Luteolin Limits Infarct Size and Improves Cardiac Function after Myocardium Ischemia/Reperfusion Injury in Diabetic Rats

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Abstract

**Background:** The present study was to investigate the effects and mechanism of Luteolin on myocardial infarct size, cardiac function and cardiomyocyte apoptosis in diabetic rats with myocardial ischemia/reperfusion (I/R) injury.

**Methodology/Principal Findings:** Diabetic rats underwent 30 minutes of ischemia followed by 3 h of reperfusion. Animals were pretreated with or without Luteolin before coronary artery ligation. The severity of myocardial I/R induced LDH release, arrhythmia, infarct size, cardiac function impairment, cardiomyocyte apoptosis were compared. Western blot analysis was performed to elucidate the target proteins of Luteolin. The inflammatory cytokine production were also examined in ischemic myocardium undergoing I/R injury. Our results revealed that Luteolin administration significantly reduced LDH release, decreased the incidence of arrhythmia, attenuated myocardial infarct size, enhanced ventricular ejection fraction and decreased myocardial apoptotic death compared with I/R group. Western blot analysis showed that Luteolin treatment up-regulated anti-apoptotic proteins FGFR2 and LIF expression, increased BAD phosphorylation while decreased the ratio of Bax to Bcl-2. Luteolin treatment also inhibited MPO expression and inflammatory cytokine production including IL-6, IL-1a and TNF-a. Moreover, co-administration of wortmannin and Luteolin abolished the beneficial effects of Luteolin.

**Conclusions/Significance:** This study indicates that Luteolin preserves cardiac function, reduces infarct size and cardiomyocyte apoptotic rate after I/R injury in diabetic rats. Luteolin exerts its action by up-regulating of anti-apoptotic proteins FGFR2 and LIF expression, activating PI3K/Akt pathway while increasing BAD phosphorylation and decreasing ratio of Bax to Bcl-2.

Introduction

The worldwide epidemic of diabetes mellitus is increasing the burden of cardiovascular disease, the leading cause of death among patients with diabetes [1]. Diabetes is now considered to be a risk equivalent of coronary artery disease for future MI and cardiovascular death [2]. Our previous study has shown that diabetes renders the heart more sensitive to I/R injury [3]. This warrants the significance of aggressive primary prevention against ischemia/reperfusion (I/R) injury in diabetic patients. Diabetes is associated with significantly increased cardiomyocyte apoptosis [4,5,6,7]. It is well documented that blocking the apoptosis process could prevent the loss of contractile cells, minimize cardiac I/R injury and therefore slow down the occurrence of heart failure [8]. FGFR2 and LIF are anti-apoptotic proteins which have been shown to be survival signal mediators in cardiomyocyte response against myocardial infarction [9,10,11]. Protective effects of LIF and FGFR2 were also related to up-regulation of the Akt Signaling [9,10,11]. Akt is known to regulate many survival pathways of the cardiac cells. Activation of Akt plays a pivotal role in fundamental cellular functions such as cell proliferation and survival by phosphorylating a variety of substrates. It has been reported that PI3K/Akt pathway regulates cardiac contractility and cardiomyocyte apoptosis [12]. Activation of PI3K/Akt pathway is an effective way to reduce cardiomyocyte apoptosis thus reduces cardiac I/R injury.

Luteolin, a flavonoid polyphenolic compound, is a widely distributed in many fruits and vegetables [13]. Studies in human beings as well as animal models have revealed the diverse beneficial effects of Luteolin, such as cardiovascular protection, antioxidant, anti-inflammatory, which suggest Luteolin is a valuable compound for many medical applications [14,15,16]. Luteolin has been shown to improve contractile function and attenuates apoptosis following I/R injury in adult rat cardiomyocytes [17]. In addition, Luteolin significantly enhanced left ventricular pressure and the global and relative coronary flow in Langendorff rabbit hearts subjected to repeated myocardial ischemia [18]. The potential effects of Luteolin on diabetes and
I/R injury prompted us to investigate whether it is capable of exerting protection effects during cardiac I/R injury in diabetic rats and the underlying mechanism responsible for its effects.

Therefore, the aims of the present study were 1) to clarify whether Luteolin protects diabetic rats from cardiac I/R injury and cardiomyocytes apoptosis; 2) to identify the underlying mechanisms of Luteolin on I/R injury and cardiomyocytes apoptosis in diabetic rats.

Methods

Animals

The experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Ethic Committee on Animal Care (Approval ID: 2009055). One hundred and fifty adult male Sprague-Dawley (SD) rats, weight 200 to 220 g were purchased from the animal center in the Fourth Military Medical University. Diabetes mellitus (DM) was induced by intraperitoneal injections (i.p.) of STZ (50 mg/kg, STZ was dissolved in 0.1 M citrate buffer, pH 4.5) as previously described [19]. Rats were randomly allocated into the following groups with n = 30 each: (1) Non-DM+sham group (Non-DM); (2) DM+sham group (Sham)/Diabetic group; (3) DM+I/R group (I/R); (4) DM+Luteolin+I/R group (Luteolin); (5) DM+Luteolin+wortmannin group+I/R (Luteolin+W).

Blood glucose concentration was determined by using a reflectance meter (Accu-Chek, Roche Diagnostics GmbH, Mannheim, Germany) 1 week after STZ injection. Random blood glucose testing was also repeated at 3 and 8weeks after STZ injection (Luteolin and Wortmannin were both dissolved in DMSO). Sham group received the same volume of DMSO for injection as previous described [3]. In brief, rats were anesthetized with 3% isoflurane. The chest was opened through a left thoracic incision. A 6–0 silk suture slipknot was placed at the distal 1/3 of the left anterior descending artery. The left ventricular pressure areas indicated infarcted myocardium. Areas of infarct size (IS) and area-at-risk (AAR) were measured digitally using Image Pro Plus software (Media Cybernetics). IS and AAR were expressed as percentages of the left ventricular area (IS/LV and AAR/LV respectively).

Measurement of Myocardial Infarct Size

Myocardial Infarct Size was evaluated by Evans Blue/TTC staining as previously described [3]. Three hours after reperfusion, the ligature around the coronary artery was retracted, and 1 ml of 2% Evans Blue dye was injected into the side arm of the aortic cannula. The heart was quickly excised after the dye was uniformly distributed, frozen at −80°C and sliced transversally into 1 mm thick sections. The slices were incubated in 1% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St Louis, Mo) for 10 min at 37°C. Evans blue stained areas indicated area-not-at-risk (ANAR). Red parts in the heart, which were stained by TTC, represented for ischemic but viable tissue. Staining negative areas indicated infarcted myocardium. Areas of infarct size (IS) and area-at-risk (AAR) were measured digitally using Image Pro Plus software (Media Cybernetics). IS and AAR were expressed as percentages of the left ventricular area (IS/LV and AAR/LV respectively).

Determination of Myocardial Apoptosis

Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining and caspase 3 activity assay as previously described [3]. TUNEL staining was performed with fluorescein-dUTP (In Situ Cell Death Detection Kit; Roche Diagnostics) for apoptotic cell nuclei and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) stained all cell nuclei. Additional staining was performed using a monoclonal antibody against Troponin I (cTnI, Santa Cruz) for the identification of myocardium. Apoptotic cell index (AI) was determined. AI = number of TUNEL-positive myocytes/total number of myocytes stained with DAPI from a total of 40 fields per heart (n = 5). All of these assays were performed in a blinded manner. Caspase-3 activity was measured with the ApoAlert Caspase-3 Assay Plate (Clontech, Mountain View, Calif) according to the manufacturer’s instructions [21]. Substrate cleavage was monitored fluorometrically with a SpectraMax Gemini spectrophotometer (Molecular Devices) with excitation and emission wavelengths of 350 and 450 nm.

Determination of Cardiac Function

Echocardiography was conducted at 24 h after infarction as previously described [19]. Sedated rats (3% isoflurane) were studied on an echocardiography system (Sequoia Acuson, Siemens; 15-MHz linear transducer). Cardiac dimensions and function were assessed by M-mode echocardiography. Left ventricular end-diastolic diameter (LVEDD) and Left ventricular end-systolic diameter (LVESD) were measured on the parasternal left ventricular long axis view. All measurements represent the mean of 3 consecutive cardiac cycles. Left ventricular end-systolic volume (LVESV), Left ventricular end-diastolic volume (LVEDV) and left ventricular ejection fraction (LVEF) were calculated by use of computer algorithms. All of these measurements were performed in a blinded manner.
Western blot evaluation

Protein was isolated from homogenized heart tissue with Trizol reagent (Invitrogen, Carlsbad, Calif) and standard Invitrogen protocols. After protein concentration quantitation with the modified Bradford assay (Bio-Rad Laboratories, Hercules, Calif), protein was then used for Western blotting with primary antibodies against caspase-3, cleaved caspase-3, FGFR2, IGF, Akt, Akt-P (Thr 308, Ser 473), BAD, BAD-P (Ser 136), Bax, Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, Calif). The blots were visualized with a chemiluminescence system (Amersham Bioscience, Buckinghamshire, UK). The signals were quantified by Image Pro Plus software (Media Cybernetics).

Determination of tissue myeloperoxidase (MPO), interleukin-6 (IL-6), IL-1α and tumor necrosis factor-alpha (TNF-α) activity

Following the 3 h reperfusion period, tissue sample were taken from the AAR zones for MPO activity analysis. The activity of MPO was measured spectrophotometrically at 460 nm and expressed from the AAR mass were lower in the diabetic group as compared with the non-diabetic group. Body mass were lower in the diabetic group as compared with the non-diabetic group. Body heart rate and blood pressure before the I/R injury. Blood glucose were expressed as means ± SD. Comparison between groups were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions as previously described [3]. Values are expressed as pg/mg of total protein.

Statistical analysis

Continuous variables that approximated the normal distribution were expressed as means ± SD. Comparison between groups were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions as previously described [3]. Values are expressed as pg/mg of total protein.

Results

Baseline parameters

No major differences were found between groups in terms of heart rate and blood pressure before the I/R injury. Blood glucose and the heart to body mass ratio were significantly higher in the diabetic group as compared with the non-diabetic group. Body mass were lower in the diabetic group as compared with the non-diabetic group (Table 1).

Luteolin reduces arrhythmia and LDH release after I/R injury in diabetic rats

Luteolin administration significantly decreased the release of lactate dehydrogenase (LDH), a biochemical marker for necrotic cell death, as compared with the I/R group (116.6 ± 19.9 vs 161.6 ± 12.1, *P* = 0.003). However, the PI3K inhibitor wortmannin pretreatment statistically increased the release of LDH compared with the Luteolin group (153.4 ± 8.9 vs 116.6 ± 19.9, *P* = 0.005) (Figure 1a). I/R injury induced frequent attacks of premature ventricular beats (PVB), ventricular tachycardia (VT) and ventricular fibrillation (VF). Luteolin significantly decreased the incidence of PVB (33.3% vs 70%, *P* = 0.009), VT (16.7% vs 50%, *P* = 0.013) and VF (3.3% vs 30%, *P* = 0.012) as compared with the I/R group. Moreover, wortmannin abolished the anti-arrhythmia effects of Luteolin (PVB: 63.3% vs 33.3%, *P* = 0.038; VT 43.3% vs 16.7%, *P* = 0.047; VF 26.7% vs 3.3%, *P* = 0.026) as compared with the Luteolin group (Figure 1b).

Luteolin decreases infarct size after I/R injury in diabetic rats

Representative images of infarct size as stained by Evans Blue and TTC were shown in Figure 2a. Luteolin administration significantly decreased infarct size at 3 h (0.199 ± 0.016 vs 0.304 ± 0.017, *P* < 0.001) after I/R injury compared with the I/R group. This effect was abolished by wortmannin treatment (0.285 ± 0.038 in wortmannin group vs 0.199 ± 0.016 in Luteolin group, *P* = 0.002) (Figure 2b). No significant difference in risk area was found between the four groups (Figure 2c).

Luteolin enhances left ventricular function after I/R injury

Hemodynamic measurements were performed 3 h after I/R injury (Figure 3a, 3b). Diabetes significantly decreased the ±LV dp/dt max compared with the non-diabetic group (Figure 3a, 3b). The +LV dp/dt max (3710.2 ± 72.5 vs 4284.6 ± 90.6 mmHg/s, *P* < 0.001) and the −LV dp/dt max (3920.4 ± 152.2 vs 4920.8 ± 231.2 mmHg/s, *P* < 0.001) were decreased after I/R injury in diabetic rats compared with the sham group. Luteolin significantly enhanced the +LV dp/dt max (4115.0 ± 210.3 vs 3710.2 ± 72.5 mmHg/s, *P* = 0.004) and the −LV dp/dt max (4592.8 ± 150.5 vs 3920.4 ± 152.2 mmHg/s, *P* < 0.001) compared with the I/R group. The PI3K inhibitor wortmannin abolished the effects of Luteolin on the +LV dp/dt max (3813.2 ± 101.1 vs 4115.0 ± 210.3 mmHg/s, *P* = 0.020) and the −LV dp/dt max (4128.3 ± 155.0 vs 4592.8 ± 150.5 mmHg/s, *P* = 0.001).

Table 1. Basic Parameters of the Rats.

| Basic Parameters | Non-DM (n = 30) | Sham (n = 30) | I/R (n = 30) | Luteolin (n = 30) | Luteolin+W (n = 30) | p |
|------------------|----------------|---------------|-------------|------------------|-------------------|---|
| Heart rate (min⁻¹) | 430.1 ± 21.7   | 432.5 ± 22.6  | 427.1 ± 19.8 | 435.9 ± 24.4     | 423.7 ± 18.2      | 0.47 |
| Blood glucose (mmol/L) | 4.9 ± 0.5     | 22.6 ± 2.7    | 23.1 ± 1.4   | 22.1 ± 2.5       | 24.3 ± 2.7       | <0.001 |
| Blood pressure (mmHg) | 82.3 ± 3.9    | 83.2 ± 4.6    | 80.9 ± 3.5   | 85.6 ± 4.7       | 84.9 ± 2.8       | 0.39  |
| Body mass (g)      | 443.9 ± 42.6  | 406.5 ± 36.7  | 398.2 ± 28.5 | 412.8 ± 35.9     | 409.1 ± 33.2     | <0.001 |
| Heart to body mass ratio (mg/g) | 1.96 ± 0.19  | 2.17 ± 0.12   | 2.23 ± 0.18  | 2.21 ± 0.25      | 2.14 ± 0.28      | <0.001 |

Values are presented as mean ± SD.

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LVEF, ESV, EDV were evaluated by echocardiography 24 h after I/R injury (Figure 3c, 3d, 3e). Diabetes decreased LVEF compared with the non-diabetic group (Figure 3c). Luteolin administration significantly enhanced LVEF as compared with the I/R group (0.761 ± 0.047 vs 0.671 ± 0.028, \( P = 0.006 \)) and the wortmannin group (0.761 ± 0.047 vs 0.669 ± 0.063, \( P = 0.031 \)). Luteolin significantly inhibited the increase of LVESV compared with the I/R group (0.252 ± 0.058 vs 0.394 ± 0.018 ml,

Figure 1. Luteolin reduces LDH release and I/R induced arrhythmia in diabetic rats. Diabetes increased LDH release compared with the non-diabetic group. Luteolin reduced the LDH release after I/R injury in diabetic mice. The PI3K inhibitor wortmannin pretreatment statistically increased the release of LDH compared with the Luteolin group (a). Luteolin decreased the incidence of PVB, VT and VF attacks as compared with the I/R group. Wortmannin abolished the anti-arrhythmia effects of Luteolin (b). PVB: premature ventricular beats; VT: ventricular tachycardia; VF, ventricular fibrillation. The columns and errors bars represent means and SD. \(* p < 0.05\) vs Non-DM, \# \( p < 0.05\) vs Sham, \( ^{\dagger} p < 0.05\) vs Luteolin.

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Luteolin Reduces I/R Injury in Diabetic Rat

Figure 2. Luteolin decreases infarct size in diabetic rats subjected to I/R injury. Representative illustrations of infarct size as stained by Evans Blue and TTC (a). Luteolin decreased infarct size 3 h after I/R injury compared with the I/R group and the wortmannin group (b). The risk of area had no statistical difference between the groups (c). The columns and errors bars represent means and SD. \(* p < 0.05\) vs Non-DM, \# \( p < 0.05\) vs Sham, \( ^{\dagger} p < 0.05\) vs Luteolin.

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Luteolin reduces I/R injury in diabetic rats.

Hemodynamic measurements indicated that diabetes decreased the ± LV dp/dt max compared with the non-diabetic group. I/R induced significant decrease of ± LV dp/dt max in diabetic rats. Luteolin enhanced the ± LV dp/dt max compared with the I/R group. The PI3K inhibitor wortmannin abolished the effects of Luteolin on the ± LV dp/dt max (a, b). Luteolin administration significantly enhanced LVEF as compared with the I/R group and the wortmannin group (c). Luteolin significantly inhibited the increase of LVESV and LVEDV compared with the I/R group and the wortmannin group (d, e). LVEF: Left ventricular ejection fraction; LVESV: Left ventricular end-systolic volume; LVEDV: Left ventricular end-diastolic volume. The columns and error bars represent means and SD. *p < 0.05 vs Non-DM, #p < 0.05 vs Sham, §§p < 0.05 vs Luteolin.

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Luteolin increases antiapoptotic protein expression and inhibits cardiomyocytes apoptosis after I/R injury in diabetic rats

The apoptotic rates were significantly higher in the diabetic group compared with the non-diabetic group. Diabetes enhanced
caspase-3 activity and caspase-3 expression compared with the non-diabetic group (Figure 4). Representative photomicrograph showed that TUNEL-positive cardiomyocytes were more frequently observed in the I/R group and the wortmannin group as compared with the Luteolin group (Figure 4a). Quantitative analyses demonstrated that the number of TUNEL-positive cardiomyocytes was significantly less in the Luteolin group than in the I/R group (0.146 ± 0.018 vs. 0.20 ± 0.03, P = 0.009) and the wortmannin group (0.146 ± 0.018 vs. 0.197 ± 0.047, P = 0.032) (Figure 4b). Concurrently, Luteolin group showed significantly decreased caspase-3 enzymatic activity compared with the I/R group (80.8 ± 8.3 vs. 133.2 ± 15.3, P < 0.001) and the wortmannin group (Figure 4c).

**Figure 4.** Luteolin decreases cardiomyocytes apoptosis in diabetic rats with I/R Injury. Representative photomicrograph showed that TUNEL-positive cardiomyocytes were more frequently observed in the I/R group and the wortmannin group compared with the Luteolin group (a). The apoptotic rate was higher in the diabetic group compared with the diabetic group. The number of TUNEL-positive cardiomyocytes (in green, DAPI in blue) was significantly reduced in the Luteolin group than in the I/R group and the wortmannin group (b). Luteolin treatment also significantly decreased caspase-3 activity compared with the I/R group and the wortmannin group (c). Luteolin decreased caspase-3 and cleaved caspase-3 expression as compared with the I/R group and the wortmannin group (d, e). Scale bar: 25 μm. The columns and errors bars represent means and SD. *p < 0.05 vs Non-DM, †p < 0.05 vs Sham, ‡p < 0.05 vs Luteolin.
Western blot analysis also demonstrated decreased caspase-3 and cleaved caspase-3 expression in the Luteolin group as compared with the I/R group and the wortmannin group (Figure 4d, 4e).

Western blot analysis revealed that the expression of anti-apoptotic proteins FGFR2 and LIF were decreased in the diabetic group as compared with the non-diabetic group. Diabetes decreased p308-Akt, p473-Akt, p136-BAD, Bcl-2 expression and Bax/Bcl-2 ratio, increased the expression of Bax as compared with the non-diabetic group (Figure 5). After 3 h of reperfusion, Luteolin treatment was associated with a significant increase in the expression of FGFR2 and LIF as compared with the I/R group (Figure 5a, 5b). Luteolin pretreatment also enhanced phosphorylation of Akt and BAD, increased Bax expression while decreased Bcl-2 expression resulted in decreased Bax/Bcl-2 ratio in cardiac tissue that were exposed to I/R injury (5c, 5d, 5e). Interestingly, the PI3K inhibitor wortmannin abolished the effects of Luteolin on anti-apoptotic proteins expression. Wortmannin administration was associated with reduced expression of FGFR2, LIF, p308-Akt, p473-Akt, p136-BAD and Bax as indicated in Figure 5.

Luteolin treatment alleviates leukocyte infiltration and reduces cytokine levels after I/R injury in diabetic rats

MPO activity was increased in the diabetic group as compared with the non-diabetic group. Following 3 h of reperfusion, the activity of MPO was significantly elevated in the I/R group when compared to the sham group (25.4±3.5 vs 8.4±0.6 U/100 mg, P<0.001). Treatment with Luteolin reduced the MPO activity compared with the I/R group (18.3±1.6 vs 25.4±3.5 U/100 mg, P=0.003) and the wortmannin group (18.3±1.6 vs 22.1±2.3 U/100 mg, P=0.016) (Figure 6a).

Elisa analysis demonstrated that diabetes increased the levels of left ventricular IL-6, IL-1β and tumor necrosis factor-α (TNF-α) as compared with the non-diabetic group (Figure 6b, 6c, 6d). I/R resulted in a noticeable increase in IL-6 (40.6±2.5 vs 23.9±2.8 pg/mg protein, P<0.001), IL-1β (70.4±5.9 vs 30.0±6.1 pg/mg protein, P<0.001) and TNF-α (115.1±9.0 vs 38.6±2.8 pg/mg protein, P<0.001) compared with the sham group. Luteolin reduced the levels of IL-6 (29.9±3.3 vs 40.6±2.5 pg/mg protein, P<0.001), IL-1β (40.2±4.4 vs 70.4±5.9 pg/mg protein, P<0.001) and TNF-α (55.9±4.8 vs 115.1±9.0 pg/mg protein, P<0.001) compared with the I/R group. Moreover, wortmannin administration significantly increased IL-6 (37.1±2.1 vs 29.9±3.3 pg/mg protein, P=0.003), IL-1β (66.0±6.5 vs 40.2±4.4 pg/mg protein, P<0.001) and TNF-α (94.7±13.4 vs 55.9±4.8 pg/mg protein, P<0.001) production compared with the Luteolin group.

**Discussion**

Coronary artery disease is associated with a less favorable outcome in diabetic than in non-diabetic patients. Accumulating
evidence indicates that diabetes is associated with 2–4 times increased risk of CHD mortality compared with patients without diabetes [3,22]. The flavone Luteolin, is widely distributed in many fruits, vegetables and traditional Chinese herbs [14]. Several pieces of evidences have shown that Luteolin is capable of protecting the myocardium against IR injury [23]. Clinical observations showed that regular Luteolin intake was associated with a reduced risk of cardiovascular diseases [24]. The beneficial effects were further supported by experimental studies in rabbit or guinea pig hearts showing enhancement of left ventricular pressure and coronary flow by Luteolin with or without regional ischemia [18,25]. However, the direct cardio-protective effects of Luteolin on I/R injury in diabetic rats and the exact mechanism of its therapeutic action are still poorly understood. This promoted an investigation of the protective effects of Luteolin on I/R injury in diabetic rats and the underlying mechanism.

The present study has provided the evidence that diabetes decreased ± LV dp/dt max, LVEF and caspase-3 expression, while increased LDH release and cardiomyocyte apoptotic rate. Luteolin reduced incidence of arrhythmia, reduced LDH release and decreased infarct size of diabetic rats subjected to I/R injury. We also demonstrated that Luteolin significantly improved the left ventricular function via increasing ± dp/dt max, LVEF and limiting the increase of LVESV and LVEDV. Similar to our results, Fang et al [17,26] reported that Luteolin inhibits apoptosis and improves cardiomyocyte contractile function in Langendorff perfused rat hearts and isolated cardiomyocyte subjected to simulated I/R injury.

Cardiomyocyte apoptosis is one of the major contributors to the development of heart failure after myocardial infarction. Blocking the apoptosis process could prevent the loss of contractile cells, minimize cardiac injury induced by I/R injury and therefore slow down the occurrence of heart failure [8,27]. Given the markedly improved recovery of left ventricular function exerted by Luteolin, we determined the effects of Luteolin on post-ischemic cellular damage. The extent of necrotic and apoptotic cell death was examined. The release of lactate dehydrogenase (LDH), a biochemical marker for necrotic cell death, was significantly decreased in the Luteolin treatment group. Luteolin administration significantly decreased TUNEL-positive cardiomyocytes and reduced caspase-3 expression in diabetic rats underwent I/R injury.

Both FGFR2 and LIF are anti-apoptotic proteins which have been shown to reduce cardiomyocyte apoptosis in myocardial I/R injury.
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...injury [28]. Most studies suggest that the Bcl-2 family is key regulators of physiological and pathological apoptosis. The family consists of both cell death promoters such as Bak, Bad, and cell death inhibitors, which include Bcl-2, Bcl-X, etc. It has been demonstrated that the high ratio of Bax/Bcl-2 is associated with greater vulnerability to apoptotic activation [29,30]. In the present study, we confirmed that Luteolin may exert its anti-apoptotic effects through up-regulating FGF2R2 and LIF expression, increasing BAD phosphorylation and Bcl-2 expression while down-regulating MPO, IL-6, IL-1β and TNF-α. Extracellular MPO can be determined as an index of polymorphonuclear leukocyte infiltration in response to inflammation. Luteolin administration significantly decreased MPO expression compared with the I/R group. Co-administration of wortmannin and Luteolin could also increase the expression of MPO, IL-6, IL-1β and TNF-α indicating that wortmannin abolishes the anti-inflammatory effects of Luteolin.

In conclusion, the salient finding of the present study is that Luteolin pretreatment reduces infarct size and improves cardiac hemodynamics after I/R injury in diabetic rats. This was through decreased cardiac apoptosis and inflammation. Luteolin exerted its protective effects by up-regulating of anti-apoptotic proteins FGF2R2 and LIF expression, which activated PI3K/Akt pathway and associated with increased BAD phosphorylation and decreased ratio of Bax to Bcl-2. These results may provide important insights for the understanding of the molecular mechanisms involved in the cardioprotective effect of Luteolin in diabetic rats underwent I/R injury.

Author Contributions
Conceived and designed the experiments: DS JH ZZ FC. Performed the experiments: DS JH ZZ HG JL MS HW. Analyzed the data: DS JH ZZ FC. Contributed reagents/materials/analysis tools: HG JL MS HW. Wrote the paper: DS FG.

References
1. Donahoe SM, Stewart GC, McCabe CH, Mohanavelu S, Murphy SA, et al. (2007) Diabetes and mortality following acute coronary syndromes. Jama 298: 765–775.
2. (2002) Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation 106: 3143–3421.
3. Cao F, Sun D, Li C, Nurskins K, Zhao I, et al. (2009) Long-term myocardial functional improvement after autologous bone marrow mononuclear cells transplantation in patients with ST-segment elevation myocardial infarction: a five-years follow-up. Eur Heart J 30: 1966–1994.
4. Liang JL, Xiao DZ, Lin XY, Lin QX, Shan ZY, et al. (2010) High glucose induces apoptosis in AG16 human cardiomyocytes via macrophage migration inhibitory factor and c-Jun N-terminal kinase. Clin Exp Pharmacol Physiol 37: 969–973.
5. Wu Y, Xia ZY, Dou J, Zhang L, Xu J, et al. (2011) Protective effect of ginsenoside Rb1 against myocardial ischemia/reperfusion injury in streptozotocin-induced diabetic rats. Mol Biol Rep 38: 4327–4335.
6. Yousef CW, Wang K, Kotakachalay PE. (2010) Hyperglycemia-induced cardiomyocyte death is mediated via MCP-1 production and induction of a novel zinc-finger protein, MCPIP. Cardiovasc Res 87: 665–674.
7. Yu XY, Geng VJ, Jiang JL, Lin QX, Lin SG, et al. (2010) High levels of glucose induce apoptosis in cardiomyocyte via epigenetic regulation of the insulin-like growth factor receptor. Exp Cell Res 316: 2905–2909.
8. Song QJ, Teng X, Cai Y, Tang CS, QI YF (2009). Activation of Akt/GSK-3beta signaling pathway is involved in medinef1(-/-) protection against myocardial apoptosis induced by ischemia/reperfusion. Apoptosis 14: 1299–1307.
9. Matonaga S, Oskigi M, Takekawa M, Matoz A, Hombo S, et al. (2009) Endothelialium-targeted overexpression of constitutively active FGF receptor increases cardioprotection in mice myocardial infarction. J Mol Cell Cardiol 46: 663–673.
10. Negoro S, Oh H, Tanos E, Komuuda K, Fujio Y, et al. (2001) Glycogen 130 regulates cardiac myocyte survival in disorders in-induced apoptosis through phosphorylation kinase/Akt phosphorylation and Bcl-2/Lcaspase-3 interaction. Circulation 103: 555–561.
11. Zou Y, Takano H, Mizukami M, Akazawa H, Qin Y, et al. (2003) Leukemia inhibitory factor enhances survival of cardiomyocytes and induces regeneration of myocardium after myocardial infarction. Circulation 108: 748–753.
12. Shirasaki I, Mezenez J, Aha Y, Skvadahl M, Murphy E, et al. (2004) Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. Circ Res 94: 884–891.
13. Li L, Henry GE, Seeran N (2009) Identification and bioactivities of resveratrol oligomers and flavonoids from Carsex folliculata seeds. J Agric Food Chem 57: 7292–7297.
14. Lopez-Lazaro M (2009) Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem 9: 31–59.
15. Park YJ, Kim HJ, Lee SJ, Choi HY, Ju C, et al. (2007) A new chromosome, 11-hydroxy-sec-O-glycosylhamaudol from Ostericum koreanum. Chem Pharm Bull (Tokyo) 55: 1055–1056.
16. Seelinger G, Merfort I, Schenpp CM (2008) Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med 74: 1667–1672.
17. Qi L, Pan H, Li G, Fang F, Chen D, et al. (2011) Luteolin improves contractile function and attenuates apoptosis following ischemia-reperfusion in adult rat cardiomyocytes. Eur J Pharmacol 668: 201–207.
18. Rump AF, Schusser M, Arac D, Cordes A, Theisohn M, et al. (1994) Functional and anti-anphischer effects of luteolin-7-glucoside in isolated rabbit hearts. Gen Pharmacol 25: 1137–1142.
19. Sun D, Shen M, Li J, Li W, Zhang Y, et al. (2011) Cardioprotective effects of thanshione IIIA pretreatment via kinin B2 receptor-Akt-GSK3-beta dependent pathway in experimental diabetic cardiomyopathy. Cardiovasc Diabetol 10: 4.
20. Walker MJ, Curtis MJ, Hearn DJ, Campbell RW, Jane MJ, et al. (1988) The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemiia infarction, and reperfusion. Cardiovasc Res 22: 447–455.
21. Lancel S, Joulain O, Favori R, Geossens JF, Khale J, et al. (2005) Ventricular myocyte caspases are directly responsible for endotoxin-induced cardiac dysfunction. Circulation 111: 2596–2604.
22. Lee WL, Cheung AM, Cape D, Zinman B (2000) Impact of diabetes on coronary artery disease in women and men: a meta-analysis of prospective studies. Diabetes Care 23: 962–966.
23. Liao PH, Hung LM, Chen YH, Kuan YH, Zhang FB, et al. (2011) Cardioprotective effects of luteolin during ischemia-reperfusion injury in rats. Circ J 75: 443–450.
24. Middleton E, Jr., Kanaskwami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52: 673–751.
25. Rump AF, Schusser M, Arac D, Cordes A, Rutke R, et al. (1993) Effects of different isomers with antioxidant properties on acute regional myocardial ischemia in isolated rabbit hearts. Gen Pharmacol 26: 603–611.
26. Fang F, Li D, Pan H, Chen D, Qi L, et al. (2011) Luteolin inhibits apoptosis and improves cardiomyocyte contractile function through the PI3K/Akt pathway in simulated ischemia/reperfusion. Pharmacology 80: 149–158.
27. Fu J, Huang H, Liu J, Pi R, Chen J, et al. (2007) Tanshinone IIA protects cardiac myocytes against oxidative stress-triggered damage and apoptosis. Eur J Pharmacol 568: 213–221.
28. Wang X, Zhang X, Ren XP, Chen J, Liu H, et al. (2010) MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury. Circulation 122: 1308–1318.
29. Chae IH, Park KW, Kim HS, Oh BH (2004) Nitric oxide-induced apoptosis is mediated by Bax/Bcl-2 gene expression, transition of cytochrome c, and activation of caspase-3 in rat vascular smooth muscle cells. Clin Chim Acta 341: 83–91.
30. Dong JW, Zhu HF, Zhu WZ, Ding HL, Ma TM, et al. (2003) Intermittent hypoxia attenuates ischemia/reperfusion induced apoptosis in cardiac myocytes via regulating Bcl-2/Bax expression. Cell Res 13: 385–391.
31. Feldman AM, Combes A, Wagner D, Kadokami T, Kubota T, et al. (2000) The role of tumor necrosis factor in the pathophysiology of heart failure. J Am Coll Cardiol 35: 537–544.
32. Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotis T, et al. (2001) Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. J Pharmacol Exp Ther 296: 181–187.