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**Original Paper**

# AMPK Contributes to Cardioprotective Effects of Pterostilbene Against Myocardial Ischemia- Reperfusion Injury in Diabetic Rats by Suppressing Cardiac Oxidative Stress and Apoptosis

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**Key Words**
Pterostilbene • Diabetes • Myocardial ischemia-reperfusion • Apoptosis • AMPK

**Abstract**
***Background/Aims:*** Pterostilbene (PT) exerts antidiabetic effects by decreasing blood glucose and modulating lipid metabolism and has been shown to attenuate myocardial ischemia-reperfusion (IR) injury in non-diabetic subjects. However, whether PT can protect against myocardial IR injury in diabetes is unknown. AMPK stimulation is indispensable in offering cardioprotection against myocardial IR injury in diabetes by limiting cardiac apoptosis. Thus, we hypothesized that PT may confer protection against myocardial IR injury in diabetes *via* AMPK activation. ***Methods:*** Sprague-Dawley rats at eight weeks of diabetes induction (induced by an intravenous dose of 65 mg/kg streptozotocin) were administered with vehicle or PT (20 and 40 mg/kg/day, p.o.) for four weeks (starting from week 9 to 12). At the end of week 12, myocardial IR injury was induced by subjecting the diabetic rats to 30 minutes of coronary artery ligation and followed by 2 hours of reperfusion. In *in vitro* studies, rat primary cardiomyocytes were incubated with low glucose (LG, 5.5 mM) or high glucose (HG, 30 mM) and exposed to 45 minutes hypoxia and 2 hours reoxygenation in the presence or absence of PT (0.5 μM) or the AMPK inhibitor compound C (CC, 5 μM). ***Results:*** PT significantly reduced post-ischemic cardiac infarct size, oxidative stress, plasma lactate dehydrogenase (LDH), creatine kinase-MB levels and apoptosis in diabetic rats. In cardiomyocytes, PT decreased hypoxia/reoxygenation-induced oxidative stress, attenuated LDH and cleaved caspase3/caspase3 ratio and increased Bcl-2/Bax ratio and AMPK phosphorylation. However, CC administration blunted

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the cardioprotective effects of PT both *in vivo* and *in vitro*. ***Conclusion:*** Suppressing cardiac oxidative stress and apoptosis *via* AMPK stimulation may represent a primary mechanism whereby pterostilbene attenuates diabetic myocardial IR injury.



## Introduction

Diabetes markedly alters the cardiac gene expression patterns of several metabolic, structural, signal transductions and stress response proteins, leading to the development of cardiac complications. These alterations accelerate the susceptibility of the heart muscle to myocardial ischemia-reperfusion (IR) injury [1, 2], resulting in poor clinical prognosis after myocardial infarction [3]. Moreover, diabetic patients have a higher risk of developing myocardial IR than non-diabetic subjects [4]. Therapeutic agents that alleviate myocardial IR injury in non-diabetic rodent models are mostly ineffective in reducing cardiac IR injury in diabetic animal models [5]. Since conventional antidiabetic therapies have severe limitations due to their adverse effects [6], it is essential to seek efficient antidiabetic drugs with negligible side effects to facilitate the treatment of myocardial IR injury in diabetes.

Pterostilbene (PT) is a naturally occurring dimethylated analogue of resveratrol that is most abundant in blueberries and *Pterocarpus marsupium* heartwood [7, 8]. Recently, PT is drawing increased attention because of its numerous health benefits including anti-oxidative, anti-inflammatory, anti-diabetic, anti-lipidemic, anti-atherosclerotic and infarct-sparing effects [9]. Furthermore, cardioprotective effects of PT centers around its suppressive effects on oxidative stress, apoptosis, and inflammation [10], which attenuates myocardial IR injury in non-diabetic rats. In a recent clinical trial, administration of PT at the daily doses of 100 mg to 250 mg for 6-8 weeks did not produce any significant adverse drug events in hyperlipidemic patients [11]. Moreover, daily administration of *Pterocarpus marsupium* extract at the dose of 450 mg in healthy volunteers resulted in detectable PT in the serum and did not produce any signs of toxicity [12]. Thus, the ultra-high safety profile of PT, coupled with its broad spectrum activities stimulated the interest to consider it as an attractive therapeutic candidate against myocardial IR injury in diabetes.

Adenosine monophosphate-activated protein kinase (AMPK) has emerged as a master regulator of metabolic energy, and is a cellular adaptive mechanism activated during metabolic stress to boost energy production and so that to salvage the failing myocardium [13, 14] and myocardial IR injury [15]. Of note, AMPK activation has been verified to confer cardioprotection against myocardial IR injury in diabetes by limiting cardiac apoptosis through attenuation of endoplasmic reticulum stress [14, 16]. Intriguingly, several AMPK activators such as metformin [17], trimetazidine [18], rosiglitazone [19] have been demonstrated to attenuate diabetic myocardial IR injury *via* AMPK stimulation. However, the serious adverse effects like hypoglycemia, lactic acidosis and gastrointestinal disturbances associated with these drugs limited their therapeutic utility in diabetic patients [6]. Recent studies have shown that PT decreases lipogenesis and fat accumulation, promotes macroautophagy and inhibits apoptosis *via* AMPK stimulation in the myocardial tissues of non-diabetic subjects [20-22]. Also, resveratrol (a metabolite of PT) has been extensively studied for its beneficial effects against diabetic myocardial IR through AMPK signaling [23]. A study in AMPK-knockout mice confirmed that AMPK is the central target for metabolic effects of resveratrol [24]. Considering that PT is a parent compound of resveratrol with superior bioavailability and stronger potency [8], high safety profile [11], it is reasonable to hypothesize that PT can also exert a protective effect against diabetic myocardial IR injury *via* AMPK stimulation.

Therefore, the present study aimed to determine whether PT can attenuate myocardial IR injury in streptozotocin-induced diabetic rats and if so, to investigate whether PT protects against myocardial IR injury in diabetes by stimulating AMPK signaling pathway.

## Materials and Methods

*Animals and diabetes induction*

The present study was performed in adherence to the principles of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication no. 86–23, revised 1996), and experimental protocols were approved by the Institutional Animal Care and Use Committee of Hong Kong University. Adult male Sprague-Dawley rats weighing 220-270 g, age-matched were selected for the study. All animals were maintained at 25°C under a 12 hour/12 hour light-dark cycle and allowed free access to food and water.

Diabetogenic streptozotocin is commonly administered through intraperitoneal and intravenous routes in rodents [25, 26]. Intraperitoneal route has been regarded as the rapid and regular mode of administration, particularly for induction of diabetes through multiple doses of streptozotocin. However, accidental administration of streptozotocin into the bowel or sub-dermal space may lead to decreased diabetogenic effect or enhanced moribundity. Additionally, we and others demonstrated that intravenous administration of streptozotocin (65 mg/kg i.v.) generates a more stable and reproducible model of type 1 diabetes than intraperitoneal administration [1, 26, 27]. Therefore, we determined to employ intravenous delivery of streptozotocin for our *in vivo* studies.

Rats were injected with 65 mg/kg dose of streptozotocin (Sigma, USA), intravenously through the tail vein, for one time. Three days after streptozotocin injection, blood glucose was measured through the tail tip cut method by using a glucose meter (OneTouch Ultra). Only those animals having fasting blood glucose level ≥16.7mM were considered as diabetic (D) and were maintained on the standard diet for eight weeks. Pterostilbene (PT) is a generous gift sample from Sami Labs Limited, Bangalore, India.

*Experimental protocol*

Rats were anaesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg). The myocardial ischemia (30 minutes) was introduced by making a slipknot (6-0 silk) around the left anterior descending coronary artery. After ischemia, the slipknot was unfastened, and the myocardium was reperfused for 2 hours. Sham-operated rats experienced the similar surgical procedures except for coronary slipknot.

The oral dose range of 20 and 40 mg/kg (approximately equivalent to one and two times of the human equivalent dose) was chosen based on previous efficacy studies in rats [28, 29] and in a recent clinical trial where 250 mg of PT demonstrated an anti-hypertensive effect [11]. Presume that the average body surface area of an adult human is 1.8 $m^2$, the dose of PT validated in the clinical trial was 138.9 mg/$m^2$. Furthermore, it seems safe to consider it non-toxic since the oral administration of 250 mg daily for 6-8 weeks in humans [30] and an ultra-high chronic dose of PT (3 g/kg, 28 days) [31] did not cause any notable side effects. Therefore, clinically appropriate doses of 20 and 40 mg/kg in rats (equivalent to 120 and 240 mg/$m^2$) were selected to investigate the impact of dose escalation on myocardial IR injury in diabetic rats.

After eight weeks, the diabetic animals were orally gavaged with either sodium methyl cellulose or PT at doses of 20 and 40 mg/kg body weight per day and designated as PT 20 and PT 40 for four weeks, respectively. Four weeks after PT treatment, myocardial IR (30 minutes ischemia/2 hours reperfusion) was employed [32]. There were four groups (n=8/group), namely, 1) D sham group (receiving vehicle); 2) D + IR group (receiving vehicle); 3) D + IR + Pterostilbene 20 mg/kg/d (PT 20) group; 4) D + IR + Pterostilbene 40 mg/kg/d (PT 40) group.

*Determination of cardiomyocyte cross-sectional area and myocardial infarct size*

At the end of the study, cardiomyocytes cross-sectional area was assessed in paraffin-embedded sections of left ventricles (5 μm) stained with hematoxylin-eosin (H-E) as previously described [33]. A minimum of 150 cells per rat were selected for the measurement of cardiomyocytes cross-sectional area using ImageJ software (downloaded from NIH website). Upon completion of reperfusion, coronary Slipknot was retied, and 0.5 ml of 2% Evans blue dye was injected into the jugular vein. Hearts were rapidly excised, and infarct size was measured using 1% 2, 3, 5-triphenyltetrazolium chloride (TTC) staining as described [34]. Infarct size was represented as a percentage of the area at risk.

*Measurement of plasma creatine kinase-MB and free 8-isoprostane levels*

Plasma creatine kinase-MB (CK-MB) and free 8-isoprostane are considered as cardiac damage and oxidative stress markers, respectively. After 2 hours reperfusion, blood samples (1 ml) were collected in heparinized Eppendorf tubes and plasma was separated. Plasma CK-MB and free 8-isoprostane levels were measured using related enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's protocol. The values of CK-MB and free 8-isoprostane were expressed as U/L and pg/ml in plasma, respectively.

*Primary adult rat cardiomyocytes culture and simulated hypoxia/reoxygenation (HR)*

Primary adult rat cardiomyocytes were isolated from four weeks old Sprague-Dawley rats and cultured as previously described [35]. We employed high glucose (HG, 30mM in culture medium) to mimic *in vivo* diabetes since 30 mM glucose concentration represents the reported peak levels of blood glucose achieved in noncontrolled diabetes [36]. The cultured cells were treated with HG (30mM glucose) for 48 hours, followed by challenge of hypoxia-reoxygenation (HR). Hypoxia was established by equilibrating a humidified chamber (37°C) containing cardiomyocytes with a gas mixture of 5% $CO_2$, 95% $N_2$ and 0.1% $O_2$ with the help of gas transfusion apparatus (BioSpherix, Redfield, NY, USA) and lasted for 45 minutes. Then, the cardiomyocytes were transferred to a $CO_2$ incubator (5% $CO_2$, 95% $O_2$) for 2 hours to achieve reoxygenation. Cells and medium were collected after HR and preserved at -80° C until analysis.

*Determination of cardiac cell viability*

Cardiac cell viability was determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. Primary cardiomyocytes were dispersed into a 96-well plate (density 1400 cells/well) and treated with PT at different concentrations (0.1, 0.5, 1 μM). At the end of the experimental treatment, MTT (5 mg/ml) was added to each well and the plates were incubated at 37°C for 4 hours in a humidified chamber. The absorbance of blue formazan derivative was calculated at 590 nm *via* a microplate reader (Bio-Rad Laboratories, CA, USA) and the assays were performed in duplicate.

*Determination of LDH levels in diabetic rats and primarily cultured rat cardiomyocytes*

For *in vivo* studies, blood was collected from experimental groups, and plasma was separated and analyzed for lactate dehydrogenase (LDH) levels. For *in vitro* studies, forty-six microliters of culture medium from primary cardiomyocytes post HR treatment was determined for LDH release by spectrophotometry *via* commercial assay kit (UV-120-02, Shanghai, China), according to the manufacturer's protocol. LDH release was expressed as the percentage of total cell LDH activity. All readings were measured in duplicate.

*Determination of apoptotic cell death in post-ischemic diabetic hearts and primary rat cardiomyocytes*

Terminal deoxynucleotidyl nick-end labelling (TUNEL) assay was employed to detect myocardial apoptotic cell death according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA). For *in vivo* studies, 5 μm thick paraffin embedded left ventricular tissue sections were deparaffinized and subsequently, the slides were permeabilized with proteinase K (30 mg/ml) for 30 minutes at 37°C. For *in vitro* studies, primary cardiomyocytes were fixed and permeabilized using acetone and 0.1% Triton-X, respectively. The slides were washed by phosphate buffered saline (pH 7.4) for three times and incubated with TUNEL reaction mixture for one hour at 37°C in the dark. To identify the nuclei of cardiomyocytes, DAPI was added to the cells and incubated for 2 min at room temperature, and observed with a fluorescence microscope. The percentage of apoptotic cardiomyocytes or apoptotic index was calculated by the ratio of the number of TUNEL-stained cardiomyocytes to the total number of DAPI-stained cardiomyocytes in a given field of observation.

*Determination of reactive oxygen species (ROS)*

ROS production was measured based on the oxidation of dihydroethidium (DHE, a superoxide indicator) to ethidium. Briefly, primary cardiomyocytes were placed in a six-well plate and were allowed to undergo HR treatment in the presence or absence of PT or the AMPK inhibitor (CC). After treatment, cells were incubated with 10 μM DHE for 30 min at 37°C in the dark. Oxidized DHE intercalates within the cell's DNA and stains the nucleus with a bright fluorescent red. Images were acquired using a fluorescence microscope (Olympus IX51).

*Western blot analysis*

Heart tissue or primary rat cardiomyocytes were homogenized and lysed in lysis buffer on ice for 30 min. Then, the lysates were centrifuged at 12, 000 × g, 4°C, 15 min (ThermoScientific™MicroClick 24 × 2 microtube rotor). The supernatant was collected, and protein concentration was quantified by Bradford protein assay kit (Bio-Rad, CA, USA). Equal samples (40 μg of total protein) were loaded onto and separated by 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Millipore, USA) by electrophoretic transfer system (Bio-Rad). The membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature and then incubated with primary antibody including Bax, Bcl-2, caspase3, cleaved caspase3, AMPK, phosphorylated AMPK, GAPDH, β-actin (Cell Signaling Technology, USA) (1:1000) overnight at 4°C. After three times of washing with TBST (15 minutes each), the membranes were incubated with secondary antibody conjugated to horseradish peroxidase for 2 hours at room temperature, then washed as earlier. The protein bands were detected by an enhanced chemiluminescent system, and the bands were scanned and measured by densitometric analysis using ImageJ software (downloaded from NIH website).

*Statistical analysis*

All values are represented as mean ± standard error mean (SEM). Statistical differences were evaluated by One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons and Repeated measures Two-way ANOVA followed by Bonferroni post-test using GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, CA). P values less than 0.05 was considered statistically significant.

## Results

*General characteristics*

As shown in Table 1, four weeks of PT treatment at both doses (20 and 40 mg/kg) significantly decreased plasma glucose levels (all $P<0.001$ vs D+IR), increased heart weight (all $P<0.001$ vs D+IR) and body weight (all $P<0.05$ vs. D+IR) when compared to diabetic rats without treatment. Intriguingly, PT (at either 20 or 40 mg/kg) significantly reduced the ratio of heart weight to body weight (an indirect indicator of myocardial hypertrophy) in diabetic rats (all $P<0.001$ vs D+IR). In line with this, PT (20 and 40 mg/kg) significantly (all $P<0.001$) decreased the diabetes-induced alteration of cardiomyocyte cross-sectional area in diabetic rats (Fig. 1A), indicating that PT can attenuate cardiac hypertrophy in diabetes.

*PT reduced post-ischemic myocardial injury in diabetic rats*

To examine whether PT decreased IR-induced myocardial apoptotic cell death, myocardial infarct size, area at risk, plasma CK-MB, LDH release, free 8-isoprostane and myocardial apoptotic index after 2-hour of reperfusion in different groups were further measured. TTC staining was employed to demonstrate infarct size (Fig. 1B). Myocardial infarct size was markedly increased in D+IR group when compared with the D group (48.60±2.75% vs 0.9±0.02%; n=8; $P<0.001$). In contrast, PT treatment significantly reduced

**Table 1.** General characteristics during the study period. Plasma glucose and body weight were measured after the completion of the treatment period. All values are represented as mean ± standard error mean (SEM). n=8/group. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. $^{*}P<0.05$, $^{***}P<0.001$ vs. D+IR; $^{\#\#\#}P<0.001$ vs. D+IR+PT20 group

| General characteristics | D | D+IR | D+IR+PT20 | D+IR+PT40 |
|---|---|---|---|---|
| Plasma glucose (mM) | 23.24±0.06 | 22.60±0.24 | 15.68±0.08*** | 17.98±0.21***### |
| Heart weight (g) | 1.18±0.01 | 1.20±0.01 | 1.38±0.02*** | 1.35±0.02*** |
| Body weight (g) | 238.75±10.14 | 250.25±12.44 | 347.50±24.39* | 336.00±14.30* |
| Heart weight/body weight (mg/g) | 4.90±0.01 | 4.85±0.01 | 3.98±0.02*** | 4.01±0.03*** |

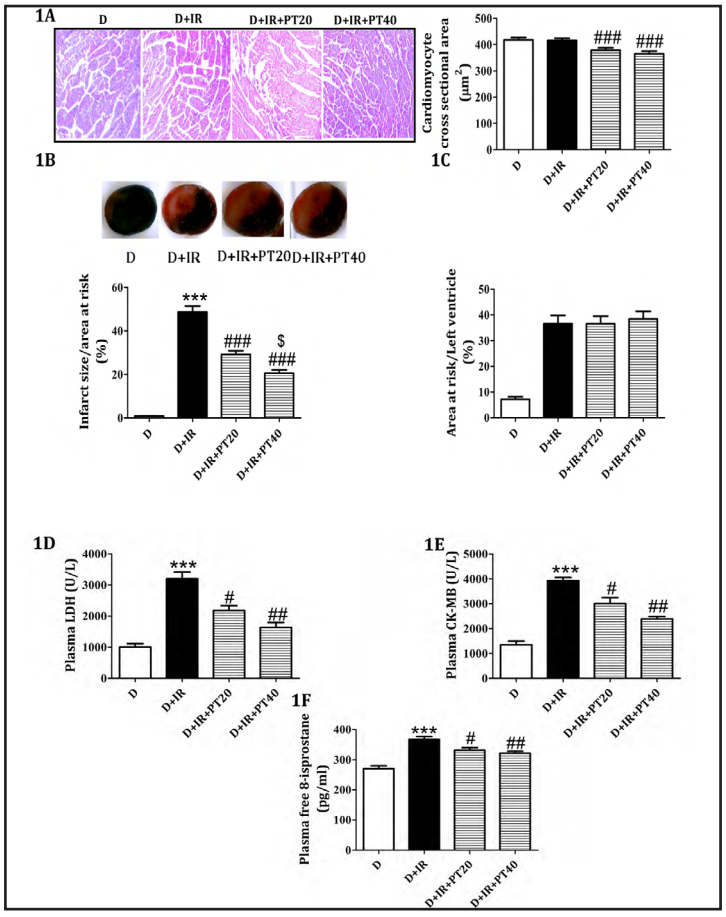


**Fig. 1.** PT reduced myocardial IR injury in diabetic rats (infarct size, necrosis, and oxidative stress). (A) Cardiomyocytes cross sectional area at 8 weeks post-treatment by H-E staining. (B) Myocardial infarct size in diabetic rats subjected to 30 minutes ischemia, followed by 2 hours reperfusion. Blue-staining demonstrates live& non-ischemic area, red-staining demonstrates the area at risk, and pale area demonstrates infracted area. Infarct size expressed as percentage of area at risk. (C) Area at risk is expressed as percentage of left ventricle (D) Plasma lactate dehydrogenase (LDH) levels. (E) Plasma creatine kinase-MB (CK-MB) levels. (F) Plasma free 8-isoprostane levels. All values are presented as mean ± SEM. n=8/group. $^{***}P<0.001$ vs. D, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. D+IR, $^{\$}P<0.05$ vs. D+IR+PT 20. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test.

myocardial infarct size, at both doses (20 and 40 mg/kg) (29.08±1.84%, 20.63±1.37% vs. 53.76±2.17%, respectively; n=8; all $P<0.001$). However, the infarct-limiting effect of PT 40 was better than that of PT 20 in diabetic rats since the post-ischemic myocardial infarct size in PT 40 group was significantly smaller than that in the PT 20 group ($P<0.05$). No significant difference in the ratio of the area at risk to left ventricle was observed among D+IR, D+IR+PT 20 and D+IR+PT 40 groups ($P>0.05$, Fig. 1C).

As shown in Fig. 1D, 1E, plasma levels of LDH and CK-MB were significantly increased in D+IR group when compared with D group. After two hours (2h) of reperfusion, treatment with PT at dosages 20 and 40 mg/kg significantly attenuated the post-ischemic levels of LDH ($P<0.05$ and $P<0.01$) and CK-MB ($P<0.05$ and $P<0.01$) in diabetic rats, in a dose-dependent manner when compared with the D+IR group.

After 2h of post-ischemic reperfusion, plasma free 8-isoprostane level was markedly higher in the D+IR than that in the D group (Fig. 1F, 367.20±9.45 vs 270.5±9.76; n=8; $P<0.001$). PT (20 mg/kg and 40 mg/kg) significantly reduced the IR-induced elevations in plasma free 8-isoprostane levels when compared to D+IR group (331.70±9.45; n=8; $P<0.05$ and 321.60±6.56; n=8; $P<0.01$, respectively).

The apoptotic index was significantly higher in the D+IR group when compared to the D group (Fig. 2A, 11.20±0.67% vs 2.77±0.38; n=6; $P<0.001$), which was markedly attenuated by treatments with employed doses of PT (20 mg/kg, 7.53±.39%; n=6; $P<0.01$ vs. D+IR; d PT 40 mg/kg, 4.93±0.44%; n=6; $P<0.001$ vs. D+IR). Taken together, these data suggested that both doses of PT (20 and 40 mg/kg/d) could protect diabetic hearts against IR-induced cell necrosis, oxidative stress, and apoptosis.

Next, the effect of PT on IR-induced cardiac apoptosis in diabetic rats was further assessed by measuring the changes of pro- and anti-apoptotic proteins. Myocardial IR injury decreased Bcl-2/Bax ratio ($P<0.001$, Fig. 2B) and increased cleaved caspase3/caspase3

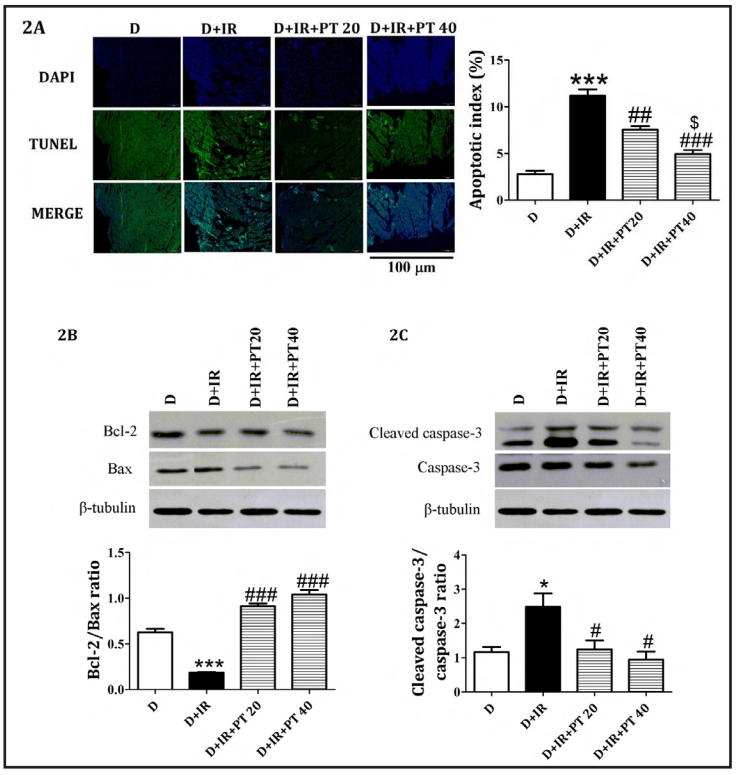


**Fig. 2.** PT decreased cardiac apoptosis in diabetic rats. (A) Terminal deoxynucleotidyl nick-end labeling (TUNEL) staining in myocardial IR heart tissue from the experimental groups. Green fluorescence indicates TUNEL-positive cardiomyocytes; blue fluorescence indicates nuclei of total primary cardiomyocytes. Apoptotic index was depicted as histogram. (B) Bcl-2/Bax ratio (C) cleaved caspase-3/caspase-3 ratio. All values are presented as mean ± SEM. n=6/group. $^{*}P<0.05$, $^{***}P<0.001$ vs. D, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. D+IR, $^{\$}P<0.05$ vs. D+IR+PT 20. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test.

ratio ($P<0.05$, Fig. 2C) when compared to sham-operated diabetic rats. PT at two doses significantly reversed the altered ratios of Bcl-2/Bax (all $P<0.001$) and cleaved caspase-3/caspase-3 (all $P<0.05$) when compared to D+IR group. These results demonstrated that PT decreased IR-induced myocardial apoptosis in diabetic rats.

*PT treatment increased phosphorylation of myocardial AMPK in diabetic rats*

We asked whether PT exerts cardioprotective effects *via* AMPK stimulation. As shown in Fig. 3, despite no change in total protein levels of AMPK in all treatment groups, p-AMPK level was significantly increased in the D+IR group when compared to the D group ($P<0.05$). As anticipated, PT treatment at both doses significantly further increased p-AMPK levels when compared to D+IR group ($P<0.01$, PT dose 20 mg/kg vs. D+IR; $P<0.05$, PT dose 20 mg/kg vs. D+IR), respectively. A slight decrease in p-AMPK levels was observed in PT 40 group when compared to PT 20 group, however, this difference did not reach statistical significance ($P>0.05$).

*PT enhanced the viability of cardiomyocytes exposed to hypoxia-reoxygenation under high glucose condition*

To investigate the underlying mechanisms of cardioprotective effects of PT on myocardial IR injury in diabetic rats, we performed *in vitro* studies in adult rat primary cardiomyocytes challenged with 45 minutes hypoxia and 2 hours reoxygenation under high glucose (HG) condition.

Primary cardiomyocytes were exposed to low glucose (LG) and varying concentrations of PT (0.1, 0.5, 1 μM) at the onset of re-oxygenation to determine the effective concentration of PT. Cell viability and LDH release, indices of primary cardiomyocytes injury, were measured by MTT and LDH assay, respectively. As shown in Fig. 4A and 4B, after being challenged with HR, cell viability in LG+HR group was significantly reduced to 66.00%±3.00%, and LDH increased to 157.60%±6.80% when compared to LG (n=6; all $P<0.001$). PT (0.1 and 0.5 μM) markedly reduced HR-induced cell death, increasing viability rate to 76.40%±1.21% (n=6; $P<0.05$) and 84.78%±3.11% (n=6; $P<0.01$) and decreasing LDH release to 139.20%±1.93%

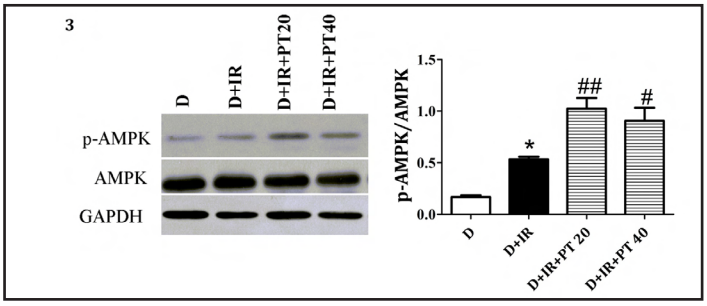


**Fig. 3.** PT treatment increases phosphorylation of AMPK in heart tissues of diabetic rats. All values are presented as mean ± SEM. n=6/group. *P<0.05 vs. D, #P<0.05, ##P<0.01 vs. D+IR. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test.

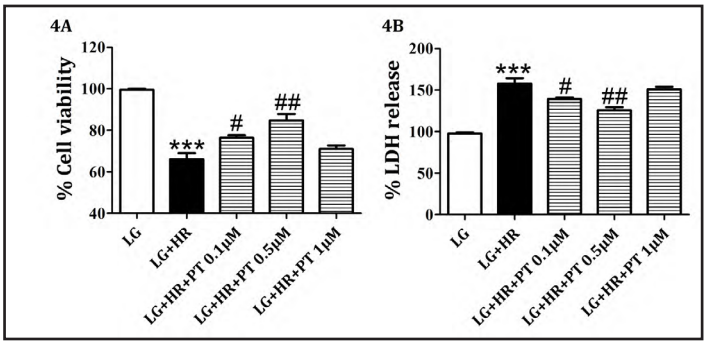


**Fig. 4.** Dose selection of PT for in-vitro cell line studies based on MTT and LDH assays. Primary cardiomyocytes exposed to low glucose (LG) or PT were challenged with hypoxia-reoxygenation (HR). Cell viability and LDH release were measured by MTT and LDH assay, respectively. (A) Cell viability in primary cells administered with PT (0.1, 0.5, 1 μM) during HR. (B) LDH assay in primary cells administered with PT (0.1, 0.5, 1 μM) during HR. ***P<0.001 vs. LG, #P<0.05, ##P<0.01 vs. LG+HR. Results demonstrated as mean ± SEM, n=6/group. Differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test.

(n=6; *P*<0.05) and 125.60%±3.78% (n=6; *P*<0.01), respectively. By contrast, PT at 1 μM failed to show protection against HR induced cell death. Also, DMSO did not affect HR induced cell injury. Taken together, these results indicated that PT markedly preserved post-hypoxic cell viability and attenuated injury at the concentrations of 0.1 and 0.5 μM, and the highest cellular viability was observed at 0.5 μM concentration of PT.

We then examined the effect of PT (0.5 μM) on primary rat cardiomyocytes exposed to LG+HR and HG+HR in the presence or absence of the AMPK inhibitor, Compound C (CC, 5 μM, administered one hour before HR stimulation). The rat primary cardiomyocytes were randomly divided into the following groups: (1) LG (5 mM glucose, Control); (2) LG+HR; (3) LG+HR+PT; (4) LG+HR+CC (5) LG+HR+PT +CC; (6) HG (30mM glucose)(hyperglycemic control); (7) HG+HR; (8) HG+HR+PT; (9) HG+HR+CC (10) HG+HR+ PT +CC. As shown in Fig. 5A, 5B, HR treatment significantly decreased cell viability (69.82%±3.20% vs. 100.4%±4.13%, n=8, *P*<0.001 vs. LG; and 49.26%±3.08% vs. 78.82%, n=8, *P*<0.001 vs HG, respectively) and increased LDH levels (35.10%±3.74% vs. 14.80%±2.70%, n=8, *P*<0.001 vs. LG; and 52.17%±3.25% vs 26.97%±2.20%, n=8, *P*<0.001 vs. HG, respectively). PT treatment significantly reversed the LG+HR-induced and HG+HR-induced damage to cardiomyocytes, as evidenced by significantly increased cell viability (88.63%±3.38%, n=8, *P*<0.01 vs LG+HR; and 69.33%±5.03%, n=8, *P*<0.01 vs HG+HR) and decreased LDH levels (35.10%±3.74%, n=8, *P*<0.01 vs LG+HR; and 52.17%±3.25%, n=8, *P*<0.001 vs HG+HR). Whereas CC treatment significantly decreased the cell viability (55.06%±2.21%, n=8, *P*<0.05 vs LG+HR; and 34.87%±2.16%, n=8, *P*<0.05 vs HG+HR) and markedly increased LDH levels (46.45%±2.1%, n=8, *P*<0.05 vs LG+HR; and 64.23%±2.2%, n=8, *P*<0.05 vs. HG+HR). In addition, the effect of PT on HR-induced cardiac damage in both LG and HG conditions was abrogated in the presence of the AMPK inhibitor CC.

Oxidative stress plays a crucial role in the pathogenesis of ischemic heart diseases in the context of diabetes. In the present study, we determined HR- and HG (diabetes)-induced myocardial $O_2^-$ production in primary cardiomyocytes by DHE staining (Fig. 5C). HR

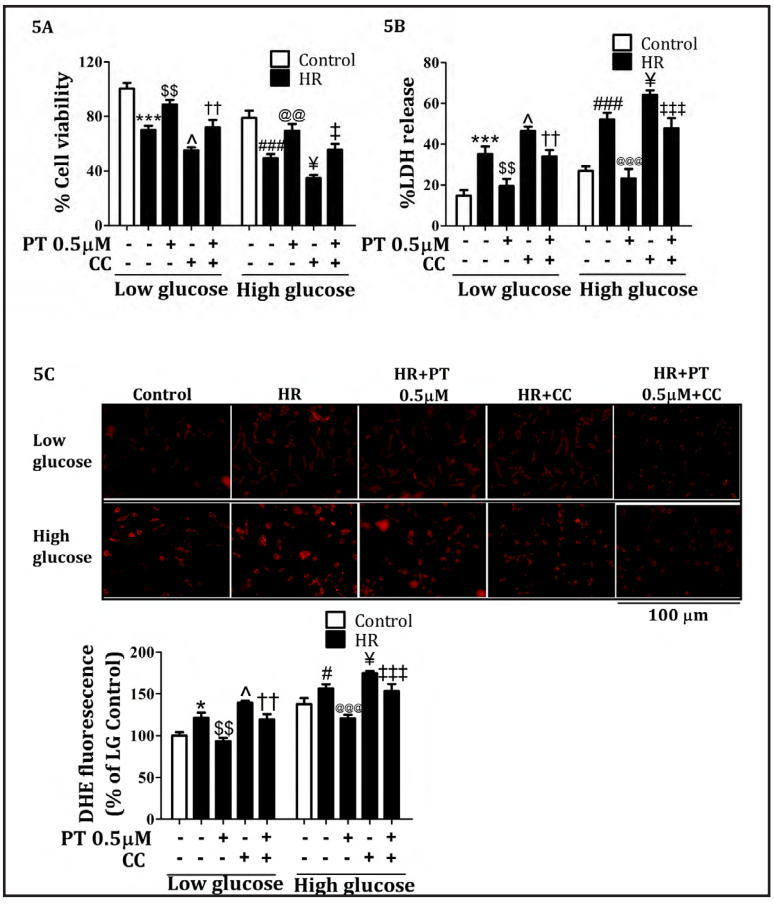


**Fig. 5.** PT ameliorated HR-induced injury in primary rat cardiomyocytes (cell viability, LDH release and oxidative stress) under normal and diabetic conditions. (A) Cell viability in different treatment groups. (B) LDH release in different treatment groups. (C) Oxidative stress determined by DHE staining. Results were expressed as fold of LG (Control) and demonstrated as mean ± SEM. n=8/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{*}$P<0.05, $^{***}$P<0.001 vs. LG, $^{\$\$}$P<0.01 vs. LG+HR, $^{\wedge}$P<0.05 vs. LG+HR, $^{\dagger\dagger}$P<0.01 vs. LG+HR+PT 0.5μM, $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. HG; $^{@@}$P<0.01, $^{@@@}$P<0.001 vs. HG+HR, $^{¥}$P<0.05 vs. HG+HR, $^{\ddagger}$P<0.05, $^{\ddagger\ddagger\ddagger}$P<0.001 vs. HG+HR+PT 0.5μM.

significantly increased the number of positive DHE-stained cardiomyocytes under normal and diabetic conditions (all $P$<0.05 vs LG and HG). PT (0.5 μM) treatment significantly attenuated the LG+HR- and the HG+HR-induced increase of positive DHE-stained cells ($P$<0.01 LG+HR+PT 0.5μM vs LG+HR; and $P$<0.001 HG+HR+PT 0.5μM vs HG+HR), while CC treatment significantly ($P$<0.05 LG+HR+CC vs LG+HR; and $P$<0.05 HG+HR+CC vs. HG+HR) enhanced DHE-stained cardiomyocytes. Furthermore, co-administration of CC reversed the PT's suppressive effect on myocardial $O_2^-$ production ($P$<0.01 LG+HR+PT 0.5μM+CC vs. LG+HR+PT 0.5μM; and $P$<0.001 HG+HR+PT 0.5μM+CC vs. HG+HR+PT 0.5μM).

*PT increased phosphorylation of AMPK in primary cardiomyocytes subjected to HG+HR*

To further explore the molecular mechanism underlying PT-mediated cardioprotection, we determined p-AMPK/AMPK expression in primary cardiomyocytes subjected to HG+HR. There was no significant difference in total protein levels of AMPK between treatment groups at baseline (Fig. 6). HR treatment significantly ($P$<0.05 vs. LG and HG) enhanced the p-AMPK levels under normal and diabetic conditions. However, PT (0.5 μM) treatment further enhanced the post-hypoxic p-AMPK and significantly increased p-AMPK/AMPK ratio (all $P$<0.001 vs. LG+HR or HG+HR), whereas CC treatment significantly diminished it (all $P$<0.001 vs. LG+HR or HG+HR). Pretreatment with CC significantly blocked PT-mediated phosphorylation of AMPK (all $P$<0.001 vs. LG+HR+PT 0.5 μM or HG+HR+PT 0.5 μM).

*PT modulated apoptosis in primary cardiomyocytes subjected to HG+HR*

Cellular apoptosis was determined by TUNEL staining (Fig. 7A). HR significantly increased the TUNEL positive cells and apoptotic index under normal glucose and high glucose conditions (all $P$<0.001 vs LG and HG). PT (0.5 μM) treatment significantly attenuated HR-induced apoptotic index in both control and diabetic/high glucose conditions ($P$<0.001

LG+HR+PT 0.5μM vs. LG+HR; and *P*<0.001 HG+HR+PT 0.5μM vs. HG+HR), whereas CC treatment further exacerbated post-hypoxic apoptosis (*P*<0.001 LG+HR+CC vs LG+HR; and P<0.05 HG+HR+CC vs HG+HR). Furthermore, co-administration with CC reversed the suppressive effect of PT on cardiomyocyte apoptosis (all *P*<0.001). These results suggested that PT had a direct cardioprotective impact on HR-induced cardiac apoptosis via AMPK activation in both normal and diabetic condition.

Next, we determined whether PT conferred protection against HR-induced apoptosis in primary cardiomyocytes by modulating proteins of the Bcl-2 family. HR significantly downregulated Bcl-2 (an anti-apoptotic protein) expression, upregulated Bax (a pro-apoptotic protein) expression, and eventually, decreased the Bcl-2/Bax ratio under normal and diabetic condition (all *P*<0.001 vs LG and HG) (Fig. 7B). Pretreatment with PT (0.5 μM) significantly increased Bcl-2/Bax ratio (*P*<0.001 LG+HR+PT 0.5μM vs. LG+HR; and P<0.001 HG+HR+PT 0.5μM vs. HG+HR), while treatment with CC decreased Bcl-2/Bax ratio (*P*<0.001 LG+HR+CC vs. LG+HR; and *P*<0.05 HG+HR+CC vs. HG+HR). Furthermore, co-administration of CC prevented PT-induced increase of Bcl-2/Bax ratio in cardiomyocytes under normal (*P*<0.001 LG+HR+PT 0.5 μM+CC vs LG+HR+PT 0.5 μM) and diabetic (*P*<0.001 HG+HR+PT 0.5 μM+CC vs HG+HR+PT 0.5 μM) condition.

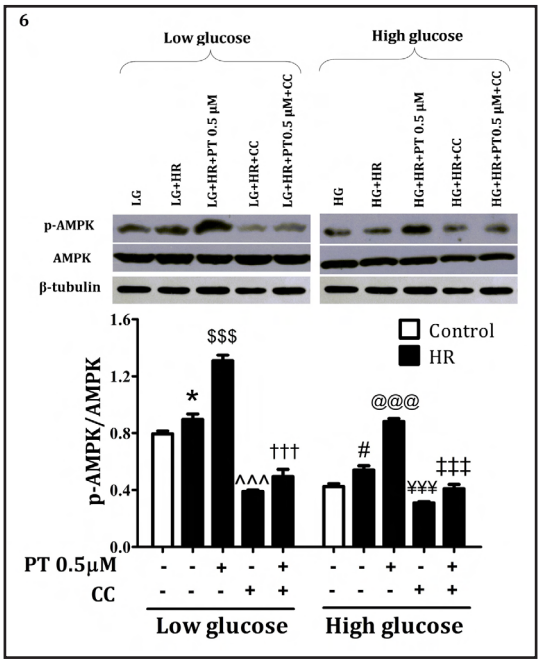


**Fig. 6.** PT activates AMPK in rat primary cardiomyocytes. Results were expressed as mean ± SEM, n=6/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. *P<0.05 vs. LG, $$$P<0.001 vs. LG+HR, ^^^P<0.001 vs. LG+HR, †††P<0.001 vs. LG+HR+PT 0.5μM, #P<0.05 vs. HG; @@@P<0.001 vs. HG+HR, ¥¥¥P<0.001 vs. HG+HR, ‡‡‡P<0.001 vs. HG+HR+PT 0.5μM.

Caspases regulate myocardial apoptosis, and caspase-3 is regarded as the final executioner of the apoptotic process. HR significantly enhanced cleaved caspase-3/caspase-3 ratio (all *P*<0.001 vs LG and HG, Fig. 7C). PT (0.5 μM) treatment significantly attenuated the cleaved caspase-3/caspase-3 ratio (*P*<0.001 vs LG+HR; and *P*<0.001 vs HG+HR), while CC treatment markedly enhanced the cleaved caspase-3/caspase-3 ratio (*P*<0.05 vs LG+HR; and *P*<0.01 vs HG+HR). However, co-treatment with CC inhibited PT-mediated decrease of the cleaved caspase-3/caspase-3 ratio (all *P*<0.001 vs. LG+HR+PT 0.5 μM and HG+HR+PT 0.5 μM).

## Discussion

In this study, we have made the following novel findings: 1) four weeks treatment with PT attenuated myocardial IR injury in rats with streptozotocin-induced diabetes (evidenced by reduced infarct size, LDH, CK-MB, free 8-isoprostane and cardiac apoptosis after myocardial IR), and decreased *in vitro* HR injury in primarily cultured rat cardiomyocytes incubated with HG (evidenced by preserved cardiomyocytes viability and decreased LDH, oxidative stress and apoptotic index). 2) AMPK activation is essential for the anti-oxidative, anti-apoptotic action of PT as evidenced by the finding that, compound C, an inhibitor of AMPK, blunted the protective effects of PT against myocardial IR injury in diabetes.

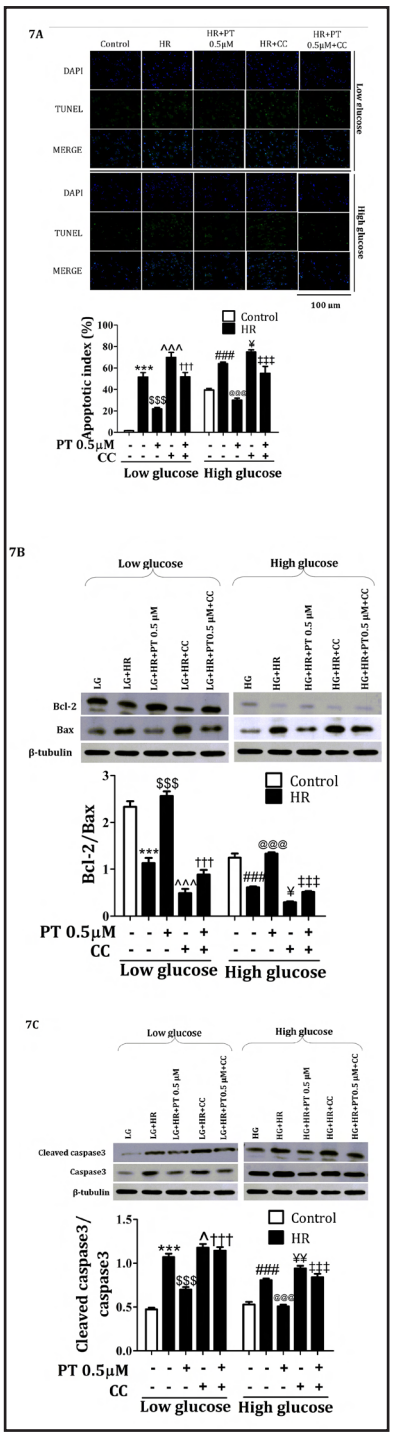


**Fig. 7.** Pterostilbene inhibits cardiac apoptosis in primary cardiomyocytes under normal and diabetic conditions. (A) HR-induced apoptosis as determined by TUNEL staining. (B) Bcl-2/Bax ratio (C) Cleaved caspase-3/caspase-3 ratio. Results were expressed as fold of LG (Control) and demonstrated as mean ± SEM, n=6/group. Differences were evaluated by Repeated measures Two-way ANOVA followed by Bonferroni post-test. $^{***}$P<0.001 vs. LG, $^{\$\$\$}$P<0.001 vs. LG+HR, $^{\wedge}$P<0.05, $^{\wedge\wedge\wedge}$P<0.001 vs. LG+HR, $^{\dagger\dagger\dagger}$P<0.001 vs. LG+HR+PT 0.5μM, $^{\#\#\#}$P<0.001 vs. HG; $^{@@@}$P<0.001 vs. HG+HR, $^{¥}$P<0.05, $^{¥¥}$P<0.01 vs. HG+HR, $^{\ddagger\ddagger\ddagger}$P<0.001 vs. HG+HR+PT 0.5μM.

Oxidative stress occurs as a consequence of enhanced generation of reactive oxygen and nitrogen species and poor antioxidant enzyme defense, and is responsible for cardiac remodeling in diabetic hearts after myocardial IR injury [37]. Prostaglandin isoprostane isomers are identified as a new class of oxidative stress markers and produced mostly from oxidative alterations of phospholipids *via* a free radical catalyzed mechanism [38]. Of these, 8-isoprostane is regarded as the sensitive quantitative measurement of the myocardial oxidative stress *in vivo* [39]. Smith et al. reported that enhanced level of myocardial 8-isoprostane, oxidized glutathione in the diabetic heart after myocardial infarction is coupled with the increased functional severity of heart failure [40]. Similarly, elevated level of myocardial 8-isoprostane is correlated with depressed indices of left ventricular hemodynamic function and decreased cardiac proteome levels of antioxidant defense and apoptotic resistance in rats with diabetic cardiomyopathy [41]. These findings provide a potential link between oxidative stress and myocardial dysfunction in diabetes after myocardial IR injury. In our study, we found that enhanced oxidative stress in the diabetic myocardium as evidenced by elevated plasma free 8-isoprostane level and DHE staining. However, PT treatment significantly alleviated myocardial IR-induced oxidative stress in diabetic rats. Interestingly, four weeks treatment with PT significantly decreased the plasma glucose levels in streptozotocin-induced diabetic rats, and therefore, PT-induced attenuation of hyperglycemia can save the diabetic myocardium from excessive oxidative stress. Thus, the protective effects of PT against diabetic myocardial IR injury may be ascribed to its potent antioxidant nature and its suppressive activity against hyperglycemia-induced oxidative damage.

It is reported that enhanced oxidative stress in the ischemic myocardium leads to the alteration of the membrane integrity which results in the release of cardiac damage markers like LDH and creatine kinase into the serum [42, 43]. The CK-MB is a sensitive marker of post-ischemic myocardial infarction in acute myocardial ischemic patients [44], while infarct size is widely accepted as the gold standard for measuring the index of IR-induced cardiac injury [44, 45]. In acute myocardial infarction patients undergoing thrombolytic therapy, serum concentrations of CK-MB attain peak level after ten hours of ischemia and

correlate well with maximal indices of cardiac infarct size after five to seven days following reperfusion [44]. Similar findings were observed in a rat model of myocardial IR injury, in which post-ischemic CK-MB level reached maximum minutes after reperfusion while significant myocardial infarct size became apparent only after a lag period of one hour following reperfusion [46]. In our study, we observed that IR-induced enhanced levels of LDH, CK-MB in diabetic rats that were correlated with increased indices of cardiac infarct size and apoptosis. However, PT treatment significantly restricted the diabetic IR-induced elevation of cardiac damage markers in serum, indicating that PT ameliorates the post-ischemic cardiac injury in diabetic rats.

Substantial evidence suggests that enhanced oxidative stress during myocardial IR can trigger cardiac apoptosis *via* the mitochondrial (intrinsic)-mediated apoptosis, and represents a significant contributor to cardiomyocyte death in diabetes [47, 48]. The Bcl-2 family proteins, consists of both pro- and anti-apoptotic members, are essential regulators of mitochondrial apoptosis in the myocardium [49]. Bcl-2, an anti-apoptotic protein that decreases cell apoptosis by opposing Bax (a pro-apoptotic protein) mediated release of cytochrome c from mitochondria to the cytoplasm and eventually inhibits caspase cascade [50]. It has been reported that down-regulation of apoptosis could diminish IR-induced cardiomyocyte damage, and rescue the contractile function and therefore delay or even inhibit the incidence of heart failure [51]. Thus, it would be interesting to investigate the direct effect of PT in diabetic myocardial IR injury related to or beyond its anti-apoptotic capacity. Western blot analysis revealed that PT attenuated cardiac apoptosis after myocardial IR injury in diabetic rats. It is noteworthy that PT (10 mg/kg, administered intraperitoneally, once a day for five consecutive days) significantly attenuated myocardial IR-induced-inflammation, oxidative stress, and myocardial apoptosis *via* up-regulating Gas6/Axl pathway in non-diabetic rats [52]. Furthermore, PT administration (10 minutes before reperfusion) markedly reduced myocardial caspase-3 activity, *via* lowering nitrative/oxidative stress by attenuating peroxynitrite production, ROS generation and inflammatory response following myocardial IR injury [53]. Previous studies also lend the support for an anti-apoptotic role of PT in non-cardiac tissues such as brain [54] and skeletal muscle [55] following IR injury. Further, we extended our interest to explore whether PT has retained its anti-apoptotic effect against *in vitro* HR injury (an *in vitro* model of IR injury) in diabetic (high glucose, HG) condition using primary rat cardiomyocytes. We found that PT decreased cardiac apoptosis in primary rat cardiomyocytes by up-regulating Bcl-2/Bax ratio, down-regulating cleaved caspase-3/caspase-3 ratio. Guo et al. demonstrated that PT attenuated HR-induced apoptosis *via* restoration of sirt1 activity in H9C2 cardiomyocytes [56]. Thus, our results suggest that PT confers cardioprotection against diabetic myocardial IR injury by suppressing cardiac apoptosis.

To gain insight into the signaling pathways responsible for the antioxidant and anti-apoptotic action of PT against diabetic myocardial IR injury, we further elucidated the role of AMPK pathway in PT-elicited cardioprotection. AMPK is an "endogenous survival mechanism"[57], and its activation plays a vital role in protecting against diabetes mellitus [58] and myocardial IR injury [14]. AMPK has been reported to exert an infarct-sparing effect by increasing glucose uptake and glucose transporter-4 translocation [14] and precluding cardiomyocyte apoptosis [14, 59]. Furthermore, AMPK activation is known to increase myocardial resistance to IR and oxidative stress of different magnitudes by up-regulating sulfonylurea receptor 2A (SUR2A), a major cardioprotective protein in the heart [60]. Mohammed Abdul et al [60]. demonstrated that AMPK-mediated activation of SUR2A enhanced the trafficking and activity of sarcolemmal ATP-sensitive K+ channels in mice, which in turn offers cardioprotection against prolonged hypoxic injury. Also, AICAR (an AMPK activator) treatment reduced the myocardial sensitivity to hypoxic insult by increasing the SUR2A expression in H9C2 cardiomyocytes [60]. Therefore, manipulation with AMPK has been suggested to be a potential therapeutic approach to cure cardiac ischemic diseases where enhanced cardiac resistance to stress is indispensable. In addition, PT can stimulate AMPK activity to induce suppressive effects on fat accumulation and apoptosis in both

adipocytes and vascular endothelial cells [21, 22]. In our study, myocardial IR significantly increased p-AMPK levels in diabetic rats, and the rise of p-AMPK was not sufficient to combat apoptosis, and this led to the enhanced cardiac oxidative stress and apoptosis in diabetic hearts. However, treatment with PT markedly potentiated IR-induced increase in p-AMPK and significantly reduced the oxidative stress and apoptosis in the myocardium of diabetic rats.

To further reveal the participation of AMPK signaling pathway in PT-induced cardioprotective effects against myocardial IR injury in diabetes, AMPK inhibitor compound C was applied to primary rat cardiomyocytes incubated with HG condition. Compound C co-administration not only prevented PT-induced AMPK phosphorylation but also decreased PT-mediated anti-oxidative and anti-apoptotic effects in diabetic cardiomyocytes exposed to HR challenge. Thus, phosphorylation of AMPK by PT and its suppression by CC bestow robust support for the contribution of AMPK pathway in PT-induced safeguard in diabetic myocardial IR injury.

Recent studies showed that restoration of AMPK activation reduced oxidative stress [18, 57], while other studies have linked AMPK stimulation to enhanced oxidative stress [61, 62]. Although the exact relationship between AMPK and oxidative stress is a matter of debate, our results support the notion that restoration of AMPK activation contributes to the amelioration of oxidative stress injury. Such an effect may be related to the attenuated fatty acid synthesis caused by phosphorylation of acetyl-CoA carboxylase enzyme [63], although further studies are essential to validate this. Furthermore, additional experiments are required to determine the mechanisms of PT induced cardioprotection from IR-induced oxidative stress and apoptosis in diabetes.

## Conclusion

In summary, these results demonstrate that PT exerts anti-oxidative and anti-apoptotic action against myocardial IR injury and limits cardiac cell damage *via* stimulating AMPK signaling in diabetic rats. These findings suggest that the PT can be considered to have a potential therapeutic value in the prevention and rescue for diabetes-associated cardiovascular complications.

## Abbreviations

PT (Pterostilbene); PT20 (Pterostilbene 20 mg/kg/d); PT40 (Pterostilbene 40 mg/kg/d); IR (Ischemia-reperfusion); LG (Low glucose); HG/HL (High glucose/High lipid); HR (Hypoxia-reoxygenation); LDH (Lactate dehydrogenase); CC (Compound C); D (Diabetic); ROS (Reactive oxygen species); DHE (Dihydroethidium); CK-MB (Creatine kinase-MB); H-E (Hematoxylin-eosin); SEM (Standard error mean); ANOVA (Analysis of variance); Gas6 (Growth arrest specific gene 6 protein); TTC (1% 2, 3, 5-triphenyltetrazolium chloride); TUNEL (Terminal deoxynucleotidyl nick-end labeling); AMPK (Adenosine monophosphate-activated protein kinase); MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide).

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## Disclosure Statement

No conflict of interests exists.

## References

1 Li H, Liu Z, Wang J, Wong GT, Cheung CW, Zhang L, Chen C, Xia Z, Irwin MG: Susceptibility to myocardial ischemia reperfusion injury at early stage of type 1 diabetes in rats. Cardiovasc Diabetol DOI: 10.1186/1475-2840-12-133.

2 Kandula V, Kosuru R, Li H, Yan D, Zhu Q, Lian Q, Ge R, Xia Z, Irwin MG: Forkhead box transcription factor 1: role in the pathogenesis of diabetic cardiomyopathy. Cardiovasc Diabetol DOI: 10.1186/s12933-016-0361-1.

3 Kim H-L, Kang S-H, Yoon C-H, Cho Y-S, Youn T-J, Cho G-Y, Chae I-H, Kim H-S, Chae S-C, Cho M-C, Kim Y-J, Kim JH, Ahn Y, Jeong MH, Choi D-J, Other Korea Acute Myocardial Infarction Registry (KAMIR) and Korea Working Group on Myocardial Infarction (KorMI) Investigators: Differential Prognostic Impacts of Diabetes over Time Course after Acute Myocardial Infarction. J Korean Med Sci 2013;28:1749-1755.

4 Hur SH, Won KB, Kim IC, Bae JH, Choi DJ, Ahn YK, Park JS, Kim HS, Choi RK, Choi D, Kim JH, Han KR, Park HS, Choi SY, Yoon JH, Gwon HC, Rha SW, Jang W, Bae JW, Hwang KK, Lim DS, Jung KT, Oh SK, Lee JH, Shin ES, Kim KS, DIAMOND investigators: Comparison of 2-year clinical outcomes between diabetic versus nondiabetic patients with acute myocardial infarction after 1-month stabilization: Analysis of the prospective registry of DIAMOND (DIabetic acute myocardial infarctiON Disease) in Korea: an observational registry study. Medicine (Baltimore) DOI: 10.1097/MD.0000000000003882.

5 Potier L, Waeckel L, Vincent MP, Chollet C, Gobeil F, Jr., Marre M, Bruneval P, Richer C, Roussel R, Alhenc-Gelas F, Bouby N: Selective kinin receptor agonists as cardioprotective agents in myocardial ischemia and diabetes. J Pharmacol Exp Ther 2013;346:23-30.

6 Li WL, Zheng HC, Bukuru J, De Kimpe N: Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. J Ethnopharmacol 2004;92:1-21.

7 Roupe KA, Remsberg CM, Yanez JA, Davies NM: Pharmacometrics of stilbenes: seguing towards the clinic. Curr Clin Pharmacol 2006;1:81-101.

8 Lin HS, Yue BD, Ho PC: Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. Biomed Chromatogr 2009;23:1308-1315.

9 Kosuru R, Rai U, Prakash S, Singh A, Singh S: Promising therapeutic potential of pterostilbene and its mechanistic insight based on preclinical evidence. Eur J Pharmacol 2016;789:229-243.

10 Remsberg CM, Yanez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM: Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. Phytother Res 2008;22:169-179.

11 Riche DM, Riche KD, Blackshear CT, McEwen CL, Sherman JJ, Wofford MR, Griswold ME: Pterostilbene on metabolic parameters: a randomized, double-blind, and placebo-controlled trial. Evid Based Complement Alternat Med DOI: 10.1155/2014/459165.

12 Hougee S, Faber J, Sanders A, De Jong RB, Van Den Berg WB, Garssen J, Hoijer MA, Smit HF: Selective COX-2 inhibition by a Pterocarpus marsupium extract characterized by pterostilbene, and its activity in healthy human volunteers. Planta Med 2005;71:387-392.

13 Young LH: AMP-activated protein kinase conducts the ischemic stress response orchestra. Circulation 2008;117:832-840.

14 Russell RR, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, Young LH: AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. J Clin Invest 2004;114:495-503.

15 Liu L, Jin X, Hu CF, Li R, Zhou Z, Shen CX: Exosomes Derived from Mesenchymal Stem Cells Rescue Myocardial Ischaemia/Reperfusion Injury by Inducing Cardiomyocyte Autophagy Via AMPK and Akt Pathways. Cell Physiol Biochem 2017;43:52-68.
16 Terai K, Hiramoto Y, Masaki M, Sugiyama S, Kuroda T, Hori M, Kawase I, Hirota H: AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. Mol Cell Biol 2005;25:9554-9575.
17 Calvert JW, Gundewar S, Jha S, Greer JJM, Bestermann WH, Tian R, Lefer DJ: Acute Metformin Therapy Confers Cardioprotection Against Myocardial Infarction Via AMPK-eNOS–Mediated Signaling. Diabetes 2008;57:696-705.
18 Liu Z, Chen JM, Huang H, Kuznicki M, Zheng S, Sun W, Quan N, Wang L, Yang H, Guo HM, Li J, Zhuang J, Zhu P: The protective effect of trimetazidine on myocardial ischemia/reperfusion injury through activating AMPK and ERK signaling pathway. Metabolism 2016;65:122-130.
19 Morrison A, Yan X, Tong C, Li J: Acute rosiglitazone treatment is cardioprotective against ischemia reperfusion injury by modulating AMPK, Akt, and JNK signaling in nondiabetic mice. Am J Physiol Heart Circ Physiol 2011;301:H895-902.
20 Lin VC, Tsai YC, Lin JN, Fan LL, Pan MH, Ho CT, Wu JY, Way TD: Activation of AMPK by pterostilbene suppresses lipogenesis and cell-cycle progression in p53 positive and negative human prostate cancer cells. J Agric Food Chem 2012;60:6399-6407.
21 Zhang L, Cui L, Zhou G, Jing H, Guo Y, Sun W: Pterostilbene, a natural small-molecular compound, promotes cytoprotective macroautophagy in vascular endothelial cells. J Nutr Biochem 2013;24:903-911.
22 Gomez-Zorita S, Fernandez-Quintela A, Lasa A, Aguirre L, Rimando AM, Portillo MP: Pterostilbene, a dimethyl ether derivative of resveratrol, reduces fat accumulation in rats fed an obesogenic diet. J Agric Food Chem 2014;62:8371-8378.
23 Yang Q, Wang HC, Liu Y, Gao C, Sun L, Tao L: Resveratrol Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Involves Upregulation of Adiponectin Levels and Multimerization in Type 2 Diabetic Mice. J Cardiovasc Pharmacol 2016;68:304-312.
24 Um JH, Park SJ, Kang H, Yang S, Foretz M, McBurney MW, Kim MK, Viollet B, Chung JH: AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. Diabetes 2010;59:554-563.
25 Szkudelski T: The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 2001;50:537-546.
26 Tay YC, Wang Y, Kairaitis L, Rangan GK, Zhang C, Harris DC: Can murine diabetic nephropathy be separated from superimposed acute renal failure? Kidney Int 2005;68:391-398.
27 Furman BL: Streptozotocin-Induced Diabetic Models in Mice and Rats. Curr Protoc Pharmacol DOI: 10.1002/0471141755.ph0547s70.
28 Zhang H, Wang C, Li X, Zhang Y: Effects of pterostilbene on treating hyperprolactinemia and related mechanisms. Am J Transl Res 2016;8:3049-3055.
29 Pari L, Satheesh MA: Effect of pterostilbene on hepatic key enzymes of glucose metabolism in streptozotocin- and nicotinamide-induced diabetic rats. Life Sci 2006;79:641-645.
30 Riche DM, McEwen CL, Riche KD, Sherman JJ, Wofford MR, Deschamp D, Griswold M: Analysis of Safety from a Human Clinical Trial with Pterostilbene. J Toxicol DOI: 10.1155/2013/463595.
31 Ruiz MJ, Fernandez M, Pico Y, Manes J, Asensi M, Carda C, Asensio G, Estrela JM: Dietary administration of high doses of pterostilbene and quercetin to mice is not toxic. J Agric Food Chem 2009;57:3180-3186.
32 Liu Y, Jin J, Qiao S, Lei S, Liao S, Ge ZD, Li H, Wong GT, Irwin MG, Xia Z: Inhibition of PKCbeta2 overexpression ameliorates myocardial ischaemia/reperfusion injury in diabetic rats via restoring caveolin-3/Akt signaling. Clin Sci (Lond) 2015;129:331-344.
33 Liu Y, Lei S, Gao X, Mao X, Wang T, Wong GT, Vanhoutte PM, Irwin MG, Xia Z: PKCbeta inhibition with ruboxistaurin reduces oxidative stress and attenuates left ventricular hypertrophy and dysfunction in rats with streptozotocin-induced diabetes. Clin Sci (Lond) 2012;122:161-173.
34 Xue R, Lei S, Xia ZY, Wu Y, Meng Q, Zhan L, Su W, Liu H, Xu J, Liu Z, Zhou B, Xia Z: Selective inhibition of PTEN preserves ischaemic post-conditioning cardioprotection in STZ-induced Type 1 diabetic rats: role of the PI3K/Akt and JAK2/STAT3 pathways. Clin Sci (Lond). 2016;130:377-392.

35 Lei S, Li H, Xu J, Liu Y, Gao X, Wang J, Ng KF, Lau WB, Ma XL, Rodrigues B, Irwin MG, Xia Z: Hyperglycemia-induced protein kinase C beta2 activation induces diastolic cardiac dysfunction in diabetic rats by impairing caveolin-3 expression and Akt/eNOS signaling. Diabetes 2013;62:2318-2328.
36 Worthley MI, Holmes AS, Willoughby SR, Kucia AM, Heresztyn T, Stewart S, Chirkov YY, Zeitz CJ, Horowitz JD: The deleterious effects of hyperglycemia on platelet function in diabetic patients with acute coronary syndromes mediation by superoxide production, resolution with intensive insulin administration. J Am Coll Cardiol 2007;49:304-310.
37 Eguchi M, Kim YH, Kang KW, Shim CY, Jang Y, Dorval T, Kim KJ, Sweeney G: Ischemia-reperfusion injury leads to distinct temporal cardiac remodeling in normal versus diabetic mice. PLoS One DOI: 10.1371/journal.pone.0030450.
38 Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJ: A series of prostaglandin F2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc Natl Acad Sci U S A 1990;87:9383-9387.
39 Delanty N, Reilly MP, Pratico D, Lawson JA, McCarthy JF, Wood AE, Ohnishi ST, Fitzgerald DJ, FitzGerald GA: 8-epi PGF2 alpha generation during coronary reperfusion. A potential quantitative marker of oxidant stress *in vivo*. Circulation 1997;95:2492-2499.
40 Smith HM, Hamblin M, Hill MF: Greater propensity of diabetic myocardium for oxidative stress after myocardial infarction is associated with the development of heart failure. J Mol Cell Cardiol 2005;39:657-665.
41 Hamblin M, Friedman DB, Hill S, Caprioli RM, Smith HM, Hill MF: Alterations in the Diabetic Myocardial Proteome Coupled With Increased Myocardial Oxidative Stress Underlies Diabetic Cardiomyopathy. J Mol Cell Cardiol 2007;42:884-895.
42 Rossoni G, Manfredi B, Civelli M, Berti F, Razzetti R: Combined simvastatin-manidipine protect against ischemia-reperfusion injury in isolated hearts from normocholesterolemic rats. Eur J Pharmacol 2008;587:224-230.
43 Deng F, Wang S, Zhang L, Xie X, Cai S, Li H, Xie G, Miao HL, Yang C, Liu X, Xia Z: Propofol Through Upregulating Caveolin-3 Attenuates Post-Hypoxic Mitochondrial Damage and Cell Death in H9C2 Cardiomyocytes During Hyperglycemia. Cell Physiol Biochem 2017;44:279-292.
44 Christenson RH, Vollmer RT, Ohman EM, Peck S, Thompson TD, Duh SH, Ellis SG, Newby LK, Topol EJ, Califf RM: Relation of temporal creatine kinase-MB release and outcome after thrombolytic therapy for acute myocardial infarction. TAMI Study Group. Am J Cardiol 2000;85:543-547.
45 Turer AT, Mahaffey KW, Gallup D, Weaver WD, Christenson RH, Every NR, Ohman EM: Enzyme estimates of infarct size correlate with functional and clinical outcomes in the setting of ST-segment elevation myocardial infarction. Curr Control Trials Cardiovasc Med DOI: 10.1186/1468-6708-6-12.
46 Xia Z, Kuo KH, Godin DV, Walker MJ, Tao MC, Ansley DM: 15-F(2t)-isoprostane exacerbates myocardial ischemia-reperfusion injury of isolated rat hearts. Am J Physiol Heart Circ Physiol 2005;289:H1366-1372.
47 Crow MT, Mani K, Nam Y-J, Kitsis RN: The Mitochondrial Death Pathway and Cardiac Myocyte Apoptosis. Circ Res 2004;95:957-970.
48 Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Cramer MJ, Lips DJ, Doevendans PA: Role of apoptosis in reperfusion injury. Cardiovasc Res 2004;61:414-426.
49 Gustafsson ÅB, Gottlieb RA: Bcl-2 family members and apoptosis, taken to heart. Am J Physiol Cell Physiol 2007;292:C45-C51.
50 van Empel VPM, Bertrand ATA, Hofstra L, Crijns HJ, Doevendans PA, De Windt LJ: Myocyte apoptosis in heart failure. Cardiovasc Res 2005;67:21-29.
51 Chen K, Li G, Geng F, Zhang Z, Li J, Yang M, Dong L, Gao F: Berberine reduces ischemia/reperfusion-induced myocardial apoptosis via activating AMPK and PI3K-Akt signaling in diabetic rats. Apoptosis 2014;19:946-957.
52 Wu M, Lu S, Zhong J, Huang K, Zhang S: Protective Effects of Pterostilbene Against Myocardial Ischemia/Reperfusion Injury in Rats. Inflammation 2017;40:578-588.
53 Yu Z, Wang S, Zhang X, Li Y, Zhao Q, Liu T: Pterostilbene protects against myocardial ischemia/reperfusion injury via suppressing oxidative/nitrative stress and inflammatory response. Int Immunopharmacol 2016;43:7-15.

54 Zhou Y, Zhang XM, Ma A, Zhang YL, Chen YY, Zhou H, Li WJ, Jin X: Orally administrated pterostilbene attenuates acute cerebral ischemia-reperfusion injury in a dose- and time-dependent manner in mice. Pharmacol Biochem Behav 2015;135:199-209.
55 Cheng Y, Di S, Fan C, Cai L, Gao C, Jiang P, Hu W, Ma Z, Jiang S, Dong Y, Li T, Wu G, Lv J, Yang Y: SIRT1 activation by pterostilbene attenuates the skeletal muscle oxidative stress injury and mitochondrial dysfunction induced by ischemia reperfusion injury. Apoptosis 2016;21:905-916.
56 Guo Y, Zhang L, Li F, Hu CP, Zhang Z: Restoration of sirt1 function by pterostilbene attenuates hypoxia-reoxygenation injury in cardiomyocytes. Eur J Pharmacol 2016;776:26-33.
57 Qi D, Young LH: AMPK: energy sensor and survival mechanism in the ischemic heart. Trends Endocrinol Metab 2015;26:422-429.
58 Coughlan KA, Valentine RJ, Ruderman NB, Saha AK: AMPK activation: a therapeutic target for type 2 diabetes? Diabetes Metab Syndr Obes 2014;7:241-253.
59 Ma H, Wang J, Thomas DP, Tong C, Leng L, Wang W, Merk M, Zierow S, Bernhagen J, Ren J, Bucala R, Li J: Impaired macrophage migration inhibitory factor-AMP-activated protein kinase activation and ischemic recovery in the senescent heart. Circulation 2010;122:282-292.
60 Mohammed Abdul KS, Jovanović S, Jovanović A: Exposure to 15% oxygen *in vivo* up-regulates cardioprotective SUR2A without affecting ERK1/2 and AKT: a crucial role for AMPK. J Cell Mol Med 2017;21:1342-1350.
61 Jung JE, Lee J, Ha J, Kim SS, Cho YH, Baik HH, Kang I: 5-Aminoimidazole-4-carboxamide-ribonucleoside enhances oxidative stress-induced apoptosis through activation of nuclear factor-kappaB in mouse Neuro 2a neuroblastoma cells. Neurosci Lett 2004;354:197-200.
62 Jiang B, Le L, Pan H, Hu K, Xu L, Xiao P: Dihydromyricetin ameliorates the oxidative stress response induced by methylglyoxal via the AMPK/GLUT4 signaling pathway in PC12 cells. Brain Res Bull 2014;109:117-126.
63 Ford RJ, Fullerton MD, Pinkosky SL, Day EA, Scott JW, Oakhill JS, Bujak AL, Smith BK, Crane JD, Blumer RM, Marcinko K, Kemp BE, Gerstein HC, Steinberg GR: Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. Biochem J 2015;468:125-132.