Diabetes mellitus (DM), a condition of chronic hyperglycemia, represents one of the greatest concerns to modern global health. DM is a well-recognized cause of cardiomyopathy and left ventricular dysfunction. It is accompanied by impaired antioxidant defenses including nitric oxide (NO). Aim of work was to; (1) evaluate the microscopic changes in the heart of diabetic rats, (2) study the possible reversibility of these diabetic-induced cardiac changes by insulin glargine. Forty male albino rats were divided into; control group, non treated diabetic group and diabetic group treated with insulin glargine. DM was induced by intra-peritoneal alloxan injection. The experiment continued for six weeks. Heart tissues were prepared and stained with H&E, PAS and immunostained against e NOS. Heart tissue of diabetic rats revealed myocardial degeneration, disarray of myocardial fibers, mononuclear cellular infiltration; decreased e NOS immunoexpression in cardiomyocytes as well as endothelial cells and smooth muscles of cardiac blood vessels. Administration of insulin glargine improved these diabetic changes to great extent and increased e NOS immunoexpression. Insulin has to be given as early as possible for insulin dependent diabetics to control diabetic cardiac complications. NO is important for cardiac functions. Insulin has a role in cardiac e NOS expression.

**Key words:** Diabetes, rats, heart, insulin glargine, e NOS

**INTRODUCTION**

Diabetes mellitus (DM) has long been identified as a leading cause for the development of ischemic heart disease within the diabetic population. Many epidemiological findings support the fact that cardiovascular problems are the primary cause of morbidity and mortality among diabetics (Tziakas et al., 2005, Cerghizan et al., 2007 and James, 2009). The diabetic state is associated with increased oxidative stress and hyperglycemia that stimulates the production of advanced glycosylated end products which subsequently increase microvascular permeability. Moreover, abnormal lipid metabolism increases oxidized low-density lipoprotein formation resulting in oxidative stress that exacerbates myocardial and vascular endothelial cell insult (Donahoe et al., 2007). The pathogenesis of diabetic vascular impairment is caused by endothelial dysfunction. The endothelium adapts to local environment by releasing large number of endogenous substances that regulate vascular tone and
cause vasodilatation. Among these substances nitric oxide (NO) is the most important (Felco et al., 2001).

Nitric oxide is an important regulatory factor for the cardiovascular system mediating endothelium-dependent vasodilatation and modulating different facets of cardiac functions including heart rate, contraction, relaxation, cell growth and survival. That is why eNOS expression was identified within various cardiac cell types; cardiac muscles, the endocardial cells, endothelial cells, and the conducting system of the sinoatrial and atrioventricular nodes (Maison et al., 2003). NO is synthesized from L-arginine by NO synthases (NOS), a family of isoenzymes with characteristic functional and regulatory properties. There are three NOS isoforms; the neural isoform (nNOS), the inducible isoform (iNOS), and endothelial isoform (eNOS). The eNOS, the most predominant vascular NO synthase isoform, is responsible for the majority of NO production in the vasculature (Zhao et al., 2008). Regulation of NO metabolism is particularly important in DM because activation of NOS is under insulin control through the phosphatidylinositol 3-kinase/protein kinase B (AKT) pathway. Thus, disturbances of NO generation may be a consequence of insulin deficiency and/or resistance affecting the vascular response. An impaired NO metabolism is found in diabetes (Tessari et al., 2010).

Under basal conditions endogenous eNOS exerts a positive inotropic effect while its increase reduces βadrenergic responsiveness (Muller, 2005).

Insulin glargine (lantus) is a new human insulin analogue with an activity that results in a relatively constant concentration/time profile over 24 h with no pronounced peak, rendering it ideal basal insulin for the treatment of DM. It is increasingly recognized to provide good glycemic control and to reduce the risk of hypoglycemia in type 1 diabetes (Hofmann et al., 2002; Rosenstock et al., 2005; Gerstein and Yusuf, 2012).

As DM is associated with decreased NO bioavailability (Masha et al., 2011) so, this study was designed to evaluate: 1) cardiac changes in diabetic heart of male albino rats, 2) the reversibility of these diabetic changes by insulin glargine.

MATERIALS AND METHODS

Chemicals used

Alloxan monohydrate (Sigma, St. Louis) used in a dose of 125 mg/kg BW. Alloxan solution 5% in normal saline was prepared fresh just before use (Trivedi et al., 2004).

- Insulin glargine (lantus, Sanofi Oventis) used in a dose of 5 IU/kg BW daily for six weeks (Hofmann et al., 2002)
- Rabbit anti-human nitric oxide synthase-endothelial (eNOS) polyclonal antibody (Spring bioscience, catalog E4110)

Animals used

Forty adult male albino rats weighing 150-250 g were used in this study. The rats were provided by Dubai Medical College, Animal House. All rats were housed at room temperature and allowed free water access and fed ad libitum. They were randomly divided into:

- Group I (control group): 10 rats received normal saline in amounts similar to alloxan treated rats.
- Group II (Non treated diabetic group): 15 rats were fasted for 18 hours but allowed free access to water. Diabetes was induced by intra-peritoneal injection, of each rat, with a single dose (125 mg/kg BW) of alloxan monohydrate 5% (dissolved in normal saline) (Trivedi et al., 2004). Thirty minutes after alloxan administration, food and water were offered to animals. None of the rats showed any abnormality in blood glucose level before alloxan injection. The presence of diabetes was confirmed by measuring the fasting blood glucose level. In order to assess chemically establish diabetic condition; an incision was done in one of tail veins using 15 scalpel blade. A blood sample was collected on a reagent strip three days after the induction of diabetes to determine the blood glucose level using a portable glucose analyzer (Hassan and Abdel Moneium 2001). Blood glucose level for each rat was measured before induction of diabetes, three days after induction; at weekly interval (to ensure the stability of diabetic status) and immediately before being sacrificed. Rats were considered diabetic when their fasting blood glucose level exceeded 200 mg/100 ml (Carvalho et al., 2003).

- Group III (Diabetic treated group): This group included 15 diabetic rats (induction of diabetes was done as before). These rats received lantus subcutaneously for six weeks in a daily dose of 5 IU/kg BW (Hofmann et al., 2002). Treatment of diabetic rats started from the first day of diabetes induction.

Methods

At the end of the experiment (6 weeks), the rats were sacrificed and the heart of all animals was excised and processed for paraffin blocks. Sections of 5µm thickness were cut and stained with:

- Hematoxylin and eosin (Bancroft and Gamble, 2007): to study the histological changes of the heart tissues.
- Periodic acid Schiff (PAS), (Bancroft and Gamble, 2007): to assess the glycoprotein content of the heart tissues.
- Immunohistochemical staining using eNOS primary antibody (Felco et al., 2001): To examine the localization of eNOS in the cardiac tissue.
- Morphometric measurements: PAS stained sections and positively eNOS immunostained sections of the cardiac specimens from different groups were
used to measure the area percent. From each section, ten randomly chosen fields were captured separately at a magnification of X 100 in a standard frame of 118476.6µm². The area % represented the percentage of the positive areas, which were masked by a binary color to the area of the standard measuring frame was recorded.

- **Statistical analysis:** The collected data was statistically analyzed using the SPSS version 18. All data were reported as mean ± standard deviation (SD). To evaluate the significant differences, the comparison of means between each group with the control was done by computer program for student- t test. Differences were considered to be statistically significant if p ≤ 0.05.

**RESULTS**

- **H&E stained sections**

Control cardiac muscles appeared branching and anastomosing multicellular fibers. The cells were connected end to end by intercalated discs. Cardiomyocytes revealed central oval vesicular nuclei and acidophilic sarcoplasm (Plate 1: A). Alloxan–induced diabetes caused degeneration and apoptosis of some cardiocytes with loss of myofibrils and disarray of muscle fibers. The nuclei of degenerated cardiac muscle fibers appeared dense, variable in size and shape (Plate 1: B). There was mononuclear cellular infiltration in between cardiac muscles (Plate 1: C). Treatment of diabetic rats with insulin glargine showed marked improvement of these degenerative changes of the myocardium as the nuclei appeared nearly equal in size with uniform shape and the cardiac muscle fibers had regular array and intact myofibrils (Plate 1:D).

- **PAS stained sections**

Cardiomyocytes of control rats showed PAS moderately positive (+++) stained granules in the sarcoplasm. The wall of small blood vessels and the interstitium showed strong positive (+++) material of little amount (Plate 2: A). Diabetic rats revealed weak to mild PAS positive (+) homogenous sarcoplasm of cardiac muscles. Moreover, the small blood vessels had thick walls with moderate to strong positive PAS staining material (Plate 2: B). There were abundant moderate to strong PAS positive strands in the interstitium among cardiac muscles (Plate 2: C). Treatment of diabetic rats with insulin glargine revealed moderate PAS positive cytoplasm of cardiomyocytes. The blood vessels revealed strong PAS positive material surrounding their thickened wall. The amount of PAS strong positive material in the interstitial tissue between cardiac muscles became less (Plate 2: D).

- **e NOS immunostained sections**

Sarcoplasm of control cardiac muscle fibers and the epicardial cells showed strong positive (+++) cytoplasmic e NOS immunostaining (Plate 3: A). Also, endothelial cells and some smooth muscles of the vascular wall revealed strong positive e NOS immunostaining (Plate 3: B). Induction of diabetes caused marked decrease in e NOS immunostaining as the endothelium and smooth muscles of cardiac blood vessels showed mild to moderate positive (+ to ++) e NOS immunostaining. Also,
Plate 2. Photomicrographs of PAS stained (x 400) sections of the heart from A) a control rat shows moderately (+++) PAS positive sarcoplasmic granules and strong (+++) PAS positive material around small blood vessels (arrow) and in the interstitial tissue (arrowhead). B) a diabetic rat reveals that the sarcoplasm appears homogenous with weak to mild (+) PAS positive reaction. Moderate to strong PAS positive material is seen around blood vessels (arrows) and in the interstitium (arrowheads). C) Also the heart of diabetic rat shows moderate to strong PAS positive strands in the interstitium. D) a diabetic rat treated reveals moderate PAS positive staining of the sarcoplasm. Strong PAS positive material is seen around thick walled blood vessel (arrow) and in the interstitial tissue (arrowhead).

more patches of cardiac muscle fibers appeared weak to mild (+) positive in their e NOS immunostaining (Plate 3: C&D). When diabetic rats were treated with insulin glargine, there was increased e NOS immunostaining in both epicardium and sarcoplasm of most cardiomyocytes (Plate 3: E). The endothelium of cardiac blood vessels revealed strong positive e NOS immunostaining but the vascular smooth muscles showed mild positive e NOS immunostaining (Plate 3: F).

Plate 3. Photomicrographs of e NOS immunostained (x 400) sections of the heart of A) a control rat shows: strong positive e NOS immunostaining of cardiac muscles' sarcoplasm (arrow-heads) as well as the epicardium (arrow). B) a control heart reveals strong positive e NOS immunostaining of both endothelium (arrowhead) and vascular smooth muscles (arrows). C) a diabetic rat showing that the sarcoplasm of large areas of cardiac muscles reveals weak to mild (+) positive e NOS immunostaining. Blood vessels reveal mild to moderate immunostaining of their smooth muscle cells (arrowheads). D) Vascular endothelial cells of diabetic heart blood vessels reveal mild to moderate positive e NOS immunostaining (arrowheads). E) a rat from the treated group reveals strong positive e NOS immunostaining of the sarcoplasm of most of cardiac muscles as well as endocardium (arrow). F) Also, the heart of insulin glargine treated group shows small patches of cardiac cells with very weak positive (-) e NOS immunostaining. Vascular endothelium shows strong positive immunostaining (arrowhead) while vascular smooth muscles (arrow) reveal mild positive immunostaining.
Morphometric measurements and Statistical analysis

Table 1. Mean area % ± SD of PAS positive stained areas in control, diabetic and treated diabetic groups

<table>
<thead>
<tr>
<th>Item/ group</th>
<th>Control</th>
<th>Diabetic</th>
<th>Treated diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>26.41 ± 1.43</td>
<td>18.99 ± 1.04</td>
<td>24.15 ± 1.32</td>
</tr>
<tr>
<td>&quot;t&quot; test versus control</td>
<td>10.2748</td>
<td>t = 2.8490</td>
<td>t = 7.5035</td>
</tr>
<tr>
<td>Versus diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>p&lt;0.0001*</td>
<td>0.0173</td>
<td>p&lt;0.0001*</td>
</tr>
</tbody>
</table>

*P ≤0.05 considered significant

Chart 1. Mean area % of PAS positively stained areas in different groups

Table 2. Mean area % ± SD of eNOS positive immunostaining in control, diabetic and treated diabetic groups

<table>
<thead>
<tr>
<th>Item / Group</th>
<th>Control group</th>
<th>Diabetic group</th>
<th>Treated diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>36.6 ± 1.39</td>
<td>16.6 ± 0.676</td>
<td>35.4 ± 2.24</td>
</tr>
<tr>
<td>'t' test versus control</td>
<td>31.8</td>
<td>1.12</td>
<td>19.68</td>
</tr>
<tr>
<td>versus diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>P=0.0001*</td>
<td>0.29</td>
<td>P&lt;0.0001*</td>
</tr>
</tbody>
</table>

* ps≤0.05 considered significant

Chart 2. Mean area % of eNOS positive immunostained areas in control, diabetic and treated groups

Table 1 showed that induction of diabetes caused statistically significant (p< 0.0001) decrease in PAS positive staining areas compared with the control. In diabetic rats treated with insulin glargine, the mean area
% of PAS was increased statistically significant (P< 0.0001) compared with the diabetics.

Table (2) revealed that induction of diabetes caused statistically significant (p=0.0001) decrease in e NOS immunoexpression in heart tissue compared with the control. Treatment of diabetic rats with insulin glargine caused statistically significant (<0.0001) increase in e NOS immunoexpression in comparison with the diabetic group.

**DISCUSSION**

Cardiac failure is a major leading cause for mortality of diabetic patients, in part due to specific cardiomyopathy, referred to as diabetic cardiomyopathy (DCM) which occurs with or without co-existence of vascular diseases. Although several mechanisms responsible for DCM have been proposed, oxidative stress is widely considered as one of the major causes for the pathogenesis of the disease (Cai, 2007). Among anti-oxidant substances NO is important especially its isoform e NO (Albrecht et al., 2003). The enzyme responsible for e NO synthesis is e NOS.

The current study revealed that alloxan-induced diabetes caused disarrayed pattern of cardiac muscle fibers, degeneration and apoptosis of some cardiomyocytes, the nuclei of cardiac muscles appeared dense and variable in size. Also, there was mononuclear cellular infiltration in between cardiac muscles. There was thickening of blood vessels' wall. Disarrayed cardiac fibers that observed in diabetic rats was also observed by Thent et al., 2012 who mentioned that was probably due to degeneration of mitochondrial structural protein that occurred as part of protein degradation related to DM. Apoptosis of myocardial cells was reported by Zhang et al., 2013, who referred it to be fundamentally ascribed to hyperglycemia and lipid disorders. That hyperglycemia induced an anaerobic state in heart muscles leading to muscle hypertrophy and degeneration. As an independent risk factor, hyperglycemia could directly cause myocardial damage and ultimately led to DCM. Intra-myocardial inflammation in diabetes was reported by Westermann et al., 2007. When diabetic rats treated with insulin glargine most, but not all, of the previous diabetic changes were reversed as some cardiocytes still exhibited degeneration and there was still some thickening of vessel's wall. This finding was also observed by Thompson, 2005, who mentioned that cardiomyopathy observed in some diabetics might at least partially reversible with insulin.

By using PAS stain, the present study showed statistically significant decrease in area% of PAS positive stained areas in heart of diabetic rats compared to control rats. The same finding was previously recorded by Narasimman et al., 2005 and Ding and Brian, 2006 who referred that to; 1) severe insulin deficiency resulted in large decrease in the activation of cardiac glycogen synthase and phosphatase which caused a shift in myocardial metabolism resulting in excessive use of free fatty acids and marked reduction in the utilization of plasma glucose. 2) Degradation of cardiac glycogen in diabetic rats. 3) Glucose metabolism was severely depressed in the heart of diabetic rats due to reduction of; glucose uptake, glycolytic activity, pyruvate oxidation and rate of glycogen storage. Also, the current study showed deposition of PAS positive material in the thickened wall of cardiac blood vessels and abundant PAS positive strands in myocardial extracellular matrix. The same results were seen by Narasimman et al., 2005 who mentioned that this PAS positive material corresponded to the distribution of antitype VI collagen reactivity which represented a major glycoprotein of myocardial extracellular matrix and implicated in diabetic cardiomyopathy. Treatment of diabetic rats with insulin glargine caused statistically significant increased area% of PAS positive staining areas compared to diabetic rats.

As regards e NOS immunostaining, the study revealed that control rats showed strong positive immunoexpression against e NOS within various cell types of the heart as cardiomyocytes, the endocardial cells, endothelial cells and vascular smooth muscles. Maison et al., 2003 mentioned the same sites plus the conducting system of the sinus and atrio-ventricular node. These results proved the presence of e NOS enzyme in cardiomyocytes, endocardium, endothelium and vascular smooth muscles. Subsequently, e NO has a regulatory effect on cardiac muscles’ contraction and relaxation. Also, it causes vasodilatation of cardiac vessels via its effect on vascular smooth muscles. Induction of diabetes caused marked diminution of e NOS immunoexpression in cardiac muscles as well as endothelium and vascular smooth muscles. That decrease in e NOS was statistically significant compared to the control. This finding was also observed by Felco et al., 2001, and Awata et al., 2004 who referred that to the direct effect of insulin on e NOS gene expression which is restricted to those anatomical structures. A defect in e NO function developed very early in DM and led to altered vascular e NOS reactivity (Chan et al., 2000). The association of DM with atherosclerotic cardiovascular disease might be a consequence, in part, of the decrease of e NOS protein in endothelial cells (Fisslthaler et al., 2003). The cause for that decreased e NOS expression might be due to reduced availability of L- arginine as a substrate for NO synthesis. The plasma concentration of L- arginine had been reported to be decreased in diabetics (Endemann and Schiffrin, 2004). Also, the activity of NOS was impaired during the progression of insulin deficient-diabetes and that was associated with decreased abundance of e NOS (Perreault et al., 2000; Naruse et al., 2006 and Xia et al., 2011). Another cause for that was accelerated degradation of NO (by its reaction with O₂) which was likely to occur in vascular
diseases. NO and O$_2^-$ reacted to form ONOO$^-$, which in turn led to eNOS uncoupling and enzyme dysfunction so, oxidative stress played a pivotal role in the development of diabetes vascular complication (Li et al., 2002 and Giacco and Brownlee, 2010). On the other hand Forsterrman and Munzel, 2006 mentioned that cardiovascular risk factors were associated with an increase rather than a decrease in eNOS expression. They referred this increase in vascular eNOS expression to be a consequence of an excess production of H$_2$O$_2$. H$_2$O$_2$, the dismutation product of O$_2^-$, could increase eNOS expression through transcription and post-transcriptional mechanisms. The present study showed that treatment of diabetic albino rats with insulin glargine increased the area % (statistically significant compared to the diabetic rats) of eNOS positive immunostaining. That observation could prove that insulin was an essential factor for cardiac eNOS immunexpression and those diabetic vascular changes might be due to insulin deficiency (Felco et al., 2001).

CONCLUSION

In conclusion, the findings of the present study proved that administration of insulin glargine early in diabetes had a blunting effect on diabetic cardiac complications. Although some changes could not be prevented completely by insulin treatment, additionally, it could be concluded that eNO was important for normal cardiac structure and subsequently cardiac function. The expression of eNOS was controlled by insulin.

REFERENCES


Li H, Wallerath T, Munzel T, Forsterrman U (2002). Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. Nitric Oxide Biol. Chem. 7:149-164.


