Bull. Egypt. Soc. Physiol. Sci. *37(2)*,92-109

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Possible Protective Role of Erythropoietin in Diabetic Cardiomyopathy in Rats

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Received: 24 April 2017
Accepted: 25 May 2017
Available online: 1 July 2017

Keywords

- Erythropoietin (EPO)
- Diabetic cardiomyopathy,
- Glibenclamide
- Oxidative stress
- High fat diet (HFD)

Abstract

There are controversial experimental works about the way of EPO in protection against DCM; whether EPO action is through making better metabolic condition and glycemic control or the antioxidant and antiapoptotic effect. So,the aim of this study was to evaluate the modulating effects of EPO on some biochemical markers in diabetic cardiomyopathy. Fifty male Sprague Dawley rats were divided into five groups of 10rats each. The rats of group **I** served as normal control. Group **II** received HFD for 1month and STZ in a concentration of 35mg/kg intraperitoneal. Group **III**:(Diab + Gliben) diabetic rats treated with glibenclamide, Gliben was given at a dose (0.6 mg/kg orally) for 4 weeks, Group **IV**: Diabetic rats treated with EPO; twice a week 4 weeks' duration. (1,000 IU/kg) and Group **V**: diabetic rats treated by both (EPO + Gliben) with the same previous doses for 4 weeks' duration. At the end of experiment serum samples and cardiac tissues were obtained from the sacrificed rats. Cardiac enzymes, blood glucose levels, serum insulin level and oxidative stress markers were estimated. Diabetic cardiomyopathy changes were confirmed by electrophysiological and histopathological results as well as increased serum activity of cardiac enzymes and high blood glucose levels. EPO exhibited myocardial protection in rats with DCM, at least in part through antioxidant effect and better glycemic control. However, this study was based on animal models in vivo, and detailed molecular mechanisms should be further investigated using in vitro studies. Clinical trials may also be considered to validate the results in humans, following further pre-clinical studies.

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Introduction

Diabetes is the third most common chronic disease of childhood, and its incidence has been increasing worldwide, with anticipated prevalence of 5.4% in 2025**[1]**.Corresponding to300 million persons are suffering from diabetes worldwide. Cardiovascular complications are considered the main cause of death in these people like hypertension , coronary artery disease or due to direct effect of diabetes mellitus on the heart, called Diabetic cardiomyopathy DCM**[2].**

Diabetic cardiomyopathy (DCM) was first described by *Rubler***,** *Dlugash* **[3];** depending on observations noticed in diabetic patients post-mortemwith heart failure but no coronary artery disease or other etiological conditions explaining heart failure.Myocardial fibrosis with LV hypertrophy was observed in those patientsand the concept of DCM was introduced for the first time **[4].**

The characters of DCM are dysfunction in myocardial contraction and relaxation, as a result of some factors like inflammatory process, oxidative stress, myocardial fibrosis and apoptosis so DCM considered a unique cardiovascular disease**[5]**.

Erythropoietin (EPO) hormone is a glycoprotein secreted mainly by the kidney about (90%) in adult, and by the liver (10%) in the fetus, its function to stimulates the bone marrow to produce red blood cells from stem cells (erythropoiesis) **[6],** Synthetic analogue of EPO, like recombinant human EPO (rhEPO), have been successfully introduced in treatment of anemia caused by diabetic nephropathy **[7].**

It's clear nowadays that EPO has many biological roles rather than regulation of red cell production. There is a focus primarily on the central nervous system along with the kidney, heart, liver, and vasculature. In the brain; EPO and Epo-receptor mRNA are widely expressed and appear throughout development in astrocytes, neurons, and endothelial cells**[8],** also, Epo administration protects against experimental cerebral ischemia in vivo**[9].** In addition, rhEpo has been proved to protect the kidney and liver from ischemia/reperfusion injury**[10-14].**

EPO act by enhancing multiple signaling pathways when binds to the EPOR in the brain, heart, liver and kidney, these pathways are involved in metabolism, survival of cells and apoptosis**[15]**EPO can protect the cell through decreasing the transition pore opening of mitochondrial permeability and prevent the passage of mitochondrial contents, such as ROS and cytochrome c, and so reducing apoptosis**[16]**. However, until now, there are controversial experimental works about the way of EPO in protection against DCM; whether EPO action is through making better metabolic condition and glycemic control or the antioxidant and antiapoptotic effect.

Materials and Methods

Experimental Animals

Fifty adult male rats of Sprague Dawley type, with average body weigh between 200-250g and aging 2-3 months were included in our study. Animals bred and housing in the animal house of the Medical Experimental Research Center (MERC), Mansoura faculty of medicine, at controlled room

temperature of 24°C and alternating (12 hrs light/dark cycles) fed ordinary laboratory chow and had a free access to water. All experimental procedures were approved by Mansoura faculty of medicine committee of animal care and ethics at 25-1-2015.

Drugs:

Human recombinant erythropoietin (Epo):preservative-free vial: each 0.4 mL of solution contains 4000 IU of EPO purchased from Julfar (Gulf pharmaceutical industries), Streptozotocin (STZ);1 gm powder, Stored at -20c, away from dry air and moisture, It is dissolved in cold citrate buffer PH 4.5 formed of 0.9gm citric acid and 1.47 gm sodium citrate dissolved in 100 ml saline, This drug was purchased from Sigma (St. Louis, MO, USA),Glibenclamide (Gliben) (Daonil) Oral antidiabetic drug Purchased from Sanofi Aventis pharmaceutical industries.

Experimental groups:

The rats were divided into five groups (n=10 in each group) Group I: Normal group, Group II: Diabetic group induced by administration of HFD for 1 month followed by STZ injection at minimal dose (35 mg/kg, intra peritoneal), Group III:Diab + Glibenrats treated with glibenclamide, Gliben was given at a dose (0.6 mg/kg orally) for 4 weeks **[17]**,Group IV: Diabetic rats treated with EPO for 4 weeks: EPO dissolved in 0.3 ml physiological saline, and then subcutaneously injected twice a week 4 weeks duration. (1,000 IU/kg) **[18]**. EPO given after induction of diabetes, Group V: diabetic rats treated by both (EPO + Gliben) with the same previous doses: 4 weeks' duration.

Experimental Model: (Type2 DM)

For induction of type2 diabetes, rats were fed a high-fat, high-carbohydrate diet for four weeks**[19]**, and theninjected with STZ (35 mg/kg, intra peritoneal)**[18].**Rats which identified to have diabetes mellitus, according to the criterion of a fasting blood glucose concentration 200 mg/dl were included in the model groups.

Collection of blood samples and harvesting heart

The blood samples were collected from the ophthalmic venous plexus by using Pasteur pipettes under halothane anesthesia, the blood was collected in test tubes and serum obtained by centrifugation at 2000 (rpm) for 10 mins. Storage at -20 c^o for cardiac enzymes assayCreatine kinase-MB (CK-MB) and Lactate dehydrogenase (LDH). At the end of the experimental protocol, rats were sacrificed using a large dose of Na+ thiopental (120 mg/Kg) and the thorax was opened then the heart from each animal was excised, then washed with saline and a sample of left ventricle was dissected, weighted and then preserved in liquid nitrogen for measurement of oxidative stress markers while, the remaining part of the heart was soused in 10% neutral formalin for further histopathological and immunostaining tests**[20]**.

1-Electrophysiological study: ECG:

ECG recording was done under light ether anesthesia using BIOPAC student lab system (software BSL 3.7.5), data acquisition unit MP45,biopac electrode lead set(SS2LA/L) and disposable vinyl electrodes(EL503), 3 electrodes per rat BIOPAC electrode gel (GEL1) and abrasive pad (ELPAD) or Skin cleanser or alcohol prep. At the start of experimental protocol ECG recording

was done for all rats, then another ECG recording was done for all groups before scarification.

2-Biochemical measurements

2.1.Assay of cardiac enzymes (CK-MB and LDH)

Cardiac enzymes (CK-MB and LDH) were measured using commercially special kits the detailed method according to the manufacturer's instructions. Kits for CK-MB were purchased from BioMérieux Diagnostics, Milan, Italy, for LDH were purchased from Bayer Diagnostics Ltd., Baroda, India.

2.2.Estimation of oxidative stress markers

Small piece of the left ventricle wall was isolated, homogenized in cold phosphate buffer saline (pH 7.4, 50 mm).GSH in the heart tissue was measured by the modified method [21] and MDA was measured by the modified method [22] using colorimetric kits (BioDiagnostics, Giza, Egypt) according to the manufacturer's instructions.

2.3. Estimation of Diabetic markers

Plasma glucose is measured by the modified method**[23]** by using commercial kit (Human), plasma insulin level was measured using special rat insulin kit (ELISA) ,obtained from company of Sun Red biological technology. Fasting blood glucose and insulin level of the same rat were measured and its *(HOMA-1)* insulin sensitivity index (ISI) was calculated by the following equation:

$$\frac{FBG \times FINS}{405}$$

FBG: fasting blood glucose in mg/L, FINS: fasting plasma insulin in IU/L.

3-Histopathological examination:

The cardiac tissues samples collected from all experimental rats, were scrubbed with saline immediately, and fixation was done by solution of 10% buffered neutral formalin. After fixation, the heart tissue was prepared by inserting in paraffin, sectioned, lastly stained with hematoxylin and eosin (H&E). light microscope was used for Histopathological assessment.

4-Statistical methods

The numerical values were expressed as the mean ± S.D. data were analyzed using the SPSS 12.0 statistic software package. One way ANOVA with Turkey post hoc test (significance at $p \leq 0.05$)

Results

1-ECG

As shown in table (1), graph (1) ECG parameters recorded showed; QT interval &QTc were significantly higher in diabetic group (p=0.0001) compared to normal group, on the other hand QT interval &QTc were significantly lower in gliben, EPO and combined treated groups(p=0.001) compared to diabetic group and there was Non-significant changes in other parameters of ECG, also QTc was significantly lower in combined treated group (p=0.0001) compared to gliben treated group.

2-Biochemical assay:

1. Cardiac enzymes

As shown in tables (2) The level of CK-MB and LDH was significantly higher in diabetic group (p=0. 001) than in normal group. On the other hand, the level of the enzymes was significantly lower in either gliben treated group, EPO treated

group and combined treated group (p=0.001) when compared with diabetic group. Also, CK-MB and LDH were significantly lower in combined treated group (p=0.002) compared to gliben group.

2-Oxidative stress markers

2.A – Malonaldehyde MDA

As shown in tables (3), the level of MDA was significantly higher in diabetic than in normal diet group. On the other hand, the level of the MDA was significantly lower in either gliben treated group, EPO treated group and combined treatment group(p=0.001) when compared with diabetic group. Also, MDA was significantly lower in combined treatment group (p=0.0001) compared to gliben treated group.

2.B- Glutathione; Reduced form (GSH)

As shown in tables (4) The level of GSH was significantly lower in diabetic group(p=0.001) than in normal diet group. On the other hand, the level of the GSH was significantly higher in either gliben treated group, EPO treated group and combined treated groups(p=0.0001) when compared with diabetic group. Also, GSH was significantly higher in combined treated group (p=0.001) compared to gliben treated group.

3-Diabetic markers

As shown in tables (5) the level of blood glucose was significantly higher in type 2 diabetic group than in normal group (p=0.001). On the other hand, the level of the glucose was significantly lower in either gliben treated group, EPO treated group and combined treated groups (p=0.001) when compared with the diabetic group. Also, glucose was significantly lower in combined treated group(p=0.001) compared to gliben or EPO treated groups.

As regard insulin, the level of insulin was significantly lower in diabetic group than normal group(p=0.001). On the other hand, the level of the insulin was significantly higher in gliben treated group (p=0.001) compared with diabetic group, but it showed insignificant decrease in EPO treated group (p=0.031) compared with the diabetic group, Also, insulin was significantly higher in combined treated group (p=0.001) compared to either diabetic or EPO treated groups.

As regard HOMA-1 it was significantly higher in diabetic group (p=0.001) as compared to normal group. On the other hand, the HOMA-1 was significantly lower in gliben and EPO treated groups(p=0.001) compared to diabetic group. Also, HOMA-1 was significantly lower in combined treated group(p=0.001) compared to EPO treated group.

3-Histopathology

A- Myocardial morphology by Hx& E

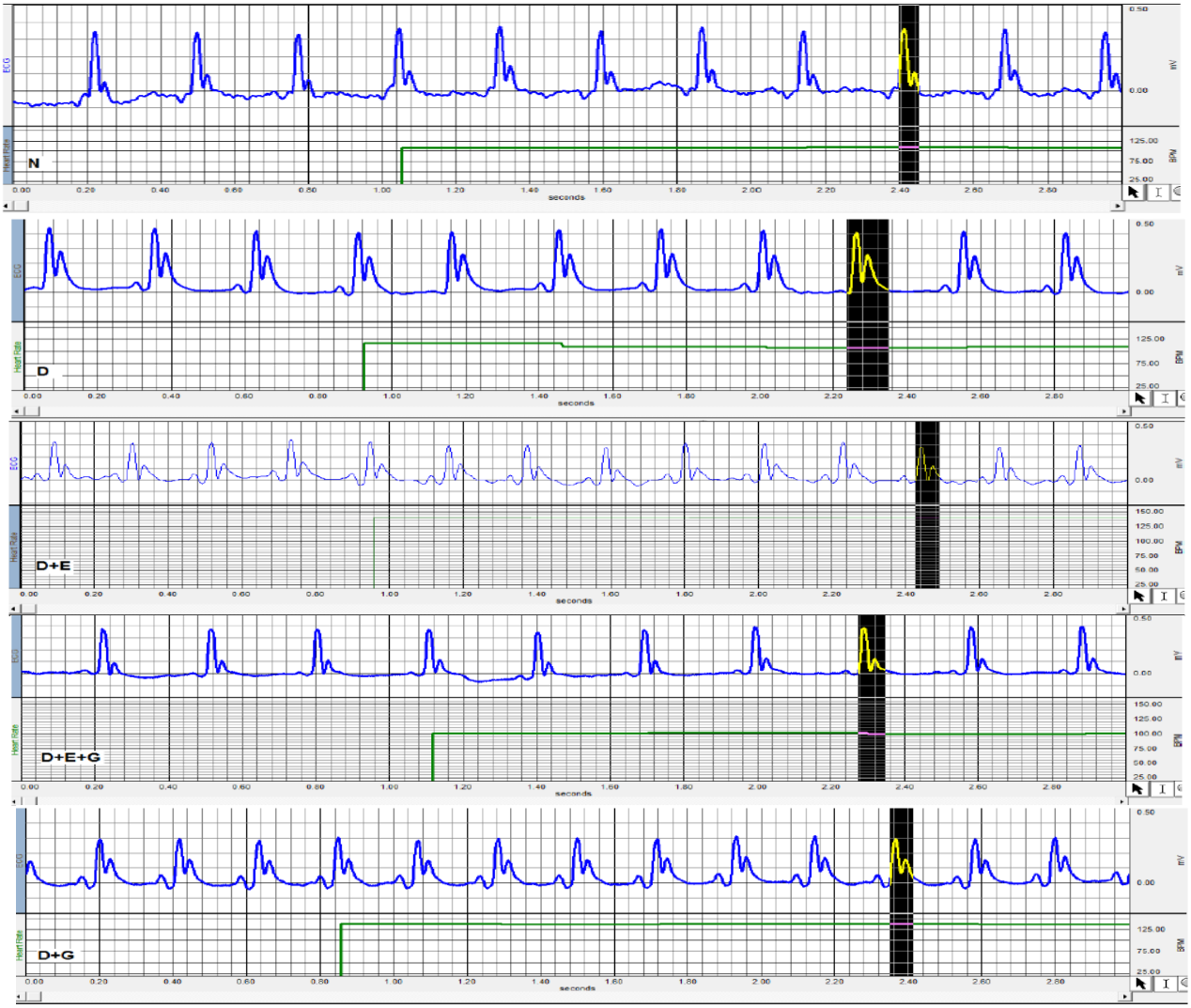
Figures (1a-e) show myocardial muscle changes in hematoxylin and Eosin stained section by light microscope.

Histological examination of normal group showed normal myocardium muscle. On the other hand, in untreated Diabetic group there was degeneration of myocardial muscle with marked inflammation and hypertrophy of muscle as compared to normal group. Tissue samples from Diabetic group treated with EPO showed marked odema of myocardial muscle with mild inflammation and mild hypertrophy of the muscle. Gliben treated group

Tables (1) ECG parameters in normal group, type2 diabetic, gliben treated, Epo treated, and combined treated rat- groups

Groups no (10)	Normal (G: Ⅰ)	Diabetic (G: Ⅱ)	Diab + Gliben (G: Ⅲ)	Diab + EPO (G: Ⅳ)	Diab + EPO + Gliben (G: Ⅴ)
PR	.0467±.002	.0483± .004	.0450± .004	.0467±.003	.0467± .004
QRS sec	.0383±.004	.0417±.004	.0367±.005	.0383±.004	.0350±.005
QRS mv	.3133 ±.05	.3267±. .05	.2533±. .05	.2300±. .07	.2617±. .08
QTR	.0800±.0126	.1183±.0116	.0883±.0116$	.0867±.0150$	.0900±.0154$
R-R	.248 3±.020	.2667±.035	.2433±.028	.2133±.010	.2833±.038
QTc	.1617±.024	.2483±.017*	.1733±.023$	.1850±.033$	.1767±.041$ €
HR	246±8.84	220±13.6	225±9.83	278±11	263±19
ST	.0100±.008	.0283±.038	.0267±.020	.0033±.005	.0217±.038

ECG parameters recorded; PR interval (m.sec), QRS duration (m.sec), Q-T interval (m.sec), R-R interval (m.sec), HR (beat per minute) and ST segment elevation (m.v). All results are expressed as mean ± SD, One way ANOVA with Turkey post hoc test (significance at p≤0.05), Diab=diabetic, gliben=glibenclamide, Epo = Erythropoietin.* significant vs normalgroup. **$** significant vs diabetic group. €significant vs gliben treated group. Tsignificant vs EPO treated group.



Graph (1) ECG traces recorded from normal control group (N), diabetic group (D), EPO treated group (D+E), combined treated group (D+E+G), gliben treated group (D+G). The highlighted part represents QT interval in different groups. The highlighted part represents QT interval in different groups

Table (2) serum LDH (ng/ml) and CK-MB (ng/ml) in normal group, type 2 diabetic, gliben treated, Epo treated, and combined treated groups

Group No (10)	Normal (G: Ⅰ)	Diabetic (G: Ⅱ)	Diab + Gliben (G: Ⅲ)	Diab + EPO (G: Ⅳ)	Diab + EPO + Gliben (G: Ⅴ)
LDH	245.9±31.5	1146.5±348*	626.3±22$	453.5±42$	**335.3±29$ € T**
CKMB	25.5± 3.4	287.3±26*	105.5±7$	198.6±15$	**72.5±12$ € T**

All results are expressed as mean ± SD, One way ANOVA with Turkey post hoc test (significance at p≤0.05), Diab=diabetic,gliben=glibenclamide, Epo = Erythropoietin.* significant vs normal group. **$** significant vs diabetic group**. €**significant vs gliben treated group. **T** significant vs EPO treated group

Table (3) Tissue MDA in normal group, type2 diabetic, gliben treated, Epo treated, and combined treated groups

Group No(10)	Normal (G: Ⅰ)	Diabetic (G: Ⅱ)	Diab + Gliben (G: Ⅲ)	Diab + EPO (G: Ⅳ)	Diab + EPO + Gliben (G: Ⅴ)
MDA	2.3±.2	14.9±.8 *	8.8±.4 $	7.5±.2 $	**3.6±.3 €$ T**

All results are expressed as mean ± SD, One way ANOVA with Turkey post hoc test (significance at p≤0.05),Diab=diabetic,gliben=glibenclamide, Epo = Erythropoietin. * significant vs normal group**. $** significant vs diabetic group. **€**significant vs gliben treated group. **T**significant vs EPO treated group

Table (4) Tissue GSH in normal group, type2 diabetic, gliben treated, Epo treated, and combined treated groups

Groups No(10)	Normal (G: Ⅰ)	Diabetic (G: Ⅱ)	Diab + Gliben (G: Ⅲ)	Diab + EPO (G: Ⅳ)	Diab + EPO + Gliben (G: Ⅴ)
GSH	7127±84.8	3549±73.2*	5369.3±24.8$	4492.3±27.7$	**6181.9±65.9€$**

All results are expressed as mean ± SD, One way ANOVA with Turkey post hoc test (significance at p≤0.05), Diab=diabetic,gliben=glibenclamide, Epo = Erythropoietin. * significant vs normalgroup**. $** significant vs diabetic group. **€**significant vs gliben treated group.**T**significant vs EPO treated group.

Table (5) Diabetic markers in normal group, type2 diabetic, gliben treated, Epo treated, and combined treated rat groups

Group No (10)	Normal (G: Ⅰ)	Diabetic (G: Ⅱ)	Diab + Gliben (G: Ⅲ)	Diab + EPO (G: Ⅳ)	Diab + EPO + Gliben (G: Ⅴ)
Glucose	105±8.26	373±62*	139±7.08$	200±.8$	**140±7.13$ T**
Insulin	11.6±.48	6.4±.74*	9.3±.61$	5.3± .94	**8.2±.19$T**
HOMA-1	2.98±.30	5.81±.72*	3.08±.13$	2.46±1.01$	**2.8±.27$**

All results are expressed as mean ± SD, One way ANOVA with Turkey post hoc test (significance at p≤0.05), Diab=diabetic, gliben= glibenclamide, Epo = Erythropoietin. * significant vs normal group. **$** Significant vs diabetic group. **€**significant vs gliben treated group**. T** significant vs EPO treated group

showed marked interstitial edema with hypertrophy of myocardial muscles and mild inflammation. Combined treated group showed normal myocardial muscles no edema, no inflammation, no hypertrophy i.e. near normal.

B-Myocardial fibrosis assessment (Masson trichrome staining)

Figures (2a-e) show histological examination of myocardial muscle stained with Masson trichrome stain. Cardiac muscle of normal group showed no evidence of fibrosis. Type2 diabetic group shows marked fibrosis (+3), gliben treated group shows moderate fibrosis, (+2). Diabetic group treated with EPO shows mild fibrosis (+1). Combined treated group showed minimal or no fibrosis.

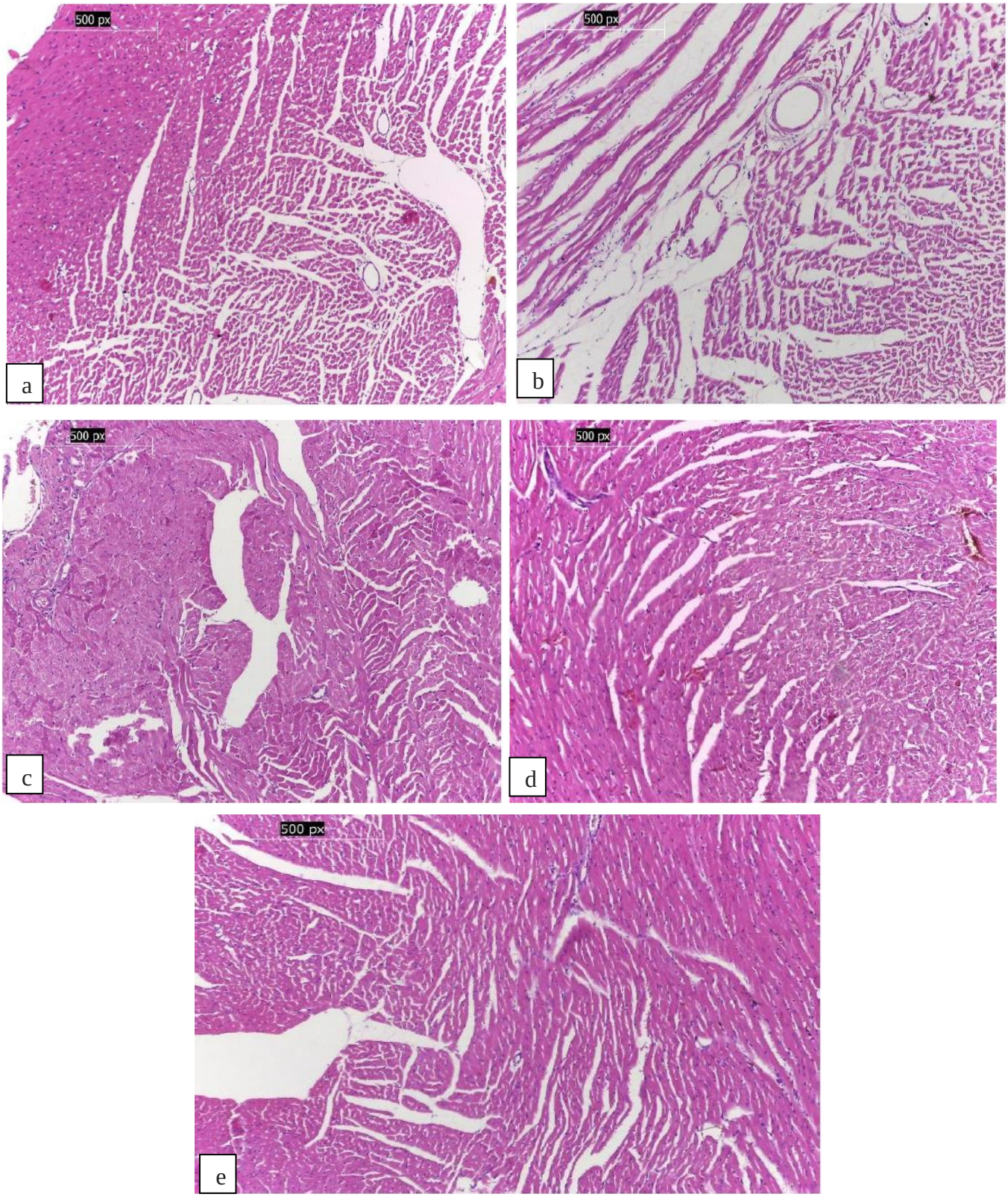


Fig (1) cardiac specimen shows normal architectures of rat myocardium (normal group (a) , heart specimen from rat treated with gliben shows marked interstitial odema with hypertrophy of myocardial muscles and mild inflammation (b), heart specimen of type2 diabetic rat shows degeneration of myocardial muscle with marked inflammation & hypertrophy of muscle (c), heart specimen of diabetic rat treated with EPO shows marked odema of myocardial muscle with mild inflammation mild hypertrophy of the muscle (d), heart specimen of diabetic rat treated with combined treatment shows normal myocardial muscles no odema, no inflammation, no hypertrophy; near normal (e) (H& E, 500px)

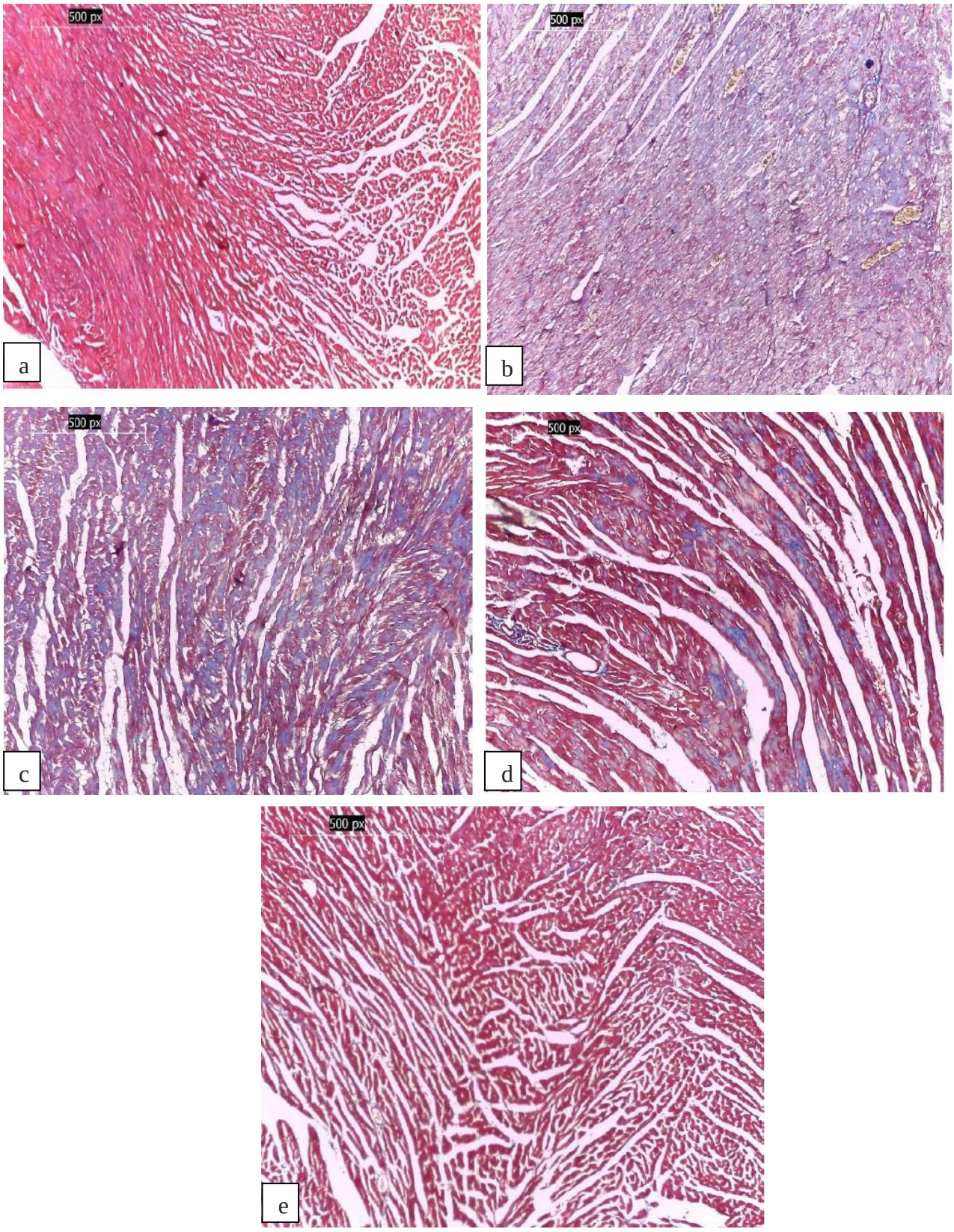


Fig. (2): Cardiac muscle of normal control group showed no evidence of fibrosis (a), heart specimen of gliben treated group shows moderate fibrosis, (+2) (b), heart specimen of type2 diabetic group shows marked fibrosis (+3) (c), heart specimen of diabetic group treated with EPO shows mild fibrosis(+1) (d) and heart specimen of combined treatment group showed minimal or no fibrosis €, (Masson trichrome, 500px).

Discussion

In the present study, we observed the DCM at the histo-morphological, functional and molecular level. Through our studies with EPO in DCM rats, we found that the administration of EPO could improve cardiac function and reverse remodeling of the heart of DCM rats by antioxidant effect and cellular preservation with improvement in diabetic markers.

At the end of the present study, it was found that cardiac enzymes (LDH, CK-MB) in untreated diabetic rat were significantly elevated in comparison to normal control group this result is consistent with *Yu, W., et al* **[24].**Also, we found that cardiac enzymes were significantly reduced in rats treated with erythropoietin only and rats treated with combined therapy in comparison to untreated diabetic group this means that long term treatment with erythropoietin may help in reducing cardiac damage that occurs in diabetic cardiomyopathy its likely due to anti apoptotic effect of erythropoietin. Results of our study showed that combined treatment group resulted in more cardio protection by more decrease in LDH and CK-MB, than single treatment with either glibenclamide or erythropoietin.

Oxidative stress is nowadays considered as underlying mechanism underlying for diabetes and diabetic complications**[25]**.Several studies have recorded an increased generation of ROS in DM **[26-28]**.Peroxidation of lipid is a diagnostic element of diabetic cardiomyopathy. It is considered the main mechanism destroying the cell membrane by free radicals. The degree of lipid peroxidation is assessed by formation of(MDA)

In our study, there was significant increase in serum level of MDA in untreated diabetic group as compared to normal control group. This result is consistent with a clinical study recently done by *Yang, Jia **[29]***.several studies have reported increased levels of lipid peroxides in the myocardium of diabetic rats**[29-31].**

Lipid peroxidation enhancement is also a reliable index of deterioration in defense mechanisms of enzymatic and non-enzymatic antioxidants**[32]**

Our results revealed that in untreated diabetic rats there was significant decrease in the serum level of non-enzymatic antioxidants (GSH) when compared with control group. These results are similar to those obtained by *Yang, Jia* **[29].**

Erythropoietin treated group showed significant decrease in MDA and increase in GSH as compared to untreated diabetic group, this result is in accordance with *Ritchie, Love* **[33]**. Role of EPO in preservation of cellular energy reserves is dependent upon the maintenance of mitochondrial integrity during DM**[34]**. Cryoprotection by EPO also is closely related to the maintenance of mitochondrial membrane potential. Loss of this potential through the opening of the mitochondrial permeability transition pore represents a significant determinant for cell injury and the subsequent induction of apoptosis**[35, 36]**. EPO has the capacity to prevent the depolarization of the mitochondrial membrane that also affects the release of cytochrome c**[37].**

So, EPO treatment reduced the lipid peroxidation present in diabetes and improved the antioxidant activity, which support the significant decrease in serum level of MDA and the increase in serum

levels of various antioxidants which occur in combined treatment with both glibenclamide and EPO when compared to untreated diabetic group, glibenclamide treated group and EPO treated group.

Regarding the electrophysiological properties, the results of this studyshowed that DM affects theelectrical activity of the heart,QRS is a sign of abnormal interventricular conduction and prolonged QT interval results in ventricular arrhythmia, an indicator of increased CVD risk**[38].**

Untreated diabetic group showed prolongation in QT interval as compared with normal control group, this result is consistent with *Tiwari and Ndisang* **[39].**The possible mechanism was the decrease of expression ATP- sensitive potassium (KATP) channels, as evidenced in both diabetic rats and high glucose treated cardiomyocytes, which increased the susceptibility to arrhythmia**[40].**EPO treated group showed significant decrease in QT interval when compared to untreated diabetic group. To our knowledge this is the first study which examine electrophysiological changes after erythropoietin treatment in diabetic cardiomyopathy. This effect may be due to limitation of hypertrophy and collagen deposition in the heart by chronic erythropoietin treatment**[18]**.

Type II DM was induced by combination of high fat diet and injection of low dose STZ, **[41].** This is a common model used to study long term diabetic complication like; cardiomyopathy, neuropathy.

Untreated diabetic group showed significant elevation in blood glucose, and (HOMA-1) in comparison to normal group, this finding associated with significant decrease in insulin level these finding are consistent with *Nath, Ghosh **[42].***The HOMA-IR provides an exact estimation of insulin sensitivity in rats**[43]**.

The progression of prediabetes to frank hyperglycemia in patients with type 2 diabetes is accompanied with decrease in secretory capacity of beta cells of the pancreas to compensate for the existing insulin resistance. But, the insulin deficiency is little in patients with type 2 diabetes when compared to the values seen in non-diabetic individuals**[44]**. In our study the evolution of disease pattern was achieved in insulin-resistant HFD rats upon injection with low dose of STZ (35 mg/ kg i.p.) which produced frank hyperglycemia in the presence of circulating insulin concentration almost comparable to normal rats (relative insulin deficiency).Many metabolic hypotheses have been advanced to explain insulin resistance in HFD fed rats. It has been shown that insulin resistance may occur as a result of defect in insulin binding caused by decrease in the number, affinity of insulin receptor, or defects at the level of effector molecules like enzymes concerned with glucose metabolism or glucose transporter

EPO treated group showed significant decrease in blood glucose decrease in insulin resistance and non-significant change in level of insulin, so our result agreed with previous studies which have documented a reduction effect of EPO on blood glucose levels[45-47]. The majority of these studies were performed on diabetic patients, a condition linked to insulin resistance[48]. Long-term EPO treatment was found to improve glucose metabolism in chronic hemodialysis patients, mainly by improving insulin resistance[45]. In rats, other study on EPO treatment was also associated

with lowered blood glucose levels, both in resting and under physical stress [46].

What could be the mechanisms of EPO effects on blood glucose levels? Decrease blood glucose level associated with exposure to high EPO levels may result from an increase in the erythrocyte counts and their higher glucose uptake[49].Another theory to be considered, is that EPO may also operate via increasing insulin sensitivity. This is in accordance with our finding that the EPO treated group was more responsive to insulin and thus maintained lower glucose levels with non-significant increase in insulin level.

The molecular mechanism by which EPO affecting insulin sensitivity is not yet resolved. EPO may act via an increase in NO [50], which is a potent vasodilator as well as insulin sensitizer, as proposed by *Trincavelli, Da Pozzo*[51]. Various aspects of EPO action in rodent models have been documented, including enhancement of wound healing [52], prevention of diabetes-induced podocyte damage[53],and promotion of vascular cell viability[54]. Taken together, our findings favor EPO as a novel potential glucose regulator. The current study intensifies an exciting potential publication for EPO as an adjunct anti-diabetic drug for reduction of blood glucose levels especially where these are associated with anemia. Definitely, introducing EPO for these indications would require further basic research studies along with clinical trials. The idea that EPO acts on glucose metabolism places this hormone in a new and critical important area, with interesting clinical applications in diabetes and obesity. These are currently great medical and social issues of interest. So, further studies are warranted to examine other metabolic effects of EPO.

Regarding pathological examination, in the present study structural changes in the cardiac muscles of untreated diabetic rats includes; degenerative changes of myocardial muscle with marked inflammation and cardiomyocyte hypertrophy these changes were significantly increased in diabetic rats as compared to control rats and this was accompanied by increased myocardial collagen (an indication of cardiac fibrosis) with masson trichrome staining. These changes are hall mark of diabetic cardiomyopathy as reported by many studies[55, 56].*Ouwens, Boer* [57] reported that high fat diet induces myocardial hypertrophy with inflammatory changes in the heart tissue.

Pan, Guleria [58] tried to find out the contributing factors of the morphological changes in diabetic cardiomyopathy which may be hyperglycemia, hypoinsulinemia, impaired lipid metabolism together with intracellular oxidative stress which leads to inflammatory infiltration.

On the other hand, there was improvement in these structural changes with treatment by either EPO or glibenclamide in this study; EPO attenuates myocardial interstitial fibrosis and cardiomyocyte hypertrophy. Interstitial and perivascular fibrosis is a histological hallmark of DCM [55], and cardiomyocytes pathological hypertrophy often accompanies it. These results were in agreement with *Lu, Yao* [59].This result may be referred to the fact that EPO improve tissue oxygenation by enhancing angiogenesis and attenuating interstitial fibrosis by anti-apoptotic action

Lu, Yao [59] in their work reported that EPO reduced the collagen type I and III deposition in DCM and down-regulated TGF-β expression. It is highly possible that EPO treatment led to a reduction in TGF-βexpression and attenuated the hypertrophic growth of the diabetic heart. TGF-β is a mediator of extracellular matrix production, can stimulate collagen production and contribute fibrosis in DCM[60].

Our results showed that in diabetic rats receiving combined treatment of EPO and glibenclamide there was significant improvement in pathological changes and the myocardium appear near normal no edema, no inflammation, no hypertrophy this may be due to the co-operative action of EPO with glibenclamide treatment.

Conclusion

Erythropoietin could be considered as a potential drug for improving diabetic cardiomyopathy due to its modulatory action through improving the antioxidant status in cardiac tissue, and better glycemic control.

Limitations of the study:
There were certain limitations to the study. As this was an animal study, it would be useful to repeat the study on other experimental models of diabetes, to verify the results. Further research is required to determine the acute effect of EPO and chronic effect in vitro study also.

Acknowledgments:

We would like to acknowledge dr.Yomna Khater, veterinary doctor, Faculty of Medicine, Mansoura University, for the animal handling and sampling technique.

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