and TGF-β

Heart tissue levels of TNF-α and TGF-β in ADR group markedly increased compared with those in the CON group. Tanshinone II A treatment significantly reduced the levels of TNF-α and TGF-β (figure 5).

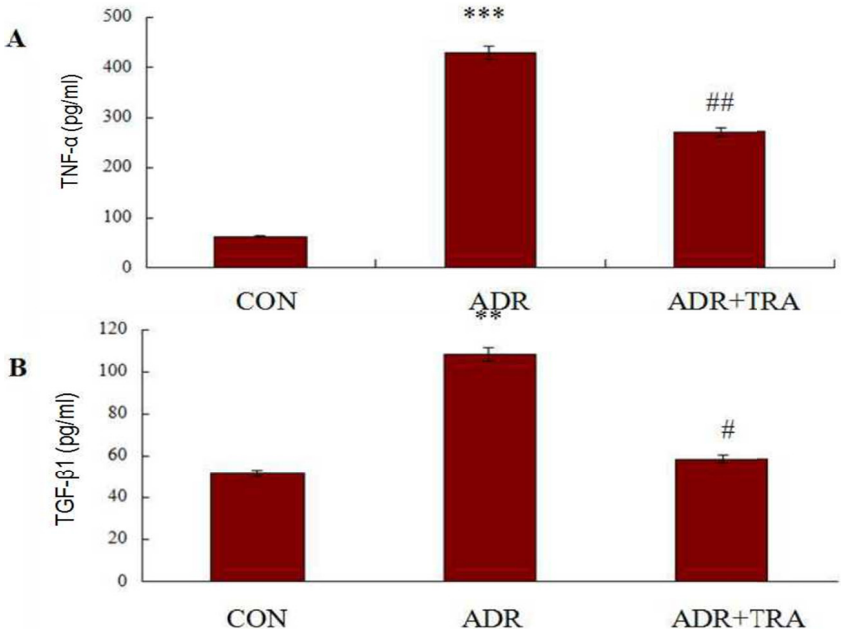

Figure 5. TNF-α and TGF-β1 serum levels in three control groups.

3.4 UltrastructuralRemodellingof Nucleus TractusSolitarii

Compared with control rats, mitochondrial damage and vacuolization were frequently found in adriamycin-treated rats hearts but minimal ultrastructural changes in similarly treated Tanshinone II A treated hearts (figure 6).

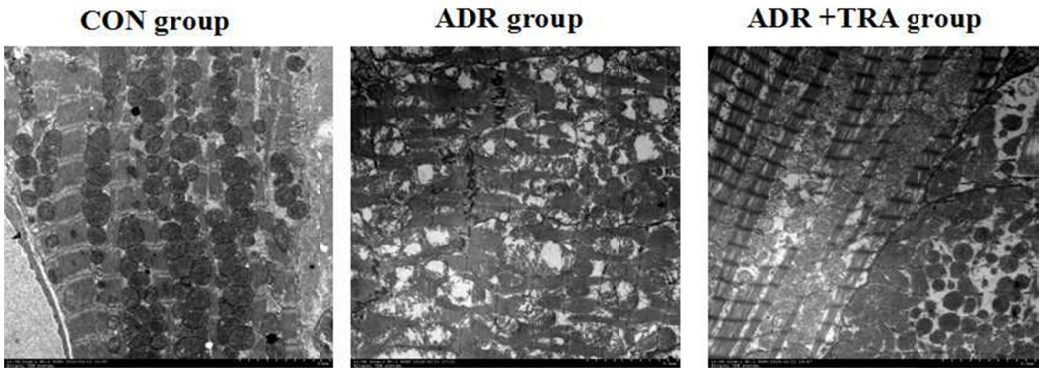

Figure 6. Transmisson electron microscopy images of hearts. Original magnification: ×1,500.

3.5 Detection of Apoptosis by TUNEL Assay

To evaluate the effect of Tanshinone II A on cardiomyocytes, the hearts of ratswere treated by adriamycin or Tanshinone II A and measurement of cell apoptosis using TUNEL analysis. From the fig. 7, adriamycin significant increased the apoptotic rate compared with control group. While the number of TUNEL-positive cells were decreased after dealing with Tanshinone II A.

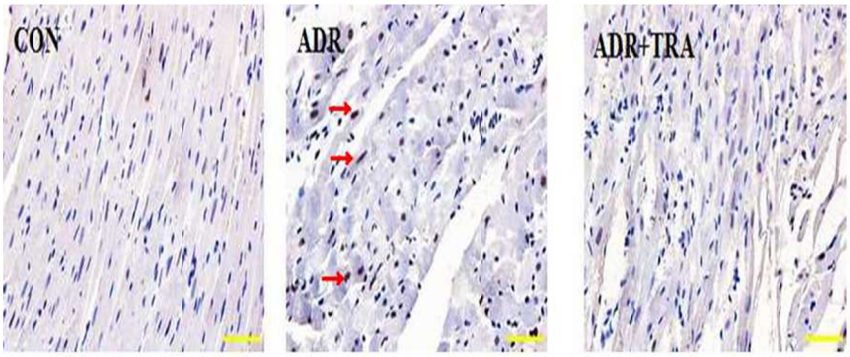

Figure 7. Apoptosis of rat myocardial cell was detected by TUNEL assays.

3.6 Western Blotting of ER Stress Associated Apoptosis Related Protein

In our study, the expression level of pro-apoptotic protein Bax was significantly higher in ADR group compared with CON and ADR+TRA group, while anti-apoptotic protein Bcl-2 in CON group did not. The ratio of Bax/Bcl-2 was elevated by adriamycin, but this change was affected by Tanshinone II A (figure 8A)

The expression level of Caspase-3 was increased in atrial of ADR group compared with those of the CON group, and this increase was prevented by Tanshinone II A (figure 8B).

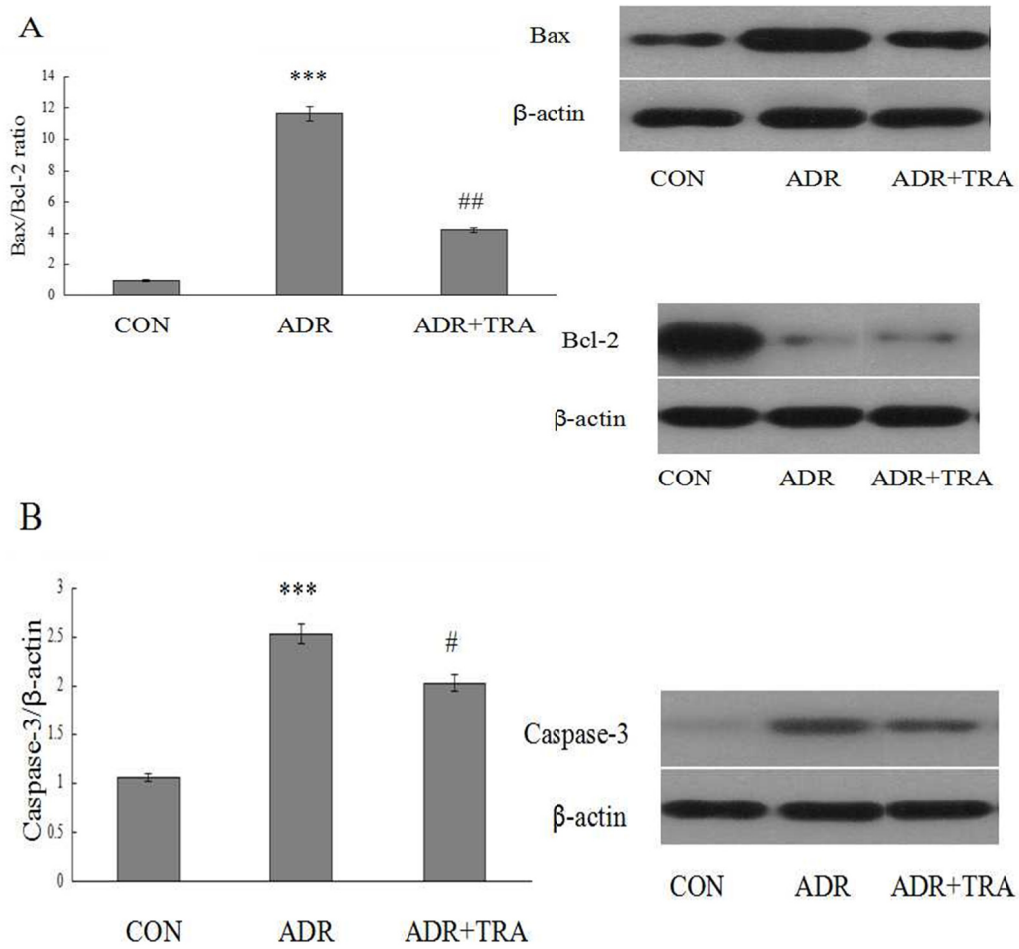

Figure 8. Tanshinone II A relieves adriamycin induced apoptosis. A/B: Western blot analyzed the expression of Bax, Bcl-2 and Caspase-3. All data are shown as mean ±SE. ***p<0.001, n=3 vs CON; ##p<0.01, n=3 vs ADR; #p<0.05, n=3 vs ADR.

The results of western blotting showed that the protein expression of p-PERK (figure 9A), p-eIF2α (figure 9B), and ATF-4 (figure 9C) in ADR group were significantly down-regulated compared with that in the CON group. However, those down-regulations were significantly inhibited by Tanshinone II A respectively.

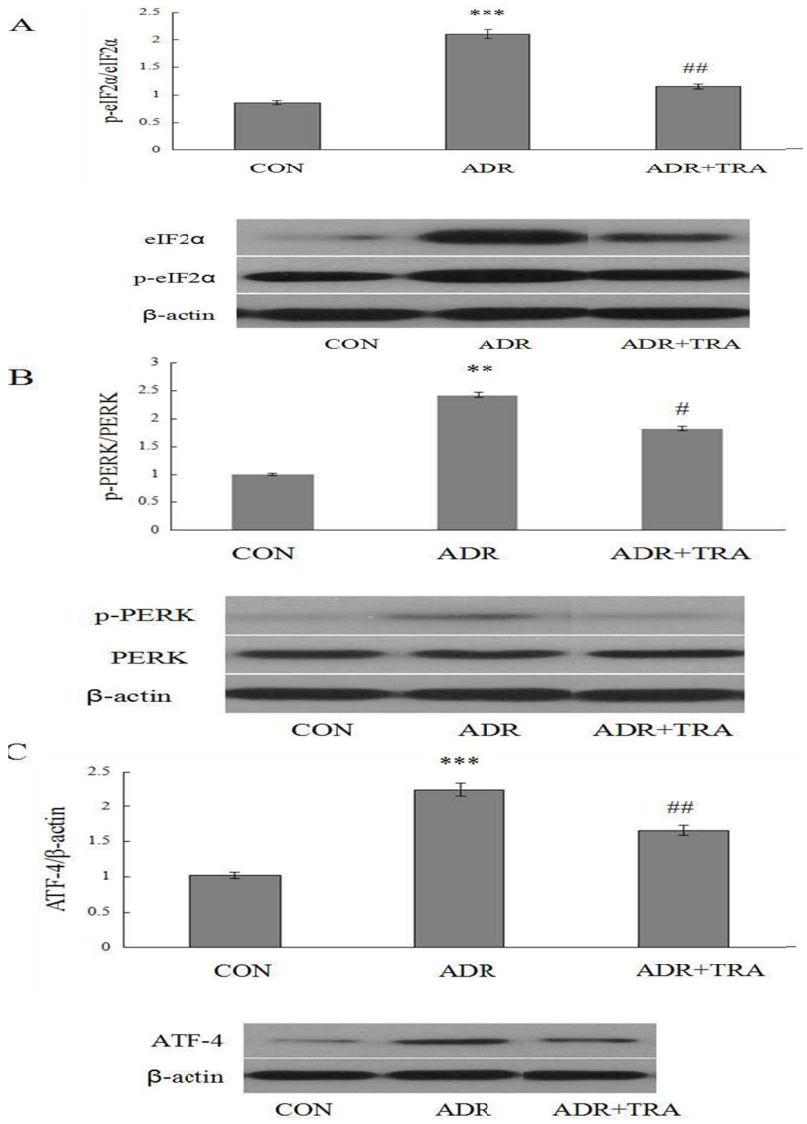

Figure 9. The expression of ERS related protein decreased by Tanshinone II A. A/B/C: western blot analyze the expression of p-PERK, p-eIF2α, ATF. All data are shown as mean ±SE. ***p<0.001, n=3 vs CON; **p<0.01, n=3 vs CON; ##p<0.01, n=3 vs ADR; #p<0.05, n=3 vs ADR.

4. Discussion

In the present study, we have observed the effects of tanshinone II A, as an ingredient of Salvia miltiorrhiza Bunge which is used for prevention or treatment of cardiovascular disease in China, on adriamycin-induced myocardial injury. The underlying mechanisms of tanshinone II A against adriamycin related cardiac function, myocardial fibrosis, mitochondrial damage, inflammatory response and ER stress associated apoptosis.

In our research, we used Transmission electron microscopy analysis to study the effect of Tanshinone II A treatment in adriamycin induced myocardial injury. We treated rats with adriamycin (2 mg per kilogram of body weight by intraperitoneal injection) or/and Tanshinone II A (2 mg per kilogram of body weight by intraperitoneal injection) and killed them 7 weeks later. We observed thicken of LVEDd and LVEDs and down-regulation of EF in adriamycin-treated rats but not in cardiomyocytes in adriamycin/Tanshinone II A-treated rats. Furthermore, for the histological detection of HE staining, the disarray, serous vacuolization of the cardiomyocyte and myofibrillar loss in ADR group more serious than CON and ADR + TRA group. Fibrosis has been reported to be related in cardiac stiffness and dysfunction in adriamycincardiotoxicity (Miyata et al., 2010). In our research, we observed that adriamycin-induced fibrosis was prevented by Tanshinone II A. This finding is consistent with Tanshinone II A inhibition adriamycin-induced myocardial injury.

Abnormality in the mitochondria has been propose for adriamycin toxicity (Papadopoulou et al., 1999). On the basis of the cardioprotective action of Tanshinone II A, we tested whether the effect would have functional effects on

mitochondria. By electron microscopy, we observed marker mitochondrial damage and vacuolization in adriamycin-treated rats' hearts but minimal ultrastructural changes in adriamycin/Tanshinone II A-treated rats. Thus, Tanshinone II A inhibited adriamycin-induced ultratructural changes in cardiomyocyte mitochondria.

To gain further insight into the treatment of Tanshinone II A, we performed TUNEL assay to explore the mechanism of Tanshinone II A inhibition adriamycin-inducted myocardial injury. From the result, it indicated that Tanshinone II A inhibited cardiomyocyte apoptosis inducted by adriamycin which was similar to previous reports (Hong-Jye et al., 2012). In addition, ER stress was suggested to contribute to cardiomyopathy (Xu et al., 2012). To investigate the reasons of Tanshinone II A prevent adriamycin-induced myocardial injury. We examined apoptosis related protein Bax, Bcl-2 and Caspase-3, the ratio of Bax/Bcl-2 and Caspase-3 increased in ADR+TRA group compared with CON and ADR group. In the present study, the observed inhibition of PERK (figure 9A), eIF2α (figure 9B), and the activation of ATF-4 (figure 9C) by Tanshinone II A suggests that the Tanshinone II A relieves adriamycin-induced myocardial injury in rat model by down-regulation ERS related apoptosis. It was evidenced that Tanshinone II A had anti-inflammatory response (Ren et al., 2010). Finally, we tested heart tissue levels of TNF-α and TGF-β, and found tanshinone II A reduce adriamycin-induced inflammatory response.

5. Conclusion

In summary, the present studies demonstrated Tanshinone II A inhibited adriamycin-induced myocardial injury in rat model. Then we explored the underlying mechanisms of this effect. We found the cardioprotective effects of Tanshinone II A involved with cardiac function, myocardial fibrosis, mitochondrial damage, inflammatory response and ER stress associated apoptosis.

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