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Molecular Approach to Repair Damage on Streptozotocin Induced Diabetic Rats

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Abstract

Streptozotocin- induced diabetic rats used as model to design drug for the treatment of diabetes and its related cardiac disease. The research was designed to investigate the differential expression of specific gene in diabetic rats treated with novel protein isolated from extract of *Eugenia jambolana* seeds used as trial therapeutic compound for drug development. Diabetes increases oxidant stress and doubles the risk of dying after myocardial infarction, but the mechanisms underlying increased mortality are unknown. Rats with streptozotocin-induced diabetes developed profound heart rate slowing and doubled mortality compared with controls after myocardial infarction Streptozotocin induces diabetic rat treated with Eugenia jambolana extracts had increased cell survival, maintained normal heart rates, and were resistant to diabetes-attributable mortality after myocardial infarction. Heart tissues from STZ induced diabetic rats were subjected to RNA extraction for gene expression by using real time RT-PCR. Three diabetic cardiac-specific genes of interest such as IGF-1, VEGF and ANG-1 were chosen and the expression level of these genes has examined and the abnormal expression of genes in STZ induced diabetic group would be rescued by the protein of *Eugenia jambolana* based therapy. Gene expression of VEGF, ANG-1 and IGF-1 was upregulated after administrated protein of *Eugenia jambolana* (EJ) evidenced by RT-PCR. Administration of protein of *Eugenia jambolana* to diabetic rats significantly enhances survival, proliferation, and the angiogenic ability to improved function in a diabetic animals confirmed plant protein might be useful for the management of the diabetic cardiomyopathy.

Keywords: *Eugenia jambolana*, Streptozotocin, diabetes, Gene expression, RT-PCR

Introduction

Diabetes mellitus (DM) is a chronic disease and major endocrine disorder caused by inherited and/or acquired deficiency in the production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. It is a growing health problem in most countries and its incidence is considered to be high (4%-5%) all over the world [1]. Chronic hyperglycemia causes complications linked to diabetes, such as heart disease, retinopathy, kidney disease and neuropathy. It is also a common cause of chronic morbidity and disability among the working population in the world [2]. Prospective studies have documented increased likelihood of sudden cardiac death and unrecognized myocardial infarctions in patients with diabetes [3]. Moreover, acute ischemic syndromes, peripheral arterial disease, and advanced cardiovascular disease (CVD) complications occur more commonly in patients with diabetes. It has been speculated that myocardial insulin resistance develops in animal models of both type 1 and type 2diabetes [4]. Various studies to date indicate multiple sites of impaired insulin signaling in various animal models; all the findings clearly support the existence of myocardial insulin resistance [5]. Biochemical processes such as glucose uptake, protein synthesis and glycogen synthesis have been stimulated in the presence of insulin which could be reduced in the cardiomyocytes of diabetic rats [6]. A single injection of STZ is widely used to generate a rat model of type I diabetes, which results from the selective toxicity of STZ towards the insulin-producing β-cells in pancreatic islets [7]. A number of factors influence the vascular dysfunction that develops in this model, such as the age of the rats, the dose of STZ administered and the duration and severity of hyperglycaemia [8].

Diabetic cardiomyopathy was associated with diastolic and systolic impairments, leading to endothelial dysfunction, alterations in glucose metabolism, increased fatty acid oxidation, and generation of free radicals [9]. Endothelial dysfunction significantly alters angiogenesis in a diabetic heart because of impaired toxic oxide production [10] that further contributes to the etiology of the disease. The management of diabetes without any side effects has still a challenge to the medical field there was growing interest in the use of natural health products as an alternative approach to current medications. Plant sources has become a target to explore new drugs and in searching biologically active compounds [11].

The streptozotocin-induced Diabetes mellitus in rats could be used as animal model to study the molecular mechanism behind the development of diabetic related cardiac diseases. The present study was designed to investigate the differential expression of specific gene precondition with novel protein from extract of *Eugenia jambolana* seed kernel and in the protective role of protein at molecular level, which may be efficiently used to design a drug for the treatment of streptozotocin-induced diabetic rat model.

Materials and Methods

Induction of diabetes

Diabetes was induced in wistar albino male rats in the weight range of 110-120g by a single intraperitoneal injection of 60 mg/kg streptozotocin(STZ, Sigma Aldrich, Tokyo, Japan), dissolved in 10 mM citrate buffer (pH 4.5).Control rats were treated with the same volume of citrate buffer. Rats were fasted 5 hours prior to injection. Diabetes was confirmed at 2 weeks after STZ injection by measuring the glucose concentration of peripheral blood obtained from the tail vein using NIPRO Free Style blood glucose monitoring system (Nipro Corporation, Osaka, Japan). Afterwards the blood glucose levels and body weight of rats were monitored weekly. The rats having plasma glucose levels more than 300 mg/dl were classified as diabetic and used for the present studies. Rats were sacrificed, coronary arteries were perfused with saline, and the heart was excised and weighed, rinsed in ice cold saline and then homogenized in Tris–HCl buffer (pH 7.4). The tissue homogenates were used for the further experiments. All protocols were performed in accordance with the Institutional Animal Ethical Committee (IAEC) as per the directions of the CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals). 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 Alagarkoill 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 [12].

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 4 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 d. At the end of the experimental period, the rats were

anaesthetized and sacrificed by cervical dislocation. The heart tissues were taken for further experiments.

Biochemical analysis

On 4th week blood samples were collected from retro-orbital plexus under light ether anesthesia and centrifuged at 2500 rpm for 20 minutes to separate serum. Blood glucose was estimated by the oxidase/peroxidase method [13] Glycosylated hemoglobin was estimated using the diagnostic kit from Biosystems, Spain [14].

Extraction of total RNA

The heart tissue was homogenized with RNA ZOL (2ml per 100mg tissue) with few strokes in a glass Telflon homogenizer. The use of guanidium to lyze cells was originally developed to allow purification of RNA from cells rich in endogenous ribonuclease. Guanidium denatures protein and thus inactivates any ribonucleases were present. RNA Extraction was carried out 0.2ml Chloroform was added to 2ml of tissue homogenate and the samples were tightly closed and shaken vigorously for 15 seconds and let them stay on ice for 15 minutes. The suspension was centrifuged at 12,000rpm (-4ºC) for 15 minutes. RNA precipitation step was continued transfer of the aqueous phase to a fresh tube, added an equal volume of iso-propanol and stored the samples for 45 minutes at -20ºC. Samples were centrifuged for 15 minutes at 12,000rpm (-4ºC). RNA precipitate (often invisible before centrifugation) formed a white pellet at the bottom of the tube. The supernatant was removed and washed the RNA pellets twice with ice cold 75% ethanol by vortexing and subsequent centrifugation for 8 minutes at 12,000rpm (-4ºC) and the pellets were dried under vacuum for 10-15 minutes. The RNA pellet was dissolved in 1mM EDTA, pH 7.0. Diethyl pyrocarbonate (DEPC) treated and RNase free solutions should be used for RNA solubilization. The final preparation was free from DNA and protein and pure RNA samples checked by measuring its OD at 260nm. The preparation was ready for dot blot hybridization gel electrophoresis to detect specific mRNA by Northern blotting. RNA blot RNA was determined by hybridizing the membrane to a specific labeled DNA probe [15]. The RNA ZOL method can be completed within 3 hours providing both high yield and purity of RNA preparation. RNA isolated with the use of RNA ZOL is non-degraded, free of DNA and protein and contains the whole spectrum of RNA, molecules, including small RNAs.

Real Time Reverse Transcriptase-PCR (RT-PCR) The assay performs using a one-step RT-PCR kit (Qiagen, Germany). A set of primer were designed for real-time RT-PCR assay. The sequences (5′ to 3′) for the primer pairs and their product lengths (bp) are mentioned below:

IGF-1(f) AGGCTATGGCTCCAGCATTC
IGF-1(r) AGTCTTGGGCATGTCAGTGTC
ANG1(f) GACACCTTGAAGGAGGAGAAAG

ANG1(r) GTGTCCATGAGCTCCAGTTGT
VEGF(f) ACCCCGACGAGATAGAGTACAT
VEGF(r) CTTC TAATGCCCCTCCTTGT

The reaction contained 5 µl of RNA, 0.6µM of each primer, 0.2 µM of the Taq Man probe. Real time-PCR machine was used with the following thermal steps: reverse transcription 30 min, initial denaturation, followed by 40 cycles of denaturation, annealing, and extension (RT-PCR kit Qiagen,Germany).Gel Electrophoresis was carried out for the assessment of PCR products using Agilent Bionalayzer system. The PCR product was visualized as a single compact band with expected size (IGF-1:166bp; ANG1:143bp; VEGF: 200bp).

Results and Discussion

In diabetic condition, the excess of glucose present in the blood reacts with hemoglobin to form glycated hemoglobin, which has altered affinity for oxygen and this may be a factor in tissue anoxia. Glycated hemoglobin was significantly increased in diabetic rats, and this increase was directly proportional to fasting blood glucose **[Figure1]**. There was increasing evidence that advanced glycation endproducts (AGEs) play a pivotal role in atherosclerosis, in particular in diabetes. **Figure 2** revealed AGE accumulation is a measure of cumulative metabolic and oxidative stress and also increased AGE accumulation was closely related to the development of cardiovascular complications in diabetes [16]. The receptor for advanced glycation end products (RAGE) was a cell surface receptor whose signaling pathway has been implicated in atherogenesis. Hemoglobin was highly susceptible to non-enzymatic glycation, Glycated hemoglobin was an effective means to screen for diabetes. The decreased level of total hemoglobin in diabetic rats is mainly due to the increased formation of glycosylated hemoglobin (HbA1c). HbA1c was found to increase in patients with diabetes mellitus and the amount of increase is directly proportional to the fasting blood glucose level. During diabetes, the excess glucose present in the blood reacts with hemoglobin to form HbA1c) HbA1c was used as a marker for estimating the degree of protein glycation in diabetes. AGE determine the acceleration of the cross linkage process of collagen, leading to the formation of links throughout the entire collagen molecule, as opposed to the more limited terminal positions for normal cross linking [17]. Diabetes mellitus can produce a stiff myocardium before the development of myocardial fibrosis. The stiff myocardium in the early stages of the development of the cardiomyopathy of diabetes mellitus was not a consequence of an increase in ventricular resistance after load and in these circumstances was associated with the formation of collagen AGEs. Data shows [18] that glucose is not only the main energy source for short periods, but also the major sources of diabetes mellitus complications, mainly by forming oxidative and proinflammatory advanced glycation end products (AGE). Further studies were needed to identify the gene expression behind the injection of active principles of the plant extract.

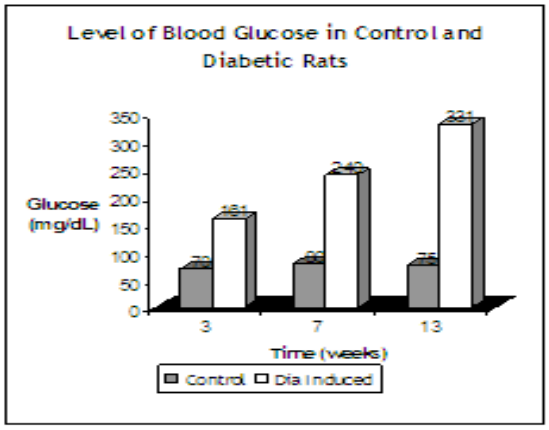

Figure1: Analysis of Blood Glucose in STZ induced diabetic Legend.

Representation of data on level of blood glucose at different time intervals after diabetic induction in experimental animals. X - Indicates experimental period in weeks; Y – Indicates blood glucose level.

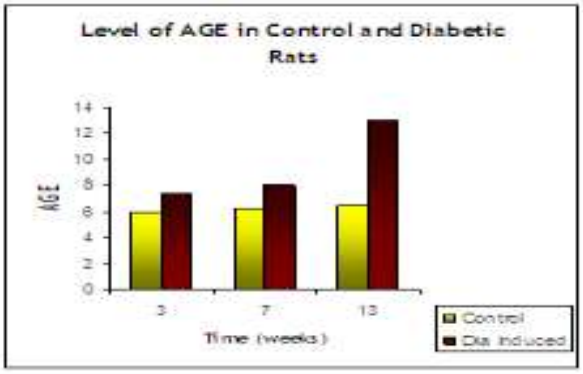

Figure 2: Estimation of advanced glycation endproducts in STZ induced diabetic.

Representation of data on level of AGE at different time intervals after diabetic induction in experimental animals. X - Indicates experimental period in weeks; Y – Indicates AGE level.

Administration of Protein purified from *Eugenia jambolana* seeds extract to STZ diabetic rats significantly increased the level of hemoglobin and significantly decreased the levels of glycated hemoglobin. Glycated hemoglobin (HbA1c) was a diagnostic marker of chronic hyperglycemia. The association of AGEs with chronic hyperglycemia has attracted a great deal of interest into the possible role of AGEs in diabetic complications. There are several potential ways that AGE-modified proteins could be damaging; the formation of AGEs may alter protein function disrupt extracellular matrix [19]. However it now appears that the predominant vascular effect of AGEs occurs through their interaction with RAGE (receptor for AGE) found on macrophages and endothelial dysfunction.

Gene-expression analysis of dmMSCs preconditioned with media from cardiomyocytes subjected to treatment with different concentrations of VEGF, IGF-1, & ANG1. STZ-induced diabetes in animals could

be used as a model for diabetic cardiomyopathy which might provide a novel insight into the prevention and treatment of this pathological process. Gene expression can be assessed by measuring the quantity of the final product for instance, the protein measuring RNA level was chosen because it was more efficient with the current technologies and easy access of information. Molecular Studies can be assessed by measuring the quantity gene expression of the final product of protein. However, measuring RNA level was critically important to have a good quality of RNA in **Figure 3**. RNA assessment was carried out to ensure the reliability of the RNA samples isolation technique yields good quality of RNA. The intact total RNA sample has shown distinct 18S and 28S subunit spikes, with 2:1 ratio (28:18S). In studies of target gene expression by RT-PCR, the use of internal reference genes were required to control for RNA quality, reverse transcription efficiency and overall transcriptional activity in samples preferably internal standards should be constitutively expressed by all cell types independent of experimental conditions and they should not be affected by any disease and to make sure the amplified product within the expected size of gene profiling analysis on STZ induce diabetic rat similar observation by [20] demonstrated many changes occurred in their gene expression of specific genes were chosen to analysis the hypoglycaemic effect of the extract treatment with protein from EJ might showed significant changes in rescue the abnormal expression of genes in STZ induced diabetic rats. Etiologically, the main cause responsible for the development of heart dysfunction was sustained hyperglycaemia which promotes the formation of advanced glycation end products [21]. Protein purified from *Eugenia jambolana* seeds extract could be used as a formulating drug in discriminating diabetic animals.

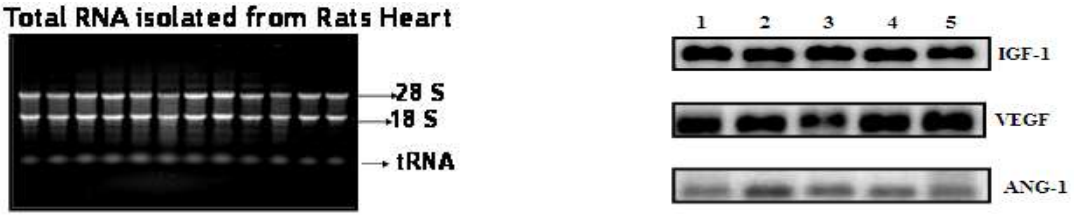

Figure 3: Gene expression in diabetic rat. Gene-expression analysis of diabetic rats pretreated with protein from *Eugenia jambolana* and cardiac tissues subjected to treatment in the presence of different concentrations of IGF-1:166bp; VEGF: 200bp ANG1:143bp. Lane 1 -5 indicates various concentrations of 0.06ng/ml, 0.07ng/ml, 0.08ng/ml, 0.09ng/ml and 0.1ng/ml IGF-1, VEGF & ANG1.

Among the treatment groups, VEGF concentration resulted in significantly higher levels of the angiogenic markers and cardiac transcription factors compared with other IGF-

1 and ANG-1 expressions in the experimental animal groups. Gene expressions were compared in **Figure 1** revealed that IGF-1, VEGF, ANG1 concentrations and significant expression in diabetic treated group and confirmation of VEGF plays an important role in remodelation of diabetic heart. Gene-expression analysis of angiogenic and cardiac markers was done for all groups of protein from *Eugenia jambolana* treated, whereas nontreated groups were used as controls. Elevated levels of VEGF, ANG-1 (angiogenic markers) were observed in protein injected groups compared with all other non treatment groups. Among the VEGF treatment groups, 0.09 ng/ml and 0.1ng/ml VEGF concentration resulted in significantly higher levels of the same angiogenic markers. Significant upregulation of angiogenic markers was observed in the protein treated group compared with VEGF in nontreated evidenced by gene-expression analysis.

Figure 4 explained the gene expression after protein treatment had an increased level on the left ventricular tissue when compared with other such as left atrium, right ventricle and right atrium. Accumulation of ROS in right ventricle tissues was equivalent to that in right ventricle in STZ-induced rats, but RA-right atrium had significantly less expression compared with STZ treated controls in right atrial tissues. An increased plasma level of soluble vascular adhesion molecule (sVCAM)-1 acts as a marker of endothelial dysfunction and increased risk of atherosclerosis.

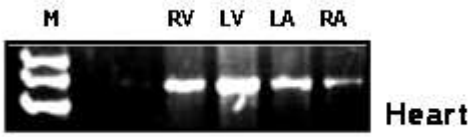

Figure 4: RT- PCR to identify the reporter gene expression in protein injected rats. Figure showed the expression of reporter gene in various tissues of heart analyzed by RT- PCR in protein injected STZ induced diabetic rats. M- Marker, RV- right ventricle, LV- left ventricle, LA- left atrium, RA-right atrium.

The present observation has suggested that protein injected in STZ induced diabetic rats reduces myocardial infarction in diabetic rats. Recent studies have suggested that prevalence of type 2 diabetes was rapidly increasing with heart failure of myocardial infarction or ischemic origin was more frequent and severe in patients with diabetes [22]. Diabetes was an independent risk factor for cardiac failure although its detrimental impact on the myocardium remains to be identified. The significant amount of myocytes loss in this model of non insulin dependent diabetes mellitus was consistent with a greater vulnerability of the diabetic heart to cardiac processes.

Development of diabetic cardiomyopathy was associated with decreased diastolic compliance, increased interstitial fibrosis, and myocyte hypertrophy. Accrual of reactive

oxygen species (ROS) in a diabetic heart leads to endothelium dysfunction characterized by vascular remodeling, disappearance of capillary endothelium, and altered gene and protein expression in endothelial cells [23]. Cell-based therapies using protein from *Eugenia jambolana* have been successfully used for treatment of the damaged heart adoptive transfer of protein in a rat model of diabetic cardiomyopathy was associated with enhanced angiogenesis, myogenesis, and cardiac function and the success of plant extract therapy for diabetic heart repair has been accompanied by reports of streptozotocin-induced diabetic rats have impaired abilities for proliferation, paracrine, antiapoptosis, and myogenic differentiation [24]. Therefore, increased paracrine factors (IGF-1, were found in the hearts of animals streptozotocin-induced diabetic rats supporting the idea that treated with protein have better survival, angiogenic ability, and could improve heart function by augmentation of a diabetic environment, possibly through a paracrine-mediated effect. Accordingly, it can be concluded that protein from *Eugenia jambolana* regenerate the damaged endocrine pancreas and thereby stimulation of insulin secretion in cells as revealed by insulin and C-peptide assays. Results represented an attractive option for plant-based therapy to repair diabetic heart failure. The continued identification and investigation of existing and novel pathways linking hyperglycemia and diabetes mellitus to atherosclerosis was important to the development of new and effective antiatherosclerotic therapies that are tailored to individuals with diabetes [25]. A great deal of research has been focused upon the role of hyperglycemia in the development and progression of atherosclerosis in cell culture and animal model systems, the AGE–RAGE interaction show potential and were actively being evaluated as targets for putative antiatherogenic therapies. However, all interventions targeting the effects of hyperglycemia, including direct glucose lowering, appear to show greater effect in the treatment of microvascular complications than in the control of macrovascular disease.

Conclusion

In the present study, an increase in the levels of serum glucose and HbA1c in STZ- treated rats confirmed the induction of diabetes mellitus. Significant was observed in the glucose and HbA1c level in diabetic rats after treatment with protein from *Eugenia jambolana* when compared with non treated rats at the end of experimental period. *Eugenia jambolana* extract could protect the heart from cardiomyopathy associated with STZ-induced diabetes. In summary plant has bioactive compound appear to have cardioprotective beneficial health effects in experimental models, Much scientific research needs to be conducted before we can begin to make science-based plant therapy recommended, Gene expression of angiogenic and cardiac markers was significantly upregulated in IGF-1 in addition reduced fibrosis, apoptosis, and increased angiogenesis were observed in diabetic hearts 4 weeks after administration of protein of *Eugenia jambolana* to diabetic rats

significantly enhances survival, proliferation, and the angiogenic ability of diabetic rats compared to untreated diabetic rats revealed the anti-diabetic properties of *Eugenia jambolana* proved its function in a diabetic animals confirmed treatment with EJ extract may show significant changes in rescue the abnormal expression of genes in STZ induced diabetic rat. The protein from *Eugenia jambolana* treated diabetic rats exhibited reduction in necrosis with less fragmentation of fibres as compared to diabetic control groups, which reflects the cardio protective effect of plant bioactive compound. This study concluded that Protein from EJ seeds at 100 mg/kg may show reduce experimentally induced myocardial infarction in diabetic rats.

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