Panax quinquefolium saponins alleviate myocardium injury from ischemia-reperfusion by inhibiting excessive endoplasmic reticulum stress

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Abstract
Panax quinquefolium saponins (PQS) protected myocardium from ischemia reperfusion (I-R) injury, but it remains unknown whether the effect is associated with the suppression of excessive endoplasmic reticulum stress (ERS). In the present study, the Sprague-Dawley rats administered with PQS or vehicle were subjected to I-R. The myocardial infarct size was measured with triphenyltetrazolium chloride (TTC) staining; apoptosis was detected with in situ TDT-mediated dUTP nick end labeling (TUNEL). Western blotting was used to examine the protein expression of the ERS-related proteins, glucose-regulated protein 78 (GRP78), calreticulin (CRT), CCAAT/enhancer-binding protein homologous protein (CHOP) and caspase-12; and Bax and Bcl-2. Pre-treatment with PQS protected myocardium against I-R-induced injury and apoptosis, and attenuated I-R-induced excessive ER stress as evidenced by decreased caspase-12 activation and expression of GRP78, CRT and CHOP. In conclusion, PQS could alleviate myocardial injury from I-R and the underlying mechanism was likely associated with inhibiting excessive ER stress induced by I-R. That will provide a more profound theoretic basis of Chinese medicine on preventing myocardium I-R injury. However, what is the downstream signal pathway on the earth and whether PQS has the same effects on clinical research. Which are needed to be explored in the future research.

Citation:

1. Introduction
Panax quinquefolium (American ginseng) belongs to the Araliaceous family and is native to the northern United States and southern Canada. It is a popular, nutritional supplement throughout the world and the leaves and stems exhibit a host of medical effects. The
chemical compositions of *P. quinquefolium* stem and leaf extracts have been studied for more than a decade, and these analyses have resulted in the identification of saponins, amino acids, carbohydrates, volatile oils, inorganic elements, and fatty acids that may contribute to these health benefits. The saponins in particular are responsible for numerous pharmacological actions. With the development of modern technology, more than 40 distinct saponin compounds have been isolated and identified, while there’s still a lot of being filtered. Both cell culture and animal models have demonstrated that *Panax quinquefolium* saponins (PQS) have potential benefits for the cardiovascular system by chelating transition metal ions, scavenging free radicals, as well as inhibiting the activation of protein tyrosine kinase induced by ischemia-reperfusion, modifying vasomotor function, and by improving serum lipid profiles. Furthermore, *Panax quinquefolium* saponins directly exerted preventative effects of myocardial ischemia and reperfusion injury induced by hyperbaric oxygen. (Kitts, D.D, et al. 2000; Dou DQ, et al. 2001; Kang SY, et al. 1995; Li JP, et al. 2000; Maffei Facino, R, et al. 1995). As a dietary supplement and a tonic, PQS has the unique ability to stimulate the immune system. Pretreatment with PQS can up-regulated the ability of treated cardiomyocytes to combat ischemia/reperfusion (I-R) injury.

The endoplasmic reticulum (ER) is the main organelle for protein synthesis, protein folding and intracellular calcium storage. Disordered ER Ca\(^{2+}\) homeostasis, ischemia, hypoxia, nutrient deprivation, ATP depletion, oxidative stress, and the accumulation of misfolded proteins all lead to endoplasmic reticulum stress (ERS). Recent evidence suggests that severe ER stress can lead to cell dysfunction following ischemia-reperfusion (Rao RV, et al. 2004). Moderate ER stress up-regulated the expression of ERS molecular indicators such as glucose-regulated protein 78 (GRP78) and calreticulin (CRT), enhances degradation of misfolded (mutant or unfolded) proteins, and inhibits protein synthesis to decrease the functional load within the ER. Prolonged and excessive ERS, however, can aggravate I-R injury by reducing ER Ca\(^{2+}\) buffering capacity, leading to Ca\(^{2+}\) overload and mitochondrial dysfunction (Boya P, et al. 2002; Breckenridge DG, et al. 2003). Under excessive or prolonged ERS, important mediators of ERS-associated death include the cleavage and activation of ER-associated caspase-12 and increased expression of CCAAT/enhancer-binding protein homologous protein (CHOP), a transcription factor that sensitizes cell to apoptosis. Therefore excessive ERS is one of the key mechanisms of I-R injury (Xu C, et al. 2005).

2. Objective of Research

Our previous study has shown a protective effect on cardiomyocyte against hypoxia-reoxygenation injury by inhibiting excessive ERS-related apoptosis (Chen Wang, et al. 2012). The aim of the present study is to investigate whether PQS can protect rat myocardium against I-R injury by suppressing excessive ERS. In the study, the Sprague-Dawley rats administered with PQS or vehicle were subjected to I-R. The myocardial infarct size and the apoptosis rate were measured and detected. Western blotting was used to examine the protein expression of the ERS-related proteins. That will provide the theoretic basis in prevention and treatment of ischemia/reperfusion.

3. Materials and Methods

3.1 Materials

PQS was provided by Yisheng Pharmaceutical Co., Ltd. (Jilin, China); Dead End™ Fluorometric TUNEL System was purchased from Promega (CA, USA); triphenyltetrazolium chloride (TTC) and evans blue were purchased from Sigma (St. Louis, MO, USA); rabbit polyclonal antibodies against calreticulin (CRT), glucose-regulated protein 78 (GRP78) and caspase-12 were purchased from Stressgen (New York, USA); rabbit polyclonal antibodies against Bcl-2 and Bax, rabbit monoclonal antibody against GAPDH, and mouse monoclonal antibody against CCAAT/enhancer-binding protein homologous protein (CHOP) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA); enhanced chemiluminescence kit was purchased from Millipore (Massachusetts, USA); HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG were purchased from Santa Cruz (California, USA).

3.2 Animals

All experimental animals were obtained from the Experimental Animal Center, General Hospital of People’s Liberation Army, Beijing, China. Male Sprague-Dawley rats, weighing 100 to 150 g, were housed five per cage at 23 °C with luminosity cycles of 12h light/12h dark and allowed free access to water and food. The protocol was approved by the Institutional Animal Care and Use Committee of Peking
University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

3.3 Methods
3.3.1 Myocardial I-R
The SD rats were randomly divided into I-R group (n = 15), PQS+I-R group (n=15) and sham group (n = 15), all rats allowed free access to water and food for six weeks excepting for rats in PQS+I-R group administered with additional PQS solution at 270mg/kg/d. The I-R protocol is as follows: the rats were fixed on the operating table under anesthetization condition with 2% pentobarbital sodium peritoneally (2.3 ml/kg). Under artificial ventilation with a rodent ventilator, the thoracic cavity was opened at the third or fourth left intercostals space to expose the heart. The proximal portion of the left coronary artery was surgically occluded for 45 minutes through ligation with a suture (size 6.0) followed by coronary reperfusion through release of the tie. Coronary occlusion was confirmed by elevation of the ST segment on the ECG obtained from a limb lead. The rats were returned to their cages and killed 24 hours later. The rats in PQS+I-R group underwent the same surgical procedure with I/R group. The rats in sham group (n = 15) were underwent the same surgical procedure excepting for the ligation of left coronary artery.

3.3.2 Hemodynamics
Twenty-four hours after coronary reperfusion, rats were anesthetized again through intraperitoneal administration of 2.3 ml /kg 2% sodium pentobarbital. ECG readings were monitored, and a polyethylene tube (PE 50; Becton-Dickinson) was inserted into the left ventricular cavity via the right carotid artery. MAP and LV±dp/dt max were measured using a polygraph system (AP601G; Nihon Koden).

3.3.3 Serum cardiac troponin T (cTnT)
The blood samples were collected from the right common carotid artery and the serum was separated and cTnT concentration was determined with automatic biochemical analyzer.

3.3.4 Infarct size
Infarct size was measured in established method. After hemodynamics were assessed at 24 hours of coronary reperfusion, 3 ml of blood was obtained from the tube for measurement of serum cTnT. Then, an intratracheal tube was inserted, and the chest was reopened under artificial ventilation. The coronary artery was again briefly occluded through ligation of the tie that remained at the site of the previous occlusion. Immediately after the ligation, 1% Evans blue solution was infused through the catheter into the beating left ventricular cavity to delineate the ischemic area at risk (underperfused and then reperfused area) of the left ventricle. After administration of an excessive dose of sodium pentobarbital into the left ventricular cavity, the heart was excised and cross-sectioned from the apex to the atrioventricular groove into five specimens of ~2 mm in thickness. Because there might be some anatomic differences in the left coronary artery of each rat, the three middle slices were prepared for morphometry to determine the ischemic area at risk. These slices were incubated with a 4% TTC solution for 30 minutes at 37 °C in a dark room. Then, ischemic but viable (TTCstained) and infarcted (TTC-unstained) zones within the non-perfused and then reperfused area (Evans blue-unstained) and the non-ischemic area (Evans blue–stained) were fixed in 10% neutral-buffered formalin for 24 h. These areas are measured with scanner and Image-Pro Plus (image analyzing software, Version 4.1, Media Cybernetics). Infarct size is defined as the ratio of TTC-unstained zones to ischemic area at risk (non-perfused and then reperfused area).

3.3.5 TUNEL-Positive Cardiomyocytes
The heart was excised and washed in saline solution. Myocardium tissues from risk area (approximately 2 mm in thickness) were removed. Samples were then fixed in 4% paraformaldehyde for 1w, embedded in paraffin wax and cut into 3 μm slices. The cell apoptosis rate in the myocardium was determined by TUNEL according to the manufacturer's instructions. Six micrographs were randomly selected and the numbers of normal or apoptotic cardiomyocytes were counted. The percentage of apoptotic cardiomyocytes was defined as the proportion of TUNEL-positive ones to total cells.

3.3.6 Histology
The heart was excised and washed in saline solution. Myocardium tissues from risk area (approximately 2 mm in thickness) were removed. Samples were then were fixed in 4% paraformaldehyde at room temperature for 1 w and embedded in paraffin for histological examination. Paraffin-embedded tissues were then sectioned into slices 3 μm thick. Sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water and stained
with hematoxylin and eosin (H&E). Images were visualized under an optical microscope at x200 magnification.

3.3.7 Western blot analysis

Myocardium from risk area (infarct margin) was used for Western blotting analysis. Total protein from myocardium tissues were extracted (150 mg/lane as determined by the Bradford method) and separated by 12% SDS-polyacrylamide gels. After electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 40 min. The membranes were then probed with primary antibodies against GRP78, CRT, CHOP, caspase-12, Bcl-2, Bax and GAPDH, respectively (all 1:500 diluted) at 4°C overnight. The antibody-tagged membranes were incubated with a secondary antibody solution consisting of either a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for GRP78, CRT, caspase-12, Bcl-2, Bax and GAPDH). An enhanced chemiluminescent detection system was used for immunoblot detection. An enhanced chemiluminescent detection system was used for immunoblot detection. The optical density of the bands (as measured in arbitrary densitometry units) was normalized against GADPH.

### Table 4.1.1: Effect of PQS on hemodynamics and cTnT level in rats (n=8)

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mmHg)</th>
<th>HR (beat/min)</th>
<th>+dp/dt\text{max} (mmHg)</th>
<th>-dp/dt\text{max} (mmHg)</th>
<th>cTnT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>60.9±3.2</td>
<td>22.6±15.1</td>
<td>1147.5±294.6</td>
<td>1067.6±253.0</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>I-R</td>
<td>94.3±15.2*</td>
<td>441±32.0</td>
<td>652.9±170.3*</td>
<td>620.1±179.0*</td>
<td>1.4±0.6*</td>
</tr>
<tr>
<td>PQS+I-R</td>
<td>64.1±15.0*</td>
<td>445.8±18.5</td>
<td>1069±2988.0*</td>
<td>839.7±151.9*</td>
<td>0.7±0.2*</td>
</tr>
</tbody>
</table>

I-R: ischemia/reperfusion; PQS: *Panax quinquefolium* saponins; *P*<0.05 vs Sham, *P*=0.05 vs I-R

4.1.3 Infarct size

The ischemic area at risk in Sham, I-R and PQS+I-R groups were 49.8±3.4%, 50.2±2.2% and 50.1±1.3% (No significant difference among them, *P*>0.05). Infarct size was 44.2±1.4% in I-R group and 15.3±4.2% in PQS+I-R group (*P*<0.05, vs. I-R).

4.1.4 Cardiomyocyte apoptosis

Apoptosis detected by TUNEL assay was shown in Fig. 4.1.4. The apoptosis rate of cardiomyocytes was 8.2±0.6 % in sham group, 56.9±6.0 % in I-R group (*P*<0.05, vs. I/R). Pretreatment with PQS resulted in a significant decrease in apoptosis rate of cardiomyocytes (54.9%± vs. I-R group, *P*<0.05).

3.4 Statistic analysis

The SAS 8.2 version was adopted for statistical analysis. The data were expressed as mean±SD. For multiple-group comparisons, one-way ANOVA followed by Newman-Keuls post-hoc analysis was performed. *P*<0.05 was considered to be statistically significant.

4. Results

4.1 Effects of PQS on Myocardial I-R injury

4.1.1 Hemodynamics

Hemodynamic parameters are shown in Table 4.1.1. HR was not significantly different among groups (*P*>0.05). The rats in I-R group showed a significant increase in MAP and a decrease in +dp/dt\text{max} and -dp/dt\text{max} compared with the rats in sham group. Pretreatment with PQS significantly reduced the increased MAP by 32% and improved decreased +dp/dt\text{max} and -dp/dt\text{max} by 64% and 35%, respectively (*P*<0.05), compared with the rats in I-R group.

4.1.2 Serum cTnT level

cTnT levels are shown in Table 4.1.1. The cTnT of I-R rats showed a higher level by 2.5-fold than rats in sham group (*P*<0.05). Pretreatment with PQS decreased the cTnT level of the rats subjected to I-R by 50% (*P*<0.05).

4.1.3 Infarct size

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4.1.5 Myocardium histopathologic changes

The histopathological changes in the ischemic heart tissue were assessed by H&E staining, shown in Figure 4.1.5. The rats in sham group showed a normal structure and shape of myocardium. The myocardial fibers were arranged in order. The rats in I-R group showed acute injury characterized by myocardial necrosis, neutrophil infiltration and interstitial edema. The ruptured and lysed myocardial fibers in infarct area were found. The rats in PQS+I-R group revealed a reduced neutrophil infiltration and interstitial edema compared with the rats in I-R group.
**Figure 4.1.4:** Effect of PQS on cardiomyocyte apoptosis. (*P<0.05 vs Sham, #P<0.05 vs I-R; n=3)

**Figure 4.1.5:** Histopathological slides of rat myocardial tissue (A: Sham group; B: ischemia/reperfusion group; C: pretreatment with Panax quinquefolium saponins group; magnification, ×200; n=3)

### 4.1.6 Bcl-2 and Bax expression

Bcl-2 and Bax protein expression were detected with Western blotting. The myocardium of rats with I-R reduced expression of Bcl-2 by 54.9% (P<0.05 vs. sham) and enhanced expression of Bax protein by 290% (P<0.05 vs. sham), while pretreatment with PQS enhanced Bcl-2 expression by 110% and decreased Bax protein expression by 47.8% compared with myocardium of rats with I-R (P<0.05). (Figure 4.1.6).

**Figure 4.1.6:** Panax quinquefolium saponins (PQS) reduced the expression of pro-apoptotic Bax and increased expression of anti-apoptotic Bcl-2 in I-R myocardium. (*P<0.05 vs. sham, **P<0.05 vs. I/R; n=6)

### 4.2 Effects of PQS on Expression of ER Stress Molecules

#### 4.2.1 GRP78 and CRT expression

I-R resulted in a 3.1-fold increase in GRP78 protein expression and a 3-fold increase in CRT protein expression relative to the sham group (P<0.05), while CRT protein was significantly reduced by 43.4% in myocardium of rats pretreated with PQS compared with I-R group (P<0.05). (Figure 4.2.1)
Figure 4.2.1: Effect of *Panax quinquefolium* saponins (PQS) on the expression of ER stress molecular indicators of GRP78 and CRT in I-R myocardium. (*P*<0.05 vs. sham, *P*<0.05 vs. I-R; n=6)

4.2.2 CHOP expression

I-R enhanced protein expression of CHOP, a pro-apoptotic factor, by 240% relative to the sham group (P<0.05), while pretreatment with PQS reduced I–R induced CHOP overexpression by 38.6% (P<0.05) (Figure 4.2.2).

4.2.3 Caspase-12 activity in myocardium

Expression of activated caspase-12 and inactive procaspase-12 were examined with Western blotting. Expression of the procaspase-12 (48-50 kDa) was not significantly different among three groups (P>0.05). The level of the 36 kDa cleaved (activated) caspase-12 was increased by 1.5-fold in I–R-treated myocardium compared with the sham group (P<0.05), while pretreatment with PQS reduced 36 kDa caspase-12 expression by 23.7% compared with I–R rats (P<0.05). (Figure 4.2.2).

Figure 4.2.2: Effect of *Panax quinquefolium* saponins (PQS) on the expression of the ERS-associated apoptotic protein CHOP and activation of the apoptotic effector enzyme caspase-12 in I-R myocardium (*P*<0.05 vs. sham, *P*<0.05 vs. I/R; n=6)

5. Discussion

Ischemia-reperfusion (I–R) injury refers to the progressive and irreversible injury caused by reperfusion after certain period of ischemia (Brawndale E, et al. 1995). Our previous study has shown that PQS alleviates cardiomyocyte hypoxia-reoxygenation injury by inhibiting excessive ERS-related apoptosis. In the present study, pretreatment with PQS showed a remarkable reduction in myocardial infarct size, serum cTnT concentration and improvement in heart function in rats subjected to I-R, suggesting that PQS protects myocardium from I-R injury.

Apoptosis in cardiomyocyte was first reported by Gottlieb et al (RA Gottlieb, et al. 1994). Accumulating evidences suggested that cardiomyocyte apoptosis contributed to the pathogenesis of ischemia-reperfusion injury (Zhao et al., 2002). Thereafter, the studies focusing on the role of ER stress in cardiomyocyte apoptosis indicated that the apoptosis is a dominant of cardiomyocyte death in ischemia-reperfusion (Fliss et al. 1996; Janice, Reeve et al. 2005). Musat-Marcu and his colleagues (Sorin Musat-Marcu, et al. 1999) observed apoptotic cells in rats’ myocardium in vitro at the early stage of reperfusion and demonstrated that the Bcl-2 family, comprised of both pro-apoptotic and anti-apoptotic members, constitutes a critical intracellular checkpoint for apoptosis within a common cell death pathway (Chao et al. 1998). This family includes proteins that predispose cells to apoptosis, such as Bax and proteins that antagonize apoptosis, such
as Bcl-2. The anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax are involved in the regulation of apoptosis during myocardial I–R injury (Borutaite et al. 2003).

In our study, we demonstrated that pretreatment with PQS significantly reduced myocardial I–R injury and apoptosis, and up-regulated Bcl-2 expression. However, pretreatment with PQS only showed a moderate down-regulation of Bax expression, indicating that the PQS’s regulatory effect on Bax expression was lower than that on regulation of Bcl-2 expression, suggesting that PQS protected myocardium from I-R injury mainly by up-regulating Bcl-2 expression.

Oxygen free radicals and calcium overload are critical downstream mediators of I–R injury. Recent studies have focused on mitochondrial pathways leading to cell death, while the role of the endoplasmic reticulum (ER) has not been extensively studied. The ER is a crucial organelle for intracellular Ca^{2+} transfer and protein synthesis, and endoplasmic reticulum stress (ERS) resulting from physical and chemical changes of ER is supposed to be a critical pathogenesis for I–R injury (Shibata et al. 2003; Mario Vilatoba, et al. 2005). The moderate ERS induces up-regulation of ER chaperones, including glucose-regulated protein 78 (GRP78) and calreticulin (CRT) (Kaufman RJ. et al. 1999), which enhance the capacity for processing of unfolded proteins that promote the functional recovery of the ER. However, excessive ERS triggered by I–R leads to over-expression of GRP78 and CRT (Marietta Flores-Diaz, et al. 2004), induced the expression and activation of the pro-apoptotic factors such as CHOP and caspase-12. Our study demonstrated that PQS suppressed I-R-induced ER stress, as shown by a decrease in GRP78 and CRT over-expression. This is consistent with the findings of Liu et al (Liu XH, et al. 2008).

The CHOP protein promotes apoptosis through direct regulation of target genes that increase the sensitivity of cells to ERS-mediated apoptosis (Oyadomari S, et al. 2004; Friedman AD. et al. 1996). The CHOP-mediated apoptosis signaling pathway is closely linked to the mitochondrial apoptosis pathway, by which CHOP can reduce Bcl-2 protein expression, leading to Bax translocation from cytoplasm to mitochondria (Karen D.McCullough, et al. 2001) and activation of the mitochondrial apoptotic pathway. The present study showed that CHOP over-expression in myocardium induced by I–R was significantly inhibited by pretreatment with PQS indicating that PQS could protect myocardium by inhibiting ER-mediated apoptosis pathways.

Pro-caspase-12 is a constitutively expressed protein located on the cytoplasmic side of the ER membrane. The inactive pro-caspase-12zymogens are cleaved and activated by TNF receptor-associated factor 2 (TRAF2) (Yoneda, et al. 2001). Cleaved caspase-12 causes apoptosis through activation of caspase-9 and caspase-3 (Nakagawa et al. 2000). Thus, caspase-12 is a key factor of the ER apoptosis pathway. The present study showed that caspase-12 activation in myocardium induced by I–R was significantly inhibited by pretreatment with PQS indicating that PQS could protect myocardium by inhibiting activation of ER-mediated apoptosis pathways.

This SD rat model with myocardial ischemia-reperfusion has been widely used in previous studies. In the present study, this model was also used to investigate the effects of PQS on the ischemia-reperfusion injury. PQS showed a myocardial protective effect against I–R injury through inhibiting excessive ERS as evidenced by the reduction in GRP78, CRT and CHOP over-expression, as well as caspase-12 activation, which is closely associated with reducing ER-related apoptosis of cardiomyocyte. Further studies are required to determine the downstream signal pathway responsible for the effects.

**Conclusion**

In conclusion, PQS could have the potential to alleviate myocardial injury from I-R which plays an important role on protecting myocardial and the underlying mechanism was likely associated with inhibiting excessive ER stress induced by I-R. Compared with the earlier report, our study is more superior. First of all, the I-R model was modified based on the traditional, we applied reversible ligation of left coronary artery and set the ischemia and reperfusion time according to the serum myocardium injury indicators’ trends; second, PQS’ myocardium protective effect through inhibiting excessive ERS was confirmed for the first time. That will provide a more profound theoretic basis of Chinese medicine on preventing myocardial ischemia/reperfusion injury and PQS therefore appear to be a promising source of useful anti-ischemia agent. However, what is the downstream signal pathway of the protective effects on the
earth and whether PQS has the same effects on clinical research. Which are needed to be explored in the future research. No conflict of interest exits in the submission of this manuscript.

**Author’s Contribution**

Xiu-Hua Liu and Chen Wang contributed equally to this work.

**References**


