Новости месяца: Дигидрокверцетин (ДКВ, Taxifolin) и диабет.

В 2013 году в журнале Food Chem. Toxicology опубликована яркая и клинически важная статья китайских ученых Sun X., Chen RC и соавт. «Taxifolin prevents diabetic cardiomyopathy in vivo and in vitro by inhibition of oxidative stress and cell apoptosis» или «Таксифолин (ДКВ) предотвращает развитие диабетической кардиомиопатии in vivo и in vitro путем ингибирования окислительного стресса и клеточного апоптоза (программированная смерть клеток)».

Показано, что диабетическая кардиомиопатия является ключевой причиной нарушений работы сердца у больных с диабетом. Аномально высокий уровень окислительного стресса играет центральную роль в развитии диабетической кардиомиопатии. В результате исследований на больных было показано, что ДКВ, снижая уровень окислительного стресса, уменьшает дисфункцию работы сердца и улучшает морфологическую структуру миокарда, снижает гибель миоцитов (клеток сердца) и повышает активность антиокислительных ферментов. Авторы делают вывод, что ДКВ обладает выраженными кардиопротективными эффектами, препятствующими развитию диабетической кардиомиопатии, и является потенциально сильным лекарственным средством в лечении больных с диабетической кардиопатией.

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Taxifolin prevents diabetic cardiomyopathy in vivo and in vitro by inhibition of oxidative stress and cell apoptosis

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ABSTRACT

Diabetic cardiomyopathy has been increasingly recognized as an important cause of heart failure in diabetic patients. Excessive oxidative stress has been suggested to play a critical role in the development of diabetic cardiomyopathy. The objective of this study was to investigate the potential protective effects and mechanisms of taxifolin on cardiac function of streptozotocin-induced diabetic mice and on hyperglycemia-induced apoptosis of H9c2 cardiac myoblasts. In vivo study revealed that taxifolin improved diastolic dysfunction, ameliorated myocardium structure abnormality, inhibited myocyte apoptosis and enhanced endogenous antioxidant enzymes activities. Interestingly, taxifolin reduced angiotensin II level in myocardium, inhibited NADPH oxidase activity, and increased JAK/STAT3 activation. In vitro investigation demonstrated that taxifolin inhibited 33 mM glucoseinduced H9c2 cells apoptosis by decreasing intracellular ROS level. It also inhibited caspase-3 and caspase-9 activation, restored mitochondrial membrane potential, and regulated the expression of proteins related to the intrinsic pathway of apoptosis, thus inhibiting the release of cytochrome c from mitochondria into the cytoplasm. In conclusion, taxifolin exerted cardioprotective effects against diabetic cardiomyopathy by inhibiting oxidative stress and cardiac myocyte apoptosis and might be a potential agent in the treatment of diabetic cardiomyopathy.

1. Introduction

The incidence of diabetes mellitus has increased dramatically worldwide in recent years. By the year 2030, there will be 439 million adults (aged 20–79 years) with diabetes, augmenting international health burden (Shaw et al., 2010). Evidences have shown that heart failure is one of the leading causes of deaths in diabetic patients, while diabetic cardiomyopathy (DCM), a distinct complication of diabetes mellitus, is a major risk of developing congestive heart failure (Voulgari et al., 2010). By definition, diabetic cardiomyopathy is diabetes-related abnormalities of structure and function of myocardium independent of other coronary artery diseases. Clinically, DCM usually manifests the earlier onset of left ventricular diastolic dysfunction than systolic dysfunction (Asghar et al., 2009).

Despite the underlying mechanism of DCM remains poorly understood, previous studies have revealed that oxidative stress plays a critical role in the pathogenesis of DCM (Khullar et al., 2010). The exquisite equilibration between the production and elimination of reactive oxygen species (ROS) is impaired by hyperglycemia, causing detrimental modification of intracellular macromolecules such as lipid, protein and DNA. These damages may ultimately result in myocardial apoptosis, hypertrophy, and fibrosis, thereby contributing to ventricular dysfunction. Therefore, antioxidant supplementation may be an effective therapeutic treatment for DCM.

Flavonoids are major effective components of most traditional Chinese herbal medicines used in cardiovascular diseases treatment. Despite the diverse pharmacological effects, they are well known for their potent antioxidative capacities. Taxifolin, also

Abbreviations: DCM, diabetic cardiomyopathy; ROS, reactive oxygen species; STZ, streptozotocin; Ang II, angiotensin II; LVVd, LV end-diastolic volume; LVVs, LV end-systolic volume; FS, fractional shortening; EF, ejection fraction; LDH, lactate dehydrogenase; CK-MB, creatinine kinase-MB isoenzyme; AST, aspartate aminotransferase; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; AT1, angiotensin receptor 1; $\Delta\Psi_m$, mitochondrial membrane potential; RAS, rennin-angiotensin system.

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known as dihydroquercetin, is a flavonoid commonly found in *Pseudotsuga taxifolia*, Dahurian larch, *syn Larix dahurica* Turoz (Pinaceae) (Weidmann, 2012). It elicits a wide range of biological effects including antitumor effects, hepatoprotective and anti-inflammatory effects, etc. (Weidmann, 2012). More importantly, taxifolin exerts effective antioxidant effects which contribute to its cardiovascular protective effects. Studies showed that taxifolin decreased the peroxidase activity of cytochome *c* with dioleyl cardiolipin and reduced the lipid radical production in a dose-dependent manner, which were critical for the onset of apoptosis; it also ameliorated cerebral ischemia–reperfusion injury by inhibiting oxidative enzymes and the overproduction of ROS (Vladimirov et al., 2009; Wang et al., 2006). In experiment of diabetes, taxifolin inhibited recombinant human aldose reductase and the sorbitol accumulation in human red blood cells; it also maintained the clarity of rat lens incubated with glucose, suggesting it might be effective in preventing osmotic stress in hyperglycemia (Haraguchi et al., 1997). However, the possible beneficial effects of taxifolin on DCM have little to be addressed till now.

Therefore, the objective of our study was to investigate the potential effects of taxifolin on cardiac dysfunction in streptozotocin (STZ)-induced diabetic mice in vivo and on 33 mM glucose-induced cardiomyoblast H9c2 cell injuries in vitro. Our results demonstrated that taxifolin effectively ameliorated diastolic dysfunction of diabetic mice and showed protective effects against H9c2 cells apoptosis induced by high glucose incubation.

2. Materials and methods

2.1. In vivo experiments

2.1.1. Animals and experimental protocols

The C57BL/6 mice (male, 6–8 weeks old) used in the present study were obtained from Vital River Laboratory Animal Technology (Beijing, China). All procedures involving animals were conducted in strict accordance with the protocols and guidelines approved by the Animal Ethics Committee of Institute of Medicinal Plant Development (Permit Number: 2012-4-23). Surgery was performed under anesthesia and all efforts were made to minimize suffering. The mice were maintained under standard environmental conditions (room temperature at 25 ± 1 °C, humidity of 60% with a 12-h light/dark cycle) and fed with a standard pellet diet and water ad libitum. After one week of adaptation, the mice were randomly divided into the following groups: (1) control group; (2) diabetic model group (DM); (3) DM + taxifolin 100 mg/kg/day group; (4) DM + taxifolin 50 mg/kg/day group; (5) DM + taxifolin 25 mg/kg/day group. Mice in control group and diabetic model group received intragastric administration of vehicle for 4 weeks. Taxifolin of indicated dosage in each group was intragastrically administered to the mice for 4 weeks. Then the mice in diabetic model group and taxifolin group were given a single intraperitoneal dose of 150 mg/kg STZ in citrate buffer (Sigma, USA); the mice in control group received intraperitoneal injection of citrate buffer. On day 3 after STZ injection, tail blood glucose were measured using OneTouch Ultra blood glucose monitoring system (LifeScan, USA). Blood glucose level ⩾20 mmol/l was considered as diabetic for the present study. After 4 weeks, M-mode echocardiography was performed and the mice were sacrificed. The hearts were harvested to perform subsequent experiments. Taxifolin (purity >99%) was obtained from Shanghai Winherb Medical S & T Development (Shanghai, China).

2.1.2. Echocardiographic measurements

M-mode echocardiography was performed using Vevo 770™ High Resolution Imaging System (VisualSonics Inc, Canada) as previously described (Gao et al., 2012). The mice were anaesthetized by abdominal injection of avertin (2,2,2-tribromoethanol, prepared as a 1.2% solution and used in mice at a dosage of 0.2 ml/10 g body weight). Then the chests of the mice were shaved and the mice were placed in the recumbent position. Left ventricle (LV) internal diameter in systole (LVIDs) and diastole (LVIDd), LV posterior wall thickness in systole (LVPWs) and diastole (LVPWd) were measured using M-mode echocardiography. LV end-diastolic volume (LVVd), LV end-systolic volume (LVVs), fractional shortening (FS), ejection fraction (EF), and LV Mass (AW) were automatically calculated by the ultrasound machine.

2.1.3. Tissue collection and histology

At the end of the experiment, hearts of the mice were excised and weighed. Left ventricles were fixed in 4% buffered paraformaldehyde and paraffin-embedded and sectioned at 5 μm. In each group, at least 10 randomly selected sections stained with hematoxylin-eosin were studied for the histological changes.

The degree of fibrosis was investigated by using Masson Trichrome method to stain heart sections for collagen. Quantification of the blue area representing collagen was performed as previously described using the Photoshop software (Dahab et al., 2004).

2.1.4. TUNEL assay

DNA fragmentation in situ was detected by TUNEL assay according to the kit instructions (Roche, Germany). 4',6-diamidino-2-phenylindole (DAPI) was included in the kit as DNA fragments could be stained by TUNEL specifically and produced green fluorescence, while DAPI could combine with complete DNA and debris and produced blue fluorescence. Briefly, tissue sections were fixed with 4% paraformaldehyde at room temperature, and then rinsed with PBS for 5 min and incubated in a permeabilization solution (1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The TUNEL reaction mixture was added and the samples were incubated for 1 h at 37 °C humidified atmosphere (5% CO_2) in the dark and then dyed with 1 μg/ml of DAPI for 15 min. After rinsing with PBS, samples were analyzed under a fluorescence microscope using an excitation wavelength in the range of 450–500 nm and detection in the range 515–565 nm. The apoptotic rate was indicated by percentage of the TUNEL-positive cell number against the total cell number (DAPI-positive) within the same area from five random selected fields in each treatment.

2.1.5. Measurement of myocardial enzymes activities, malondialdehyde (MDA) level and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)

Serum myocardial enzymes activities of lactate dehydrogenase (LDH), CK-MB and AST, level of MDA and activities of SOD, CAT and GSH-Px in myocardium tissue were measured with the corresponding detection kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering, China).

2.1.6. ELISA for detection of angiotensin II level in plasma and myocardium

The concentration of angiotensin II in plasma and myocardium was detected by Mouse-Angiotensin II ELISA Kit according to the manufacturer's brochure (ShangHai WuHao Trade, China). Briefly, the serum or the homogenate of heart tissue was added to plate wells. The enzyme conjugation solution was then added and mixed. After 60 min of incubation reaction at 37 °C, the liquid in the wells was removed and the plates were washed with cleaning liquid. The substrates were then added and the plates were kept from light at room temperature for 15 min reaction. O.D value was read by the ELISA reader at 450 nm.

2.1.7. Determination of NADPH oxidase activities

The activities of NADPH oxidase were investigated by NADPH Oxidase Activity Detection Kit (Genmed Scientifics, USA) according to the manufacturer's brochure. Briefly, the protein concentration was determined by BCA assay. Reactive solution was mixed into buffer solution, and 100 μl of sample was added and placed the system in the incubator for 3 min. The substrate solution was added and mixed (within 3 s) and the absorbance was then measured at 550 nm on microplate reader (BioTek, USA).

2.1.8. Western blot analysis

Cardiac tissue homogenate was lysed. Protein concentrations were measured by BCA assay. Equal amounts of protein (40 μg) from each sample were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Millipore Corporation, USA). Non-specific sites were blocked by the incubating membranes (2 h, room temperature) in 5% non-fat milk powder in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBS-T). Then, the membranes were incubated overnight at 4 °C with the primary antibodies from Santa Cruz Biotechnology (Bcl-2, 1:200; Bax, 1:250; JAK2, 1:1000; p-JAK2, 1:500; STAT3, 1:1000; p-STAT3, 1:500). The membranes were washed with TBS-T and incubated with the appropriate secondary HRP-conjugated antibodies at a 1:4000 dilution. Following 30 min wash, the membranes were visualized by enhanced chemiluminescence. The band intensity was measured and quantified.

2.2. In vitro experiments

2.2.1. Cell culture and treatment

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 mg/ml of streptomycin and maintained in a humidified incubator of 95% air/5% CO_2 at 37 °C (Park et al., 2003). The cells were divided into following groups: (1) Control group (normal glucose concentration, 5.5 mM); (2) High glucose group (HG, 33 mM); (3) HG + Taxifolin 40 μg/ml group; (4) HG + Taxifolin 20 μg/ml group; (5) HG + taxifolin 10 μg/ml group; (6) 33 mM Mannitol group. The cells were pretreated with different concentrations of taxifolin for 12 h before incubated with 33 mM D-glucose for 48 h (Cai et al., 2002; Kumar et al., 2012). Taxifolin was freshly dissolved in DMSO as a stock solution and diluted with MDEM when used. The concentration of DMSO was equal in all groups of 0.1% (v/v). Normal concentration (5.5 mM) of glucose served as control. Effect of high osmolarity was ruled out by adding same concentrations of

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mannitol (33 mM) in the cultures in the present study. In some experiments, the cells were grown on slides, which were used for Hoechst 33342 staining, TUNEL assay and JC-1 staining.

2.2.2. Morphological assessment and quantification of apoptotic myocytes

Hoechst 33342 staining was used for the morphological analyses of the apoptosis of cells, with apoptotic cells exhibiting nuclear chromatin condensation and fragmentation. After treatment, the cells were incubated with 5 mg/ml Hoechst 33342 for 15 min, washed twice with phosphate-buffered saline (PBS), and visualized by fluorescence microscopy (Leica, Germany).

2.2.3. Flow cytometric detection of apoptosis

After the cells were treated with high concentration of glucose, apoptosis was evaluated with Annexin V-FITC/PI Apoptosis kit according to the manufacturer's brochures (Invitrogen, USA). In brief, the cells were harvested, washed twice with cold PBS, incubated with the 5 μl FITC-Annexin V and 1 μl PI working solution (100 μg/ml) for 15 min in the dark at room temperature, and then cellular fluorescence was measured by flow cytometry analysis with a FACSCalibur Flow Cytometer (BD Biosciences, USA).

2.2.4. Detection of intracellular ROS production

The production of intracellular ROS was analyzed using Reactive Oxygen Species detection kit according to the manufacturer's brochures (Invitrogen, California). Briefly, cells were washed with 1× wash buffer after treatment, and then ROS detection solution was added. The cells were stained at 37 °C in the dark for 30 min and visualized by fluorescence microscopy (Leica, Germany). The fluorescence was read on a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific, USA) at 495 nm excitation and 529 nm emission wavelengths. The fold-increases in ROS level were determined by comparing the results with the level of the control group.

2.2.5. Determination of mitochondrial transmembrane potential ($\Delta\Psi_m$)

5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Invitrogen, USA) was used to determine the changes in mitochondrial transmembrane potential. 10 μl of 200 μM JC-1 (2 μM final concentration) was added to the cells on the coverlips, incubated for 30 min in the dark and washed twice with PBS. The cells labeled with JC-1 were observed under fluorescence microscopy (Leica, Germany). JC-1 fluorescence was measured from a single excitation wavelength (488 nm) with dual emission (a shift from green at 530 nm to red at 590 nm).

2.2.6. Analysis of caspase-3 and caspase-9 activities

Caspase-3 and caspase-9 activities were measured using Fluorometric Assay Kits (BioVision, USA) according to the manufacturer's instructions respectively. The cells were resuspended in lysis buffer and kept on ice for 10 min. Then, 50 μl of 2× reaction buffer containing 10 mM dithiothreitol was added to each sample. 5 μl of 1 mM substrate (DEVD-AFC or LEHD-AFC for caspase-3 or caspase-9, respectively) was added and incubated at 37 °C for 1.5 h. The samples were read on a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific, USA) at 400 nm excitation and 505 nm emission wavelengths. The fold-increases in caspase activities were determined by comparing the results with the level of the control group.

2.2.7. Western blot analysis

Cultured H9c2 cells were harvested, washed with PBS, and lysed with cell lysis buffer containing 1% phenylmethylsulfonylfluoride. The lysate was centrifuged at 12,000*g* for 15 min to remove the insoluble materials. Supernates were collected. For cytochrome *c*, the cytosolic and mitochondrial fractions were separated by Cell Mitochondrial Isolation Kit (Beyotime, China) according to the manufacturer's instructions. Primary antibodies from Santa Cruz Biotechnology (Bcl-xL, 1:500; Bad, 1:500; Bak, 1:500; cytochrome c, 1:500; cleaved caspase-3, 1:500; cleaved caspase-9, 1:500) were used for indicated proteins.

2.3. Statistical analysis

Data from at least three independent experiments were expressed as means ± SD. Statistical comparisons between different groups were measured by using one-way ANOVA followed by Student–Newman–Keuls test. The level of significance was set at $p < 0.05$.

3. Results

3.1. In vivo experiments

3.1.1. Effects of taxifolin on blood glucose, water intake, food consumption and heart weight/body weight ratio

72 h after STZ injection, blood glucose of the mice markedly increased and the mice also manifested classical symptoms of diabetes, including increased water intake and food consumption and polyuria. High glucose increases the osmotic pressure of the urine and results in increased fluid loss, causing dehydration and increased thirst; the body cannot make full use of glucose due to insulin deficiency, which leads to lack of energy and results in polyphagia. For the entire experiment duration, taxifolin showed no effect on blood glucose or food consumption of the mice. However, taxifolin treatment decreased water intake of diabetic mice (Table 1). Body weight (BW) and heart weight (HW) of mice in diabetic group were lower than those in control group, while HW/BW ratio significantly increased in diabetic mice. Taxifolin increased body weight and heart weight, and decreased HW/BW ratio in taxifolin treatment group (Table 2).

3.1.2. Effects of taxifolin on cardiac pathology in diabetic mice

Hematoxylin and eosin (H & E) staining of the heart tissue (Fig. 1A) showed that the myocardial fibers arranged regularly and the cardiac myocytes showed normal morphology with distinct cell borders and homogeneous oval nuclei in control group mice. However, in diabetic model group, the arrangement of cardiac fibers was disrupted, loss of nuclear existed in some of cardiomyocytes and the intercellular border was obscure. Taxifolin treatment ameliorated the structural abnormalities in the hearts of diabetic mice. Cardiac fibrosis resulting from collagen deposition was investigated by Masson staining. As seen in Fig. 1A and B, interstitial collagen deposition increased significantly in diabetic mice, while taxifolin treatment decreased cardiac fibrosis by inhibiting collagen deposition.

TUNEL assay was performed to investigate the effects of taxifolin on cardiomyocyte apoptosis. Few TUNEL-positive cells were detected in control group, while TUNEL-positive cells increased dramatically in diabetic model group (1.37% ± 0.22% and 24.48% ± 0.84% respectively). The treatment of taxifolin considerably decreased the amount of TUNEL-positive cardiac myocytes (Fig. 1A and B).

3.1.3. Effects of taxifolin on heart function in diabetic mice

To investigate the effect of taxifolin treatment on heart function of diabetic mice, M-mode echocardiography was used to measure cardiac parameters. LVVd significantly decreased in diabetic model group compared with control group, while taxifolin treatment prevented LVVd decrease in diabetic mice. No difference between groups existed as far as LVVs was concerned. There was no statistical difference in EF and FS between groups, although EF and FS in diabetic mice tended to be lower than in control group. LV mass in the diabetic model group was lower than that in control group, LV mass/body weight ratio was more higher in diabetic model group than in control group. Taxifolin decreased LV mass/body weight (Fig. 2 and Table 3).

3.1.4. Effects of taxifolin on myocardial enzymes, lipid peroxidation and antioxidative enzymes activities

Oxidative stress, i.e., the imbalance of reactive oxygen species (ROS) production and elimination has been indicated to play an important role in the pathogenesis of diabetic cardiomyopathy (Khullar et al., 2010). Serum LDH, CK-MB and AST levels were higher in diabetic model group than in control group, which were decreased by taxifolin treatment. Results showed that MDA level increased significantly in diabetic mice myocardium. The activities of myocardial antioxidative enzymes SOD and GSH-Px were decreased in diabetic mice, indicating that antioxidant capacity was compromised in diabetic myocardium. CAT activities in diabetic model group showed no difference when compared with control group. Taxifolin enhanced myocardial antioxidative enzymes activities and inhibited lipid peroxidation as indicated by the decrease of MDA level (Table 4).

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Table 1
Effects of taxifolin (Tax) on general characteristics in control and diabetic mice.

	Group	Blood glucose (mM)	Water intake (ml/kg/day)	Food consumption (g/kg/day)	Body weight (g)
Day 1	Control	8.16 ± 0.72	240.21 ± 23.21	110.21 ± 14.16	22.37 ± 1.14
	DM	8.39 ± 1.76	238.99 ± 30.15	108.65 ± 13.64	23.11 ± 0.89
	DM + Tax 25 mg/kg	9.08 ± 1.3	237.68 ± 29.19	115.75 ± 10.97	22.09 ± 1.01
	DM + Tax 50 mg/kg	8.64 ± 1.79	231.41 ± 32.94	112.62 ± 11.44	22.60 ± 1.47
	DM + Tax 100 mg/kg	8.81 ± 1.68	227.38 ± 30.04	117.23 ± 12.53	22.21 ± 1.67
Day 28	Control	8.32 ± 1.02	257.65 ± 30.23	123.21 ± 10.33	26.33 ± 1.31
	DM	8.21 ± 1.76	242.37 ± 25.96	131.64 ± 15.54	26.76 ± 1.73
	DM + Tax 25 mg/kg	8.65 ± 1.43	262.42 ± 33.51	128.76 ± 13.32	26.82 ± 1.55
	DM + Tax 50 mg/kg	8.42 ± 1.53	259.63 ± 25.57	123.43 ± 16.57	27.02 ± 1.46
	DM + Tax 100 mg/kg	8.17 ± 1.65	252.06 ± 32.11	123.21 ± 14.83	26.74 ± 1.52
Day 32	Control	8.52 ± 1.22	256.42 ± 25.62	121.67 ± 15.37	25.45 ± 1.78
	DM	30.8 ± 0.84#	1292.55 ± 38.17#	342.25 ± 18.29#	25.54 ± 1.54
	DM + Tax 25 mg/kg	31.4 ± 1.7	1313.41 ± 41.33	330.86 ± 23.74	26.12 ± 1.92
	DM + Tax 50 mg/kg	30.88 ± 0.78	1279.66 ± 30.48	335.62 ± 16.28	26.94 ± 1.34
	DM + Tax 100 mg/kg	29.63 ± 1.76	1155.33 ± 27.94*	341.86 ± 18.97	25.87 ± 1.88
Day 56	Control	8.28 ± 1.54	271.33 ± 30.23	140.79 ± 10.32	29.64 ± 2.00
	DM	31.76 ± 2.11	1363.64 ± 40.43#	368.00 ± 20.22#	18.92 ± 1.22#
	DM + Tax 25 mg/kg	32.44 ± 1.82	1349.54 ± 34.12	364.57 ± 21.65	19.81 ± 1.53
	DM + Tax 50 mg/kg	31.12 ± 1.65	1232.24 ± 39.77*	377.98 ± 19.98	22.37 ± 0.83*
	DM + Tax 100 mg/kg	32.09 ± 0.99	1173.96 ± 27.02*	357.29 ± 27.05	25.33 ± 1.78*

DM: diabetic model group. Data are means ± SD; $n = 10$ per group.
# $P < 0.05$ vs control group.
* $P < 0.05$ vs DM group.

Table 2
Effects of taxifolin (Tax) on heart weight and heart weight/body weight ratio.

	Control	DM	DM + Tax 25 mg/kg	DM + Tax 50 mg/kg	DM + Tax 100 mg/kg
Heart weight (g)	0.153 ± 0.006	0.107 ± 0.004#	0.111 ± 0.005	0.119 ± 0.003*	0.133 ± 0.006*
Body weight (g)	29.42 ± 0.98	18.77 ± 1.21#	19.82 ± 0.98*	22.45 ± 1.11*	25.09 ± 1.31*
Heart weight/body weight ratio	0.0052 ± 0.0002	0.0057 ± 0.0001#	0.0056 ± 0.00012	0.0053 ± 0.00013*	0.0053 ± 0.00011*

DM: Diabetic model group. Data are means ± SD; $n = 10$ per group.
# $P < 0.05$ vs control group.
* $P < 0.05$ vs DM group.

3.1.5. Effects of taxifolin on angiotensin II (Ang II) level

Recent studies showed that angiotensin II is involved in the cardiac myocyte apoptosis in diabetic cardiomyopathy (Nemer et al., 2006; Singh et al., 2008). Angiotensin concentration was analyzed by ELISA assay. As shown in Fig. 3B, circulating Ang II level showed no difference between groups; however, angiotensin level increased twofold in diabetic mice myocardium compared with control mice (Fig. 3A). Taxifolin treatment decreased Ang II level in myocardium of diabetic mice.

3.1.6. Effects of taxifolin on NADPH oxidase activities

NADPH oxidase activation has been reported to contribute to the overproduction of ROS in the pathogenesis of diabetic cardiomyopathy. Fig.4 showed that NADPH oxidase activities increased significantly after STZ treatment, which was decreased by pretreatment with taxifolin.

3.1.7. Effects of taxifolin on expression of proteins associated with diabetic cardiomyopathy

The effects of taxifolin on apoptosis-related Bcl-2 family and JAK/STAT pathway in diabetic mice hearts were investigated by Western blot analysis. Bcl-2 protein level was decreased and bax level was increased in diabetic mice heart, resulting in a higher Bax/Bcl-2 ratio than that in normal control group. The results indicated that the mitochondrial pathway of apoptosis might be involved in the pathogenesis of diabetic cardiomyopathy. Taxifolin treatment significantly lowered the bax/bcl-2 ratio by increasing bcl-2 expression and decreasing bax expression (Fig. 5). Results showed that the protein expression of p-JAK was significantly increased in mice myocardium after STZ injection, while taxifolin treatment further increased the expression of p-JAK (Fig. 6A). Similar tendency was observed in the protein expression of p-STAT3 (Fig. 6B).

3.2. In vitro experiments

3.2.1. Effects of taxifolin on high-glucose induced H9c2 cells apoptosis

Hoechst 33342 staining and Annexin V-FITC/PI staining for flow cytometry analysis were performed to investigate the protective effects of taxifolin on high-glucose induced H9c2 cells apoptosis. As shown in Fig. 7A, normal H9c2 cells exhibited homogeneous fluorescence intensity of nuclei, while heterogeneous intensity and chromatin condensation of nuclei appeared in high-glucose treated cells. Quantitative analysis by flow cytometry showed that 19.89% ± 2.13% of cells were apoptotic in high-glucose treated cells. Taxifolin significantly reduced the percentages of apoptotic cells down to 14.67% ± 1.22% and 11.40% ± 1.05% in 20 μg/ml and 40 μg/ml, respectively (Fig. 7B).

3.2.2. Effects of taxifolin on intracellular ROS level

Intracellular ROS exhibited green fluorescence under the microscope. In normal control cells, few cells exhibited green fluorescence, indicating that intracellular ROS level was low. 33 mM glucose treatment significantly increased intracellular ROS level. Taxifolin decreased ROS level in a dose-dependent manner (Fig. 8).

3.2.3. Effects of taxifolin on caspase-3, caspase-9 activities

Caspase enzymes are important factors modulating apoptotic cascade. Results showed that caspase-3 and caspase-9 activities were significantly higher in 33 mM glucose group than in control group. This result is consistent with the results that apoptosis

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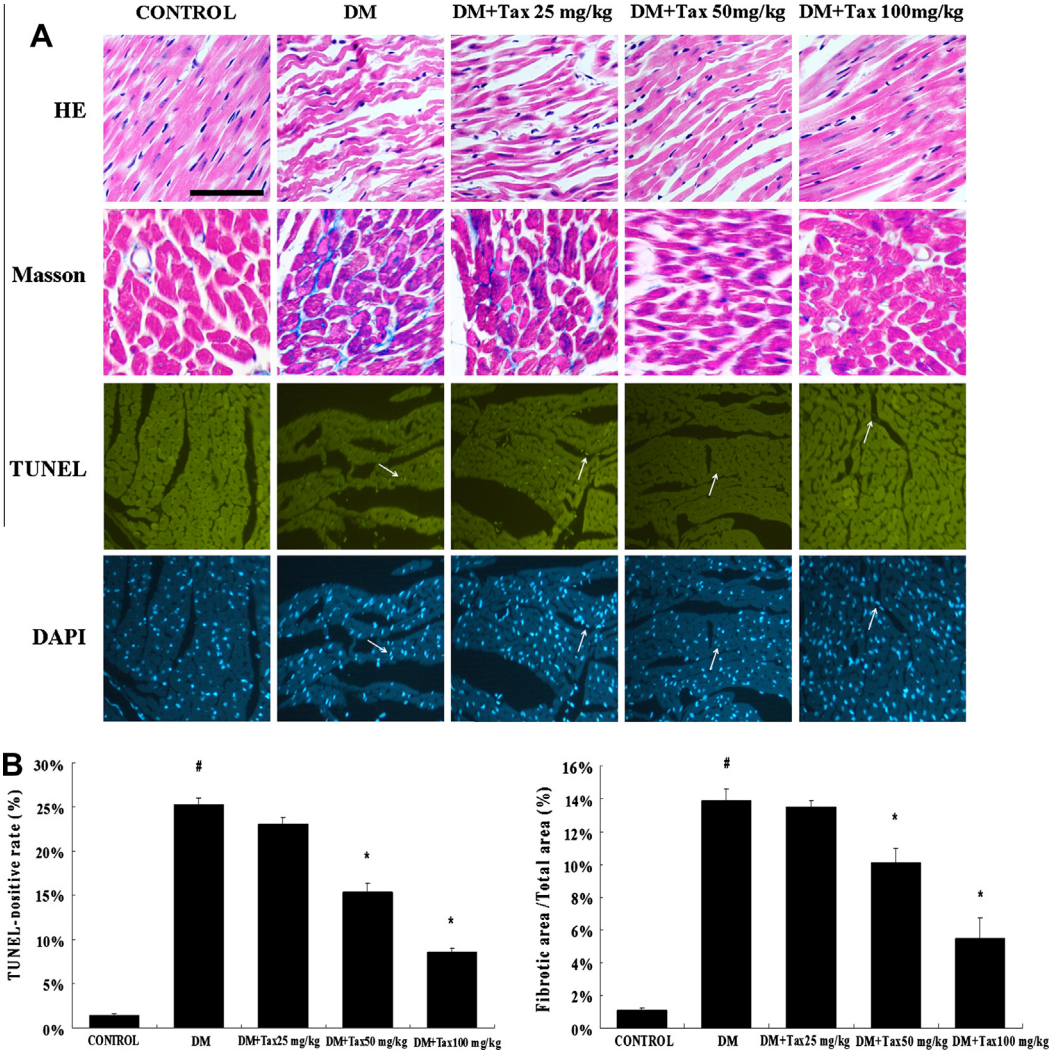

Fig. 1. Taxifolin attenuated pathological changes in the heart of diabetic mice. (A) Representative pictures of hematoxylin and eosin (HE) staining (1A first row), Masson staining (1A second row), TUNEL (1A third row) and DAPI (1A fourth row) staining of myocardium tissue. The bar represents 50 μm. Arrow indicated TUNEL-positive cells and the nuclei showed green fluorescence. (B) Left: Quantification of TUNEL-positive cells. Right: Quantification analysis of fibrosis. Data are means ± SD; $n = 6$ per group; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

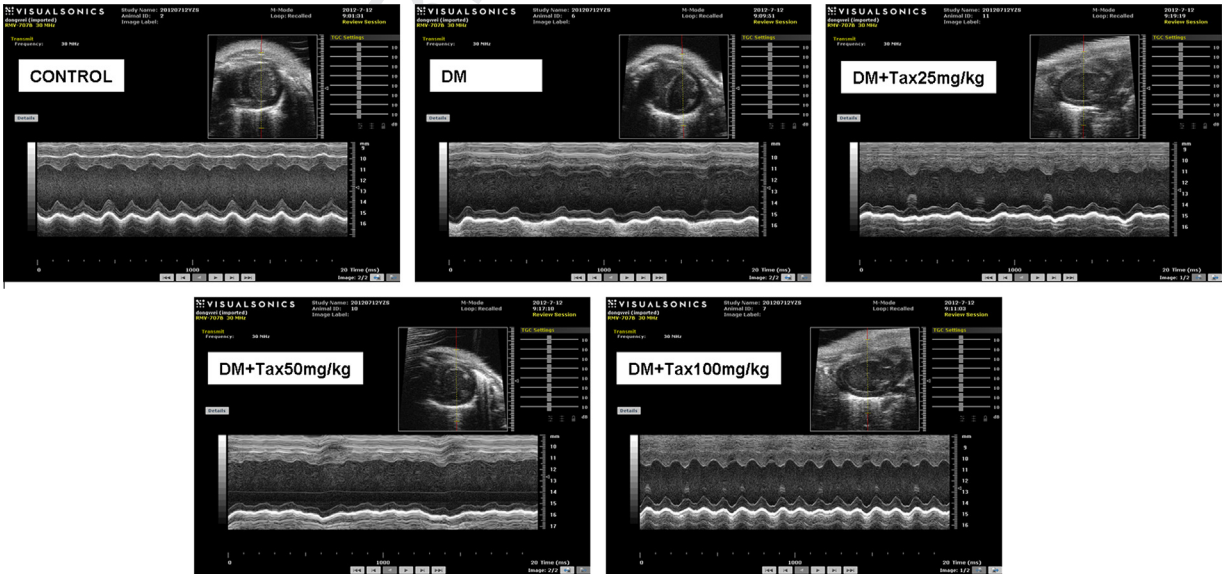

Fig. 2. Representative images of M-mode echocardiogram.

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Table 3
Effects of taxifolin (Tax) on left ventricular functions in mice.

	Control	DM	DM + Tax 25 mg/kg	DM + Tax 50 mg/kg	DM + Tax 100 mg/kg
LVVd (μl)	93.86 ± 4.23	57.04 ± 2.76#	55.33 ± 4.19	64.26 ± 2.66*	75.76 ± 3.21*
LVVs (μl)	30.01 ± 2.21	32.75 ± 1.54	31.54 ± 1.65	29.05 ± 2.23	29.53 ± 1.32
%EF (%)	56.34 ± 4.33	52.05 ± 5.54	54.32 ± 3.23	55.54 ± 2.78	56.17 ± 3.44
%FS (%)	27.32 ± 1.35	26.28 ± 1.55	25.13 ± 1.89	24.18 ± 1.77	25.78 ± 1.03
LV mass (AW) (mg)	109.21 ± 5.32	80.85 ± 2.12#	82.59 ± 2.22	89.08 ± 2.81*	98.20 ± 5.9*
LV mass/body weight	3.68 ± 0.14	4.27 ± 0.12#	4.17 ± 0.15	3.98 ± 0.08*	3.88 ± 0.13*

DM: diabetic model group. LVVd, LV end-diastolic volume; LVVs, LV end-systolic volume; FS, fractional shortening; EF, ejection fraction and LV Mass (AW) were automatically calculated by the ultrasound machine. Data are means ± SD; $n = 6$ per group.
# $P < 0.05$ vs control group.
* $P < 0.05$ vs DM group.

Table 4
Effects of taxifolin on lipid peroxidation and antioxidative enzymes activities.

	Control	DM	DM + Tax25 mg/kg	DM + Tax50 mg/kg	DM + Tax100 mg/kg
LDH (U/L)	416.67 ± 49.22	1333.33 ± 56.74#	1278.89 ± 38.98	942.59 ± 43.21*	833.33 ± 20.54*
CK-MB (U/L)	39.76 ± 4.22	78.44 ± 3.56#	76.98 ± 4.76	62.13 ± 5.55*	47.12 ± 4.74*
AST (IU/ml)	16.38 ± 1.12	35.28 ± 2.97#	35.41 ± 2.22	27.28 ± 2.33*	20.53 ± 1.78*
MDA (nmol/mg)	5.99 ± 0.98	12.81 ± 0.56#	10.39 ± 0.38*	8.51 ± 0.77*	6.88 ± 0.37*
SOD (U/mg)	88.49 ± 3.11	56.13 ± 2.12#	57.14 ± 4.54	69.66 ± 4.11*	75.39 ± 3.67*
GSH-Px (U/mg)	28.16 ± 2.11	14.41 ± 1.23#	18.82 ± 1.56*	22.76 ± 1.76*	24.35 ± 2.22*
CAT (U/mg)	2.50 ± 0.26	2.52 ± 0.22	2.51 ± 0.14	2.66 ± 0.22	3.16 ± 0.13*

DM: diabetic model group. Data are means ± SD; $n = 10$ per group.
# $P < 0.05$ vs control group.
* $P < 0.05$ vs DM group.

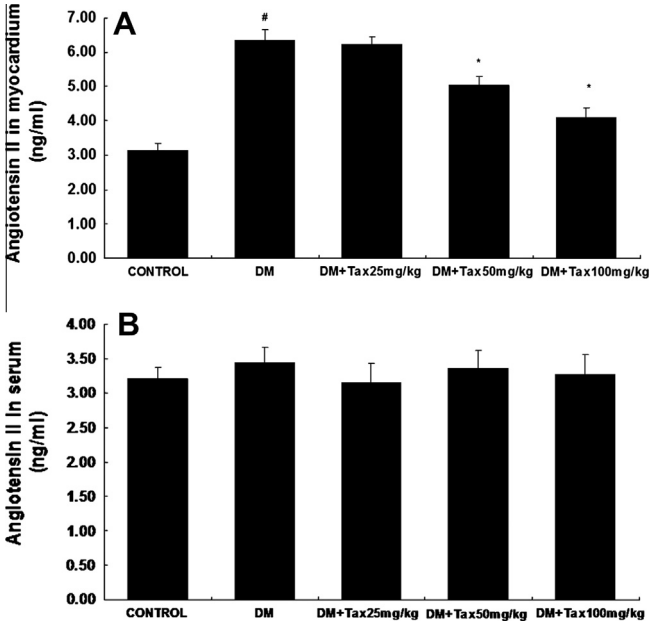

Fig. 3. Effects of taxifolin on angiotensin II level. (A) Taxifolin decreased angiotensin II level in myocardium of diabetic mice. (B) Taxifolin showed no effects on angiotensin II level in serum of diabetic mice. Data are means ± SD; $n = 10$ per group; #$P < 0.05$ vs control group; *$P < 0.05$ vs DM group.

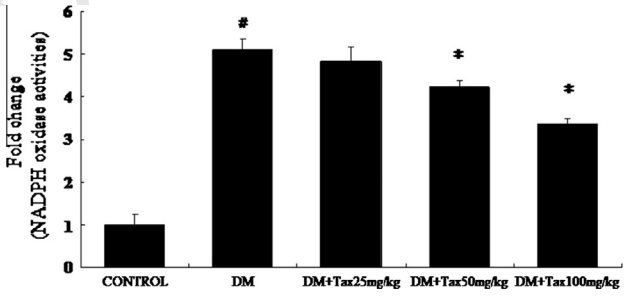

Fig. 4. Effects of taxifolin on NADPH oxidase activities. Data are means ± SD; $n = 10$ per group; #$P < 0.05$ vs control group; *$P < 0.05$ vs DM group.

increased upon high glucose treatment. Taxifolin showed inhibitory effects on activation of caspase enzymes (Fig. 9A and B). Results from western blot analysis of cleaved caspase-3 and caspase-9 protein expression further confirmed the effects of taxifolin (Fig. 9C and D).

3.2.4. Effects of taxifolin on mitochondrial transmembrane potential ($\Delta\Psi_m$)

The disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) is one of the early events of mitochondrial pathway activation of apoptosis. As shown in Fig. 10, mitochondria in normal H9c2 cells emitted red fluorescence after the cells were stained by JC-1. High glucose caused an increase in green fluorescence in cells, indicating the depolarization of mitochondrial transmembrane potential. Pretreatment with taxifolin maintained mitochondrial transmembrane potential.

3.2.5. Effects of taxifolin on expression of proteins associated with apoptosis

Proteins related to mitochondrial pathway of apoptosis were further examined by Western blot analysis. Cytochrome *c* in the cytoplasm was increased in 33 mM glucose treated cells. Taxifolin treatment decreased the release of cytochrome *c* into the cytoplasm. The expression of Bcl-x_L, an anti-apoptotic protein member of bcl-2 family, was decreased after high glucose treatment. The expressions of pro-apoptotic members of bcl-2 family such as bad and bak were higher in glucose model group, while taxifolin showed effects of decrease (Fig. 11).

4. Discussion

Considerable evidence suggests that overproduction of ROS induced by hyperglycemia is a key factor in the development of

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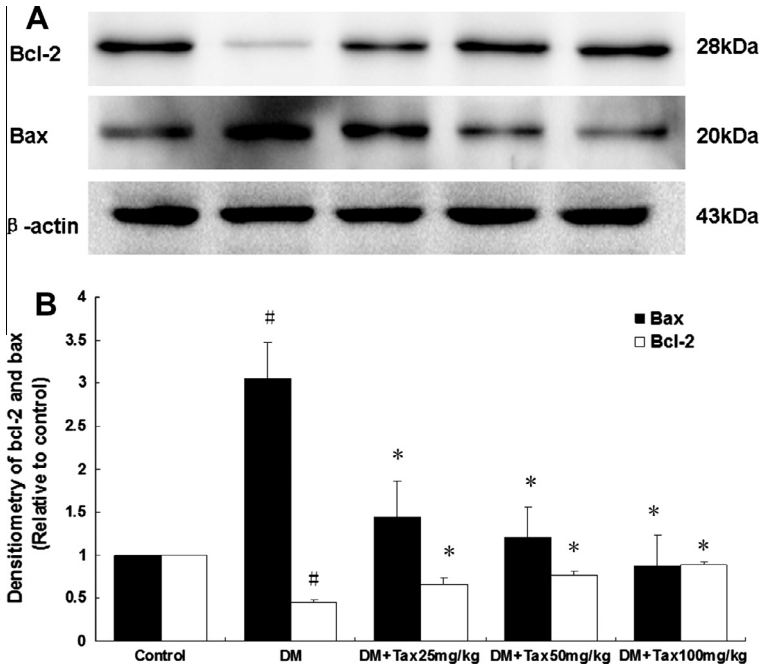

Fig. 5. Effects of taxifolin on protein expression of Bcl-2 and Bax. (A) Representative western blot image. (B) Quantification of Bcl-2 and bax compared with control. Taxifolin increased anti-apoptotic protein Bcl-2 expression and decreased pro-apoptotic protein Bax expression. Data are means ± SD; $n = 3$ per group; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

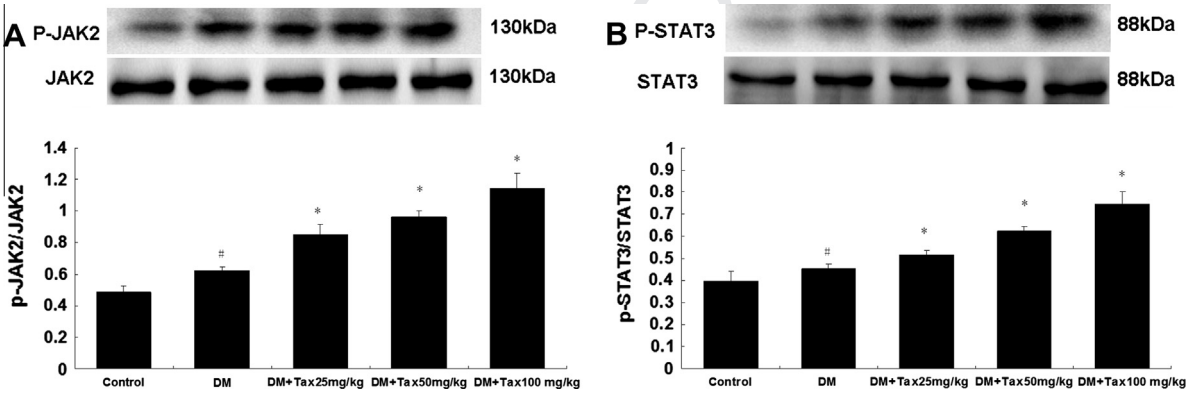

Fig. 6. Effects of taxifolin on protein expression of JAK2 and STAT3. (A) Western blot analysis of p-JAK2 and JAK2. (B) Western blot analysis of p-STAT3 and STAT3. Data are means ± SD; $n = 3$ per group; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

diabetic cardiomyopathy (DCM) (Khullar et al., 2010; Watanabe et al., 2010). In the present study, the results of in vivo and in vitro experiments showed that the antioxidant taxifolin protected the heart from structural and functional changes in STZ-induced diabetic mice. It significantly inhibited the apoptosis of cardiac myoblast H9c2 cells treated with high glucose by inhibiting oxidative stress and modulating the mitochondrial pathway. These findings indicated that taxifolin might be a potential therapeutic agent to treat diabetic cardiomyopathy.

Left ventricle (LV) diastolic dysfunction is an early sign of diabetic cardiomyopathy, preceding the onset of systolic dysfunction (Schannwell et al., 2002). The pathogenesis of LV dysfunction in diabetic heart has not been fully elucidated. In the present study, STZ injection caused morphological changes of heart tissue, increased interstitial collagen deposition, decreased heart weight and increased heart/body weight ratio and left ventricle (LV) mass/body weight ratio, indicating cardiac hypertrophy and fibrosis which lead to impaired relaxation and decreased compliance of heart muscle. M-mode echocardiography confirmed that LV end-diastolic volume (LVVd) significantly decreased in diabetic model group compared to control group, which suggested the impairment of LV diastolic function. No difference between groups was observed as far as LV end-systolic volume (LVVs), ejection fraction (EF) and fractional shortening (FS) were concerned although EF and FS in diabetic mice tended to be lower than in control group. Taxifolin treatment attenuated cardiac morphological changes, inhibited interstitial collagen deposition, decreased heart/body weight ratio and left ventricle (LV) mass/body weight ratio, and thus attenuated cardiac diastolic dysfunction.

Oxidative stress is defined as the imbalance between the production and the elimination of free radicals, which plays a critical role in the development of heart failure and left ventricular remodeling in DCM. The decrease of endogenous antioxidant capacity in myocardium contributes to the oxidative stress in the pathogenesis of DCM. In the present study, STZ injection induced myocardium damage as indicated by elevated serum LDH, CK-MB and

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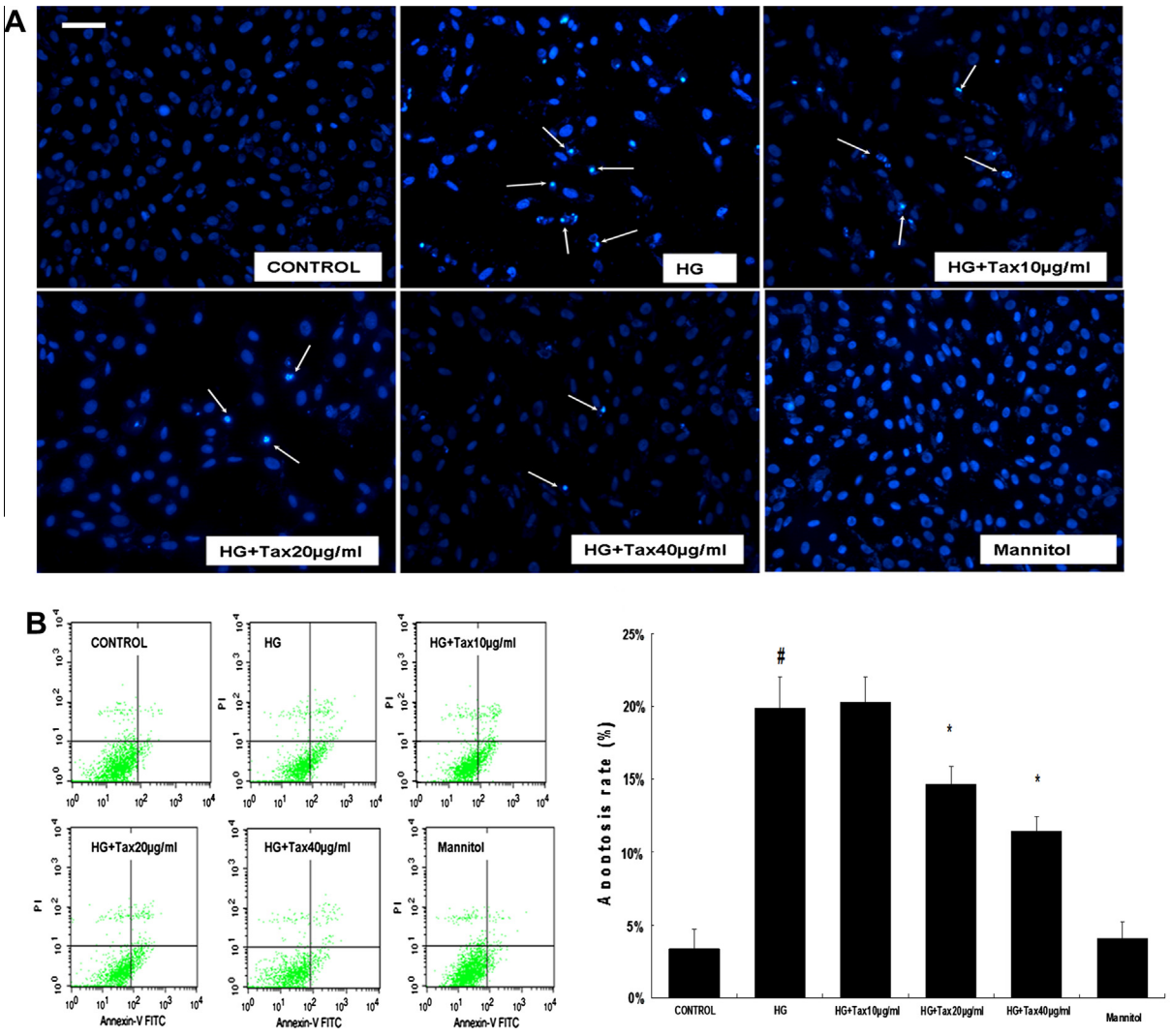

Fig. 7. Effects of taxifolin on high-glucose induced H9c2 cells apoptosis. (A) Representative images of Hoechst 33342 staining. Normal cells exhibited homogeneous fluorescence intensity of nuclei, while apoptotic cells showed heterogeneous intensity and chromatin condensation of nuclei. The bar represents 50 μm. Arrow indicated apoptotic H9c2 cells. (B) Representative images (Left) and quantitation (Right) of flow cytometry analysis. Data are presented as means ± SD from three independent experiments; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

AST levels as well as increased MDA content in diabetic mice myocardium. The marked decrease in SOD and GSH-Px activities was observed, while no significant change was observed in CAT activities. Interestingly, both increase (Ebrahimi et al., 2009; Shirpoor et al., 2009) and decrease of enzyme activities of SOD and GSH-Px (Kaul et al., 1996, 1995) in diabetic myocardium have been reported in previous literatures. There were also reports of CAT activities increase in DCM (Stefek et al., 2000). The inconsistency may be caused by differences in animals used, the severity of DCM and duration of experiments. The administration of taxifolin reduced the release of myocardial enzymes into the blood and decreased MDA content by enhancing the antioxidative activities of SOD, GSH-Px and CAT. Our in vitro experiments also confirmed that taxifolin treatment inhibited the increase of intracellular ROS level of H9c2 cells treated with 33 mM glucose. Thus, the protective effects of taxifolin might be related to its antioxidant capabilities.

The existence of apoptosis in the hearts of patients with diabetes and animals with STZ-induced diabetes has been reported in many studies (Cai et al., 2002; Fiordaliso et al., 2000; Frustaci et al., 2000). Apoptosis of cardiac myocytes contributes to cardiac remodeling and the progression of heart failure. Cardiomyocyte apoptosis is also the predominant change in diabetic cardiomyopathy (Frustaci et al., 2000). In the present study, hyperglycemia induced apoptosis both in the hearts of STZ-induced diabetic mice and in H9c2 cells treated with high glucose, while taxifolin significantly inhibited hyperglycemia-induced cardiomyocytes apoptosis. Taxifolin alone showed no detrimental effects on the myocardium of the mice or on H9c2 cells in our study (see Supplementary Material Table 5 and Figs. 9 and 10). To clarify the molecular basis of the effects of taxifolin, we studied the apoptosis signaling pathways. Results showed that caspase-3 and caspase-9 activities were significantly increased in H9c2 cells treated with 33 mM glucose, indicating the involvement of mitochondrial pathway. Taxifolin treatment suppressed caspase-3 and caspase-9 activation and restored the depolarization of mitochondrial transmembrane potential ($\Delta\Psi_m$). Furthermore, taxifolin also showed effects on Bcl-2 family proteins, which are the major regulators of mitochondrial permeability. The expressions of pro-apoptotic proteins Bax and Bak were increased by hyperglycemia, while the expressions of anti-apoptotic proteins Bcl-2 and Bcl-xL were decreased. Taxifolin downregulated bax and bak expressions and upregulated bcl-2 and bcl-xl expressions, thus monitoring the release of pro-apoptotic factors such as cytochrome *c* from

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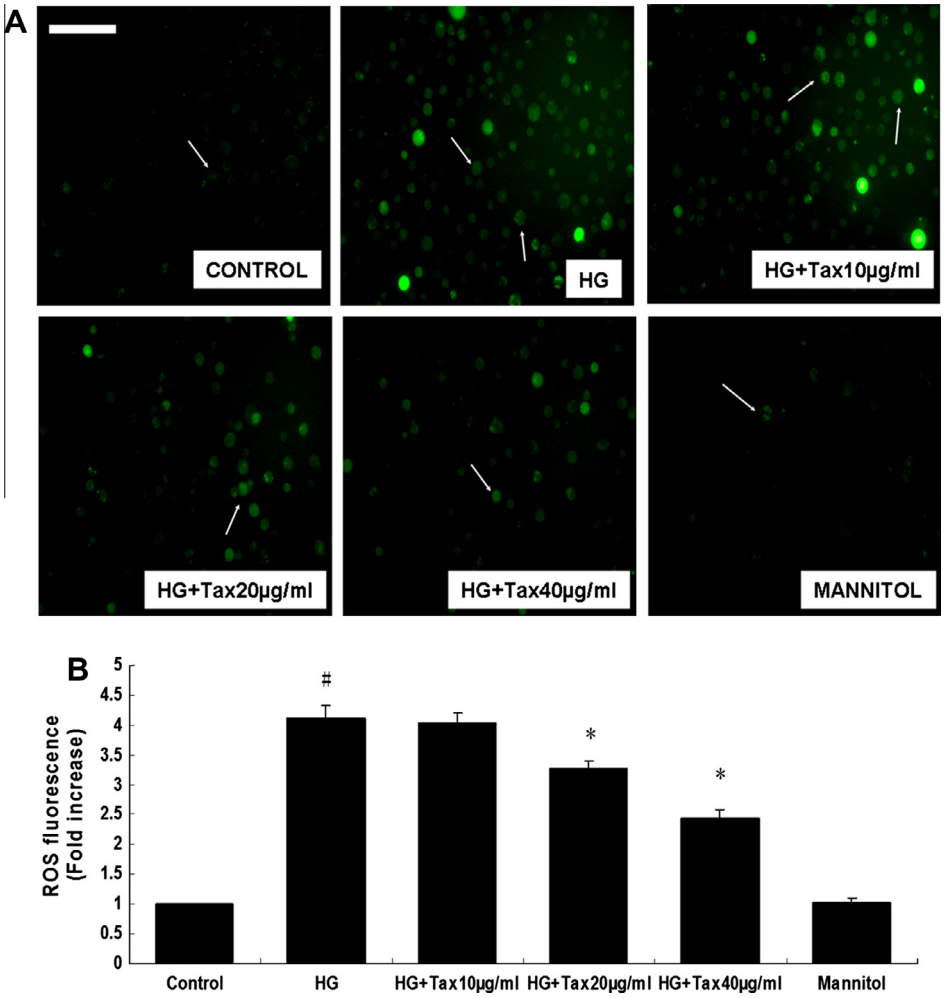

Fig. 8. Representative images of intracellular ROS staining. (A) Representative images of ROS staining. Cells with green fluorescence (Arrow) indicated elevated intracellular ROS level. (B) Quantitative analysis of ROS staining. Taxifolin treatment inhibited intracellular ROS level dose-dependently. The bar represents 50 μm. Data are means ± SD; $n = 10$ wells per group; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

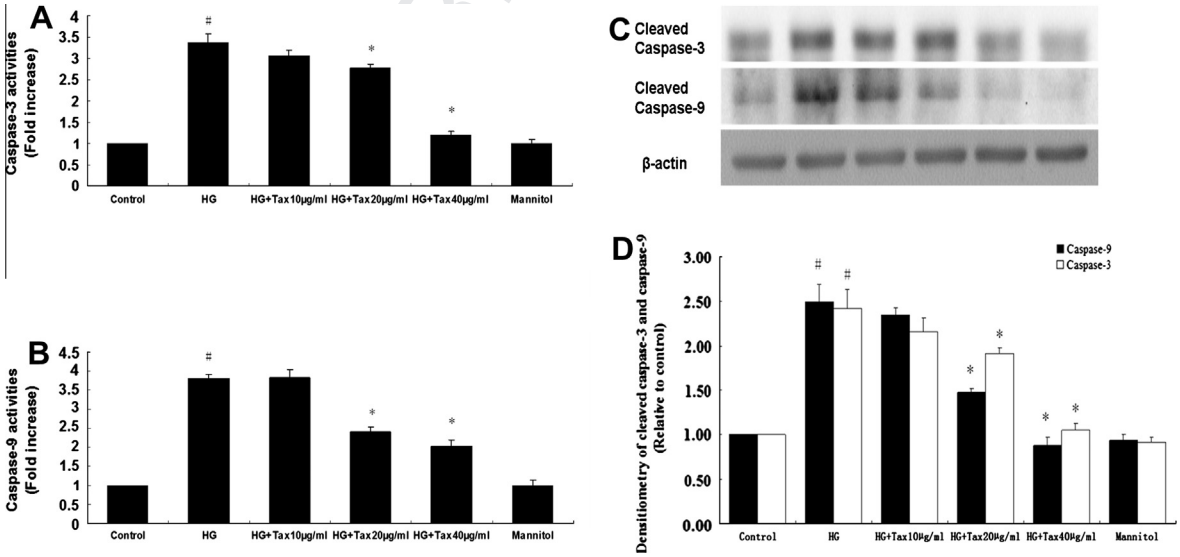

Fig. 9. Effects of taxifolin on caspase-3 and caspase-9. A and B: Caspase-3 and caspase-9 activities measured by Fluorometric Assay. C and D: Western blot analysis of cleaved caspase-3 and cleaved caspase-9. Data are means ± SD; $n = 10$ wells per group; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

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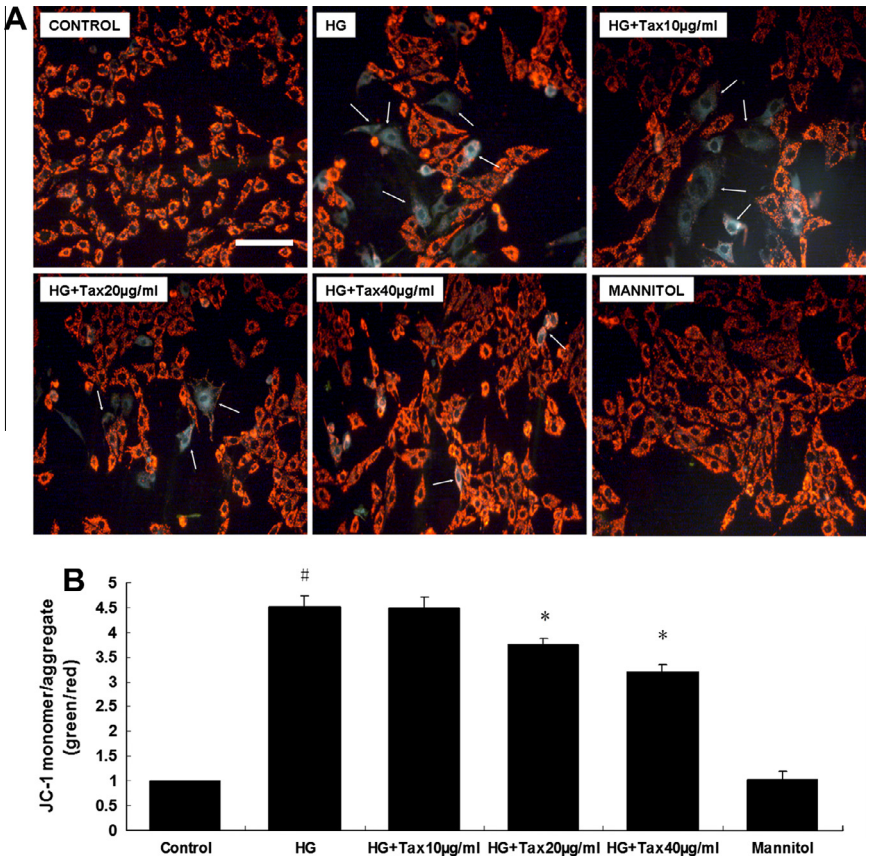

Fig. 10. Effects of taxifolin on mitochondrial transmembrane potential (JC-1 staining). (A) Representative images of JC-1 staining. Normal mitochondria exhibited red fluorescence, while apoptotic cells presented with green fluorescence of mitochondria (Arrow). (B) Quantitative analysis of JC-1 staining. Taxifolin treatment inhibited depolarization of mitochondrial memebrane potential caused by 33 mM glucose incubation. The bar represents 50 μm. Data are means ± SD; $n = 10$ wells per group; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

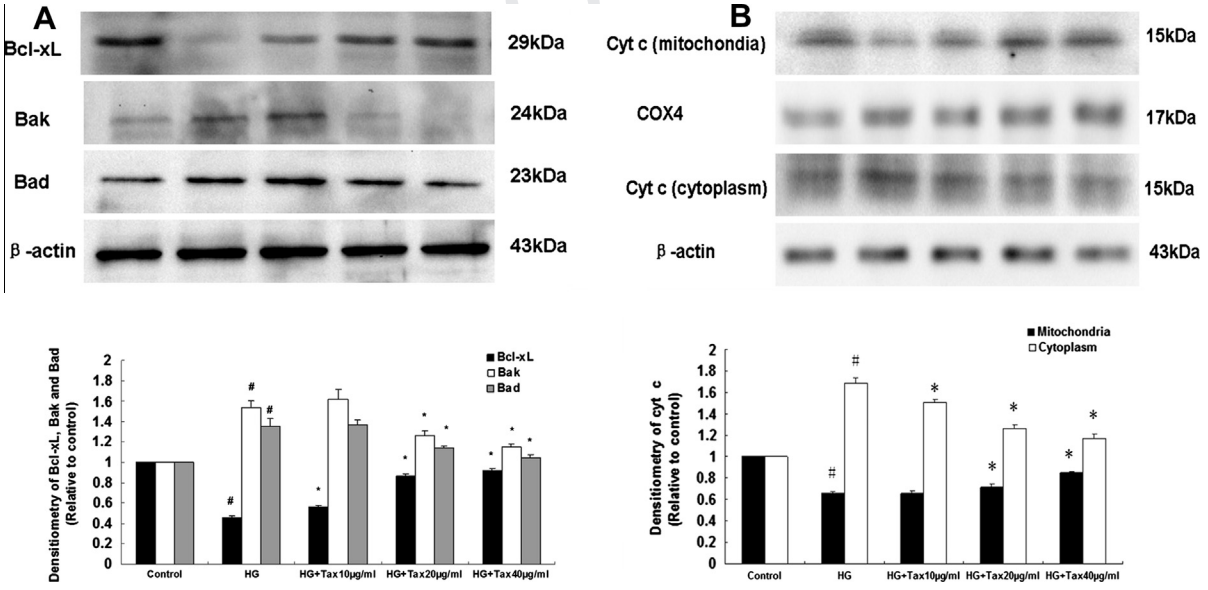

Fig. 11. Effects of taxifolin on expression of apoptosis-related proteins. (A) Western blot analysis of Bcl-Xl, Bak and Bad. (B) Western blot analysis of cytochrome c in mitochondria and in cytoplasm. Data are presented by means ± SD from three independent experiments; $^{\#}P < 0.05$ vs control group; $^{*}P < 0.05$ vs DM group.

mitochondria. Taken together, our results showed that taxifolin treatment inhibited hyperglycemia-induced apoptosis by modulating the mitochondrial pathway.

Enhanced tissue RAS action is thought to be related to heart failure and diabetic cardiomyopathy at the molecular level. In our study, circulating angiotensin II (Ang II) level showed no difference between groups; however, the elevation of Ang II level in myocardium was significant. The treatment of taxifolin reduced Ang II level in myocardium of diabetic mice. The unchanged level of circulating Ang II might be attributed to the reduced renin level

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in diabetic mice (Connelly et al., 2007). Interestingly, our results are consistent with those in previous reports that Ang II could be synthesized intracellularly, which acted in an autocrine or paracrine way (Sadoshima et al., 1993; Singh et al., 2008). The effect of taxifolin might be related to the inhibition of intracellular production of Ang II. However, the underlying mechanism should be further investigated. Recent studies have shown that NADPH oxidase is the primary source of O_2^- in diabetic heart and inhibition of NADPH oxidase provides beneficial effects on diabetes-induced myocardial dysfunction (Aronson, 2008; Heymes et al., 2003; Roe et al., 2011). Ang II exerts a direct effect on cardiomyocytes through signaling via the AT1 receptor, resulting in increased NADPH oxidase activity and elevation of ROS, which causes oxidative damage to cardiomyocytes injuries including apoptosis. In the present study, NADPH oxidase activities were significantly enhanced in myocardium of STZ-injected mice, whereas taxifolin treatment showed inhibitory effects on NADPH oxidase. Therefore, taxifolin inhibited ROS production in diabetic myocardium by inhibiting the RAS system and NADPH oxidase activities.

JAK/STAT cascade was reported to convey Ang II signals from plasma membrane to nucleus via stimulation of AT1 receptor (Mehri et al., 2011). The activation of Janus-activated kinase (JAK)-2, a soluble tyrosine kinase, is found to be closely related to Ang II-induced ROS generated by NADPH oxidase system (Schieffer et al., 2000). Expression of STAT3 in the heart is associated with cardiac survival, which is required for normal cardiac function. During cellular stress, STAT3 is an anti-apoptotic factor regulating the gene expressions of Bcl-2, Bcl-xL and MnSOD, etc. (Wagner and Siddiqui, 2009). In the present study, diabetes caused increase of p-JAK2 and p-STAT3 expression. This might be the results of a compensational mechanism of the heart to cope with oxidative stress. In a very recent study, *silico* screening was conducted using a shape similarity method to find the potential molecular target of taxifolin. Their results found that shape similarity score of JAK2 is 0.82, which means that JAK2 is the potential target of taxifolin (Oi et al., 2012). Our results showed that taxifolin treatment significantly increased the expressions of these two proteins, indicating that taxifolin might be able to activate JAK2 directly, thereby activating STAT3. These results suggested that the protective effects of taxifolin might be related to its effects on JAK2/STAT3 cascade.

In conclusion, our study demonstrated that taxifolin showed cardioprotective effects against diabetic cardiomyopathy by decreasing angiotensin II production, inhibiting NADPH oxidase, and activating JAK2/STAT3 cascade. Taxifolin also inhibited high glucose-induced cardiac cell apoptosis by suppressing oxidative stress and modulating mitochondrial function. Therefore, taxifolin might be a potential therapeutic medicine to prevent diabetic cardiomyopathy.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.11.013.

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