G-CSF Has a Therapeutic Effect on Cardiomyopathy in Diabetic Rats

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Introduction

The major cause of morbidity and mortality in diabetes mellitus is cardiovascular disease. Coronary atherosclerosis and cardiomyopathy occur as a result of metabolic abnormalities and hyperglycemia associated with diabetes [1]. An abnormal increase in blood glucose was found to be related with cardiomyocyte to death by apoptosis. Apoptosis, result of myocardial abnormalities might be an important cause of cardiomyopathy [2]. Consequences of such abnormalities occur in diabetes and the contractile function of the heart is ultimately altered. The electro-cardiogram of many diabetes patients show several alterations from normal patterns. Increase of QT interval duration is one of them. Increase of QT interval reflects the abnormalities of ventricular myocardial re polarization. Prolongation of QT interval has been associated with sudden death in diabetes patients [3-4].

It was found that impaired fasting glucose and acute hyperglycemia was associated with QT prolongation. Also QT prolongation was found to be associated with hypertension and left ventricular hypertrophy, these conditions are related with cardiovascular diseases in diabetes mellitus [5].

Granulocyte colony stimulating factor (G-CSF) is a glycoprotein and a member of hematopoietic growth factor family which is capable of mobilizing bone marrow derived hematopoietic stem cells into the blood stream [6]. Recent years it's been involved in investigating different treatment techniques for varied of diseases as a novel regenerative strategy. It's been used in treatment of various diseases due to its role on multiplication of hemapoietic stem cells. In our previous study, we demonstrated that G-CSF has nephro-protective effect in diabetic rats [7]. This evidence is important in nephropathy which is one of the most frequent complications seen in advanced diabetes. Cardiomyopathy is another most common complication of diabetes. Increased hematopoietic stem cells induced by G-CSF could contribute to the tissue healing process. Our aim in this study is, inspired by our previous study, investigate the possible effects of G-CSF on QT-prolongation in diabetic rats and shed light on whether G-CSF could have a protective effect from cardiomyopathy.

Material and Methods

Animals
21 male Sprague Dawley albino mature rats were used in this study. Rats were 8 weeks old and weighing 200-220 g. All animals were fed ad libitum and housed in pairs in steel cages having a temperature-controlled environment (22 ± 2°C) with 12-h light/dark cycles. The protocol of this study was approved by the Committee for Animal Research of Ege University. All animal studies are strictly conformed to the animal experiment guidelines of the Committee for Human Care.

Experimental Protocol

Diabetes was induced by intraperitoneal (i.p.) injection of streptozocin (STZ, Sigma-Aldrich, Inc.; Saint Louis, MO, USA) (60 mg/kg in 0.9% NaCl, adjusted to a pH 4.0 with 0.2M sodium citrate) for 14 rats. Control group didn’t receive any drugs (n=7), (control group). Diabetes was verified after 24 hours by evaluating blood glucose levels with the help of glucose oxidase reagent strips (Boehringer- Mannheim, Indianapolis). The rats with blood glucose levels of 250 mg/dl and higher were included in the study. 14 diabetic rats were randomly divided into 2 groups; diabetes group was treated with 1 mL/kg saline (Diabetes) (n=7), and diabetes+ G-CSF group was treated with 100 µg/kg/day G-CSF (Neupogen*, 48 MIU/0.5 ML) (Diabetes +G-CSF) (n=7) by i.p. injection for four weeks.

At the end of this four week, the animals were euthanized and electrocardiography (ECG) was taken as derivation I, blood samples were collected by cardiac puncture for blood glucose and removal of the heart were performed for histopathological examination.

Histopathological Examination of Heart Tissue

For histological and immunohistochemical studies, all animals were anesthetized by an i.p. injection of 40 mg/kg ketamin

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(Alfamine®, Ege Vet, Alfasan International B.V., Holland)/4 mg/kg xylazine (Alfazyne®, Ege Vet, Alfasan International B.V., Holland) and perfused with 200 ml of 4% formaldehyde in 0.1 M phosphate-buffer saline (PBS). Formalin-fixed heart sections (5μm) were stained with hematoxylin and eosin (H&E). All sections were photographed with Olympus C-5050 digital camera mounted on Olympus BX51 microscope.

Morphological analysis was assessed by computerized image analysis system. The degree of heart muscle cell hypertrophy were examined by light microscopy. Thickness of muscle cells was calculated from the cross-sectional image. The cross section yielding the maximum diameter of the muscle fiber was photographed and converted into a digital image by an examiner blinded to the source of the tissue. Muscle fibers were measured with the image analysis software Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA. Fifty cardiac muscle cell from each animal were examined, and the average was used for analysis.

**TGF-β1, CD-34, Akt Immuno Expression**

For immunohistochemistry, sections were incubated with H₂O₂ (10%) for 30 min to eliminate endogenous peroxidase activity and blocked with 10% normal goat serum (Invitrogen) for 1 hour at room temperature. Subsequently, sections were incubated in primary antibodies (TGF-β1, CD-34, Akt, Bioss, Inc.; 1/100) for 24 h at 4°C. Antibody detection was performed with the Histostain-Plus Bulk kit (Bioss, Inc) against rabbit IgG, and 3,3’ diaminobenzidine (DAB) was used to visualise the final product. All sections were washed in PBS and photographed with an Olympus C-5050 digital camera mounted on Olympus BX51 microscope. Brown cytoplasmic staining was scored positive for immunoexpression. The number of immunoexpression positive cell was assessed by systematically scoring at least fifty cardiac muscle cell per field in 10 fields of tissue sections at a magnification of 100x.

**Assessment of Cardiac Autonomic Neuropathy**

QT interval and T wave duration was recorded electrocardiographically (Derivation I). While the length of the QT interval is rate dependent, the QT analysis was realized on the transformed parameter QTc according to Bazett's formula:

$$QTc = \frac{QT \text{ (in milliseconds)}}{\sqrt{R - R_{\text{interval}}} \text{ (in seconds) }}$$

**Statistical Analysis**

All quantitative data were analyzed by using non-parametric (Mann-Whitney U) test. Student’s-t test was used to evaluate the differences between the groups. Data are presented as mean values ± standard error of the mean (SEM). p values of 0.05 or less were regarded as statistically significant.

**Results**

In diabetic rats, cardiac muscle cell thickness (hypertrophy), TGF-β1 expression, QT interval, T wave duration and blood glucose levels were increased significantly when compared to control group (p<0.05). Administration of G-CSF in diabetic rats causes a significant reduction both in cardiac muscle cell thickness, TGF-β1 expression and QT interval and T wave duration in these rats (p<0.05). In terms of CD 34+ and Akt expression, there was no difference between control and diabetic rats (p>0.05). Akt expression increased significantly in G-CSF administrated diabetic groups when compared to diabetic rats (p<0.05). CD 34+ expression was shown no difference between diabetic rats and G-CSF administered diabetic rats (p>0.05). Briefly, G-CSF administrated diabetic rats there was a decrease in cardiac muscle cell thickness and TGF-β1 expressions; Akt expressions were increased significantly when compared to diabetic rats (p<0.05). CD 34+ expression showed no difference between G-CSF administrated groups and diabetic rats (p>0.05).
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Discussion

In this study we showed the beneficial effects of G-CSF on both cardiac muscle and ECG on diabetic rats. Diabetes mellitus is a common disease affects many organs in the body adversely and the heart is one of them. Cardiomyopathy is one of the consequences of advanced diabetes, Especially “death in bed” syndrome in diabetes patients has been suggested to be caused by hypoglycemia. It was hypothesized that fatal cardiac arrhythmia has been triggered by hypoglycemia [8], researchers have found that hypoglycemia is associated with prolongation of QT interval and this is related to increased risk of sudden cardiac death [9]. QT interval prolongation reflects the abnormality in ventricular myocardial re polarization [10]. In summary, difficulty in re polarization which is shown in ECG as QT interval prolongation and very common in diabetic patients cause arrhythmia and this situation triggers sudden death in diabetes mellitus.

There is plenty of data in literature demonstrating the connection between QT interval prolongation an diabetes. Jankyova et al searched out that diabetic rats have prolonged QT interval [11]. In two different studies Veglio et al found the relationship between QT prolongation and increased risk of death in diabetes mellitus [12-13]. We hypothesized that if we could fix QT interval prolongation, sudden deaths could be decrease among diabetes patients.

We administered G-CSF to the diabetic rats to carry out the effects of G-CSF on QT interval and other important factors as TGF-β1,
Akt and CD 34+ cells. We chose G-CSF due to our previous studies. In our latest study we demonstrated the protective effect of G-CSF on kidneys in diabetic rats. G-CSF is a glycoprotein and a member of growth factor family. G-CSF mobilizes hematopoietic stem cells from bone marrow into the blood stream [14]. In our study we found that G-CSF causes a significant reduction both in cardiac muscle cell thickness, QT interval and T wave duration in diabetic rats. Among diabetic patients besides QT interval duration increase in T wave duration and cardiac muscle cell thickness are other risk factors for cardiomyopathy and sudden death. Since T wave in ECG reflects the re polarization in diastole, increase in T wave duration demonstrates the difficulty in re polarization and resting phase of heart in diabetics. Hypertrophy is one of the risk factors for coronary failure [15-16]. We found that diabetic rats cardiac muscle cell thickness (hypertrophy) was increased when compared to control group. Administration of G-CSF decreased the cardiac muscle cell thickness in these rats.

G-CSF has been involved in diabetic and cardiovascular studies recently. Protective effects of G-CSF was demonstrated on acute myocardial infarction (G-CSF- MI). In another study, Shin et al studied on OLETF rats and they demonstrated that G-CSF might have a cardio-protective effect in diabetic cardiomyopathy. Our data is parallel to other findings in literature. We found that G-CSF administered diabetic rats QT interval was decreased than regular diabetic rats.

To find out the mechanism of G-CSF’s therapeutic cardiac effects on diabetic rats, we studied, CD34+, TGF-β1 and Akt expression levels in these rats. CD34+ cells are bone marrow derived immature cells in blood stream. It’s been reported that circulating CD34+ cell levels are associated with cerebral infarction [17]. Makino et al found that increased CD 34+ levels in circulation might have anti-atherosclerotic effects [18]. Akt (Protein kinase B) is a molecule activated downstream of the PI3K(Phosphoinositide-3-OH kinase) signaling pathway [19]. Akt includes in regulation of cellular processes as cell survival, growth and proliferation and metabolism as well [20]. Akt also regulates the glucose uptake into tissues as a response to insulin. Akt deregulation has been reported in diabetes [21]. Studies on animal models demonstrated that Akt is an important molecule in regulating cardiovascular function and plays a role in pathologic cardiac hypertrophy [22].

In our study there were no differences between control and diabetic rats in terms of CD 34+ and Akt expressions. After G-CSF administration Akt expression increased significantly in diabetic rats. Previous data showed that transforming growth factor beta-1 (TGF-β1) is a fibrogenic and an inflammatory cytokine that plays role in nephropathy in diabetes [22]. Also it has been demonstrated that Because TGF-β1 is a pivotal mediator of matrix accumulation it plays a key role in fibrosis in the heart [23]. According to the study of Yu et al down regulation of TGF-β1 in myocardial tissue might be protective against cardiomyopathy [24]. We found over-expression of TGF-β1 in diabetic rats. TGF-β1 expression significantly decreased in diabetic rats after G-CSF administration.

Our results show that G-CSF prevents diabetic rats from cardiomyopathy via increasing Akt and TGF-β1 expression but not via increment of CD 34+ cells.

In diabetic rats, cardiac muscle cell thickness (hypertrophy), TGF-β1 expression, QT interval and T wave duration were increased significantly when compared to control group. Administration of G-CSF in diabetic rats causes a significant reduction both in cardiac muscle cell thickness, TGF-β1 expression and QT interval and T wave duration in these rats. In terms of CD 34+ and Akt expression, there was no difference between control and diabetic rats. Akt expression increased significantly in G-CSF administered diabetic groups when compared to diabetic rats. CD 34+ expression there was no difference between control and diabetic rats.

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