Protective mechanisms of rats heart in the acute phase of streptozotocin-induced diabetes in functional remodeling

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Abstract
Streptozotocin-induced diabetes was manifested by compromised ventricular contraction and prolonged relaxation attributable to multiple causative factors including oxidative stress and to elucidate the role of these changes in adaptation of the heart to acute streptozotocin induced diabetes-cardiomyopathy contributes to high morbidity and mortality in diabetic populations. This study was designed to examine the effect of cardiac expression after injection of the protein purified from Eugenia jambolana on cardiac contractile function, intracellular Ca2+ cycling proteins and the myosin heavy chain (MHC) isozyme in diabetes. Diabetes depressed the level of SERCA2a, Na+-Ca2+ exchanger and triggered a β MHC isozyme switch. All of these STZ-induced alterations with the exception of depressed SERCA2a and enhanced (MHC) isozyme data suggest a beneficial effect of Eugenia jambolana extract in the therapeutics of diabetic cardiomyopathy, possibly through a mechanism related to SERCA2a and MHC isozyme switch appear as the manifestation of endogenous protective mechanisms participating in the functional remodeling which contributes to adaptation of the heart to diabetes.

Keywords: Cardiomyopathy, Eugenia jambolana, Gene expression, MHC isozyme,

Introduction
Diabetes mellitus is the world’s fastest-growing disease with high morbidity and mortality rates predominantly as results of cardiovascular diseases [1]. Diabetic cardiomyopathy was known as independent of any macro- and micro-vascular diseases and believed to be responsible for the high incidence of heart failure and cardiovascular mortality in diabetes. It was characterized by diminished ventricular function, reduced wall compliance and rate of myocardial relaxation [2]. Several reports have been postulated in an effort to interpret the pathogenesis of diabetic cardiomyopathy including glucose metabolism, oxidative stress and intracellular Ca2+ mishandling [3]. The heart possesses a relatively low antioxidant capacity contributed by enzymatic and non-enzymatic free radical scavengers or antioxidants, thus making it a prime target for oxidative insult [4]. Research evidence have indicated the involvement of oxygen free radicals in the onset and development of diabetic cardiomyopathy in the diabetic state [5]. This was further supported by the observation that antioxidant...
treatment is proven to be beneficial for patients with diabetes [6]. However, most of the antioxidant approaches against diabetes were essentially limited to exogenously administered antioxidants such as α-tocopherol and flavonols [7], which may suffer from pitfalls such as route of absorption and cardiac tissue availability/specificity. In cardiomyocytes, two members of the glucose transporter family are present GLUT1 and GLUT4 [8]. While GLUT1 is regarded as the basal glucose transporter, GLUT4 is responsible for the increase in glucose uptake upon insulin stimulation and elevated contractile activity. Insulin resistance is an important risk factor for the development of hypertension, atherosclerotic heart disease, left ventricular hypertrophy and dysfunction, and heart failure [9]. Mitochondria play an important role in apoptosis under a variety of proapoptotic conditions, such as oxidative stress. Mitochondrial cytochrome c release is a key event in the activation of caspase-3, a downstream pivotal step to initiate apoptosis. A correlation between ROS generation and the pathogenesis of various diabetic complications has been observed. Therefore, it is possible that ROS accumulation occurs in diabetic myocardium, in which apoptosis may take place and lead to cardiomyopathy [10].

The green fluorescent protein (GFP) is a 27kDa polypeptide which converts the blue chemiluminescence of the Ca²⁺ sensitive photoprotein, aequorin into green light. GFP based reporter system was developed in which visible fluorescence was created by molecular biological techniques. The GFP can be used as reporter for the visualization of green expression and protein subcellular localization. In 1994, a novel marker system, the GFP become available [11], has also reviewed properties of the native GFP, and the expression of GFP in heterologous system. Although plasmid based gfp vectors have been used in eukaryotic systems, and some gfp –based host range plasmids have been successfully used to label certain species of bacteria. For more information reader is directed to a comprehensive review of the biochemistry of GFP published by Tsien[12].

The present study was designed to examine the influence of protein from Eugenia jambolana on cardiomyocyte contractile and evaluated generation of reactive oxygen species (ROS), oxidative stress, apoptosis and expression of the main cardiac intracellular Ca²⁺ regulating proteins [sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), in the streptozotocin (STZ) induced diabetic model. Since diabetes was known to trigger the MHC isozyme switch from α- isoform to β-isoform [13], the MHC isozyme distribution was also evaluated in control and diabetic rats with or without protein of Eugenia jambolana injected, we previously reported when this protein was injected in streptozotocin induced diabetic rats it showed a compensatory hypoglycemic effect by minimizing the cellular damage [14]. Goals of the present study are to investigate whether injection of Eugenia jambolana seed kernel protein in diabetic rats has elucidate the role of protective mechanisms securing the adaptation of heart to streptozotocin-induced diabetes and/or remodeling.

Materials and Methods
Induction of Diabetes:
Wistar rats of both sexes weighing 100-150gm were used for study (Mahaveer Enterprises, Hyderabad). All protocols were performed in accordance with the Institutional Animal Ethical Committee (IAEC) as per the directions of the CPCSEA approved number UCP/IAEC/2008/027(Committee for the purpose of Control and Supervision of Experiments on Animals).The animals were fasted overnight and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ 55 mg/kg body weight) in 0.1M citrate buffer pH 4.5 [15]. The animals were allowed to drink 5% glucose solution overnight to overcome the drug -induced hyperglycemia. Control rats were injected with citrate buffer alone. The animals were considered as diabetic, if their blood glucose values were above 250mg/dl on the 3rd day after STZ injection. The treatment was started on the 4th day after STZ injection and this was considered as 1st day of treatment. On the third day of STZ-injection, the rats were fasted for 6 h and blood was taken from tail artery of the rats [16]. Rats with moderate diabetes having hyperglycemia were taken for the experiment. The blood was collected from sinocular puncture. Streptozotocin was purchased from Sigma-Aldrich, St. Louis, USA. All other commercial reagents used were of analytical grade.
Preparation of Plant Extract:
*Eugenia jambolana* fruits were collected from a tree in Alagarokoll Hills, Tamil Nadu, India. The fruits of jambolana pulp was removed and washed with distilled water to remove the traces of pulp from the seeds. The seeds were dried and the kernel was powdered in an electrical grinder and stored at 5 °C until further use. Kernel powder (100 g) was extracted with petroleum ether (60—80 °C) to remove lipids. It was then filtered and the residue was extracted with 95% ethanol by Soxhlation. Ethanol was evaporated in a rotary evaporator at 40—50 °C under reduced pressure. The yield of kernel was 5 g/100 g of dried seeds. *Eugenia jambolana* extracts were used to precipitated protein and purified using DEAE-sepharose CL6B column chromatography and HPLC by the method of [17].

Experimental Design:
In the experiment, a total of 24 rats (18 diabetic surviving rats and 6 normal rats) were used. The rats were divided into four groups comprising of 6 animals in each group as follows: The rats were divided into four groups comprising of five animals in each group and designated as follows: Group I: Control animals receiving 0.1 M citrate buffer (pH 4.5); Group II: Diabetic Control animals; Group III: STZ-diabetic rats given *Eugenia jambolana* extract (100 mg / kg b.w/d) in aqueous solution orally for 3 times at 10 days interval for 30 days; Group IV: STZ-diabetic animals given glibenclamide (10mg/kg b.w/d) in aqueous solution orally 3 times at 10 days interval for 30 days. At the end of the experimental period, the rats were anaesthetized and sacrificed by cervical dislocation, the heart tissues were taken for further investigations. Isolation of ventricular myocytes:
Hearts were rapidly removed from anesthetized rats and immediately mounted on a temperature-controlled (37°C) Langendorff perfusion system. After perfusion with modified Tyrode solution (Ca2+ free) for 2 min, the heart was digested for 10 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) in modified Tyrode solution. The modified Tyrode solution (pH 7.4) contained the following (in mM): NaCl 13.5, KCl 4.0, MgCl2 1.0, HEPES 10, NaH2PO4 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO2 ~95% O2. The digested heart was then removed from the cannula and the left ventricle was cut into small pieces in the modified Tyrode solution. These pieces were gently agitated and the pellet of cells was resuspended in modified Tyrode solution and allowed to settle for another 20 min at room temperature during which time extracellular Ca2+ was added incrementally back to 1.20 mM. Cell viability was approximately 80% in all four animal groups. Isolated myocytes were used for experiments within 8 hours after isolation. Only rod-shaped myocytes with clear edges were selected for recording of mechanical properties and intracellular Ca2+ transients as described [18].

Intracellular reactive oxygen species (ROS):
Production of cellular ROS was evaluated by analyzing changes in fluorescence intensity resulting from oxidation of the intracellular fluoroprobe 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). In brief, isolated myocytes from each group were loaded with 1 μmol/L of the non-fluorescent dye 2′7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. The myocytes were rinsed and the fluorescence intensity was then measured using a fluorescent micro-plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Molecular Devices, Sunnyvale, CA, USA). Untreated cells showed no fluorescence and were used to determine background fluorescence, which was subtracted from the treated samples. The final fluorescent intensity was normalized to the protein content in each myocyte group [19].

Western blot analysis:
The total protein was prepared as described previously [20]. In brief, tissue samples from the heart ventricles were removed and homogenized in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Samples were then sonicated for 15 sec and centrifuged at 12,000 × g for 20 min at 4°C. The protein concentration of the supernatant was evaluated using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Equal amounts (50 μg protein /lane) of the protein and prestained molecular weight markers (Gibco-BRL, Gaithersburg, MD) were separated on 10% or 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad); they were
then transferred electrophoretically to Nitrocellulose membranes (0.2 μm pore size, Bio-Rad Laboratories, Inc, Hercules, CA, USA). Membranes were incubated for 1 hr in a blocking solution containing 5% milk in TBS-T buffer, and incubated with anti-SERCA2a (1:1000), and anti-β-actin (1:5000) antibodies at 4°C overnight and antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and a monoclonal antibody against β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). After washing blots to remove excess primary antibody binding, blots were incubated for 1 hr with horseradish peroxidase (HRP)–conjugated secondary antibody (1:5000). Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia), and film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (Model: GS-800). For all Western blot analysis experiments, β-actin was used as an internal loading control.

**MHC isofrom analysis by gel electrophoresis:**
Samples were prepared for gel electrophoresis according to method described [21]. Each sample (20–30 mg heart tissue) was placed in a microcentrifuge tube and 30 μl of sample buffer was added per mg of tissue. The sample was homogenized for 10 sec, heated for 2 min at 95°C, chilled on ice for 5 min before being centrifuged. The supernatant was saved and diluted 1:10 with sample buffer. Three μl of the diluted samples were loaded for electrophoresis. The methods for gels and the running conditions were identical to those described by [22]. Gel electrophoresis was performed at 8°C in a PROTEAN II unit (Bio-Rad, Hercules, CA, USA). Stacking gels consisted of 4% acrylamide (acrylamide:bis = 50:1) and 5% glycerol (v/v, pH 8.8). Gels were run at a constant voltage of 200 V for 30 hr, fixed for a minimum of 2 hr in 5% glutaraldehyde before being silver stained and scanned with a Bio-Rad Calibrated Densitometer (Model: GS-800) to determine the amount of MHC-α and MHC-β.

**Results and Discussion**
Diabetic cardiomyopathy has been demonstrated to be closely linked to up-regulation of the angiotensin II receptor, interrupted function of the key intracellular Ca2+ regulatory/cycling proteins including SERCA2a, To causally relate these protein markers to change of cardiomyocyte contractile function in diabetes with or without STZ-diabetic rats given *Eugenia jambolana* extracts examined the expression of SERCA2a, and MHC in myocardium from control or STZ-induced and protein injected rats [Figure1]. Our results indicated that STZ treatment significantly up-regulated SERCA2a without affecting expression of SERCA2a. The SERCA2at was significantly lower in the STZ induced diabetic group. *Eugenia jambolana* extracts itself did not elicit any overt effect on the levels of intracellular Ca2+ cycling proteins [Figure 1]. Results revealed that STZ-induced diabetes triggers an α- to β-MHC isozyme switch, consistent with a previous report [23]. Interestingly, protein injection restored the low levels of β- MHC expression in STZ-treated diabetic myocardium and itself reduced β-MHC isozyme distribution under the nondiabetic condition. Diabetic cardiomyopathy has been demonstrated to be associated with enhanced ROS generation, oxidative stress and apoptotic cell death [24]. To verify the antioxidant effect of protein injected in diabetic hearts, ROS generation, oxidative stress status and apoptosis in myocardium or cardiomyocytes from control and diabetic rats were examined by DCF fluorescence. Results shown that STZ treatment enhanced ROS generation as expected, the antioxidant potential of plant extract effectively alleviated STZ-induced ROS generation, oxidative stress and apoptosis [Data not given]. The major findings of the present studies were the plant based antioxidant biomolecules act as scavenger to rescue STZ induced diabetic cardiomyocyte contractile and intracellular Ca2+ handling dysfunctions. Diabetes-induced cardiac contractile and intracellular Ca2+ defects were associated with enhanced ROS generation, oxidative stress and apoptosis, NADPH oxidase subunit and down-regulation of SERCA2a (both SERCA2a expression and SERCA2a-tophospholamban ratio) and Na+-Ca2+ exchanger. However, diabetes induced changes in intracellular Ca2+ cycling protein SERCA2a, our data suggest that STZ-induced diabetes may elicit cardiac contractile dysfunction and intracellular Ca2+ mishandling likely through enhanced oxidative stress and cell injury.
Figure 1: Western blot analysis

![Western blot analysis](image)

Legend: Western blot analysis exhibiting expression of SERCA2a and myosin heavy chain (MHC) isozyme (α-MHC, β-MHC) distribution in ventricles from control and (untreated) and injection of *Eugenia jambolana* extracted protein (Treated) rats. Represent gel blots depicting expression of above mentioned proteins using specific antibodies.

When this protein was injected in streptozotocin induced diabetes it has showed a compensatory effect by minimizing the cellular damage. To know whether it has any protective effect by inducing hypertrophy in Diabetic heart, we performed experiments by injecting plant protein in STD induced diabetic rats. *Eugenia jambolana* extracts showed compensatory mechanism in Myocarditis and diabetic heart and highly prevalent during adaptive phase, secreted in serum of remodeling heart of rats. Plant protein showed its specificity, adaptive role and targeting properties towards heart prompt to explore the possibility of using this protein as a vehicle to deliver genes into myocardium [Figure 2]. Cardiac α2M was crosslinked with pCMV-LacZ at 254 nm of UV light for 40 seconds and checked in 0.5% gel. The Standardized DNA-Protein Complex (DPC) was used for injection studies. Formation of DNA-Protein Complex (Cardiac α2M-PLL with GFP plasmid) Purified cardiac α2M was coupled with Poly (L) lysine (PLL) with an average chain length of 450 lysine by heterobifunctional crosslinker SPDP.

Figure 2: In vivo gene transfer into the myocardium Left ventricle

<table>
<thead>
<tr>
<th>Control</th>
<th>DNA</th>
<th>DPC</th>
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![Control](image) | ![DNA](image) | ![DPC](image) |

Legend: Direct injection protein as a vehicle to deliver genes into myocardium. Control, GFP Plasmid DNA, DNA-Protein Complex (DPC) were indicated its conjugated green fluorescence.
The cardiac α2M-PLL conjugate thus obtained were added in an increasing serial (2x) from 80ng to 20 μg to 0.2μg GFP plasmid DNA in HEPES buffered saline (150mM NaCl, 10 Mm HEPES, pH 7.5) in a final volume of 25 μl. After incubation for 30 min at room temperature with constant mixing, samples were analyzed for retardation of their electrophoretic mobility on a 0.8% agarose gel [Figure 3]. A human gene for insulin can be cloned into a bacterial plasmid and then put into bacteria in a process called bacterial transformation. Under the right conditions, these bacteria can then make human insulin, which can be purified and used to treat diabetes. In this lab you will be transforming bacteria with a plasmid vector containing the gene for Green Fluorescent Protein (GFP), which comes from bioluminescent jellyfish Aequorea victoria. The gene codes for a protein that causes the jellyfish to glow and fluoresce green in the dark or under ultraviolet light. The biotechnology company Bio-Rad has developed this plasmid vector, called pGLO (figure 1), into which the GFP gene is cloned. In addition to the GFP gene, pGLO contains the gene that encodes the enzyme for beta-lactamase, bla, which breaks down the antibiotic ampicillin. pGLO also includes a positive gene regulation system, which can be used to control expression of the green fluorescent protein in transformed cells.

**Figure 3: Gel mobility retardation analysis (0.7% agarose gel)**

Legend: Gel electrophoresis indicated the expression of the green fluorescent protein in transformed cells. Lane 1. DNA: Lane 2. UV-DPC in 30mM NaCl (50 μg of CA2M + 50 μg of DNA): Lane 3. UV-DPC in 30mM NaCl (25 μg of CA2M + 50 μg of DNA)
In Vivo gene delivery:
The rats were anesthetized with diethyl ether and DPC was injected intravenously through caudal vein. In controls buffer (PBS) was given. After 72 hours rats were sacrificed and their heart, kidney were harvested for further investigation [Figure 4]. Because the typical cardiac symptoms often are masked in patients with diabetes, the diagnosis of cardiac diseases are commonly missed or delayed [25]. In such diabetic patients, cardiac 2M may act as a novel diagnostic marker in identifying diabetic patients with cardiac problems and helpful in discriminating from those without cardiac ailments.

Figure 4: In vivo gene transfer into the myocardium intravenous injection via tail vein

<table>
<thead>
<tr>
<th>Control</th>
<th>DPC-RV</th>
<th>DPC-LV</th>
<th>DPC-Kidney</th>
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Legend: Figure 4 shows cardiac 2M may act as a novel diagnostic marker
1. Control: 2. DPC-Right ventricle: 3. DPC-left ventricle; 4. DPC-Kidney

Diabetic hearts had a reduced maximum inotropic effect to increased extracellular Ca$^{2+}$ under control aerobic perfusion conditions. The improved recovery of ventricular function during reperfusion of ischemic hearts from diabetic animals was highly correlated with reduced Ca$^{2+}$ uptake, and regression lines relating depressed ventricular function to Ca$^{2+}$ overload showed that data from control and diabetic hearts fell on the same line; that is, when depressed function occurred it was related to increased Ca$^{2+}$ uptake to the same extent in both control and diabetic hearts. The resistance to ischemia in diabetic hearts was not related to higher tissue levels of high energy phosphates during reperfusion nor to lactate accumulation during ischemia. The observations suggest a role of increased reperfusion Ca$^{2+}$ influx in ischemic damage and that alteration of sarcolemmal Ca$^{2+}$ transport systems in diabetic myocardium may account for the greater resistance of these hearts to ischemia. The expression SERCA2a and myosin heavy chain (MHC) isozyme ($\alpha$-MHC, $\beta$-MHC) distribution in myocytes was enhanced in response with an increased synthesis of receptor are apparent, but their quantities do not change with diabetes. *Eugenia jambolana* seed protein injected in diabetic as well as non diabetic animals showed cardioprotective effect without influencing glycemic control and dyslipidemia associated with STZ diabetes. Present study investigated the molecular mechanisms underlying improved cardiac function in the left ventricle of the heart from diabetic and treated with plant protein. There was an observed decrease in diabetic cardiac tissue triglyceride towards normal, possibly through improvement of the structure and stability of the mitochondria. Only a small number of changes in gene expression were detected after treatment using microarray technology and none were detected using real time-quantitative PCR.

Conclusion
The remodeling is usually understood as structural and functional deviations from the normal state that are caused by some pathological stimuli. The present observations of depressed ATPase of contractile proteins provide one explanation for the diminished contractile function of hearts of diabetic rats. It is unlikely that the altered ATPase activity totally explains the mechanical abnormality. The decreased rates of relaxation observed in the myocardium from
these animals indicate a change at the level of the sarcoplasmic reticulum have reported such an abnormality. Similarly, it is possible that sarcolemmal dysfunction might contribute to diminished function of diabetic hearts. Nevertheless, it was already demonstrated that not all remodeling-associated deviations from normal function are noxious. Some may belong to endogenous protective mechanisms and represent compensatory or even adaptation changes alleviating the effect of the given pathology. The plant based protein has compensatory process was more significant in terms of increase in the number of functional contact sites integrated gene expression may be summarized that the increase in the stabilization of transmembrane potential in heart which are associated with acute functional remodeling of these organelles in streptozotocin-induced diabetes participate in endogenous protective mechanisms alleviating the effect of the disease. Protein purified from *Eugenia jambolana* seeds extract has efficiency in a treatment of diabetes related cardiac problem, based on this information drug may be designed that would utilize the endogenous protective mechanisms in the myocardium.

**References**

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