

Original Article

20(S)-ginsenoside Rg3 protects myocardium by inhibiting AGE-RAGE-mediated oxidative stress in diabetic rats

Baoxin Ma^{1,3*}, Jinning Gu^{2*}, Hongyu Jiang³, Jie Li³, Suisheng Wu³

¹Department of Cardiology, Affiliated Hospital of Binzhou Medical University, Binzhou, P.R. China; ²Department of Geriatrics, The Second Bethune Hospital of Jilin University, Changchun 130021, Jilin, P.R. China; ³Department of Geriatrics, The First Bethune Hospital of Jilin University, Changchun 130021, Jilin, P.R. China. *Equal contributors.

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Abstract: This study aimed to investigate the potential mechanisms underlying the protective effects of 20(S)-ginsenoside Rg3 on diabetes-related myocardial injury. Diabetes was induced in rats by a single dose of streptozotocin (45 mg/kg). The rats were then treated with 20(S)-ginsenoside Rg3 for 12 weeks. The lipids, glucose levels, cardiac enzymes and antioxidant system parameters of the rats were recorded at 12 weeks after the treatment. Myocardial samples were analyzed using transmission electron microscopy. The expression levels of receptor for advanced glycation end products (RAGE), nuclear factor- κ B (NF- κ B), transforming growth factor- β 1 (TGF- β 1) and connective tissue growth factor (CTGF) were also determined. Induction of the diabetic model significantly enhanced extracellular matrix deposition and increased the levels of type I and III collagen. The level of malondialdehyde (MDA) in the cardiac tissue of the diabetic rats was significantly increased, while the levels of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) were decreased compared to the control rats. Treatment with 20(S)-ginsenoside Rg3 resulted in an obvious recovery of these indexes, decreased accumulation of collagen, improved structure of the muscular fiber bundle and restored morphology of mitochondria of the LV myocardium. Compared to the control group, the expression levels of RAGE, NF- κ B, TGF- β 1 and CTGF proteins were significantly increased in the DM group, while 20(S)-ginsenoside Rg3 significantly reduced their expression. This study shows that the AGE-RAGE pathway may be involved in the mechanism of myocardial injury in diabetic rats and that 20(S)-ginsenoside Rg3 protects the myocardium via the AGE-RAGE pathway.

Keywords: 20(S)-ginsenoside Rg3, RAGE, oxidative stress, diabetic cardiomyopathy

Introduction

Diabetes augments the development of left ventricular hypertrophy, increases the susceptibility of the heart to ischemic injury and increases the overall likelihood of developing heart failure [1]. The concept of diabetic cardiomyopathy was first introduced by Rubler *et al.* [2] and is defined as ventricular dysfunction without evidence of coronary artery disease (CAD) or hypertension [3]. Increased production of reactive oxygen species (ROS), mainly produced by mitochondria in myocardial tissue, is a major effect of diabetic cardiomyopathy [3, 4].

Advanced glycation end products (AGE) have been implicated in the pathogenesis of diabetic

cardiomyopathy via interactions with receptors for AGE (RAGE). Hyperglycemia and oxidative stress both contribute to the generation of AGE [5]. AGE and RAGE interactions activate multiple cellular signaling pathways, including nuclear factor- κ B (NF- κ B), which subsequently leads to the activation of intracellular transforming growth factor (TGF)- β 1 and connective tissue growth factor (CTGF) [5, 6]. CTGF is a well-known mediator of the TGF- β 1 system [7]. CTGF and TGF- β 1 have been shown to induce the synthesis of extracellular matrix and the accumulation of collagen [8, 9].

Ginseng has been widely used to treat diabetes in traditional Chinese medicine [10]. Among more than 30 ginsenosides, 20(S)-ginsenoside

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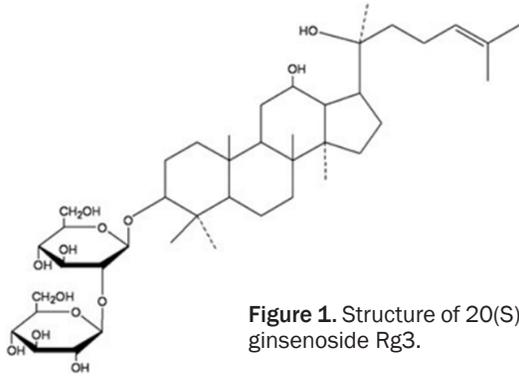


Figure 1. Structure of 20(S)-ginsenoside Rg3.

Rg3 (20(S)-Rg(3)) (**Figure 1**) is one of the major biologically active components in ginseng and is regarded as the main compound responsible for the many pharmacological actions of ginseng. Several studies have reported that 20(S)-Rg3 exhibits protective effects against diabetic renal damage [11], brain infarction after cerebral ischemia [12] and endothelial cell apoptosis [13] via the antioxidative stress process. Whether 20(S)-Rg3 can protect against myocardial damage in diabetic rats is unknown. This study aimed to investigate the protective effects of 20(S)-Rg3 on diabetic-related myocardial injury and the potential mechanisms.

Materials and methods

Animals and ethics

Sixty-four male Wistar rats (180-220 g) were housed at a constant temperature of 22-28°C with a 12-h light/dark cycle and were fed standard pellet chow with water provided ad libitum. All procedures were approved by the Ethics Committee for the Use of Experimental Animals in Jilin University.

Induction of diabetes and treatment

Diabetes was induced by a single dose of streptozotocin (STZ, Sigma Chemical Co, USA) (45 mg/kg) as previously described [14, 15]. Age-matched healthy control rats received the vehicle only. Diabetic rats were obtained based on the criterion of glucose levels >16.7 mmol/l. Rats were divided into six different groups: (1) healthy rats treated with a low dose of 20(S)-Rg3 (5 mg/kg/d) (n=10); (2) control healthy rats (n=10); (3) untreated diabetic rats (n=11); (4) diabetic rats treated with a low dose of 20(S)-Rg3 (5 mg/kg/d) (n=11); (5) diabetic rats

treated with a medium dose of 20(S)-Rg3 (10 mg/kg/d) (n=11); and (6) diabetic rats treated with a high dose of 20(S)-Rg3 (20 mg/kg/d) (n=11). Rats were administered 20(S)-Rg3 for 12 consecutive weeks. The final numbers of the surviving rats in groups 1 through 6 were 7, 6, 8, 9, 9 and 8, respectively. After 12 weeks of treatment, blood samples were collected from the abdominal aorta. The serum was immediately separated from the blood samples by centrifugation. Subsequently, rats were euthanized, and the left ventricles (LVs) were isolated. One-half of the sample was fixed in 4% paraformaldehyde for histological studies, and the other half was frozen in liquid nitrogen for protein and enzymatic activity assays.

Blood evaluation

The fasting serum total cholesterol (TC), triglyceride (TG), and free fatty acid (FFA) concentrations and cardiac enzymes were analyzed by enzymatic methods using an automatic analyzer (JCA-BM8060, JEOL Ltd, Tokyo, Japan).

Chemicals, assay kits and biochemical procedures

STZ was obtained from Sigma Chemical Co. (St. Louis, MO). 20(S)-Rg3 with a purity of 99% was obtained from the School of Pharmaceutical Sciences, Jilin University. The chemical structure of 20(S)-Rg3 is shown in **Figure 1**. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione activity (GSH) assay kits were obtained from Nanjing Jiancheng Bioengineering Company (Nanjing, China). The frozen heart tissue samples were weighed and homogenized (Ultra Turrax T25, Germany) (1:10, w/v) in 50 mmol/l phosphate buffer (pH 7.4) and kept in an ice bath. The homogenate and supernatant were frozen at -20°C in aliquots until use in biochemical assays. The protein content of the supernatant was determined using the Lowry method. The MDA level and the activities of SOD, CAT and GSH were measured.

Histology and immunohistochemistry techniques

Myocardium samples were embedded in paraffin. Four-micrometer paraffin sections were stained with Masson's trichrome and hematoxylin-eosin (H/E) following the manufacturer's

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Table 1. Characteristics of animals and cardiac enzymes (mean \pm SD)

Animal group/Parameters	I	II	III	IV	V	VI
	Control+20(S)-Rg3 5 mg	Control	DM	DM+20(S)-Rg3 5 mg	DM+20(S)-Rg3 10 mg	DM+20(S)-Rg3 20 mg
Glucose (mmol/l)	5.66 \pm 1.842	5.88 \pm 0.855	24.34 \pm 10.73 ^a	24.3 \pm 9.42	23.8 \pm 7.19	21.68 \pm 11.56
Total cholesterol (TC) (mmol/l)	0.453 \pm 0.092	0.448 \pm 0.081	6.473 \pm 1.533 ^a	6.449 \pm 1.348	6.111 \pm 1.962	5.460 \pm 0.834
Triglycerides (TG) (mmol/l)	1.209 \pm 0.125	1.147 \pm 0.128	4.654 \pm 1.381 ^b	4.404 \pm 2.108	4.108 \pm 3.24	3.718 \pm 0.197
Free fatty acids (FFA) (mmol/l)	0.55 \pm 0.08	0.56 \pm 0.10	1.22 \pm 0.12 ^a	1.05 \pm 0.13	0.84 \pm 0.16 ^c	0.62 \pm 0.07 ^d
Creatine kinase isoenzyme (CK) (U/L)	137.5 \pm 36.90	153.2 \pm 24.13	2142 \pm 587.3 ^a	1904 \pm 421.3	1793 \pm 235.2 ^c	1728 \pm 353.3 ^c
Creatine kinase-MB (CK-MB) (U/l)	0.43 \pm 0.024	0.53 \pm 0.094	2.1 \pm 0.62 ^a	1.7 \pm 0.097	1.63 \pm 0.084	1.58 \pm 0.89
Lactate dehydroge-nase (LDH) (U/l)	157.8 \pm 32.55	198.4 \pm 25.34	684 \pm 28.4 ^a	582.2 \pm 41.07 ^c	543.9 \pm 32.79 ^c	492.2 \pm 36.22 ^d
Body weight (g)	342 \pm 21.03	343.4 \pm 30.25	253.2 \pm 9.98 ^a	261.6 \pm 14.8	274.2 \pm 13.37	296.6 \pm 23.43 ^c
Cardiac weight/body weight (mg/g)	0.00347 \pm 0.0002	0.00345 \pm 0.0002	0.00521 \pm 0.001 ^b	0.00458 \pm 0.0012	0.00402 \pm 0.001	0.00365 \pm 0.0002 ^b

^aP<0.01 vs. control group; ^bP<0.05 vs. control group; ^cP<0.05 vs. DM group; ^dP<0.01 vs. DM group.

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Table 2. Malondialdehyde content (MDA), superoxide dismutase activity (SOD), glutathione activity (GSH) and catalase activity (CAT) in the myocardium of control rats (Control), rats with STZ-induced diabetes (DM) and diabetic rats receiving 5 mg, 10 mg or 20 mg of 20(S)-Rg3 (DM+20(S)-Rg3) (mean \pm SD)

Animal group	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/g protein)	GSH (U/mg protein)
I-Control+20(S)-Rg3 5 mg	3.17 \pm 0.76	158.43 \pm 21.21	4.67 \pm 1.91	0.29 \pm 0.033
II-Control	3.96 \pm 0.63	148.07 \pm 21.07	5.24 \pm 1.18	0.31 \pm 0.083
III-DM	12.29 \pm 1.52 ^b	113.82 \pm 23.21 ^b	1.86 \pm 0.83 ^b	0.11 \pm 0.064 ^b
IV-DM+20(S)-Rg3 5 mg	9.93 \pm 1.58 ^a	162.36 \pm 17.52 ^a	2.40 \pm 0.56 ^a	0.16 \pm 0.055
V-DM+20(S)-Rg3 10 mg	8.32 \pm 1.64 ^a	173.72 \pm 25.85 ^a	3.18 \pm 1.50 ^a	0.18 \pm 0.025 ^a
VI-DM+20(S)-Rg3 20 mg	6.21 \pm 0.92 ^a	180.78 \pm 17.38 ^a	3.41 \pm 0.79 ^a	0.22 \pm 0.045 ^a

^aP<0.05 vs. DM group; ^bP<0.01 vs. control, control+20(S)-Rg3 group.

instructions. Cardiac fibrosis was quantified on Masson's trichrome-stained sections with Image-Pro Plus 6.0 software (Media Cybernetics, USA). For electron microscopy, the heart tip sample was fixed in 2.5% glutaraldehyde and then fixed in 1% Ngok acid. After dehydration with ethanol, the sample was embedded in Epon812 epoxy resin. After semi-thin sectioning, ultrathin sections were observed by JEM-100EX-type transmission electron microscopy. For immunohistochemistry (IH), myocardial tissue sections were immunohistochemically stained using the streptavidin-peroxidase method to detect the protein expression of collagen I and collagen III. Cells were defined as positive if the cytoplasm stained brown.

Western blot analysis

Cardiac tissue was lysed in RIPA buffer [0.1% SDS, 1% deoxycholate, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.4, 10% protease inhibitor cocktails (Sigma-Aldrich)] for 30 min at 4°C and then centrifuged at 12,000 rpm for 5 min. The supernatant fluid was collected, and the protein content was quantified using the Bio-Rad Protein assay reagent (Bio-Rad, Regents Park, NSW, Australia). Equal quantities of protein in each sample were separated on 8% sodium dodecyl sulfate polyacrylamide gels, transferred to Immobilon-PVDF membranes (Millipore, Billerica, MA, USA) for 2 h at 4°C and blocked in 5% skim milk (Difco, Sparks, MD, USA) for 30 min at room temperature. Membranes were incubated with mouse anti-RAGE, anti-NF- κ B, anti-TGF- β 1 and CTGF monoclonal antibodies (Santa Cruz Biotechnology, CA, USA) overnight at 4°C, washed in Tris-buffered saline with 0.1% Tween-20 (TBST)

and incubated for 1 h with the appropriate HRP-conjugated secondary anti-mouse IgG (Santa Cruz Biotechnology, CA, USA). After washing with TBST, antibody binding was detected using an enhanced chemiluminescence (ECL) reagent (Amersham Biosciences/GE Healthcare, Rydalmere, NSW, Australia). Western blotting against β -actin (Santa Cruz Biotechnology) was used as a control to assess equal protein loading.

Statistical analyses

The results were analyzed by the Mann-Whitney U test and expressed as the mean values \pm standard deviation (SD). *P* values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS 11.5 software (IBM-SPSS, Inc., Armonk, New York, USA).

Results

Glucose concentration, triglycerides, cholesterol, body weight, cardiac weight/body weight and cardiac enzymes

The glucose concentration in the blood serum of STZ-diabetic rats was considerably higher than that in the control group after 12 weeks. However, in the three 20(S)-Rg3-treated groups, the glucose concentration was not significantly elevated. The activities of creatine kinase (CK), CK-MB and lactate dehydrogenase (LDH) were measured in STZ-diabetic rats. Compared to the control group, the levels of CK, CK-MB and LDH were increased in STZ-diabetic rats at 12 weeks (all *P*<0.01, **Table 1**). However, the CK and LDH activities were decreased in diabetic rats treated with 20(S)-

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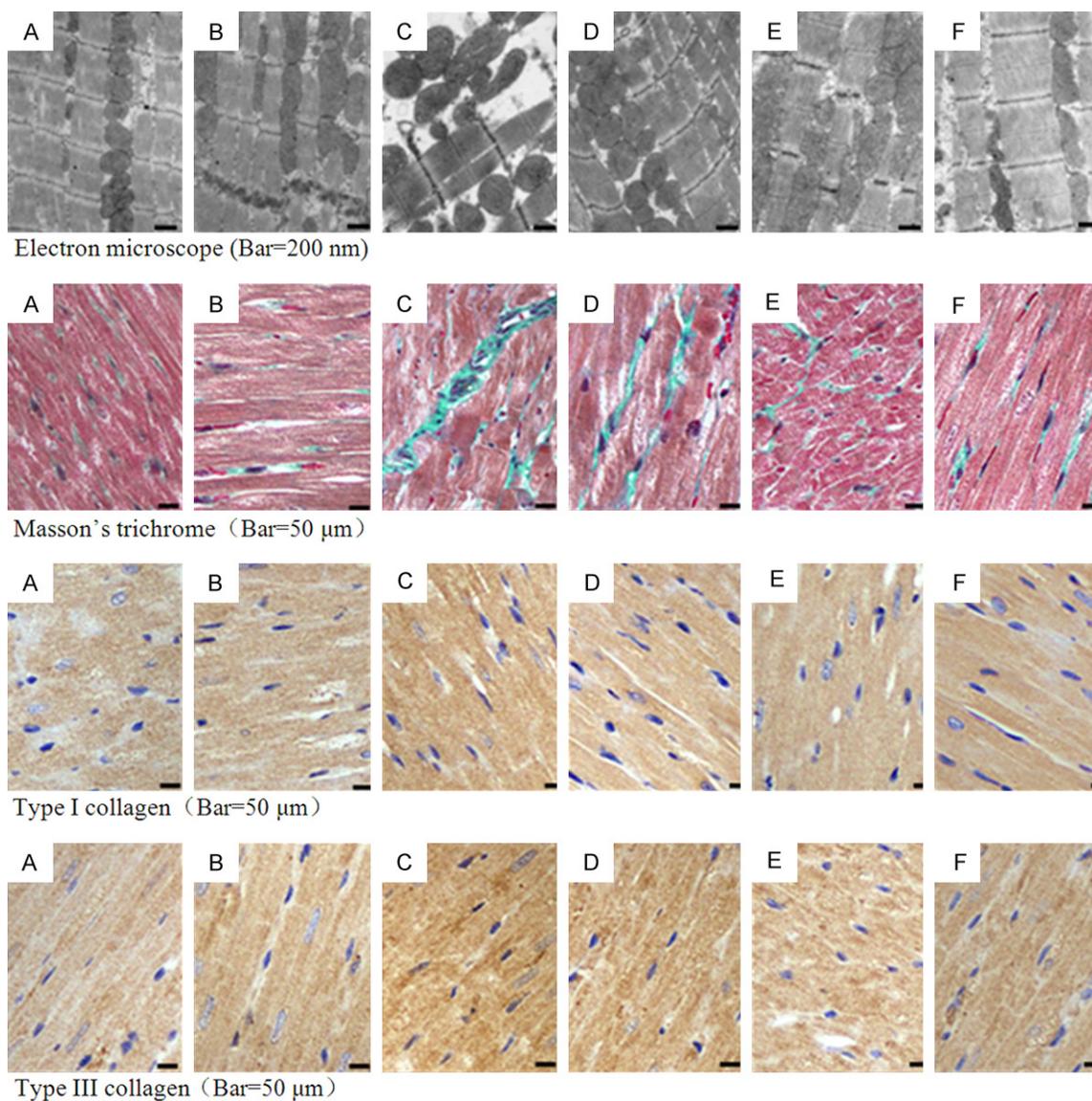


Figure 2. Morphological changes in different groups. Cells were defined as positive if the cytoplasm was stained brown. Sections are counterstained with hematoxylin (Bar=50 μm). A. Healthy rats treated with a low dose of 20(S)-Rg3; B. Healthy rats; C. Diabetic rats; D-F. Diabetic rats treated with 5, 10 and 20 mg/kg body weight/day of 20(S)-Rg3, respectively.

Rg3 compared to diabetic rats that received no treatment. The levels of serum TC, FFA and TG observed at 12 weeks were markedly elevated in STZ-diabetic rats compared to the control group. Nevertheless, treatment with 20(S)-Rg3 significantly lowered the serum FFA level but did not significantly decrease the levels of serum TC and TG compared to diabetic rats. The body weight of diabetic rats was significantly lower than that of control rats. Treatment with 20(S)-Rg3 significantly alleviated the weight loss. The cardiac weight/body weight ratio was significantly higher in the STZ-treated

rats than in the control rats. After 12 weeks, a statistically significant attenuation of this ratio was found in the 20(S)-Rg3-treated STZ-diabetic rats compared to the diabetic rats that received no treatment ($P<0.05$).

Antioxidant system evaluation in myocardial tissue

As shown in **Table 2**, the level of MDA in the heart was increased in diabetic rats compared to rats in the control group ($P<0.01$). The groups that received three different dosages of 20(S)-

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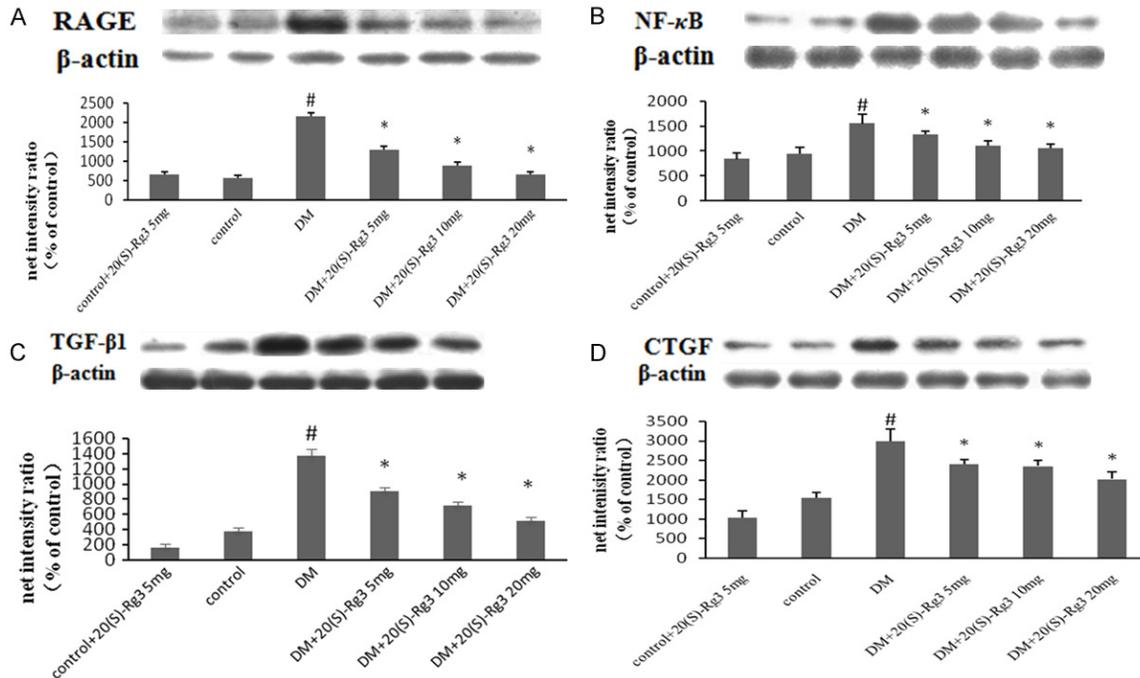


Figure 3. Western blot analysis of RAGE, NF- κ B, TGF- β 1 and CTGF protein expression in cardiac tissue. Healthy rats treated with a low dose of 20(S)-Rg3 (control+20(S)-Rg3 5 mg); healthy rats (control); diabetic rats (DM); and diabetic rats treated with 5, 10 and 20 mg/kg body weight/day of 20(S)-Rg3; # $P < 0.05$ compared to control rats and controls treated with a low dose of 20(S)-Rg3; * $P < 0.05$ compared to DM rats.

Rg3, particularly the high-dose group, exhibited significantly reduced MDA levels in myocardial tissue compared to the diabetic group ($P < 0.05$). The SOD, GSH and CAT levels were significantly lower in diabetic rats than those in the control rats. The SOD, GSH and CAT levels were significantly increased in the 20(S)-Rg3-treated diabetic rats compared to the diabetic rats ($P < 0.05$), especially those in the high-dose group.

Histopathology and immunohistochemistry

Masson staining was used to examine the morphological and collagen matrix changes in the cardiac tissue. The myocardium of control rats showed a normal structure. However, STZ-diabetic rats presented increased extracellular matrix deposition. 20(S)-Rg3 treatment gradually decreased the accumulation of collagen in a dose-dependent manner. Electron microscopy showed that the myocardial structure was intact, the muscular fiber bundle was tightly packed, and mitochondria were long and oval in myocardium of the control rats. At 12 weeks in STZ-diabetic rats, the muscular fiber bundle in the cytoplasm was decreased, and the mito-

chondria of myocytes were small and round. 20(S)-Rg3 treatment improved the structure of the muscular fiber bundle and restored the morphology of mitochondria in the LV myocardium in a dose-dependent manner. The immunohistochemical results showed a significant increase in type I and III collagen at 12 weeks in STZ-diabetic rats compared to the control group. 20(S)-Rg3 significantly decreased the expression of type I and III collagen proteins. These ameliorations were dose-dependent. As the dose increased, the effect became more obvious (**Figure 2**).

RAGE, NF- κ B, TGF- β 1 and CTGF protein expression

Compared to the control group, expression levels of the four proteins were significantly increased in the DM group, while 20(S)-Rg3 significantly reduced their expression levels in the treatment groups. These reduced levels were dose-dependent (**Figure 3**).

Discussion

Diabetes mellitus is characterized by reduced glucose and enhanced fatty acid (FA) metabo-

lism [16]. Increased FA uptake exceeds oxidation rates in the heart, resulting in lipid accumulation in the myocardium, which may promote myocardial lipotoxicity [17-19]. In the present study, we evaluated the effect and possible mechanism of action of 20(S)-Rg3 on myocardial injury in diabetic rats. The current investigation indicated that 20(S)-Rg3 significantly improved the structure of muscular fiber bundles and restored the morphology of mitochondria in the LV myocardium, confirming that 20(S)-Rg3 could be beneficial for the myocardium via the AGE-RAGE pathway.

Diabetes mellitus increases the frequency and severity of myocardial infarction (MI) and increases mortality after acute MI [20]. Diabetic patients have a high risk of congestive heart failure due to a pathologic condition known as diabetic cardiomyopathy [20]. According to our results, the levels of CK, CK-MB and LDH were increased in STZ-diabetic rats. The cardiac weight/body weight ratio was also significantly higher. Diabetic rats presented increased extracellular matrix deposition and damage to the myocardial structure. 20(S)-Rg3 decreased the levels of CK and LDH, attenuated the cardiac weight/body weight ratio and improved the structure of the myocardium.

Regarding the different possible mechanisms by which myocardial fibrosis may be triggered, it has been postulated that oxidative stress may be a key player in such induction [21-23]. Increased ROS generation and impaired antioxidant defenses could both contribute to oxidative stress. SOD, GSH and CAT all belong to the antioxidant defense system [24-26]. In the present study, we found that the level of MDA in the cardiac tissue of diabetic rats was significantly increased, while the levels of SOD, GSH and CAT were decreased compared to the control rats. Treatment with 20(S)-Rg3 obviously recovered these indices. Our results demonstrated the antioxidative effects of 20(S)-Rg3 in diabetic rats.

The formation of AGEs is closely correlated with oxidative stress and higher glucose levels [27]. STZ-induced diabetes is associated with colocalized AGE formation and enhanced cell-surface receptor (RAGE) expression in cardiomyocytes [28], resulting in the activation of post-receptor signaling, the generation of intra-

cellular ROS and the activation of gene expression [29]. NF- κ B is a transcriptional factor that plays an important role in the expression of genes involved in the response to injury and infection. It has been reported that the oral administration of 20(S)-Rg3 tends to reduce NF- κ B protein expression in response to LPS-induced acute oxidative damage in the liver and kidney [30]. TGF- β 1 is one of several cytokines whose gene expression is enhanced by diabetes [31]. Increased CTGF expression and collagen deposition have also been observed in mouse models of STZ-diabetes [32].

Our results showed that the expression of RAGE and NF- κ B was significantly downregulated in 20(S)-Rg3-treated diabetic rat hearts compared to untreated diabetic rat hearts. The expression of TGF- β 1 and CTGF was significantly decreased in 20(S)-Rg3-treated diabetic rat hearts compared to untreated diabetic rat hearts in a dose-dependent manner. These results indicated that the mechanism underlying the beneficial effects of 20(S)-Rg3 involved minimizing oxidative stress by reducing the activation of RAGE.

In summary, our study suggested that 20(S)-Rg3 could protect against myocardial injury in diabetes. This beneficial effect of 20(S)-Rg3 may result from its inhibitory effect on activation of the AGE-RAGE pathway.

Disclosure of conflict of interest

None.

Address correspondence to: Jie Li, Department of Geriatrics, The First Bethune Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, Jilin, P.R. China. Tel: 86-0543-3256722; E-mail: lijie2000_2009@163.com

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