Resveratrol protects against early cardiomyopathic changes-induced by Type-1 diabetes through a heme-oxygenase-1 dependent mechanism

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Abstract

Diabetes mellitus can be considered as a state of chronic inflammation and oxidative stress. Excessive oxidative stress is implicated in the pathogenesis of diabetic complications. Heme oxygenase-1 is an antioxidant enzyme that is upregulated in response to a variety of insults as a protective mechanism. Resveratrol (RSV) is a polyphenol that exhibits promising pharmacological effects in a variety of disease models. In this study, we investigated the role of RSV in prevention of early pathological changes in the diabetic heart as well as the role of hemeoxygenase-1 in this regard. To achieve this aim, diabetes was induced by ip injection of streptozotocin (50 mg/kg). Rats were divided into 5 experimental groups; normal control (CTR), diabetic control (DM), diabetic rats treated with RSV (DM+RSV); 10 mg/kg) in drinking water, diabetic rats treated with a combination of RSV and an HO-1 inhibitor, Zinc protoporphyrin (ZnPP), (DM + RSV+ZnPP); 10 μmole/Kg/week I.P) and diabetic rats treated with ZnPP alone (DM + ZnPP). Induction of diabetes was accompanied by a significant increase in cardiac MDA level and TGF-β protein expression as well as a significant reduction in eNOS and HO-1 expression. Chronic treatment with RSV significantly attenuated the abovementioned pathological changes in addition to alleviation of diabetes-induced histopathological alterations. Furthermore, the effect of RSV was diminished when the HO-1 activity was blocked by ZnPP. In conclusion, these results demonstrate the role of HO-1 in mediating RSV protective effects against diabetes-induced early cardiomyopathy.

1. Introduction

Diabetes mellitus (DM) represents one of the most prevalent diseases worldwide. Diabetic complications (such as, cardiovascular and renal complications) are the major cause of morbidity and mortality among diabetic patients [1]. In diabetes, the risk for developing heart failure increases even in the absence of clinically significant cardiac ischemia or systemic hypertension [2]. The hallmark of DM is sustained hyperglycemia, which leads to excessive production of reactive oxygen species (ROS) and impaired nitric oxide (NO) production causing cell injury and death. Hyperglycemia induces several other pathways leading to a state of chronic inflammation [3]. All these metabolic changes contribute to the development of cardiomyopathy [2]. Moreover, increased fibrosis and collagen deposition in cardiac tissue have been reported [4] and are linked to increased transforming growth factor (TGF)-β expression [5]. Impaired cardiac function was also related to decreased cardiac expression of endothelial nitric oxide synthase (eNOS) but an upregulation of the inducible isoform of nitric oxide synthase (iNOS) with progression of time in a model of STZ-induced diabetes [6].

Recent studies have demonstrated the role of the heme-oxygenase (HO) system in inflammation, oxidative stress and apoptosis [7, 8]. HO is the primary enzyme for heme catabolism converting heme to iron, biliverdin and carbon monoxide. Two HO isoenzymes are now identified; HO-1 and HO-2. The constitutive form is HO-2 which is responsible for HO activity in normal conditions while the inducible HO-1 is upregulated in response to various tissue insults such as oxidative stress, ischemia and hypoxia [7]. In a type-1 diabetic model induced by streptozotocin (STZ), upregulation of HO-1 leads to amelioration of glomerular injury [9] , diabetic neuropathy [10] as well as reduction of blood pressure in spontaneously hypertensive diabetic rats [11].

Resveratrol (RSV; 3, 4', 5-trihydroxystilbene) is a natural compound synthesized in response to infection in some plants and is also produced in response to some environmental stresses such as ozone, climate, and UV light. It can be found in peanuts and different types of berries [12]. Studies using RSV in animal models of diabetes showed a protective role against different types of diabetic complications including diabetic nephropathy [13] and neuropathy [14]. RSV also showed cardioprotective effects both *in vitro* [15] and recently in *in vivo* studies [16-18]. Numerous studies proposed different mechanisms by which RSV can act to protect against various diseases such as activation of transcription factors such as nuclear factor (erythroid derived-2)-like 2 (Nrf2), reduction of inflammatory biomarkers such as nuclear factor kappa B (NFκB) and the inducible form of nitric oxide synthase (iNOS) as well as induction of antioxidant enzymes (e.g., HO-1) [12]. Many of these mechanisms have been already linked to the

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cardioprotective effects of RSV against diabetic cardiomyopathy. One study, demonstrated the protective effect of RSV on cardiac function of diabetic rats, partially attributed the beneficial effects of RSV to induction of HO-1. However, the role of HO-1 was not clearly investigated since RSV produced significant reduction in blood glucose level. Therefore, in our study, we attempted to clarify the role of HO-1 induction in mediating the protective effects of RSV aside from its antidiabetic effect. Thus, we hypothesized that RSV will reduce cardiac injury induced by diabetes via induction of HO-1 expression. In order to test this hypothesis, we evaluated the cardioprotective effect of RSV alone or in combination with an HO-1 inhibitor; Zn protoporphrin (ZnPP) in an STZ-induced type-1 diabetes in rats.

2. Methods

2.1. Chemicals

Resveratrol was purchased from Nutravit, USA. While, Zinc protoporphyrin IX was obtained from Santa Cruz, USA. Streptozotocin was purchased from Sigma Aldrich, USA. Other chemicals were of highest available grade.

2.2. Animals

Wistar male rats (200 - 220 g) were purchased from the Experimental Animal Center of Nahda University (Beni Suef, Egypt). Rats were subjected to an acclimatization period of one week and were kept under constant environmental conditions throughout the experiment. Animals were exposed to a 12:12 h dark: light cycle. Standard rat chow diet and tap water were available ad libitum. Since resveratrol was provided in drinking water, rats were single-housed and drinking water was provided using special non-spell bottles.
The study protocol was approved by members of The Research Ethics Committee at the Pharmacology & Toxicology Department, Faculty of Pharmacy, Minia University, Egypt (approval number 023/18).

2.3. Induction of Type-1 diabetes

Overnight fasted rats were intraperitoneally injected with STZ (Sigma Aldrich, USA) in a dose of 50 mg/kg [19]. Blood glucose level measurement was carried out on day 3 through tail tipping using a commercial blood glucometer (Precicheck, FIA Biomed, Germany). Rats having blood glucose level greater than 200 mg/dl were considered diabetic and included into the study.

2.4. Experimental design

Three days after STZ injection, diabetic rats were randomized into 4 groups (blood glucose greater than 200 mg/dl); diabetic control (DM; vehicle treated), diabetic rats treated with RSV (10 mg/kg/day; DM-RSV) for 4 weeks [20], diabetic rats treated with a combination of RSV and ZnPP (10 μmole/kg/week; I.P; DM-RSV+ZnPP) [21], diabetic rats treated with ZnPP only (10 μmole/kg/week; DM -ZnPP). In addition, normal rats of similar body weight were considered as control group; CTR and treated with vehicle.

2.5 Blood and tissue collection

At the end of the experimental period, rats were anaesthetized using thiobarbital and blood samples were collected by exsanguination. Hearts were rapidly isolated, blotted dry on a filter paper then weighed. Heart weight index was calculated as ratio of heart weight to body weight [22].
A portion of heart tissues was flash frozen in liquid nitrogen and stored at -20 °c until the time of homogenization, while another was fixed in 10% formalin for the standard hematoxylin and eosin staining.

2.6. Determination of cardiac lipid peroxidation

Measurement of thiobarbituric acid reactive species (TBARS) as a marker of lipid peroxidation was performed using a commercial kit (Biodiagnostic, Egypt) according to the instructions of the manufacturer.

2.7. Western blot analysis for detection of eNOS, HO-1 and TGF-β protein expression

Samples with equal protein content were loaded on polyacrylamide gel and protein separation took place using TGX Stain-Free™ FastCast™ Acrylamide Kit (SDS-PAGE) obtained from Bio-Rad Laboratories, TNC, USA Catalog. No. 161-0181. The membrane was blocked in tris-buffered saline with Tween 20 (TBST) buffer and 3% bovine serum albumin (BSA) at room temperature for 1 hr.
For detection of eNOS, HO-1 and TGF-β-1, membranes were incubated with primary antibodies from Thermo Fisher Scientific; Catalog No.PA1-037, MA1-112 and MA5-15065, respectively. All primary antibodies were diluted 1:1000 using TBST.
Incubation was done overnight in each primary antibody solution, against the blotted target protein, at 4°C. Detection of bands was done using HRP-conjugated secondary antibody; thermo Fisher Scientific, goat anti-Mouse IgG Catalog No. NB7539; 1:5000 for detection of hemeoxygenase and goat anti-Rabbit IgG Catalog No. NBP2-30348H; 1:1000 for detection of TGF-β and eNOS.
The chemiluminescent signals were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins against control sample after normalization by beta actin on the Chemi Doc MP imager.

2.8. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) and were analyzed for statistically significant differences using one way analysis of variance (ANOVA) followed by the Tukey–Kramer post analysis test to compare all groups at $p<0.05$. Graph Pad Prism® was used for statistical calculation (Version 5.00 Windows, GraphPad Software, San Diego California USA, *www.graphpad.com*).

3. Results

3.1. Initial and final blood glucose levels of diabetic groups

After induction of diabetes by injection of STZ, the diabetic rats were randomly assigned into 4 groups so that there was no significant difference in the blood glucose of different groups. At the end of experiment, blood glucose levels were measured and showed that none of the treatments had a significant effect on blood glucose levels. Data is presented as mean ± SEM in **(Table 1)**.

3.2. Effect of RSV, RSV+ZnPP or ZnPP on heart weight index

After 4 weeks of hyperglycemia, no statistically significant difference in heart weight index was observed in diabetic group compared to normal rats and none of the tested treatments resulted in a change in this parameter. Data is presented as mean ± SEM in **(Table 1)**.

Table1: Effect of diabetes and various treatments on blood glucose level and heart weight index.
A table showing blood glucose levels of five experimental groups at the end of the study as well as the heart weight index (mg/g). Data are presented as mean ± S.D. CTR= control, DM= diabetic control, DM (RSV) = diabetic rats treated with resveratrol 10 mg/Kg/day, DM (RSV+ZnPP) = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn Protoporphyrin IX (10 µmole/kg/week), DM (ZnPP) = diabetic rats treated with zinc ProtoporphyrinIX(10 µmole/kg/week).

Groups	Final Blood glucose level (mg/dl)	Heart weight index
CTR	94.6 ± 42.56	3.000 ± 0.2526
DM	263.0 ± 85.9	3.198 ± 0.3728
DM (RSV)	245.4 ± 97.54	3.108 ± 0.1972
DM (RSV + ZnPP)	222.5 ± 86.42	3.446 ± 0.709
DM (ZnPP)	141.8 ± 23.85	2.885 ± 0.502

3.3. Histopathological examination of heart tissues

Despite the lack of effect of short-term diabetes on heart weight index, histopathological examination of the heart revealed microscopically-evident changes within the myocardium. The myocardium from diabetic animals showed focal haemorrhages, sclerosis and narrowing in the myocardial blood vessels. There was focal inflammatory cell infiltration in the myocardium as well as focal brown to yellow pigments (haemosiderosis). Cardiac sections from RSV treated group showed a lesser extent of tissue damage, while combination with ZnPP showed similar changes compared to diabetes **(Figure 1)**.

3.4. Effect of RSV, RSV+ZnPP or ZnPP on cardiac lipid peroxidation

As shown in **(Figure 2)**, induction of type I diabetes resulted in a significant elevation (p<0.05) in cardiac levels of TBARS as a marker of lipid peroxidation when compared to control rats. Daily administration of RSV (10 mg/kg) for 28 days produced a

significant reduction (p < 0.05) in serum lipid peroxidation compared to DM rats. However, this effect was abolished upon using ZnPP in combination with resveratrol. In addition, no change in TBARS was observed in diabetic rats treated with ZnPP compared to diabetic control.
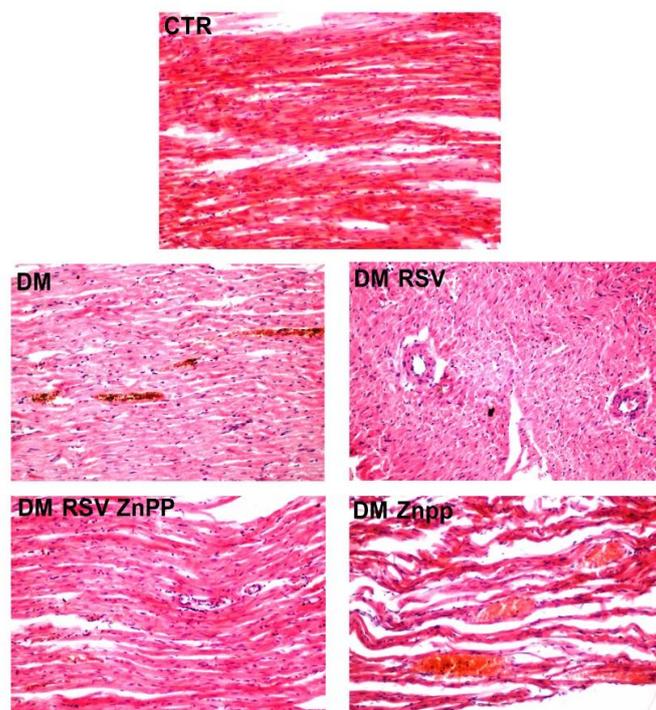
Figure 1: Histopathological examination of the myocardium of the five experimental groups.
Representative photomicrographs showing sections of the hearts of the five experimental groups. Hearts from diabetic rats showed focal haemorrhage, sclerosis and narrowing in the myocardial blood vessels in addition to inflammatory cells infiltration.CTR= control, DM= diabetic control, DM (RSV) = diabetic rats treated with resveratrol 10 mg/Kg/day, DM (RSV+ZnPP) = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn Protoporphyrin IX (10 µmole/kg/week), DM (ZnPP)=diabetic rats treated with zinc ProtoporphyrinIX(10 µmole/kg/week).
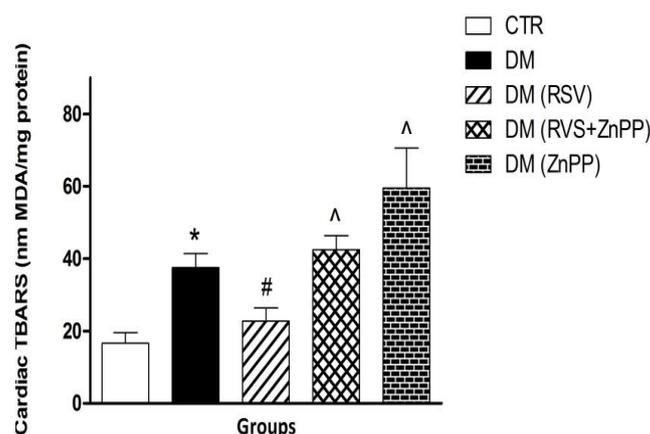
Figure 2: Effect of diabetes on Cardiac lipid peroxidation and its alteration by various treatments.
Bar graphs showing levels of cardiac TBARS of experimental group. Data are presented as mean ± S.E.M. * Significantly different from normal control (CTR) at p<0.05. # Significantly different from diabetic control (DM). ^ Significantly different from diabetic rats treated with RSV (DM (RSV)).
CTR= control, CTR+RSV = control rats treated with resveratrol, DM = diabetic control, DM (RSV)= diabetic rats treated with resveratrol 10 mg/Kg/day, DM (RSV+ZnPP) = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn Protoporphyrin IX (10 µmole/kg/week), DM (ZnPP) = diabetic rats treated with zinc Protoporphyrin IX (10 µmole/kg/week).

3.5. Effect of RSV, RSV+ ZnPP or ZnPP on cardiac HO-1 expression

Investigation of the effect of diabetes on expression levels of heme oxygenase-1 showed a significantly lower HO-1 expression compared to control group. However, treatment of diabetic rats with RSV led to a statistically significant attenuation of diabetes-induced reduction in HO-1 expression levels. Moreover, RSV treatment restored its expression to the level in non-diabetic control (**Figure 3**).

When ZnPP was used in combination with RSV, there was a significant reduction in HO-1 expression when compared to RSV-treated rats. Meanwhile, diabetic rats treated with ZnPP alone showed no significant difference in HO-1 expression when compared to non-treated diabetic rats.
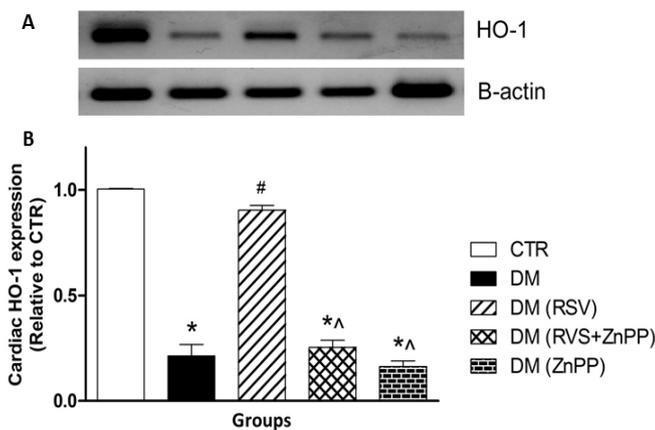
Figure 3: Effect of diabetes on heme oxygenase-1 expression in the heart and its alteration by various treatments.
Bar graphs showing change in expression of HO-1 induced by diabetes and its alteration by various treatments. Data are presented as mean ± S.E.M.
* Significantly different from normal control (CTR) at p<0.05. # Significantly different from diabetic control (DM). ^ Significantly different from diabetic rats treated with RSV (DM (RSV)).
CTR= control, CTR+RSV = control rats treated with resveratrol, DM = diabetic control, DM (RSV) = diabetic rats treated with resveratrol 10 mg/Kg/day, DM (RSV+ZnPP) = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn Protoporphyrin IX (10 μmole/kg/week), DM (ZnPP) = diabetic rats treated with zinc Protoporphyrin IX (10 μmole/kg/week).

3.6. Effect of RSV, RSV+ ZnPP or ZnPP on cardiac eNOS expression

Induction of diabetes caused a significant reduction in eNOS expression when compared to control group. However, treatment of diabetic rats with RSV resulted in a statistically significant increase in its protein expression compared to non-treated diabetic rats (**Figure 4**).

When RSV was combined with, an HO-1 blocker; ZnPP, there was a significant reduction in eNOS expression when compared to rats treated with RSV alone. However, eNOS expression was statistically elevated in ZnPP diabetic rats compared to non-treated diabetic rats.

3.7. Effect of RSV, RSV+ ZnPP or ZnPP diabetes on cardiac TGF-β expression

Induction of diabetes resulted in a significant increase in TGF-β expression when compared to normal rats. Chronic treatment with RSV significantly prevented diabetes induced-elevation in TGF-β expression. Meanwhile, ZnPP when used in combination with RSV abrogated the positive modulatory effect of RSV. However, diabetic rats treated with ZnPP alone did not show a significant change in TGF-β expression (**Figure 5**).
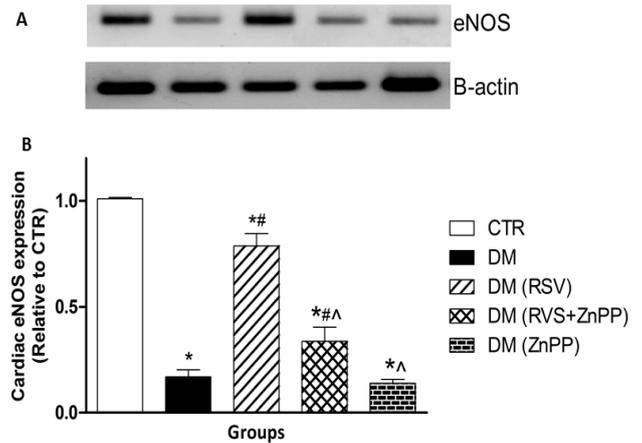
Figure 4: Effect of diabetes on endothelial nitric oxide synthase (eNOS) expression in the heart and its alteration by various treatments.
Bar charts showing the reduction in expression level of eNOS induced by diabetes and its alteration by various treatments.
Data are presented as mean ± S.E.M. * Significantly different from normal control (CTR) at p<0.05. # Significantly different from diabetic control (DM). ^ Significantly different from diabetic rats treated with RSV (DM (RSV)).
CTR= control, CTR+RSV = control rats treated with resveratrol, DM = diabetic control, DM (RSV) = diabetic rats treated with resveratrol 10 mg/Kg/day, DM (RSV+ZnPP) = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn Protoporphyrin IX (10 μmole/kg/week), DM (ZnPP) = diabetic rats treated with zinc Protoporphyrin IX (10 μmole/kg/week).
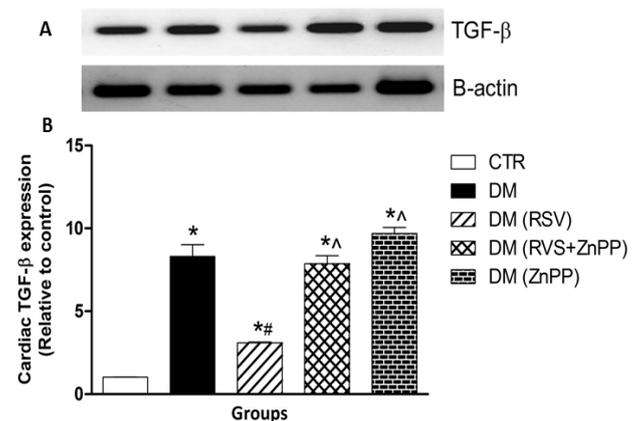
Figure 5: Effect of diabetes on TGF-β expression in the heart and its alteration by various treatments.
Bar charts showing the significant increase in TGF-b expression induced by diabetes and amelioration of this effect by resveratrol treatment. Data are presented as mean ± S.E.M. * Significantly different from normal control (CTR) at p<0.05. # significantly different from diabetic control (DM). ^ Significantly different from diabetic rats treated with RSV (DM (RSV)).
CTR= control, CTR+RSV = control rats treated with resveratrol, DM = diabetic control, DM (RSV) = diabetic rats treated with resveratrol 10 mg/Kg/day, DM (RSV+ZnPP) = diabetic rats treated with resveratrol (10 mg/kg/day) and Zn Protoporphyrin IX (10 μmole/kg/week), DM (ZnPP) = diabetic rats treated with zinc Protoporphyrin IX (10 μmole/kg/week).

4. Discussion

In this study, we have shown that type-1 diabetes induced oxidative stress with accompanied reduced eNOS and HO-1 expression in the cardiac tissues of diabetic rats and elevated expression of the fibrotic signal TGF-β along with deteriorated histological appearance of the cardiac tissue. These observed deteriorative effects of hyperglycemia are consistent with previous findings [23-25]. Thus, targeting these pathways during the progression of diabetes can be considered as a therapeutic option for managing hyperglycemia and diabetes-induced cardiovascular complications.

In our study, chronic treatment of diabetic rats with RSV (10 mg/Kg) had no effect on blood glucose level. The lack of RSV effect of blood glucose was previously reported in other studies [26, 27]. In contrast, Thirunavukkarasu *et al* demonstrated a hypoglycemic effect of RSV on STZ diabetic rats but used a different dose than ours (2.5 mg/kg) [28]. Moreover, Su *et al* showed that RSV reduces blood glucose level in a dose range 0.1- 0.75 mg/kg [29], which emphasizes that the hypoglycemic effect of RSV is evident upon the use of low doses. For this reason, we used the higher dose to investigate the role of HO-1 induction in RSV mediated cardio-protection without affecting blood glucose level.

In the present study, induction of diabetes did not cause apparent cardiomegaly. This was excepted as previous studies showed that the increase in heart weight and associated change in cardiac parameters could be only observed after 8-weeks of induction of diabetes in rats [30, 31]. However, histopathological examination revealed the presence of inflammatory cells infiltration, numerous focal haemorrhages, sclerosis and narrowing in the myocardial blood vessels. These changes indicate early diabetic cardiomyopathic changes as previously reported [32]. Roslan et al showed distortion in the myocardial cells with increased intercellular gap and interstitial and vascular extracellular matrix in hearts of diabetic rats after 28 days of diabetes [33]. RSV treatment but not ZnPP inhibited these pathological changes. However, ZnPP when administered in combination with RSV, inhibited the protective effect of RSV which suggests the role of HO-1 induction by RSV in its preventive effect on diabetic induced early cardiomyopathic changes.

Consistent with numerous studies [34, 35], our results showed that induction of diabetes was associated with elevation in oxidative stress as represented by an increased MDA level. Chronic treatment with RSV reduced oxidative stress. This can be attributed to the well-established effect of RSV in the activation of Nrf2; a transcription factor that translocates into the nucleus after exposure of the cells to oxidative stress to transactivate the genes of antioxidant enzymes such as SOD and HO-1 [36]. The reduction in oxidative stress was attenuated by combining RSV with the HO-1 blocker, ZnPP. Therefore, HO-1 activity is essential for the antioxidant effect of RSV, since ZnPP when administered alone did not significantly increase the oxidative stress. A large body of evidence supports the role of RSV in counteracting various pathways involved in cardiovascular disorders, either induced by diabetes or by other types of insults [37]; for example, RSV showed an ability to reduce oxidative stress in a myocardial ischemia –reperfusion model [38]. In addition, RSV in combination with glibenclamide protected the diabetic heart from reperfusion-induced arrhythmias [39].

After 4-weeks of STZ injection, cardiac expression of HO-1 was reduced. This can be attributed to the effect of sustained hyperglycemia which increases intracellular ROS production. A recent review showed that diabetes-induced generation of ROS results in an initial compensatory transient upregulation of HO-1 expression followed by a decrease in its activity [40]. Diabetes-induced upregulation of HO-1 has been also observed in liver and kidney [41, 42]. Meanwhile, Quan and coworkers have reported a decrease in endotheial HO activity in the early stages of diabetes in rats [43]. Another study showed the reduction in HO-1 expression in heart of diabetic rats [28]. The down regulation of HO-1 is associated with deterioration of cardiac functions as demonstrated in HO-1 deficient mice [31]. In addition to reduced HO-1 expression, cardiac eNOS was also reduced in diabetic rats. Cumulative evidences in literature show that diabetes-induced production of ROS activates PKC resulting in reduction of eNOS expression and activity. Additionally, the state of hyperglycemia activates hexosamine pathway resulting in O-acetylglucosaminylation of eNOS protein at serine 1177 and suppression of eNOS activity [5, 35]. In the present study, treatment with RSV elevated HO-1 and eNOS expressions. Interestingly, the use of HO-1 inhibitor in combination with RSV did not only inhibit HO-1 but also partially inhibited eNOS expression.

A cross talk between NO and HO-1 is reported in the literature. It is well established that NO induces HO-1 activity however, the effect of carbon monoxide (CO), an HO-1 product, on eNOS is controversial. The stimulatory effect of RSV on HO-1 carries physiological significance under conditions of high oxidative stress. NO is a free radical that is highly susceptible to inactivation by the increased ROS. In contrast, CO is essentially a stable diatomic gas with longer half life compared to NO. Both NO and CO stimulates cGMP and produces cytoprotective effects. Thus, NO which is inactivated under conditions of elevated level of ROS such as in diabetes, produces cytoprotection via generation of CO through HO-1 induction [44]. On the other hand, evidence from literature showed that a CO releasing molecule (CORM-2) increases eNOS activity through increase in intracellular calcium and activation of PI3/AKT pathways [45]. However, CO in high doses inhibits eNOS activity [46]. In our study, we demonstrated that RSV also induces eNOS expression via induction of HO-1 but, this effect was not completely blocked by ZnPP suggesting the presence of other mechanisms that mediates RSV effect in this setting. Gracia-Sancho *et al* reported that RSV increases eNOS expression via activation of SIRT1/KLF-2 pathway in human vascular endothelial cells [47].

Cardiac fibrosis is a major process that mediates cardiac remodeling. Fibrosis is promoted by the formation of myofibroblasts and excessive deposition of extracellular matrix.

Fibrotic response occurs in response to a combined action of hormones such as aldosterone, ET-1 and angiotensin II and cytokines such as transforming growth factor beta (TGF-β). TGF-β represents a large family of cytokines that play a pivotal role in the control of cell differentiation and proliferation. Activation of TGF-β receptors was associated with upregulation of genes responsible for extracellular matrix deposition and concomitant suppression of matrix metalloproteinases [48]. TGF-β was found to be upregulated in diabetic hearts through various mechanisms including; angiotensin II, and ROS mediated activation of PKC [49-51]. In our study, induction of diabetes elevated the cardiac expression of TGF-β while chronic RSV treatment prevented this effect. Inhibition of HO-1 activity by ZnPP partially suppressed RSV effect. Wu *et al* demonstrated that treatment of diabetic rats with an antioxidant such as N-acetyl cysteine produced comparable suppression in TGF-β to RSV treatment in diabetic mice [52]. The partial downregulation of TGF-β can be attributed to the antioxidant effects of RSV through its ability to induce HO-1 given that RSV can inhibit TGF-β/Smad3 pathway via activation of SIRT3 [53].

In conclusion, we investigated the cardioprotective effect of RSV on early DCM. Our 4-weeks experimental period of diabetes was only sufficient to induce some biochemical changes associated with DCM. We did not expect any change in cardiac functions since Zhao *et el* showed no change in cardiac function even after 8-weeks of induction of diabetes [31]. RSV protected the heart from the adverse effects of hyperglycemia and this protection was mostly dependent on its ability to induce HO-1 expression.

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