Taxifolin protects rat against myocardial ischemia/reperfusion injury through modulating mitochondrial apoptosis pathway

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Background: Taxifolin (TAX), a flavonoid has been reported to play an underlying protective role on the cardiovascular disease. Thus, this study is to evaluate the effect and potential mechanisms of TAX on ischemia/reperfusion (I/R) injury. **Methods:** Healthy heart of rat was subjected to I/R using Langendorff apparatus. The hemodynamic parameters were recorded during the perfusion, including heart rate (HR), the left ventricular developed pressure (LVDP), the maximum/minimum rate of left ventricular pressure rise ($+dp/dt_{max}$ and $-dp/dt_{min}$) and Rate pressure product (RPP). Histopathological examination of left ventricular was measured by using Hematoxylin-Eosin (HE) staining. Creatinekinase-MB (CK-MB) and lactate dehydrogenase (LDH) activities in effluent perfusion, and malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) levels in tissue were assayed. Apoptosis related proteins, such as B-cell lymphoma-2 (Bcl-2), Bcl2-associated X (Bax) and cytochrome c (Cyt-c) also were assayed by elisa. Western blot was employed to determine apoptosis-executive proteins, including caspase 3 and caspase 9. **Results:** The results demonstrated that TAX treatment significantly improve ventricular functional recovery, as evident by the increase in LVDP, $+dp/dt_{max}$, $-dp/dt_{min}$ and RPP, increased the levels of SOD, GSH-PX, whereas suppressed the levels of LDH, CK-MB, MDA. Furthermore, TAX upregulate the level of Bcl-2 protein and downregulate the level of Bax, Cyt-c, caspase 3, caspase 9 protein. **Discussion:** Our results indicated that treatment of TAX remarkably improve cardiac function, regulate oxidative stress and attenuate apoptosis. It is concluded that TAX has a cardioprotective effect against I/R injury through the modulation of mitochondrial apoptosis pathway.

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ABSTRACT

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Methods: Healthy heart of rat was subjected to I/R using Langendorff apparatus. The hemodynamic parameters were recorded during the perfusion, including heart rate (HR), the left ventricular developed pressure (LVDP), the maximum/minimum rate of left ventricular pressure rise ($+dp/dt_{max}$ and $-dp/dt_{min}$) and Rate pressure product (RPP). Histopathological examination of left ventricular was measured by using Hematoxylin-Eosin (HE) staining. Creatinekinase-MB (CK-MB) and lactate dehydrogenase (LDH) activities in effluent perfusion, and

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Results: The results demonstrated that TAX treatment significantly improve ventricular functional recovery, as evident by the increase in LVDP, $+dp/dt_{max}$, $-dp/dt_{min}$ and RPP, increased the levels of SOD, GSH-PX, whereas suppressed the levels of LDH, CK-MB, MDA. Furthermore, TAX upregulate the level of Bcl-2 protein and downregulate the level of Bax, Cyt-c, caspase 3, caspase 9 protein.

Discussion: Our results indicated that treatment of TAX remarkably improve cardiac function, regulate oxidative stress and attenuate apoptosis. It is concluded that TAX has a cardioprotective effect against I/R injury through the modulation of mitochondrial apoptosis pathway.

INTRODUCTION

Ischemic heart disease is a threat to human health. Extracorporeal circulation and coronary bypass surgery are usually carried out to improve myocardial ischaemia after myocardial infarction occurs. However, when blood supply returns to heart after a period of ischemia will cause additional damage to the heart. The condition was referred to as cardiac ischaemia/reperfusion (I/R) injury (*Braunwald et al., 2012*). Nowadays, I/R injury is a major factor, which often cause to death. During myocardial I/R injury, cardiomyocytes undergo death at a higher frequency, mainly including necrosis and apoptosis (*Gottlieb et al., 1994*). Apoptosis is programmed cell death, which is considered to be the vital pathological processes in acute reperfusion injury (*Konstantinidis et al., 2012*). When the amount of cardiomyocyte decreases, the heart may undergo ventricular remodelling, compensatory cardiac hypertrophy and eventually lead to heart failure (*Pangonyte et al., 2008*; *Du et al., 2010*). Therefore, exploring the detailed mechanisms that trigger cardiomyocytes death and how to prevent it during I/R injury is still a public issue.

In the I/R injury, the morphological changes of cardiomyocytes were observed include microvascular damage, myocardial cell edema, and the body symptoms include myocardial enzyme release, arrhythmias and weak systolic function (*Naito et al., 2000*). It is generally believed that this reperfusion damage is caused by increased free radical activity. When circulating blood decreases, the level of oxygen supply can't keep up with oxygen demand by cardiomyocytes, the aerobic metabolism turns into anaerobic metabolism (*Giordano et al., 2005*). Anaerobic metabolism leads to the production of lactic acid which results in disturbances of ionic homeostasis. A timely reperfusion is crucial for recovery of an ischemic myocardium, but by sudden re-appearance of circulating blood to the dying myocardium, increased reactive oxygen species (ROS) will be produced in reaction to hyperoxia which can make the functional

situation of organization worse (*Akhlaghi et al., 2009*).

Flavonoid is the most prevalent class of naturally occurring compound and ubiquity in woody and herb plants. It exerts multiple biochemical properties and wide pharmacological effects (*Moon et al., 2006*). Epidemiological studies have shown that flavonoids is associated with a reduced risk of cardiovascular diseases (*Raj Narayana et al., 2001*; *Bjorklund et al., 2017*). Fisetin, a plant-derived bioflavonoid, significantly attenuated the I/R-induced tissue injury, blunted the oxidative stress, and restored the structure and function of mitochondria (*Shanmugam et al., 2018*). Quercetin has been demonstrated improves post ischemic recovery of isolated heart of rats after global ischemia (*Bartekovᾶ et al., 2010*). Previous research has revealed that TAX exerts anti-inflammatory effects and protects against oxidative stress-induced injury in human endothelial cells (*Guo et al., 2015*) and rat peritoneal macrophages (*Arutyunyan et al., 2016*). It also exhibited to possess free radical scavenging, antioxidant and anti-inflammatory effects (*Sun et al., 2014*; *Xie et al., 2017*). Recent studies demonstrated that TAX involved in the amelioration of cardiac disorders associated with against cardiac hypertrophy and fibrosis (*Guo et al., 2015*). TAX is structurally similar to quercetin potentially beneficial effects on the cardiovascular system. But its potential for I/R protection remain unclear. TAX is a potential candidate for the prevention or treatment of I/R injury. However, there was no previous report about whether TAX could influence the injury of I/R in isolated rat hearts. In present study, we aimed to evaluate the cardioprotective effects of TAX and investigated the mechanisms underlying these effects in isolated hearts of rats.

MATERIALS & METHODS

Experimental animals and treatment

Male SD rats (280-300 g each) were obtained from the Laboratory Animal Center of Heilongjiang Medicine University Medical (License Number: SCXK (hei) 2016-0002). The rats were housed under standard conditions with natural light (12 hr) and dark (12 hr) and in a suitable temperature at 22 ± 2 °C. The rats were fed common feedstuff and drank tap water freely during the experimental period. The investigation conformed to Guide for the Care and Use of Laboratory Animals (resvised, 1996). All animal experiments were approved by College of Pharmacy of Heilongjiang University of Chinese Medicine, Animal Ethics Committee (Approval number: IACUC-20170903019).

Reagents and antibodies

TAX (purity ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Creatinekinase-MB (CK-MB), lactate dehydrogenase (LDH), malondialdehyde (MDA) glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Enzyme-linked immunnosorbent assay kit about mitochondrial apoptosis (B-cell lymphoma-2 (Bcl-2), Bcl2-associated X (Bax) and cytochrome c (Cyt-c)) was obtained from Cloud-Clone Corp (Houston, TX, USA).

Monoclonal primary antibobys anti-b-actin, anti-active caspase 3 and anti-active caspase 9 was purchased from Abcam (MA, USA). Fluorescent-labeled goat anti-rabbit IgG secondary antibodie was obtained from LI-COR Biosciences (Lincoln, Nebraska, USA).

Experimental protocol

The rats were randomly divided into four groups (n=8): Normal control group (Control); Myocardial I/R control group (I/R); I/R + TAX treatment group (TAX 5 μM); I/R + TAX treatment group (TAX 15 μM). Experimental protocol is shown in Fig. 1. Control group: The hearts were subjected a continuous perfusion of Krebs–Henseleit (K–H) solution for 120 min. I/R group: The hearts were equilibrated for 30 min, subsequently, global ischemia was performed at 37 °C for 30 min, and next, re-perfused with K–H solution for 60 min. TAX 5 μM group: The hearts was equilibrated for 30 min. subsequently, global ischemia was performed for 30 min at 37 °C, and next, perfused with 5 μM of TAX-saturated K–H solution for 60 min. TAX 15 μM group: The hearts was equilibrated for 30 min. Subsequently, global ischemia was performed for 30 min, and next, perfused with 15 μM of TAX-saturated K–H solution for 60 min.

Langendorff preparation

After anesthetization via intraperitoneal injection (chloral hydrate solution, 300 mg/kg), rat hearts were quickly removed and subsequently perfused in the Langendorff apparatus. The perfusion was performed for 30 min in a modified K-H buffer gassed with 95 % O_2 and 5 % CO_2 at a constant flow velocity and constant temperature (37 °C). The composition of K-H buffer as the following (mM): NaCl 118, $MgSO_4$ 1.2, KCl 3.2, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 5.5. After equilibration, 30 min global ischemia was induced followed by 60 min of reperfusion. Control group utilizing the same protocol, but with no ischemia induction was used Water-filled balloon inserted into the left ventricular cavity was used to monitor hemodynamic parameters. The left ventricular end-diastolic pressure (LVEDP) was maintained at 5-10 mmHg by adjusting the size and position of the balloon. The whole procedure was completed within 2 min. The inclusion criteria of experimental samples were a heart rate (HR) of > 250 beats/min and a left ventricular developed pressure (LVDP) of > 75 mmHg in equilibrium phase. The hemodynamic parameters were recorded during the perfusion, including heart rate (HR), LVDP, the maximum/minimum rate of left ventricular pressure rise ($+dp/dt_{max}$ and $-dp/dt_{min}$). Rate Pressure Product (RPP) =HR × LVDP.

Histopathological evaluation of left ventricle sections

For histopathological examination, the cut left ventricle of heart tissues fixed in 10 % neutral formalin at room temperature. After two hours, the flat tissue blocks were embedded in paraffin to make it blocks and then cut into 3 μm thick tissue sections and stained with H&E. At least three samples from each group were evaluated. The tissue sections were visualized under light microscope (Dewinter technologies, Italy).

Estimation of cardiac damage

In present study, heart tissue injury was assessed by determining the concentration of LDH and CK-MB in the perfusate. The LDH and CK-MB content in the perfusate were measured with the assay kit as per the manufacturer's instructions. Samples of the perfusate were collected from the isolated heart at 25 min, 63 min, 90 min and 120 min of perfusion.

Measurements of anti-oxidant indices

The hearts tissue was cut into small pieces of tissue and then was grinded with lysate using glass homogenizer. Supernatant of tissue homogenate was frozen for each tissue analysis. MDA, SOD and GSH-PX activity were assessed using commercial elisa kits following the manufacturer's instructions. All enzyme activities were normalized to the total protein concentrations, which were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China).

Estimation of Cyt-c, Bcl-2 and Bax levels

Heart tissue samples were weighed, and then minced the tissues to small pieces and homogenized them in specific lysis buffer (w: v = 1:30, 1 mL lysis buffer is added in 30 mg tissue sample) with a glass homogenizer on ice. Then, the homogenates were centrifugated for 5 min and collect the supernatant. The levels of Bcl-2 and Bax protein were measured according to manufacturer's instructions of commercial kits (Cloud Clone Corp, Houston, USA).

Western blotting analysis

Myocardial tissue samples were lysed with RIPA buffer containing protease inhibitors for 15 minutes on ice. The total lysates were clarified by centrifugation, and supernatants were collected. Protein samples (20-25 mg per lane) were loaded on the gels and then separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred onto the nitrocellulose membrane (Roche, Mannheim, Germany). The membrane was washed with PBS with 0.1% Tween-20 (PBST) and blocked with 5% skim milk in shaking table for 2 h at room temperature. Then the membrane was washed with PBST and incubated with antigen-specific rabbit IgG antibodies (anti-caspase 3, anti-caspase 9, anti-β-actin; Abcam) diluted 1:1000 in PBST. Next, the membrane was washed with PBST and incubated with fluorescent-labeled goat anti-rabbit secondary antibodies (Lincoln, Nebraska, USA) diluted 1:2500 in PBST for 2 h at 4 °C. The target protein bands were scanned using the blot imaging system GelLogic 212 PRO (Carestream, Rochester, NY, USA) after washed in PBST. The obtained images were quantified as final results by image J 1.4.3 (www.imagej.nih.gov/ij). The results were expressed as the fold induction, which compared with the normal control.

Statistical Analysis

SPSS16.0 for Windows (SPSS Inc., Chicago, USA) was used for statistical analysis. All data were expressed as mean ± standard deviation (SD). For comparisons between groups, the one-

way ANOVA or student T-test was used where appropriate. The statistical differences was considered significant when $P < 0.05$. # $P < 0.05$ and ## $P < 0.01$ vs. Control group. * $P < 0.01$ and ** $P < 0.001$ vs. IR group.

RESULTS

Effects of TAX on Cardiac Parameters of Isolated Hearts

We examined whether TAX could protect the hearts of rat against ex vivo I/R injury. The results showed that there was no obvious alteration in average HR of isolated heart in period of reperfusion with TAX or without TAX. In addition, there was no significant HR change between I/R and normal groups during 30 min ischemia and 60 min reperfusion periods (Fig. 2A). After reperfusion, LVDP, $+dp/dt_{max}$ and $-dp/dt_{min}$ from different treatment groups were decreased in varying degrees. For instance, LVDP was significantly increased in TAX 15 μM group compared with I/R group at the end periods of reperfusion (LVDP = 68 mmHg vs. 52 mmHg, $P < 0.05$, Fig. 2B). Compared with the I/R group, TAX 15 μM treatment also significantly improved the heart functional recovery in rat at 60 min of reperfusion (RPP = 15294 mmHg × beats/min in TAX vs. 10643 mmHg × beats/min in I/R, $P < 0.01$, Fig. 2E). The results showed that TAX treatment improved the cardiac function recovery of rats during myocardial I/R injury.

TAX Down-Regulated the Release of LDH and CK-MB

At different time points of perfusion, the heart effluents were perfusate. LDH level in total reperfusion process was not conspicuously altered in normal control group. Perfusate LDH activity in the I/R group was improved compared with that of the normal control group after reperfusion and significantly increased at 60 min of reperfusion ($P < 0.01$), and TAX highly reduce enhanced the LDH levels compared with I/R group at 60 min of reperfusion (Fig. 3A). CK-MB release was similar to LDH release. Total expression in the perfusate was not conspicuously change in the control Group, and CK-MB level was not significantly altered in different group in baseline. However, levels in I/R group were markedly higher at 30 min of reperfusion compared with baseline ($P < 0.05$). Interestingly, the CK-MB release in both TAX 5 μM and 15 μM Group was significantly decreased at end of reperfusion compared with I/R Group ($P < 0.05$ or $P < 0.01$) (Fig. 3B). These results indicated that TAX could protect cardiac function against I/R injury.

Effect of Taxifolin on Myocardial Morphology

Histopathological examination of myocardial tissue was assessed by hematoxylin-eosin (HE) staining. Typical micrographs of the myocardial structure are shown in Fig. 4. In control group

(Fig. 4A), morphology of myocardial tissue is normal. Cardiomyocytes were arranged closely, intercellular space is small, no edema between cells. In contrast, the I/R group (Fig. 4B) showed degenerated muscle fibers and obvious contraction band, severe obvious cells edema, many infiltrated inflammatory cells. As shown in Fig. 4C, TAX at 5 μM maintained the myocardium with only slight irregularly arranged of fibers and a few contraction bands. As shown in Fig. 4D, TAX 15 μM group showed orderly cardiomyocytes but a few cells dissolve and degeneration. The results show that treatment with 15 μM TAX significantly reduced I/R injury in comparison with I/R group.

Effect of TAX on I/R-Induced Oxidative Stress in the Myocardium

To explore the cardio-protective mechanism of TAX, the effects of TAX on SOD, GSH-PX and MDA activity were investigated in myocardial tissue in response to I/R injury. As shown in Fig. 5, in TAX 15 μM group, the SOD and GSH-PX activity were increased significantly in comparison with those of the I/R group ($P < 0.01$), whereas the group pretreated with 5 μM TAX showed no significant differences. Conversely, these TAX-treated groups showed that MDA production was reduced significantly ($P < 0.01$) in comparison with I/R group.

TAX Protects Myocardial Cell from I/R-Induced Mitochondrial Damage

To evaluate if the effect of TAX is mediated through attenuation of the mitochondrial damage, we determined cytochrome c (Cyt-c) in cytosol. Fig. 6A shows that I/R increased cytosolic Cyt-c levels ($P < 0.01$). By comparison, TAX at different doses could reduce I/R-induced increase of Cyt-c levels ($P < 0.01$). The result suggested that TAX attenuated I/R induced Cyt-c release to cytosol. The bcl-2 family protein modulates the release of Cyt-c by regulating mitochondrial transmembrane potential. We also determined Bcl-2 and Bax protein expression levels. Compared with the control group, I/R down-regulated Bcl-2 but up-regulated Bax protein expression (Figs. 6B-6C). TAX increased Bcl-2 levels but decreased Bax levels compared with I/R. These results indicate that TAX may protect mitochondria attenuate apoptosis by regulating the expression of the Bcl-2 family proteins.

TAX Attenuates Myocardial I/R-Induced Apoptosis

To explore the potential mechanism of TAX in rats subjected to I/R-induced myocardial injury, we investigated the proteins expressions of active-caspase 3 and active-caspase 9. Compared with control group, the expression of active-caspase 3 was up-regulated in I/R group. While compared with I/R, TAX treatment group significantly reduced the level of active-caspase 3 (Fig. 7C). In TAX treated group, it was showed that the expression of active caspase 9 was

down-regulated in 15 μM ($P< 0.05$), but did not changed significantly in 5 μM (Fig. 7D). These results indicated that TAX inhibited apoptotic level in heart injury induced by I/R.

DISCUSSION

Growing evidence indicates a therapeutic action of TAX in cardiovascular disease. However, implications of TAX in I/R injury are unclear. This is first study to evaluate the cardioprotective effects of TAX in isolated rat heart subjected to I/R injury. We demonstrated that an important role for TAX improved cardiac function, inhibited oxidative stress and apoptosis in a model of I/R injury in vitro.

In the late 19th century, Oscar Langendorff was studying on perfecting isolated heart model. Since then, the procedure has been used to probe pathophysiology of I/R and with the dawn of molecular biology (*Bell et al., 2011*). Today, the Langendorff heart assay is a predominant in vitro technique used in physiological and pharmacological research. It allows the examination of cardiac contractile strength and heart rate without the complications of an intact animal (*Herr et al., 2015*). Therefore we determine the effect of TAX to cardiac function parameters of isolated heart in myocardial I/R injury using Langendorff equipment. Cardiac functions mainly depend on the contraction and relaxation properties of the ventricular muscle. Changes in cardiac function are strongly linked to the severity of I/R injury (*Mehdizadeh et al., 2013*). Our analyze data showed I/R can cause marked myocardial dysfunction, including reduction of LVDP, $+dp/dt_{max}$ and $-dp/dt_{min}$, While the TAX treatment groups significantly improved cardiac diastolic dysfunction but did not alter the average HR in isolated heart.

LDH is a marker of cellular damage and common disease due to its mass release to plasma during tissue injuries, such as heart failure. CK-MB, expressed extensively in cardiac myocyte, often tested in the serum as an indicator of damage of rhabdomyolysis, myocardial damage and acute kidney injury in clinic (*Moghadam-Kia et al., 2016*). The increase in the levels of LDH and CK-MB in the organ perfusate after ischemia is direct evidence of cardiac damage (*Houshmand et al., 2009*). Compared with control group, LDH and CK-MB activity significantly increased in I/R group during myocardial I/R injury. Perfusate LDH and CK-MB activity in the TAX treatment groups, particularly in the 15 μM group, were remarkable reduction than those of the I/R group, which is consistent with the observation of changing cardiac function parameters. In addition, histopathological examination was implemented to assess the effect of TAX on myocardial morphology. The results of pathomorphological research of heart in I/R group show acute myocardial damage and TAX causes favourable morphological changes in heart in I/R injury. The above results support the potential application of TAX as a cardioprotective agent in myocardial I/R injury.

Under normal conditions, tissues could maintain the balance between generation and clearance of ROS. However, the balance was disrupted during I/R and cause ROS increase significantly

(*Becker et al., 2004*). Excess ROS can oxidize lipids, proteins and DNA, which cause to dysfunction of these molecules and then result in the degeneration of tissue function (*Kleikers et al., 2012*). Minimizing ROS production is an important strategy to prevent cardiomyocyte I/R injury (*He et al., 2016*). Therefore, activation of the anti-oxidant enzyme system is a necessary strategy to reduce oxidative stress-induced tissue damage (*Matsushima et al, 2013*). The levels of SOD and GSH-PX rate are used to evaluate tissue per-oxidative injury (*Maciejczyk et al., 2017*). In addition, MDA, an index to evaluate the severity of lipid peroxidation, produced by lipid peroxidation results in destruction of structural proteins and cellular structures (*Pizzimenti et al., 2013*). Our results exhibited that SOD and GSH-PX activity was conspicuous increased, whereas MDA level was dramatically decreased by TAX, especially in 15 μM group. Therefore, we speculated that the TAX exhibits cardioprotective effects by enhancing antioxidase activity and inhibiting free radicals peroxidation.

Mitochondrial damage plays an important role in I/R-induced injury. It considered to as the final arbitrators for I/R-induced cell apoptosis (*Powers et al., 2007*). During ischemia and mainly during early phases of reperfusion, excessive of ROS is generated, causing myocardial Ca2+ overload and opening of the mitochondrial permeability transition pore (mPTP), which can reduce mitochondrial function and finally result in an increase in myocardial cell apoptosis (*Garciarena et al., 2011*; *Halestrap et al., 2015*). One of the ways of cell apoptosis is activated by release Cyt-c from the mitochondria to cytosol. In our study, the results showed that TAX is capable of weakening the observed increase in expression of Cyt-c in cytosol. It is very likely that the increased cytosolic content of Cyt-c, which mediates apoptosis, while its expression in mitochondria was not changed (*Lundberg et al., 2004*). Therefore it can make an assumption that down- regulation of Cyt-c as result of TAX whittled apoptotic processes.

As an important mitochondrial regulator during myocardial apoptosis, Bcl-2 exerts anti-apoptotic effects by blocking the release of Cyt-c and reducing caspase activity.34 Apoptosis-related proteins, caspase 3 and caspase 9, are also play crucial roles in apoptosis. The caspase apoptotic pathway responds to death signals by releasing apoptosis-inducing factor from the mitochondrial and then translocated to the nucleus (*D'Amelio et al., 2012*). In this study, Bcl-2, an anti-apoptotic protein and Bax, a pro-apoptotic protein were used to assess the effects of TAX on cardiomyocytes apoptosis. The result demonstrated that TAX treatment increases protein expression of Bcl-2, significantly reduces Bax expression when compared with I/R group. Caspase 3 and Caspase 9 were tested to measure the apoptotic level in the isolated heart after I/R injury. We found that increased expression of active form of caspase 3 and caspase 9 under ischemmic conditions and decreased expression of them in TAX group. Consistent with those results, treatment with TAX significantly decreased myocardial apoptosis through regulating expression of apoptosis-related proteins, including Bax, Bcl-2, caspase 3 and caspase 9. These findings suggested that the inhibition of apoptosis is closely related to the underlying beneficial effect of TAX in I/R injury.

CONCLUSIONS

In conclusion, TAX exerted cardioprotective effects against I/R injury by inhibiting oxidative stress and cardiac myocyte apoptosis. These phenomena highlight that the mechanism associated with this protection maybe involves modulation of mitochondrial apoptosis pathway. Our find provides a novel of thought for therapeutic development as an adjuvant therapy to I/R injury.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support provided by Outstanding Innovative Talents Support Plan of Heilongjiang University of Chinese Medicine (2012RCD05), Outstanding Talent Cultivation Fund Project of Heilongjiang University of Chinese Medicine (2012cj02).

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Figure 1

Figure 1 Experimental protocol

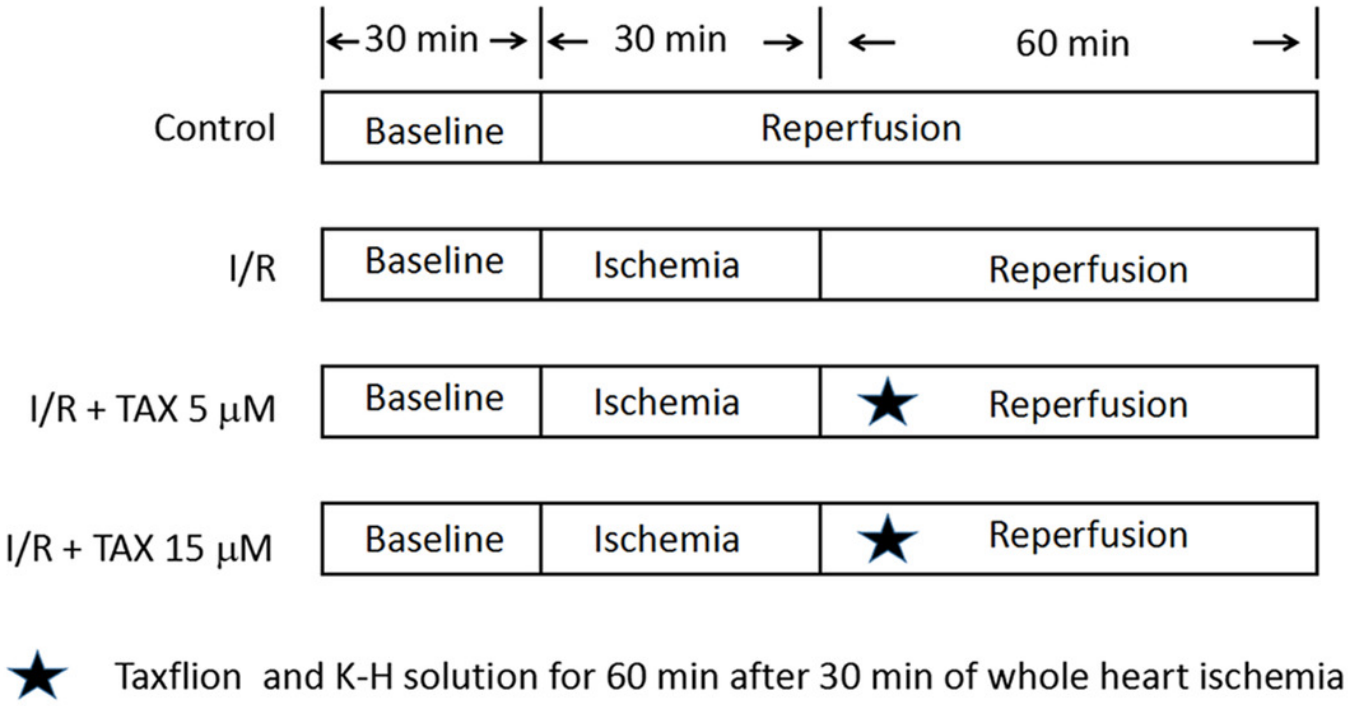


Figure 2(on next page)

Figure 2. TAX treatment improved the cardiac function recovery of rats during myocardial I/R injury in vitro model.

(A) Heart rate (HR, beat/per min); (B) left ventricular developed pressure (LVDP, mmHg); (C) maximum rate of left ventricular pressure (+dp/$dt_{max,}$ mmHg/s); (D) minimum rate of increase of left ventricular pressure (-dp/dt_{min}, mmHg/s); (E) Rate pressure product (RPP, mmHg × bpm); (F) representative left ventricular pressure records in experimental protocol form different experiment groups.

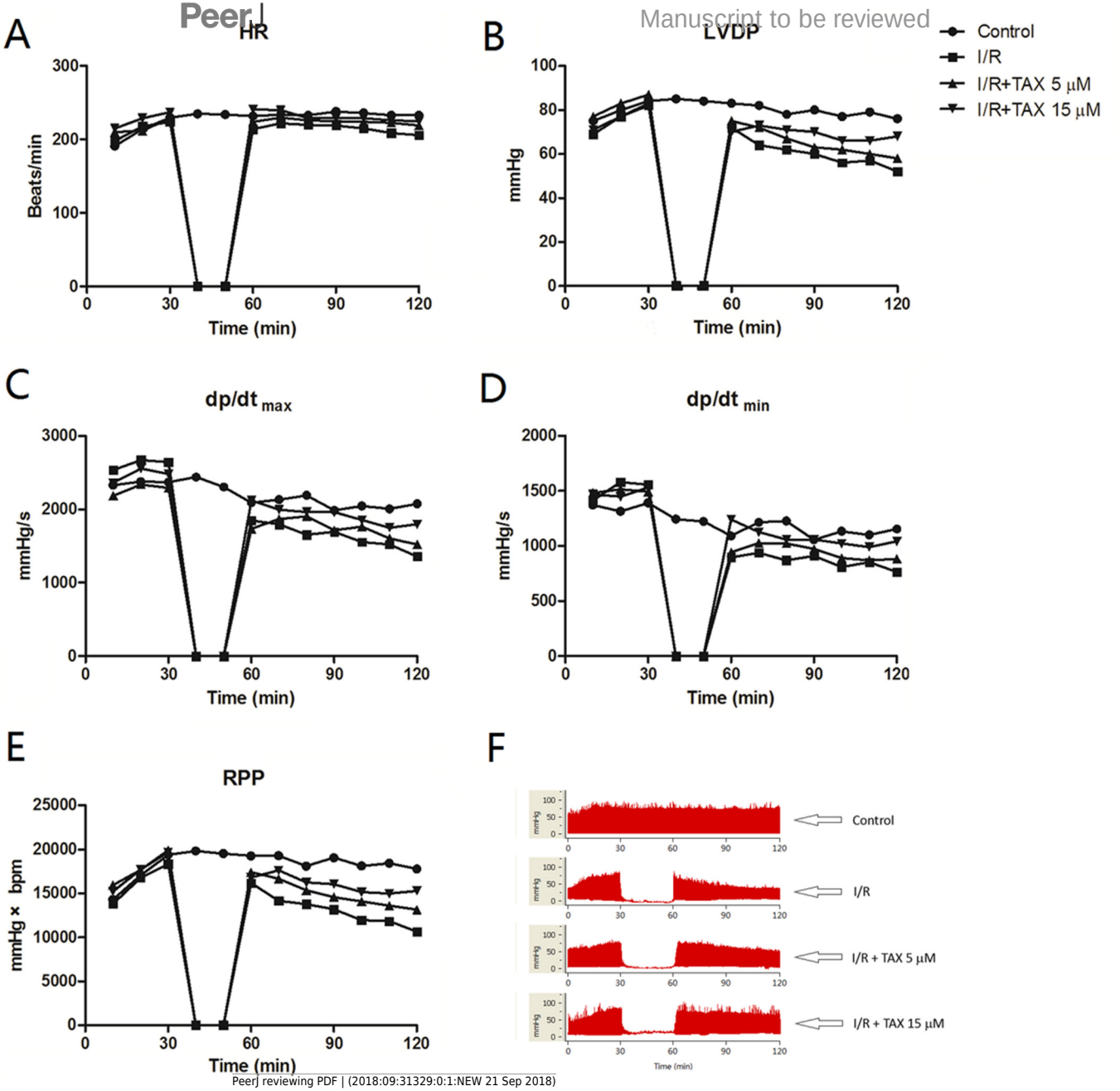

A
HR
Beats/min
300
200
100
0
0
30
60
90
120
Time (min)
B
LVDP
mmHg
100
80
60
40
20
0
Time (min)
Control
I/R
I/R+TAX 5 μM
I/R+TAX 15 μM
C
dp/dt max
mmHg/s
3000
2000
1000
0
Time (min)
D
dp/dt min
mmHg/s
2000
1500
1000
500
0
Time (min)
E
RPP
mmHg × bpm
25000
20000
15000
10000
5000
0
Time (min)
F
Control
I/R
I/R + TAX 5 μM
I/R + TAX 15 μM
Time (min)

Figure 3(on next page)

Figure 3. Effect of TAX on injury of cardiomyocytes by measurement of LDH and CK-MB.

The levels of perfusate LDH and CK at different time points in the control, I/R and TAX-treat group (5 μM and 15 μM) are shown. # $P < 0.05$ and ## $P < 0.01$ compared with the control group; * $P < 0.05$ and ** $P < 0.01$ compared with the I/R group; U/L: international enzyme activity unit per liter.

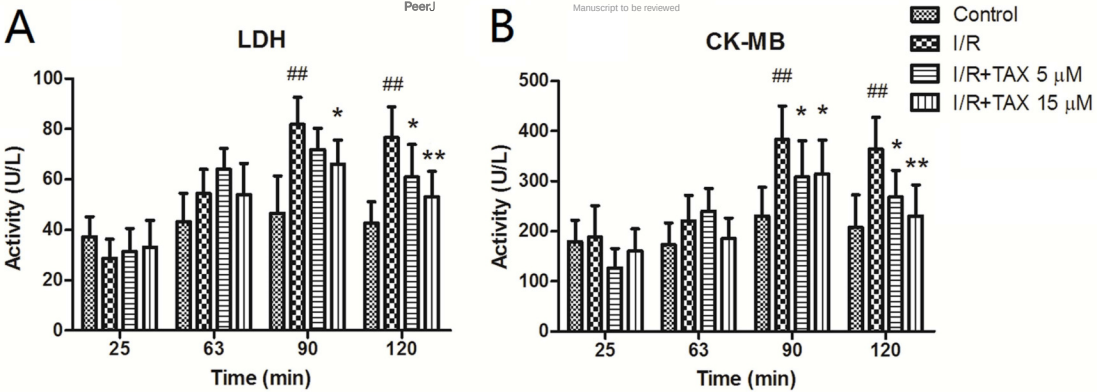

A
LDH
Activity (U/L)
100
80
60
40
20
0
25
63
90
120
Time (min)
##
##
*
*
**
B
CK-MB
Activity (U/L)
500
400
300
200
100
0
25
63
90
120
Time (min)
##
##
*
*
*
**
Control
I/R
I/R+TAX 5 μM
I/R+TAX 15 μM

Figure 4

Figure 4. Representative micrographs of HE staining results in various experimental groups.

(A) control group; (B) I/R group; (C) treated with TAX 5 μM group; (D) treated with TAX 15 μM group, (magnification, ×400).

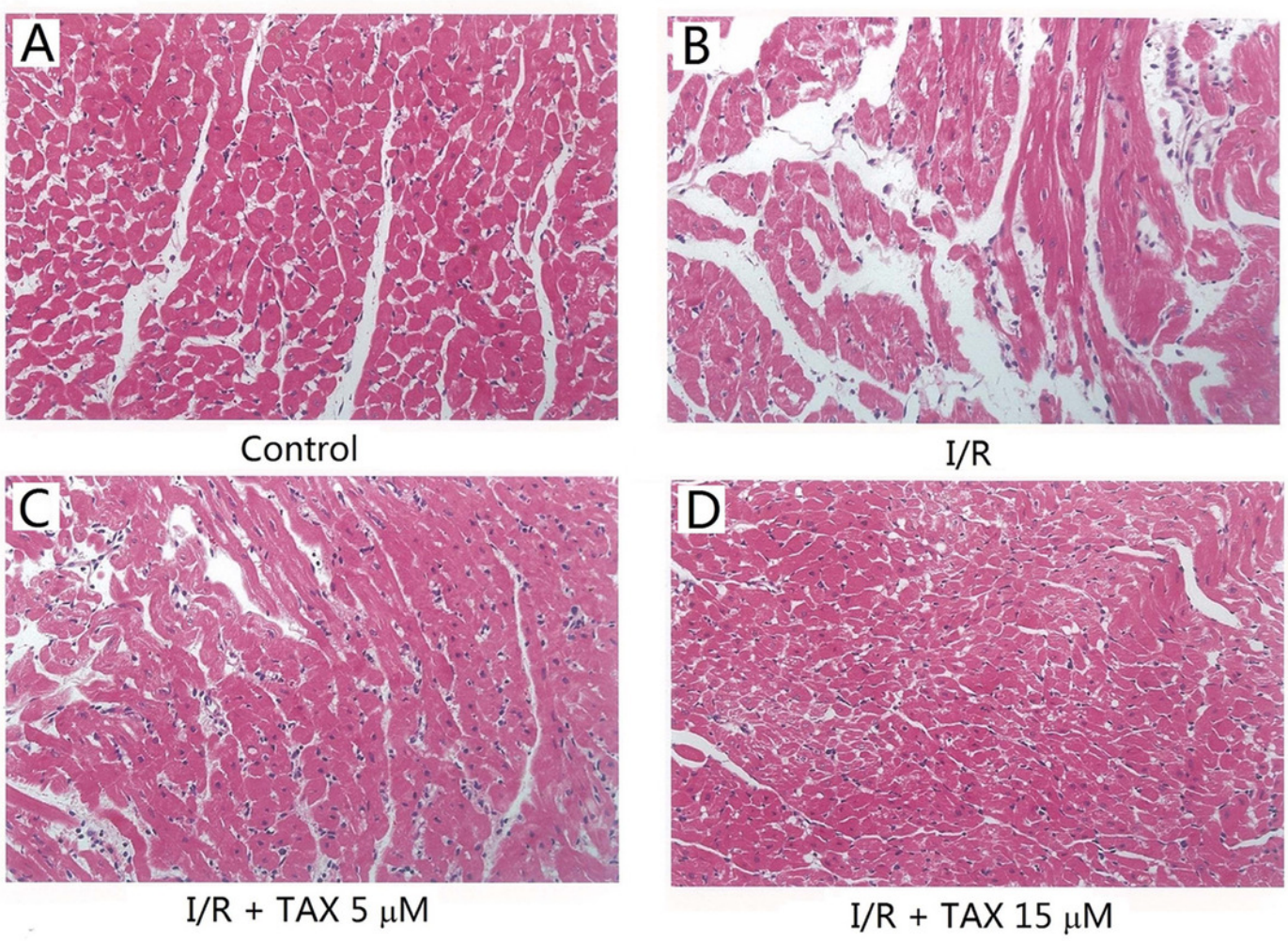


Figure 5(on next page)

Figure. 5. Effect of TAX on cardiac the activity of SOD and GSH-PX, contents of MDA.

Values are presented as mean ± SD. # $P < 0.05$ and ## $P < 0.01$ compared with the control group; * $P < 0.05$ and ** $P < 0.01$ compared with the I/R group. U/mgprot: international enzyme activity unit per milligram tissue protein.

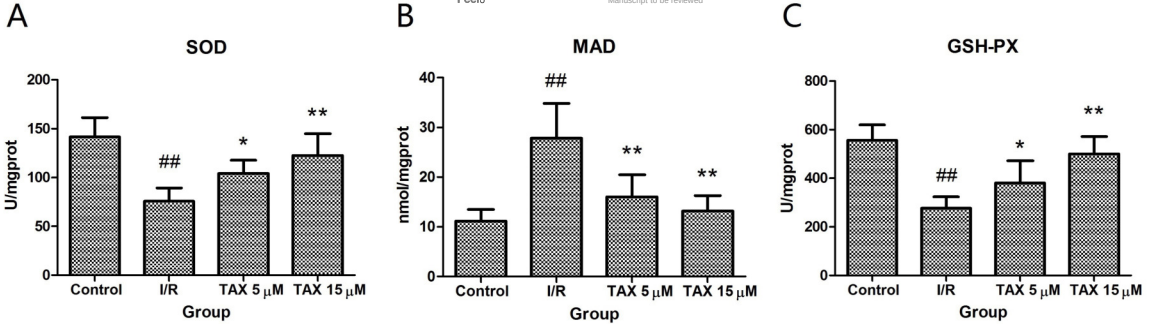

A
SOD
U/mgprot
200
150
100
50
0
##
*
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group
B
MAD
nmol/mgprot
40
30
20
10
0
##
**
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group
C
GSH-PX
U/mgprot
800
600
400
200
0
##
*
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group

Figure 6(on next page)

Figure 6. Effect of TAX on the expression of Cyt-c, Bax and Bcl-2 protein.

# P < 0.05, ## P < 0.01 vs. Control group; * P < 0.05, ** P < 0.01 vs. IR group. ng/mgprot indicate the nanogram level of the target protein per milligram total protein.

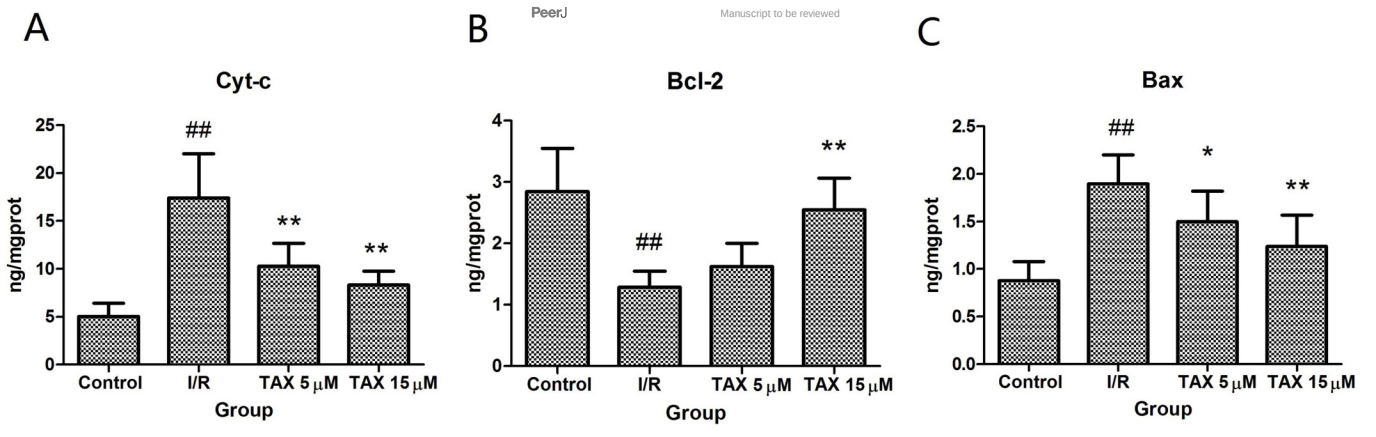
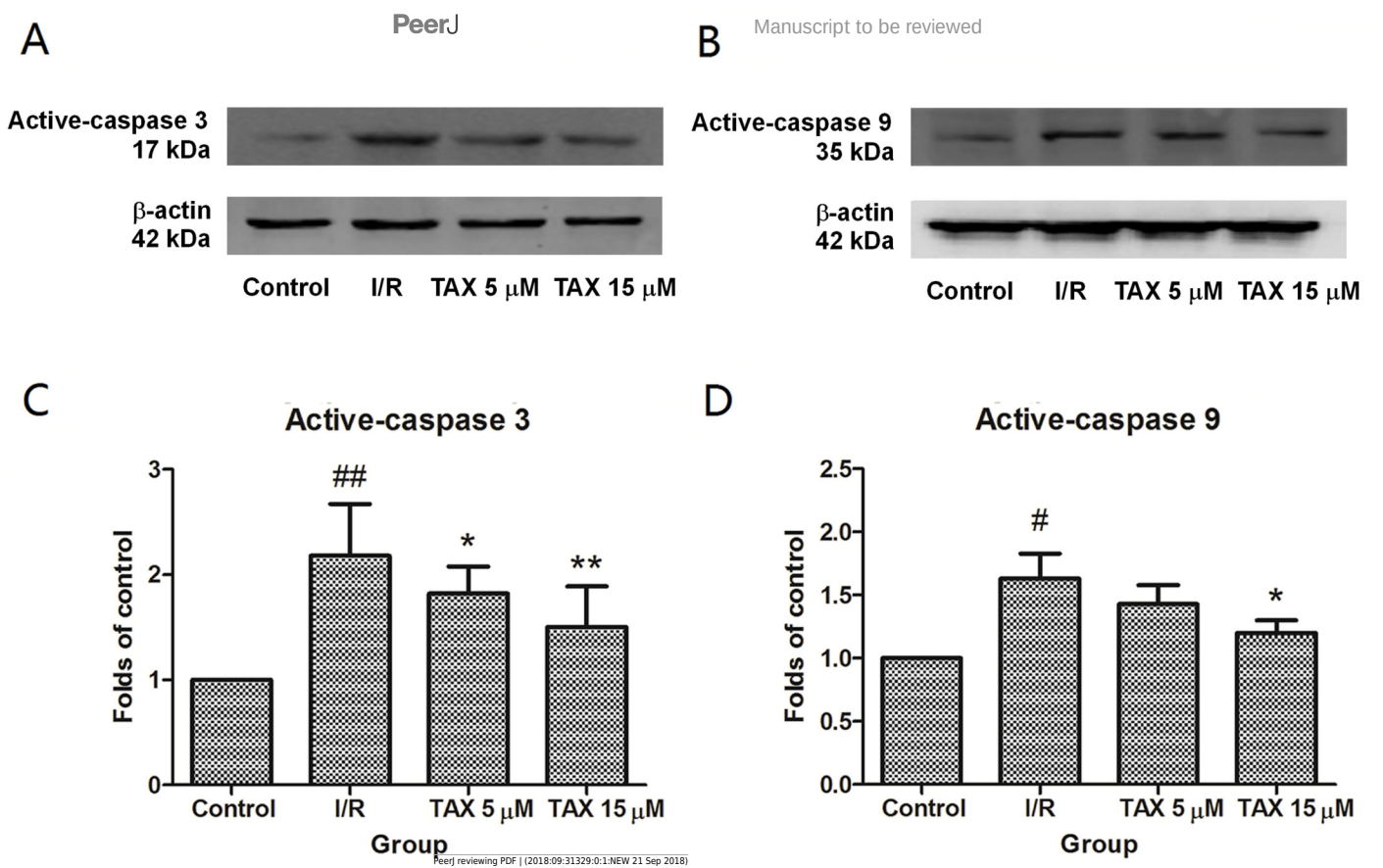

A
Cyt-c
ng/mgprot
25
20
15
10
5
0
##
**
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group
B
Bcl-2
ng/mgprot
4
3
2
1
0
##
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group
C
Bax
ng/mgprot
2.5
2.0
1.5
1.0
0.5
0.0
##
*
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group

Figure 7(on next page)

Fig. 7. The changes in the levels of caspase 3, and caspase 9 at the end of reperfusion.


A
Active-caspase 3
17 kDa
β-actin
42 kDa
Control
I/R
TAX 5 μM
TAX 15 μM
B
Active-caspase 9
35 kDa
β-actin
42 kDa
Control
I/R
TAX 5 μM
TAX 15 μM
C
Active-caspase 3
Folds of control
0
1
2
3
##
*
**
Control
I/R
TAX 5 μM
TAX 15 μM
Group
D
Active-caspase 9
Folds of control
0.0
0.5
1.0
1.5
2.0
2.5
#
*
Control
I/R
TAX 5 μM
TAX 15 μM
Group