INTRODUCTION

Diabetic cardiomyopathy (DC) is a common complication mainly in patients with type 1 and type 2 diabetes mellitus (T1DM) and is a leading cause for the development of heart failure (HF) and mortality among them (1, 2). However, DC also develops in patients and animals with T1DM even in the absence of any other cardiac disorder or risk factors (3-6). In both types of DM, the clinical manifestation associated with DC involve left ventricular (LV) hypertrophy and dilation, adverse fibrosis, and impaired systolic and diastolic function (7). To date, several studies have sought to identify the precise mechanism underlying DC. In this view, a consensus in literature has indicated that hyperglycemia-induced oxidative stress is the central mechanism for the development of DC (8-10). Accordingly, overproduction of reactive oxygen species (ROS) from the mitochondria and non-mitochondrial resources (i.e. ROS-generating enzymes) is the major contributor that activates all other damaging pathways observed in the diabetic hearts, including inflammation, hypertrophy, fibrosis, necrosis, and apoptosis (11, 12). However, suppressing oxidative stress by the overexpression or pharmacological activation of antioxidants afforded protection against DM-induced cardiac damage by suppressing all the above-mentioned pathways (11-15).

This study investigated the protective effect of ellagic acid (EA) against diabetic cardiomyopathy (DC) in streptozotocin (STZ)-treated rats and examined if the mechanism of protection involves modulating silent information regulator 1 (SIRT1). Adult male rats were divided into 5 groups (n = 12/each) as control, control + EA, diabetes mellitus (DM), STZ + EA, and STZ + EA + EX-527 (a SIRT1 inhibitor). With a hypoglycemic and insulin-releasing effect, EA preserved cardiomyocyte structure and suppressed the increase in heart weights and collagen deposition in the left ventricle (LV) of DM rats. Concomitantly, EA improved LV systolic and diastolic functions; reduced serum levels of creatinine kinase-MB (CK-MB), brain natriuretic peptide (BNP), and troponin-I, downregulated transforming growth factor beta 1 (TGF-β1), smad3, and cleaved caspase-3, and increased Bax/Bcl-2 ratio. Of note, EA increased the expression and activity of SIRT1 and suppressed the acetylation of nuclear factor erythroid-derived 2-like 2 (Nrf2), nuclear factor kappa B (NF-κB), smad2, and forkhead box, class O (FOXO1) in the LVs of both the control and diabetic groups. These effects were associated with a significant reduction in the levels of reactive oxygen species (ROS), malondialdehyde (MDA), tumor necrosis factor kappa (TNF-κ), and interleukin 6 (IL-6) levels and activity of NF-κB but with increased activity Nrf2 and levels of glutathione (GSH), superoxide dismutase (SOD), and Bcl-2. All these effects were abolished by EX-527. In conclusion, EA protected against DC by its hypoglycemic, antioxidant, anti-inflammatory, and anti-fibrotic, and anti-apoptotic effects through upregulation and activation of SIRT1.

Key words: ellagic acid, apoptosis, diabetic cardiomyopathy, oxidative stress, silent information regulator 1, nuclear factor erythroid-derived 2-like 2, nuclear factor kappa B, caspase
disorders (CVD) by stimulating the activity of SIRT1 has received substantial attention (25, 26). Ellagic acid (EA), a phytochemical mainly present in nuts and some fruits (i.e., raspberries, grapes, and pomegranates), exhibits a wide array of renal, hepatic, and cardiac protective effects in a variety of chronic disorders with many thanks to its antioxidant and anti-inflammatory effects (27-30). The cardioprotective effects of EA have been also demonstrated in several animal models including those induced by high-fat diet (HFD)-induced T2DM, myocardial infarction (MI), L-NAME-induced hypertension, and ischemia/reperfusion (IR) injury, as well as doxorubicin and cisplatin, and arsenic trioxide-induced cardiotoxicity (27-33). In all these studies, the protection afforded by EA was attributed to its potent antioxidant and anti-inflammatory potential mediated, at least, by upregulation of Nrf2 and suppression of nuclear factor kappa B (NF-κB).

Despite these findings, the mechanistic protective effects of EA against T1DM induced cardiac damage and DC is not well-established. In a single study, EA prevented the increase in triglycerides levels and suppressed oxidative stress and inflammation in the hearts of streptozotocin (STZ)-diabetic mice (5). In this study, it was of our interest to go further to reevaluate the cardioprotective effect of EA in STZ-induced diabetic rats and characterize some possible mechanisms of action by targeting SIRT1 signaling.

According, we are showing, for the first time, that chronic administration of EA at a dose of 100 mg/kg for 8 weeks, was able to prevent cardiac damage, inflammation, fibrosis, and apoptosis in the hearts of STZ-induced diabetic rats. Besides, we are also providing a piece of evidence that all these protective effects of EA are mediated by upregulation/activation of SIRT1 signaling and subsequent deacetylation of Nrf2, NF-κB p65, Smad3, and FOXO-1.

MATERIALS AND METHODS

Animals

All experiments conducted in this study have been approved by the Official Review Board of the Princess Nourah University, Riyadh, KSA (IRB Number: 20-0096). Adult male Wistar rats (150 ± 10 g/6 weeks old) from the same breed were obtained from the Experimental Animal Care Center at King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA) and were kept there throughout the whole study period under 23 ± 1°C, 60% humidity, and 12-h light/dark cycle housing conditions. All rats had ad libitum access to normal chow and drinking water.

Drugs

EA (cat E2250), EX-527 (cat E7034), and hydrochloride/xylazine hydrochloride solution (cat K-113) were purchased from Sigma Aldrich (St Louis, MO, USA). STZ (cat Ab142155) was purchased from Abcam, UK, Cambridge, EA was prepared in 0.01 M NaOH to a final concentration of 100 mg/ml. EX-527 was prepared in DMSO and diluted in phosphate buffer saline (PBS/ph 7.4) to a final concentration of 2.5 mg/ml. However, STZ was freshly prepared in 0.5 M citrate buffer (pH 4.5).

Chemicals, reagents, and antibodies

Radioimmunoprecipitation (RIPA) (cat 156034) buffer was purchased from Abcam, Cambridge, UK. Protein determination kit (cat 23200) and the protease inhibitor cocktail (78429) were purchased Thermo-Fisher Scientific, Waltham, MA, USA. Assay kit to measure glucose levels (cat 81693) were purchased from Crystal Chem, El Grove Village, IL, USA. An ELISA kit to measured plasma insulin levels (cat ERINSA) was purchase from ThermoFisher Scientific, Waltham, MA, USA) as per the manufactures’ instruction. A nuclear/cytoplasmic isolation kit (cat. 113474), an assay kit to measure SIRT1 activity (cat Ab165065), and an ELISA kit to measure serum troponin-I levels (cat ab246529) were purchased from Abcam, Cambridge, UK. ELISA kits to measure creatinine kinase-MB (CK-MB) (cat MBS2515061), superoxide dismutase-2 (SOD2) (cat MBS4502586), and malondialdehyde (MDA) (cat MBS2540409) levels were purchased from MyBioSource, San Diego, CA, USA. An ELISA kit to measure the levels of the brain natriuretic peptide (BNP) (cat ELAM-BNP) was purchased from RayBiotech Life, Reachtree Corners GA, USA. An assay kit to measure the total levels of reduced glutathione (GSH) (cat 7511-100-K) was purchased from ThermoFisher Scientific, USA.

Table 1. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Cat. No.</th>
<th>Manufacturer/Country</th>
<th>kDa/dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>9475</td>
<td>Cell Signalling technology, USA</td>
<td>120/1:1000</td>
</tr>
<tr>
<td>NF-κB p65</td>
<td>8242</td>
<td>Cell Signalling Technology, USA</td>
<td>65/1:500</td>
</tr>
<tr>
<td>Acetyl FXB (Lys310) (Cat. No. 3045, 65 kDa)</td>
<td>3045</td>
<td>Cell Signalling Technology, USA</td>
<td>65/1:500</td>
</tr>
<tr>
<td>Acetyl Nrf2 (Lys599)</td>
<td>IKT0063</td>
<td>Gbioscience, MO, USA</td>
<td>61/1:500</td>
</tr>
<tr>
<td>Acetyl smad2/3 (Lys19)</td>
<td>PA576015</td>
<td>ThermoFisher Scientific, USA</td>
<td>70/500</td>
</tr>
<tr>
<td>Collagen 1A</td>
<td>72026</td>
<td>Cell Signalling Technology, USA</td>
<td>220/1:1000</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>sc-130348</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>13/1:1000</td>
</tr>
<tr>
<td>Becl2</td>
<td>sc-7382</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>26 kDa/1:1000</td>
</tr>
<tr>
<td>Bax</td>
<td>sc-70408</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>23 kDa/1:1000</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>9664</td>
<td>Cell Signalling Technology, USA</td>
<td>17/19/1:500</td>
</tr>
<tr>
<td>β-actin</td>
<td>3700</td>
<td>Cell Signalling Technology, USA</td>
<td>45/5000</td>
</tr>
<tr>
<td>Lamin A</td>
<td>sc-293162</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>69 kDa/1:4000</td>
</tr>
</tbody>
</table>
purchased from R&D Systems, Minneapolis, MN, USA. A fluorescent kit to measure total ROS levels (cat BML-AK555-0001) was purchased from ENZO Life Sciences, AG, Lausen, Switzerland. ELISA kits to measure interleukin-6 (IL-6) (cat K4145) and tumor necrosis factor-alpha (TNF-α) (cat K1052) levels were purchased from BioVision, Milpitas, CA, USA. ELISA kits to measure nuclear levels of NF-κB p65 (cat 50296) and SIRT1 (cat 31102) were purchased from TransAM, Active Motif, Tokyo, Japan. All antibodies used for the Western blotting study and shown in Table 1.

Induction of diabetes mellitus

T1DM was induced to rats using a single intraperitoneal (i.p.) bolus of STZ solution (65 mg/kg) as described by others to induce T1DM in rodents (34, 35). To prevent the death from adverse sudden hypoglycemia, all rats were orally supplemented with 0.5% glucose. Three days later, plasma glucose levels were measured using the provided kits and all rats having glucose levels higher than 320 mg/dl were considered to have T1DM and were included in this study. Control rats received an equivalent volume of 0.5 M citrate buffer (pH 4.5).

Experimental design

Directly after the establishment of DM (day 3 post-STZ administration), the diabetic and non-diabetic rats were randomly selected and divided into the following five groups (n = 12/group):

(1) Control group: were non-diabetic rats that received an equivalent volume of 0.1% DMSO as a vehicle;
(2) Control + EA-treated group: were non-diabetic rats that received EA alone (100 mg/kg/day);
(3) STZ-treated group: were rats with pre-established T1DM that received an equivalent volume of 0.1% DMSO as a vehicle;
(4) STZ + EA-treated group: were rats with pre-established T1DM that received EA (100 mg/kg/day);
(5) STZ + EA + EX-527-treated group: were rats with pre-established T1DM that received EX-527 (5 mg/kg/i.p./twice per week).

Vehicle or EA administration in all groups was conducted daily and orally via gavage. All treatments were conducted for 8 weeks, daily. Any death in any group was replaced by others. The summary of the experimental procedure including the group classification and time frame of this experiment is shown in Fig. 1.

Dose selection

The dose and treatment period for 8 weeks was adopted from the study of Zhang et al. (36) who have demonstrated that a single i.p. dose of STZ impairs cardiac function and induces fibrosis (peaked) by weeks 4 and 8, respectively. Similar authors who used a single i.p. dose of STZ (55 mg/kg) have also shown impaired cardiac function and increased LV fibrosis by week 8 post-STZ administration. Monitoring of the development of cardiac dysfunction and LV fibrosis has been also tested in our study by adding an extra diabetic group (n = 8 rats) and measuring the cardiac function-related markers and hemodynamic parameters, as well as examining the pathological development of LV fibrosis (by histological technique) in randomly selected 2 rats every 2 weeks, during the experimental procedure. The dose of EA was based on the study of Zhou et al. (37) who showed a potent nephroprotective effect in STZ-diabetic rats mediated by antioxidant and anti-inflammatory effects. Besides, we have tested different doses of EA in our preliminary (20, 50, 100, and 200 mg/kg) data and the doses of 100 and 200 mg/kg showed similar protective and maximum effects. However, the in vivo administration of EX-527 was based on the study of Eid et al. (38).

Cardiac function evaluation

By the end of week 8, all rats were fasted for 12 h and then anesthetized all rats with a mixture of 80/12 mg/kg ketamine/xylazine solution (intramuscular, i.m.). Measurement of LV hemodynamics was performed as previously described in some describes in the previous study by Eid et al. (39). In brief, the rats were placed in a supine position on a heated table (37°C).
Thereafter, the neck was opened and the carotid artery was located and isolated from the surrounding tissues and nerves. A Millar pressure catheter (model SPR-320) was inserted through the carotid artery and forwarded into the left ventricle (LV). The pressure signal was obtained and analyzed using a PowerLab system (Model 16/35, AD Instruments, Bella Vista, Australia) and associated LabChart software (version 8). The following parameters were subsequently recorded:

Table 2. Body and heart weights, as well as fasting levels of glucose insulin in all groups of rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control + EA</th>
<th>STZ</th>
<th>STZ + EA</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weights (g)</td>
<td>333 ± 17</td>
<td>329 ± 18</td>
<td>253 ± 12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>301 ± 14&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>261 ± 9&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.48 ± 0.15</td>
<td>1.38 ± 0.14</td>
<td>1.92 ± 0.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.53 ± 0.19&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.86 ± 0.3&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart/BW ratio (%)</td>
<td>0.44 ± 0.03</td>
<td>0.41 ± 0.05</td>
<td>0.76 ± 0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.51 ± 0.04&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.71 ± 0.04&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fasting plasma glucose (mg/dl)</td>
<td>109 ± 6.9</td>
<td>88 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>312 ± 17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>154 ± 11.6&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>321 ± 21.3&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fasting plasma insulin (mIU/ml)</td>
<td>5.1 ± 0.76</td>
<td>5.2 ± 0.68</td>
<td>1.2 ± 0.37&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.9 ± 0.38&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.3 ± 0.42&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>37.6 ± 6.4</td>
<td>61.4 ± 7.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.73 ± 0.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.4 ± 4.5&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.81 ± 0.43&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation of n = 12 rats/group, were analyzed using two-way ANOVA, and were considered significantly different at P < 0.05. a: significantly different when compared to control rats. b: significantly different when compared to control + ellagic acid-treated rats. c: significantly different when compared to streptozotocin-induced diabetic rats, and d: significantly different as compared to DM + EA-treated rats. EX-527: a selective SIRT1 inhibitor. The homeostasis model assessment of β-cell function (HOMA-β) was measured for each rats using the following equation: [360 × fasting insulin (µU/ml) / (fasting glucose (mg/dl) - 63)].

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Fig. 2. Morphological photomicrographs of the left ventricles (LVs) stained with hematoxylin and eosin (H&E) in all groups. (A) and (B): image from LVs obtained from the control and control + ellagic acid (EA)-treated rats showing normal architectures characterized by horizontally running intact muscle fibers, intact centrally located oval nuclei (long arrow), endothelial cells (short arrow), and intercalated disk (arrowhead). (C): image obtained from the LV of streptozotocin (STZ)-induced diabetic rat (DM) showing severe pathological changes in the LV, including hypertrophied muscle fibers (long arrow), abnormally shaped nuclei (thick short arrowhead), damaged cardiomyocyte fibers (arrowhead), wavy fibers (short thin arrow) and infiltration of immune cells (curved arrow). (D) and (E): images were obtained from LVs of STZ + EA-treated rats showing much improvement in LV structure with almost normal cardiomyocyte architecture, similar to that observed in control rats. (F): image was taken from the LV of STZ + EA + EX-527 (a selective SIRT1 inhibitor)-treated rats showing severe alteration in LV structure with almost the same changes observed in the LV of STZ-treated rats. These LVs showed severe cardiomyocyte damage (short thin arrow), hypertrophic muscle fibers (long arrow), infiltration of immune cells (curved arrow), wavy fibers (curved arrow), and abnormal nuclei (thick short arrow).
(1) Left ventricle systolic pressure (LVSP),
(2) maximum derivative of the change in systolic pressure over time (dP/dt max),
(3) minimum derivative of the change in diastolic pressure (dP/dt min); and
(4) left ventricle end-diastolic pressure (LVEDP).

The recording of the pressure signal was conducted over 20 min where the first 5 min were excluded from analysis and considered the stabilization period.

**Plasma collection and measurements**

Blood samples (3 ml) were directly collected from the LVs via the cardiac puncture protocol, drawn into EDTA-containing tubes, and centrifuged at 1200 x g for 10 min at room temperature to collect the plasma, which was stored at -20°C until use. These plasma samples were used to measure fasting plasma glucose and insulin levels using the provided kits. The homeostasis model assessment of β-cell function (HOMA-β) was measured for each rat using the following equation (40-42):

\[ 360 \times \text{fasting insulin} (\mu U/ml) / (\text{fasting glucose} (mg/dl) – 63) \]

**Tissue collection, preparation, and measurements**

Directly after blood collection, all rats were killed by cervical dislocation and their hearts were rapidly excised on ice and cut into smaller pieces. Parts of these LVs were snap-frozen in liquid nitrogen and stored at -70°C for later use. To prepare total tissue homogenates, parts of the frozen LVs were homogenized in 9 volumes PBS (pH 7.4), centrifuged (11000 x g/10 min/4°C), and all supernatants were collected and stored at -70°C and used later to measure levels of ROS, MDA, GSH, SOD2, TNF-α, and IL-6. Some parts were used for the preparation of the nuclear fraction which was used to measure the nuclear activities of Nrf2, NF-kB p65, and SIRT1. All measurements were performed using the above-mentioned kits for n = 8/group and as per the manufacturers’ instructions.

**Western blotting**

Portions of the LVs (75 mg) were homogenized in 0.5 mL RIPA buffer and centrifuged at 11000 x g (10 min/4°C) to collect the supernatants containing total proteins of total cell homogenates. Total or nuclear proteins (40 µg) were separated SDS-PAGE (8 – 12%) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% milk and then incubated with the primary antibodies (Table 1) and then with the secondary antibodies (room temperature/2 h). Detection of the bands was conducted using the chemiluminescent (ECL) detergent. Images were captured, scanned, and analyzed using the C-Di Git blot scanner (LI-COR, NE, USA). Membranes were stripped up to three times. Internal control was run between the gels for standardization. Proteins detected in the total cell homogenates were normalized against β-actin, while nuclear proteins were normalized against lamin A.

**Histological evaluation**

Portions of freshly collected LVs were directly fixed in 10% buffered formalin. After 18 h, all tissues were dehydrated in ascending ethanol concentrations (70 – 100%), cleared in xylene, embedded in wax, and then sectioned (4 – 5 µm diameter). LV sections were then stained with either hematoxylin & eosin for routine morphology or Masson’s trichrome stain to determine the

![Fig. 3.](image-url)
extent of fibrosis. All examinations and photography were conducted using light microscopy (200×) by an investigator blinded to the experimental group.

**Statistical analysis**

GraphPad Prism statistical software (V8) was used for statistical analyses. Normality was tested using the Shapiro-Wilk test. All analyses were conducted using one-way analysis of variance (ANOVA 1) followed by Tukey’s post hoc t-test. The values were presented as mean ± standard deviation (SD) and P < 0.05 were considered significantly different.

**RESULTS**

**Changes in final body and heart weights, as well as fasting glucose and insulin levels**

Final body weights, heart weights, fasting plasma insulin levels, and heart weight/body weight ratios did not significantly differ, fasting plasma glucose levels and values of HOMA-β were significantly decreased in the control + EA-treated rats as compared to control rats (Table 2). A significant reduction in rats’ final body and heart weights, fasting insulin level, and HOMA-β with a concomitant increase in fasting blood glucose levels and the ratio of the heart/BWT were observed in STZ-treated rats as compared to control rats. All these parameters were significant reversed in STZ + EA-treated rats as compared to STZ-treated rats. However, final body and heart weights, fasting insulin levels, and values of HOMA-β were significantly decreased but fasting glucose levels, and heart/BWT ratio were significantly increased in STZ + EA + EX-527-treated rats as compared to STZ + EA-treated rats, levels which were not statistically different as compared to STZ-induced rats (Table 2).

**Histological alterations**

Normal cardiomyocyte structures were observed in the LVs of both the control and control + EA-treated rats (Fig. 2A and 2B). Numerous pathological alterations, including dysregulated, damaged, and hypertrophied muscle fibers, abnormal nuclei

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**Fig. 4.** Levels of (A): reactive oxygen species (ROS); (B): malondialdehyde (MDA); (C): reduced glutathione (GSH); (D): superoxide dismutase-2 (SOD2); (E): tumor necrosis factor-alpha (TNF-α); and (F): interleukin-6 (IL-6) in the left ventricles of all groups. Values are presented as mean ± standard deviation of n = 12 rats/group, were analyzed using two-way ANOVA, and were considered significantly different at P < 0.05. a: significantly different compared to control rats; b: significantly different compared to control + ellagic acid (EA)-treated rats; c: significantly different compared to streptozotocin (STZ)-induced diabetic rats (DM) and d: significantly different compared to STZ + EA-treated rats. EX-527, selective SIRT1 inhibitor.
structures, immune cell infiltration, the disappearance of the intercalated disk, and several wavy muscle fibers, were observed in the LVs of STZ-induced rats (Fig. 2C). All these alterations were disappeared in LVs sections obtained from STZ + EA-treated rats (Fig. 2D and 2E). However, LVs of STZ + EA + EX-527 showed similar pathological alterations to those observed in the STZ-treated rats (Fig. 2F).

Changes in cardiac markers

Serum levels of all cardiac markers are depicted in (Fig. 3A-3C). Normal levels of CK-MB, troponin-I, and BNP-1 were seen in the serum of control + EA-treated rats as compared to control rats. The levels of all these cardiac markers were significantly increased in the serum of STZ-treated rats as compared to control rats. However, lower serum levels of CK-MB, troponin-I, and BNP-1 were observed in STZ + EA-treated rats when compared to STZ-treated rats. On the other hand, the levels of all these cardiac markers measured in the serum of STZ + EA + EX-527 were significantly higher than their corresponding levels measured in control, control + EA, and STZ + EA-treated rats but were not significantly different as compared to STZ-treated rats.

Alterations in left ventricle hemodynamic parameters

The changes in the LV hemodynamic parameters are demonstrated in Fig. 3D-3F. While LVEDP and dp/dt_min remained significantly not changed, levels of LVSP and dp/dt_max were significantly increased in the control + EA-treated rats as compared to control rats. STZ-treated rats showed a significant reduction in the levels of LVSP, dp/dt_min, and dp/dt_max with a concomitant increase in the levels of LVEDP as compared to control rats. The levels of all these parameters were significantly reversed in STZ + EA-treated rats as compared to STZ-treated. However, LVEDP levels were significantly increased and the levels of the other measured hemodynamic parameters were significantly reduced in the STZ + EA + EX-527 as compared to STZ + EA-treated rats, levels which were not significantly different as compared to STZ-treated rats.

Effect on antioxidant, oxidative stress and inflammatory markers

All changes in the LV markers of oxidative stress and inflammation are depicted in Fig. 4A-4F. Significantly higher levels of ROS, MDA, TNF-α, and IL-6 with a parallel reduction in the levels of GSH and SOD were observed in the
LVs of STZ-induced rats as compared to control rats. However, control + EA or STZ + EA-treated rats showed significantly higher levels of GSH and SOD but reduced levels of ROS, MDA, TNF-α, and IL-6 in the LVs as compared to either the control or STZ-induced rats. On the opposite, levels of ROS, MDA, TNF-α, and IL-6 were significantly increased and levels of GSH and SOD have significantly decreased in the LVs of STZ + EA + EX-527-treated rats as compared to STZ + EA-treated rats.

**Effect on left ventricle collagen deposition and activity of TGF-β1/smad3 pathway**

The changes in LV collagen deposition and the protein levels of TGF-β1, Smad3, and acetylated Smad3 (Lys19) are shown in Fig. 5A-5G. No significant change in the number of collagen fibers nor protein expression of TGF-β1 and Smad3 but a significant reduction in the protein levels of acetylated Smad3 were seen in the LVs of the control + EA-treated rats as compared to control rats (Fig. 5A, 5B and 5G). However, increased collagen deposition with a significant increase in protein levels of TGF-β1, Smad3, and acetylated Smad3 (Lys19) were seen in the LVs of STZ-treated rats as compared to control rats (Fig. 5C and 5G). On the opposite, STZ + EA-treated rats showed a significant reduction in their LV collagen deposition and protein levels of TGF-β1, Smad3, and acetylated Smad3 (Lys19) as compared to STZ-induced diabetic rats, effects that were significantly reversed in the LVs of STZ + EA + EX-527 (Fig. 5D-5G). Of note, no significant variations in the LV protein levels of TGF-β1, Smad3, and acetylated Smad3 (Lys19) were seen when STZ + EA + EX-527 were compared with STZ-treated rats (Fig. 5D-5G).
Changes in apoptotic/anti-apoptotic markers

Markers of intrinsic (mitochondria-mediated) cell apoptosis are shown in Fig. 6A-6D. Protein levels of Bax and cleaved caspase-3 remained significantly not altered but protein levels of Bcl2 were significantly increased and the ratio of Bax/Bcl2 was significantly reduced in the LVs of control + EA-treated rats as compared to control rats. The protein levels of Bax, and cleaved caspase-3, as well as the ratio of Bax/Bcl2, were significantly increased but protein levels of Bcl2 were significantly reduced in the LVs of STZ-treated rats as compared to control rats. The levels of all these markers were significantly reversed in the LVs of STZ + EA-treated rats as compared to STZ-treated rats. However, the protein levels of Bax, and cleaved caspase-3, as well as the ratio of Bax/Bcl2, were significantly higher but protein levels of Bcl2 were significantly reduced in the LVs of STZ + EA + EX-527 as compared to treated EA-treated rats but were not significantly different as compared to STZ-induced rats.

Alteration in the levels/activities of SIRT1, Nrf2, and NF-κB p65

LVs of STZ-treated rats showed a significant reduction in the total protein levels of SIRT1, as well as the nuclear activities of SIRT1 and Nrf2 but had significantly higher nuclear activities of NF-κB p65 as compared to control rats (Fig. 7A-7D). On the other hand, the control + EA-treated rats, as well STZ + EA-treated rats showed significantly higher total protein levels of SIRT1, the nuclear activity of Nrf2, and nuclear activity of SIRT1 but had a significant reduction in the activities of NF-κB p65 when they were compared to either the control rats or STZ-treated rats, respectively (Fig. 7A-7D). On the contrary, total protein levels of SIRT1, as well as the nuclear activities of Nrf2 and SIRT1 were significantly reduced but the nuclear activities of NF-κB p65 were significantly increased in the LVs of STZ + EA + EX-527 as compared to STZ + EA-treated rats, levels which were not significantly different as compared to STZ-treated rats (Fig. 7A-7D).

Fig. 7. Nuclear activity of NF-κB p65 (A), Nrf2 (B), and SIRT1 (C), as well as the total protein levels of SIRT1 (D) in the left ventricles of all groups. Values are presented as mean ± standard deviation of n = 12 rats/group, were analyzed by 2-way ANOVA, and were considered significantly different at P < 0.05. a: significantly different compared to control rats; b: significantly different compared to control + ellagic acid (EA)-treated rats; c: significantly different compared to streptozotocin (STZ)-induced diabetic rats (DM) and d: significantly different compared to STZ + EA-treated rats. EX-527, a selective SIRT1 inhibitor.
Changes in the acetylation levels of Nrf2, NF-κB p65, and FOXO1

The effect of all treatments on protein acetylation of Nrf2, NF-κB p65, and FOXO1 in LVs of all groups of rats is demonstrated in Fig. 8. Acetylated protein levels of Nrf2, NF-κB p65, and FOXO1 were significantly increased in the LVs of STZ-treated rats as compared to control rats. The acetylated protein levels of Nrf2, NF-κB p65, and FOXO-1 were significantly decreased in the LVs of both the control + EA-treated rats and STZ + EA-treated rats as compared to either control rats or STZ-treated rats, respectively. However, the acetylated levels of Nrf2, NF-κB p65, and FOXO1 have significantly increased in the LVs of STZ + EA + EX-527-treated rats as compared to STZ + EA-treated rats but were not significantly different as compared to STZ-treated rats.

DISCUSSION

Limited research has investigated the protective effects of EA on DC in experimental animals, especially in animal models of T1DM. Therefore, this study was conducted to investigate if chronic administration of EA could ameliorate DC in STZ-treated rats and investigated if the possible mechanisms of action involve modulating SIRT1 signaling. Accordingly, our data support our hypothesis and show that chronic EA supplementation at a dose of 100 mg/kg for 8 consecutive weeks was able to not only suppressed hyperglycemia but also preserved cardiac systolic and diastolic function and prevented STZ-treated oxidative stress, inflammation, and fibrosis in the LVs of rats. All such effects were SIRT1-dependent and were associated with the deacetylation of multiple targets, including FOX01, smad3, NF-κB, and Nrf2. Notably, the hypoglycemic and cardioprotective effects of EA were abolished by the co-treatment with EX-527, a selective SIRT1 inhibitor. A summary of these effects is shown in the graphical abstract (Fig. 9).

In this study, we have validated DC in our animal model by the end of week 8 by the obvious hyperglycemia, hypoinsulinemia, impaired systolic and diastolic functions, increased cardiac injury markers, and LV fibrosis. Similar to our data, impaired cardiac function was reported in STZ-induced diabetic rats after 3, 4, 8, and 12 weeks post-STZ-induction, in which cardiac fibrosis was peaked by the end of week 8 (55 – 65 mg/kg) (3, 4, 7, 36, 46). On the other hand, final body weights and heart weights are typically reduced in STZ-diabetic rats whereas the heart/BWT ratio is increased indicating hypertrophy (44). Also, higher circulatory levels of BNP, a well-known marker for cardiac hypertrophy were observed in STZ-diabetic hearts (48, 49). This has been also confirmed in our animal model where we have found a similar reduction in heart and final body weights and increased heart/BWT ratio, and higher serum levels of BNP.

On the other hand, hyperglycemia-induced ROS due to impaired oxidative phosphorylation, overwhelming of antioxidant, or activating ROS-generating enzymes, is the major mechanism that leads to all damaging effects including inflammation, fibrosis, and apoptosis in the heart of T1DM and T2DM rats (10, 12, 50). Indeed, ROS can induce cardiac inflammation by stimulating NF-κB. Besides, ROS and inflammatory cytokines induce cardiac fibrosis by stimulating the TGF-β1/Smad2/3 signaling pathways (46). Besides, ROS can activate both intrinsic and extrinsic cell death by different hyperglycemia and various other characteristics that are observed in DM (43). In vivo, as well as in isolated heart preparations, cardiac contraction and structural disturbances are observed after long-term and short term administration of STZ due to alterations in several factors including abnormalities in cellular Ca^{2+} homeostasis, oxidative stress, mitochondria dysfunction, and activation of renin-angiotensin-aldosterone system, inflammation, apoptosis, fibrosis and remodeling of the myocardium (9-13, 44-46). In turn, these endogenous processes interact with each other and lead to LV hemodynamic dysfunction including reduced percentage of fractional shortening, LVSP, stroke volume, ejection fraction, and cardiac output and increased diastolic pressure, isovolumetric relaxation (i.e. low dp/dt\textsubscript{max} values), and cardiac stiffness (amplified LVEDP) (3, 44, 47).

In Fig. 8, Acetylated nuclear protein levels of FOXO-1, Nrf2, and NF-κB p65 (A) in the left ventricles of all groups. Values are presented as mean ± standard deviation of n = 12 rats/group, were analyzed using two-way ANOVA, and were considered significantly different at P < 0.05. *: significantly different compared to control rats (lane 1); †: significantly different compared to control + EA-treated rats (lane 2); ‡: significantly different compared to STZ-treated rats (lane 3) and §: significantly different compared to STZ + EA-treated rats (lane 4). EX-527, a selective SIRT1 inhibitor.
mechanisms (50, 51). All these damaging pathways were previously described in the hearts of STZ-diabetic hearts (3, 4, 7, 12, 36, 52). Indeed, LVs of STZ of this study showed a significant increase in the levels of ROS, lipid peroxides (MDA), the activity of NF-κB, and inflammatory cytokines, as well as a significant reduction in the levels of SOD, GSH, and Nrf2. Besides, increased expression of Bax and cleaved caspase-3 and the reduction in the protein levels of Bcl2 confirm the role of intrinsic cell death mediating STZ-induced cardiac damage in the LVs of diabetic rats of this study.

On the other hand, Chao et al. (5) have previously shown a protective effect of EA against STZ-induced cardiac oxidative stress and inflammation in mice. The findings of our study widen this knowledge and further show that EA also preserved cardiac function, and inhibit cardiomyocytes oxidative stress, inflammation, fibrosis, and apoptosis in the hearts of STZ-induced rats. Besides, the data in our hand provide a mechanistic effect of the protection afforded by EA and confirm it is mediated, mainly, by stimulating SIRT1 signaling. In general, the role of SIRT1 in the regulation of diverse cellular functions is well-reported in literature. In this regard, SIRT1 can stimulate the GSH synthesis and phase II antioxidant enzymes (i.e. SOD and catalase) and anti-apoptotic genes by deacetylating and activating Nr2 and FOXO transcription factors (53-55). Furthermore, SIRT1 can also suppress cellular fibrosis by deacetylating p300 and Smad2/3 (23). Also, SIRT1 can directly suppress the synthesis of several inflammatory cytokines and indirectly reduce ROS generation by deacetylating NF-κB p65 (56). Furthermore, SIRT1 can also suppress intrinsic cell death by downregulating Bax mediated by deacetylating p53 (57). In addition, SIRT1 stimulates cardiac contractility by modulating intracellular and sarcoplasmic reticulum Ca²⁺ levels through modulating the activities of several targets including the Homer proteins, Na⁺/K⁺ ATPase, and SERCA proteins (24).

In this study, STZ significantly reduced the activities and levels of SIRT1 which support many other previous studies (18-23). Besides, treatment with STZ significantly increased the acetylation of many SIRT1 targets including Nr2, NF-κB p65, smad3, and FOXO1. Such impairment in SIRT1 signaling in the LVs of these rats may participate significantly in the cardiac oxidative damage of STZ via suppressing antioxidant (i.e. GSH and SOD) and cardiac contractility (i.e. reduction in LVSP and LVdP/dtmax) and activating the inflammatory, fibrotic, and apoptotic responses. In support, knocking down the cardiac SIRT1 was sufficient to induce a cardiac phenotype that is similar to DC, which was accompanied by cardiomyocyte loss, oxidative stress, inflammation, cardiac hypertrophy, and irreversible fibrosis (21). On the other hand, our data show that EA stimulated the levels and activities of SIRT1 and reduced the acetylation of all above-mentioned transcription factors not only in the LVs of diabetic hearts but also in the LVs of control hearts, which indicates a stimulatory effect of EA on this cellular molecule. Interestingly, all the observed cardiac beneficial effects of SIRT1 in STZ-induced rats were prevented by co-administration of EX-527, a SIRT1 inhibitor. Therefore, it seems reasonable to us that the cardiac protective effect of EA is mediated by activating SIRT1 signaling. Similar to our data, EA and its metabolite, urolithin-A were shown to activate SIRT1 in other non-cardiac tissues (39).

Part of the observed cardiac protective effect of EA could be also attributed to its hypoglycemic and insulin-releasing effects. As discussed before, hyperglycemia is the key player mediating all the damaging effects in diabetic hearts including oxidative stress, inflammation, fibrosis, and apoptosis. In this study, our data suggest the ability of EA to reduce fasting blood glucose levels under basal and diabetic conditions. Besides, EA stimulated insulin release only in diabetic rats. Such hypoglycemic effect of EA has been previously reported in numerous studies and was attributed to suppressing hepatic gluconeogenesis, improving peripheral insulin action, and stimulating AMPK-induced GLUT-4 membranous translocation in the skeletal muscles (30, 58). However, the significant increment in the levels of insulin in diabetic rats may be related to suppressing STZ-induced pancreatic cell damage or

Fig. 9. Graphical abstract presenting the mechanism by which ellagic acid (EA) protects against diabetic cardiomyopathy in rats. In brief, hyperglycemia induces diabetic cardiomyopathy by increasing the production of reactive oxygen species (ROS) and suppression of SIRT1. The reduction in SIRT1 levels and activities results in increased acetylation of numerous transcription factors including Nr2, NF-κB, Smad3, and FOXO-1. Together, ROS and increased acetylation of all these transcription factors result in cardiac inflammation, oxidative stress, fibrosis, and apoptosis. EA prevents these events by three mechanisms: 1) hypoglycemic effect, 2) scavenging ROS, 3) activation and upregulation of SIRT1.

EA

Hyperglycemia

SIRT1

ROS

Acetylated
Nr2
Acetylated
NF-kB
Acetylated
FOXO
Acetylated
Smad-3

Inflammation
(TNF-α/IL-6)

GSH, SOD

TGF-β1

apoptosis

DC

Fig. 9. Graphical abstract presenting the mechanism by which ellagic acid (EA) protects against diabetic cardiomyopathy in rats. In brief, hyperglycemia induces diabetic cardiomyopathy by increasing the production of reactive oxygen species (ROS) and suppression of SIRT1. The reduction in SIRT1 levels and activities results in increased acetylation of numerous transcription factors including Nr2, NF-κB, Smad3, and FOXO-1. Together, ROS and increased acetylation of all these transcription factors result in cardiac inflammation, oxidative stress, fibrosis, and apoptosis. EA prevents these events by three mechanisms: 1) hypoglycemic effect, 2) scavenging ROS, 3) activation and upregulation of SIRT1.
increasing the generation of the pancreatic cells. In support, Fatima et al. (58) also showed that EA can inhibit β-cell apoptosis to stimulate insulin secretion.

SIRT1 is a novel molecule that can stimulate the expression of GLUT and increase insulin signaling and glucose uptake in the muscles and adipocytes (59, 60). SIRT1 is a potent stimulator of AMPK which also able to stimulate peripheral glucose uptake and insulin sensitivity (61, 62). Besides, SIRT1 can stimulate insulin release from the β-cells and promotes the regeneration of β-cells by activating the endocrine progenitor cells through activating AMPK-mediated fatty acid oxidation (63). Indeed, SIRT1 activation protected pancreatic β-cells from palmitic acid and cytokine-induced damage (64, 65).

Another novel observation reported in this study is that the hypoglycemic and insulin-releasing effects of EA in diabetic rats were prevented by the co-administration of EX-527. Indeed, higher levels of glucose with reduced levels of insulin were depicted in the plasma of STZ + EA + EX-527-treated rats as compared to STZ + EA-treated rats. These data may indicate that hypoglycemic and insulin-releasing effects of EA are mainly due to a mechanism that is related to activation of SIRT1. Unfortunately, we are not certain where this activation occurred (i.e. in the liver, skeletal muscles, or β-cells). This is a serious limitation in this study and requires further future investigations. Hence, at this stage, we can assume that EA exerts its cardioprotective effect by producing these active metabolites. Nonetheless, this needs further investigation and more organized studies. Also, DC is most common in T2DM. Therefore, it will be worthy to examine the protective effect of EA in these animal models to confirm its effectiveness and widen its therapeutic use. On the other hand, it has been reported that the impaired diastolic function in the cardiomyocytes of STZ-induced diabetic rats is mainly due to reduced relaxation and Ca2+ transient decays, mainly due to reduction in SERCA activities (71). However, EA, attenuated the reduced relaxation and Ca2+ transient decays, mainly due to cardiomyocytes of STZ-induced diabetic rats is mainly due to and widen its therapeutic use. On the other hand, it has been studied subtype and has potent anti-obesity, antioxidant, anti-inflammatory, and anti-fibrotic effects with potent cardioprotective effects (67-70). Therefore, it could be possible that EA exerts its cardioprotective effect by producing these active metabolites. Nonetheless, this needs further investigation and more organized studies. Also, DC is most common in T2DM. Therefore, it will be worthy to examine the protective effect of EA in these animal models to confirm its effectiveness and widen its therapeutic use. On the other hand, it has been reported that the impaired diastolic function in the cardiomyocytes of STZ-induced diabetic rats is mainly due to reduced relaxation and Ca2+ transient decays, mainly due to reduction in SERCA activities (71). However, EA, attenuated the impairment in such diastolic function in LVs strips obtained from STZ-diabetic LVs by accelerating the Ca2+ transient decays and relaxation by shortening the time to 90% relaxation, and the time to peak tension, as well as reducing the basal Ca2+ concentration and through activating SERCA. Although this important mechanism was not investigated in this study, it will be very encouraging to investigate if the EA-induced SERCA activation is also mediated by a SIRT1 mechanism.

In conclusion, the data presented in the current animal-based study is the first to show that dietary supplementation of EA could suppress the development of DC in STZ-treated rats by controlling fasting hyperglycemia and suppressing cardiac oxidative stress, inflammation, and apoptosis mediated by scavenging ROS, upregulating of cellular antioxidants, suppressing the TGFβ1/Smad3 axis, and upregulation of anti-apoptotic genes (Bcl2). However, all these mechanisms of protection require EA-induced activation of SIRT1 activation and deacetylation of numerous transcription factors including Nrf2, FOXO1, Smad2/3, and NF-κB. Based on these data, further translational and clinical studies are encouraged to confirm these observations in a trial to identify inexpensive and more effective therapeutic drugs.

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Author’s address: Dr. Dalal H. Aljabryn, Riyadh, 11671, P.O. Box 84428, Saudi Arabia.
E-mail: dhaljabryn@pmu.edu.sa