Study of the Possible Cardioprotective Effects of Erythropoietin in Diabetic and Uremic Cardiomyopathy in Rats

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Abstract

Introduction: Cardiomyopathy is a common chronic complication associated with diabetes and chronic kidney disease. Erythropoietin (EPO) was reported as a broad spectrum cardioprotective agent.

Aim of Work: In this study, we aim to compare between the ability of EPO to exert its beneficial role as cardioprotective agent in two different models of cardiomyopathy; diabetic and uremic cardiomyopathy.

Material and Methods: This study was applied on 48 male albino rats that were divided into 6 equal groups: Control group, diabetic group, diabetic + (EPO) group, nephrectomy group, (neph + EPO) group and sham operation group. The study proceeded for 12 weeks for diabetic model and for 16 weeks for uremic model. Diabetes was induced by Intraperitoneal (IP) injection of 65mg/kg STZ. Uremia was induced by 5/6 subtotal nephrectomy. EPO treated groups were injected with 1000IU/kg of EPO (IP) twice weekly till the end of the study. Echocardiographic assessment was done at the 12 th and 16 th weeks for diabetic and uremic groups respectively also, heart tissues were collected for pathological examination.

Results: Echocardiographic assessment of cardiac function of uremic group showed that there was significant decrease in EF% & FS% by the 12 th week in untreated nephrectomy group compared with their baseline value, EPO treated nephrectomy and sham operated group. Further decrease was observed at 16th week. However, in EPO treated nephrectomy group EPO maintained the EF% and FS% normal as there was no significant decrease compared with sham operated group up to the 12 th week. By the 16th week EF% of EPO treated nephrectomy group was significantly lower than sham group but still significantly higher than untreated group. In addition, echocardiographic assessment of cardiac function of diabetic group showed that there was significant decrease in EF% & FS% by the 4 th week in both untreated and EPO treated diabetic groups compared to control group. Cardiac functions of both diabetic groups continued to decrease up to the end of the study (12 th week). Pathological examination of both nephrectomy group by hematoxylin and eosin staining showed signs of cardiac muscle hypertrophy with more cardiac degeneration in untreated group. While in EPO treated group, there was increase in number of sprouting capillaries compared with untreated and sham groups which further proved with trichrome staining. Also, trichrome staining showed significant increase in the area percent of fibrosis in untreated nephrectomy group compared with both EPO treated and sham groups. Hematoxylin and eosin staining of the heart tissues of diabetic group there was no signs of cardiac hypertrophy. The cardiac muscles appear with signs of hyaline and fatty degeneration. There was apparent infiltration with fatty vacuoles inside and in between muscle fibers. The blood vessels appear dilated and some was congested with blood. The vessel walls also show excess vacuolation with perivascular edema.

Conclusion: Through these results we can observe that in uremic model EPO treatment succeeded in preserving the cardiac function and delaying the development of uremic cardiomyopathy and this might be due to limiting fibrosis or increasing capillary number with further improvement of cellular oxygenation. But these beneficial effects could not be exerted with the diabetic model and the state of insulin insufficiency. The matter that ensure that the underlying systemic disease can differentially activate or inactivate the molecular signaling pathways of EPO.

Key Words: Cardiomyopathy – Erythropoietin – Cardioprotective – Diabetic cardiomyopathy – Uremic cardiomyopathy.

Introduction

DCM is defined as the cardiovascular damage present in diabetic patients, which is characterized by myocardial dilatation and hypertrophy, as well as a decrease in the systolic and diastolic function of the left ventricle, and its presence is independent of the co-existence of ischemic heart disease or hypertension. It is the consequence of the direct effect of metabolic dysregulation and oxidative stress on cardiac architecture during the course of DM. DCM may be subclinical for a long time, before the appearance of clinical symptoms or signs. According to the molecular theory of DCM, hyperglycemia is the main pathogenic factor, which causes abnormalities at the cardiac myocyte level,
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eventually leading to structural and functional abnormalities [1].

The coexistence of Chronic Kidney Disease (CKD) and cardiac complications, known as the Cardio-Renal Syndrome (CRS), creates a cardiac phenotype unique to the uremic heart, described as uremic cardiomyopathy. The pathogenesis of uremic cardiomyopathy is complex and incompletely understood. However, the association and persistence of hemodynamic factors, including anemia and hypertension, in addition to metabolic and endocrine abnormalities, contribute to the pathophysiological cardiac phenotype observed in patients with CKD. Uremia gives rise to multiple risk factors all of them share in the development of cardiac dysfunction found with uremia. These risk factors include anemia, LV hypertrophy, volume overload, hypertension, insulin resistance, diabetes, oxidative stress, chronic inflammation, secondary and tertiary hyperparathyroidism, uremic toxins, hypoalbuminemia and hyper-homocysteinaemia [2].

Erythropoietin (EPO) is a glycoprotein hormone that is essential for erythrocyte development [3]. It is synthesized primarily by kidneys in adults and by kidneys and liver in the fetus. Lack of EPO is the primary cause of the anemia associated with chronic renal failure. Before recombinant human EPO (rHuEPO) became available 15 years ago, about 25% of renal patients on dialysis needed regular transfusions of red cells. Tissue hypoxia is the main stimulus of EPO production, which is regulated through Hypoxia Inducible Factor 1 (HIF 1).

Beyond erythropoiesis EPO was found to act as a cardioprotective agent whether with in vivo or in vitro study. In addition, different studies have reported the important role of EPO in cardiac development as its defect during fetal development leads to fatal cardiac anomalies [4].

Prevention of apoptosis, decreasing oxidative stress, limiting fibrosis and angiogenesis are thought to be the different mechanisms by which EPO could exerts its protective effect on the heart. In addition, different signaling pathways are involved in these actions of EPO including Jak/STAT, PI3K/AKT and ERK/MAPK pathways. These different actions make some consider that EPO can act as a broad spectrum anti-heart failure [5].

However, many studies have reported that the beneficial role of EPO on the heart such as in case of ischemia reperfusion injury was blocked in presence of diabetes mellitus [6]. This give rise to another debate around if these actions of EPO are ubiquitous or differentially activated or deactivated according to the underlying disease.

Material and Methods

Experimental animals:

Forty-eight male healthy albino rats (10-12 weeks), weighing 160 to 200 grams, were used in the current study. They were obtained from Animal House Unit, Kasr Al-Ainy Faculty of Medicine, Cairo University (Egypt) and were housed at room temperature in cages with ordinary light/dark cycle, and left to acclimatize to environment for two weeks prior to inclusion in the experiment. The experiment was done during the year 2015.

Experimental design:

The forty-eight rats were randomly divided into 6 groups, each is 8 rats, as following:

Group I (diabetic untreated group): The animals were injected with a single dose of streptozotocin STZ 65mg/kg body weight. Fasting blood glucose was measured 5 days later and rats below 250 mg/dl were excluded. Rats were then left untreated for 12 weeks.

Group II (diabetic + EPO group): The rats were injected by STZ, a week later they received EPO (1 000IU/kg) treatment twice weekly for 11 weeks.

Group III (control vehicle group): The animals were accessed to standard diet of commercial rodent chow and tap water ad libitum. They were injected with an equivalent volume (as EPO injection) of normal saline intraperitoneally twice a week for 12 weeks.

Group IV (nephrectomy group): The animals underwent 5/6 subtotal nephrectomy and then housed for 16 week after operation.

Group V (nephrectomy + EPO group): The animals underwent 5/6 subtotal nephrectomy and received EPO (1 000IU/kg) treatment twice weekly throughout the study for 16 weeks.

Group VI (sham operated group): The animals underwent sham operation and were kept under the same experimental conditions 16 weeks.

Biochemical assays:

Serum creatinine was estimated by QuantiChrom™ creatinine Assay Kit (Bergman and Ohm-
an, 1980). Serum urea was estimated by QuantiChrom™ Urea Assay kit (DIUR-500) [7].

The blood glucose was assayed by kits supplied by “diamond diagnostics”. For all biochemical assays, the manufacturer instructions were followed. Blood samples were collected for measurement of blood glucose, urea and creatinine at the start of the study then at 4, 8, 12 weeks (groups: 1, 2 and 3) and 4, 8, 12, 16 weeks (groups: 4, 5 and 6).

- **Induction of uremia:**

  Uremia was induced surgically via a one-step 5/6th nephrectomy [8]. Uremia was diagnosed through significant elevation in serum creatinine for both untreated (neph) and treated (neph + EPO) groups compared with sham group [9].

- **Induction of diabetes:**

  Experimental diabetes mellitus had been induced in groups 1 & 2 by Streptozotocin (STZ) in a single dose of 65mg/kg body weight intraperitoneally [10]. Diagnosis of diabetes was done by measurement of fasting blood glucose [11]. Hyperglycemia was seen in adult rats within 5 days of Streptozotocin treatment and the hyperglycemic state remained all over the study indicating a stable model of experimental diabetes, which indicates irreversible destruction of Langerhans islets cells [12]. Special care was taken in dealing with diabetic rats according to the Canadian Council on Animal Guide to Use and Care of Experimental Animals (Vols. 1 and 2).

**Echocardiography:**

The echocardiography was used to evaluate the cardiac functions in vivo to provide a correct image of the state of the heart through measuring of EF% and FS%. Echocardiography was performed at the start of the study then at 4, 8, 12 weeks (groups: 1, 2 and 3) and 4, 8, 12, 16 weeks (groups: 4, 5 and 6). Each rat was lightly anesthetized with an injection of ketamine hydrochloride (25mg/kg, intraperitoneally) and xylazine (5mg/kg, intraperitoneally) [13].

**Pathological examination of cardiac tissue:**

Twelve weeks’ post-induction of diabetes and 16 weeks after induction of uremia, heart tissue was removed from all groups of animals then cut vertically as two halves. One half was put in tubes containing formalin (10%) in a ratio of 40% tissue to 60% formalin for pathological examination and the other half was stored at –80ºC.

**Masson trichrome technique:**

This technique was done for the heart tissues of both nephrectomy and sham operated groups to demonstrate the proliferation of fibrous connective tissue and to detect the newly formed capillaries. These two processes were done through image analysis.

**Image analysis:**

The images were analyzed using Image J 1.45s, a Java-based image processing program commonly used for biological image analysis. This software allows for analysis of images, cell count and area percentages of fibrosis [14].

**Statistical analysis:**

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) Version 22. Data was summarized using mean ± standard deviation. Comparisons between quantitative variables were done using one way analysis of variance (ANOVA) with post hoc Tukey test [15] p-values less than 0.05 were considered as statistically significant.

**Results**

**Echocardiographic examination results:**

Table (1) shows the changes in EF% and FS% values in the DM group (D), (D + EPO) group and control group (C). At the beginning of the study there was no statistically measured differences in EF% and FS% values among those groups. While diabetic untreated (D) group showed significant deterioration in EF% and FS% values at the 4th week compared to baseline and control (C) group values. Control (C) group did not show any significant change from baseline values. Significant functional deterioration in cardiac function in diabetic untreated (D) group was further observed during the following 8th and 12th weeks intervals. At the end of the 12th week both EF% and FS% values were significantly lower than corresponding baseline values and compared to control (C) values at the same time.

Diabetic rats treated with erythropoietin (D + EPO) did not show any statistically significant improvement in EF% and FS% values as compared with diabetic untreated (D) group.

Table (2) shows the changes in EF% and FS% values in the nephrectomy group (neph), nephrectomy + Epo group (neph + EPO) and sham operated...
group (sh). Erythropoietin treatment resulted in significantly higher EF% and FS% values in rats of (neph + EPO) group at the 12th and 16th week of study. Furthermore, there was no significant decline over time in echo parameters in EPO treated group.

By comparing the therapeutic effect of erythropoietin in both models of experimental diabetic and uremic cardiomyopathy; results of the present work demonstrated significant improvement in EF% and FS% values in EPO treated nephrectomy group as compared to EPO treated diabetic cardiomyopathy observed at the 8th, 12th week of the study Figs. (1,2).

• Body weight measurement:

Measurement of body weight at the beginning of the experiment showed no statistical difference between all groups. At the end of the study, the diabetic group (D) and (D + EPO) group show significant decrease in body weight compared with control (C) group at the end of the study. In addition, (neph) group and (neph + EPO) group show significant decrease in body weight compared with the sham operated group at the end of the study as well.

On comparing the body weight measurements between untreated diabetic and untreated renal group we observed that at the beginning of the study there was no statistical difference. While at the end of the study, body weight of the renal untreated (neph) group was statistically higher than that of diabetic untreated (D) group. In addition, there was no statistical difference between treated diabetic (D + EPO) and treated renal (neph + EPO) groups at the beginning of the study. While at the end of the study body weight of the (neph + EPO) group was statistically higher than that of (D + EPO) group.

• Blood glucose level:

As regard fasting blood glucose, at the beginning of the study up to 12th week to both DM group (D) and (D + EPO) group comparing these results with that of control group (C) demonstrated that there was significant increase in the level of fasting blood glucose in both groups compared with the baseline values and control group at 4th week and this was observed until the end of the study. On the other side, fasting blood glucose in (neph) group and (neph + EPO) group compared to (sh) group demonstrated that there was no statistical difference in EPO treated group compared with sham and untreated groups.

Comparing the results of fasting blood glucose of (neph) group with that of diabetic (D) group at baseline and over all the study the data demonstrated that there was no significant difference between both groups at baseline. While at 4th, 8th and 12th weeks of the study the diabetic untreated group showed significant increase in fasting blood glucose compared with renal untreated one.

Comparing the results of fasting blood glucose of (neph + EPO) group with that of (D + EPO) group at baseline and over all the study the data demonstrated that there was no significant difference between both groups at baseline. While at 4th, 8th and 12th weeks of the study the diabetic treated group show significant increase in fasting blood glucose compared with renal treated one.

• Urea and creatinine levels:

Blood urea and serum creatinine levels at baseline, 4th, 8th and 12th week in DM group (D) and (D + EPO) group and comparing them with the results of control group (C) did not show any statistically significant difference. Also, blood urea level at 4th week after nephrectomy in (neph) group and comparing with the results of sham group at the same time interval did not show any statistical significant difference.

Blood urea level at 8th, 12th and 16th week after nephrectomy in (neph) group was significantly higher than the baseline value and sham control group at same the time interval. In addition, serum creatinine level at the 4th, 8th, 12th and 16th weeks after nephrectomy in (neph) group was significantly higher than the baseline value and sham control group at same the time interval.

Meanwhile, blood urea and serum creatinine levels at 4th, 8th, 12th and 16th weeks in (neph + EPO) group was significantly higher than the baseline value and sham control group at the same time intervals.

Comparing the results of blood urea and serum creatinine levels of (neph) group with that of diabetic (D) group showed that there was no significant difference between both groups at baseline. While at 4th, 8th, and 12th week of the study the (neph) group show significant increase blood urea compared with diabetic (D) group.

Comparing the results of blood urea and serum creatinine levels of (neph + EPO) group with that of (D + EPO) group showed that there was no
significant difference between both groups at baseline. While at 4th, 8th, and 12th week of the study the renal treated group show significant increase in blood urea and serum creatinine levels compared with diabetic treated one.

**Results of pathological examination:**

The pathological examination to the heart tissues from rats of untreated diabetic (D) group 12 weeks after STZ injection have reported that there was loss of the characteristic striations of muscle fibers and some cells appear without nucleus. Other muscle cells appear brightly eosinophilic with pyknotic nuclei. Some cells show vacuolar degeneration mostly of fat globules as they are rounded, well circumscribed and pushing nucleus aside. Others show coagulative necrosis. The walls blood vessels show marked vacuolations mostly lipid deposition.

The pathological examination to the heart tissues from rats of (D + EPO) group 12 weeks after STZ injection have reported that muscle cells appear brightly eosinophilic with pyknotic nuclei while others showed loss of striations and absent nuclei and focal area of Zinker necrosis. There was focal area of coagulative necrosis in sub endocardial area. In addition, there was swelling in endothelial lining with vacuolations in the vascular wall and perivascular edema. There was focal area of coagulative necrosis in sub endocardial area.

Pathological examination of control (C) group showed that the cardiac muscle fibers appear normal in size with normal average intermuscular space with little fibrous connective tissue cells in between, normal cigar shaped nucleus while coronary vessels appeared normal with no congestion or dilatation.

Hematoxylin and eosin Staining of the heart tissues from rats of (neph) group 16 weeks after nephrectomy have demonstrated that: There was slight proliferation of fibrous connective tissue cells with its characteristic spindle shape nucleus between muscle fibers and around the coronary blood vessels recommended for trichrome stain. About cardiac muscles, some muscles showed hyalinosis in cytoplasm with pyknotic nuclei. Some showed hypertrophy with increase in muscle fiber size and decrease in intermuscular space while preservation of normal cigar shaped nucleus, vacuolation in other bundles indicating fat or hydropic degeneration, some bundles show loss of striations with proliferation of fibrous connective tissue cells. Some muscle bundles show Zinder necrosis with homogenous brightly eosinophilic cytoplasm and pyknotic nuclei and there was severe coronary congestion and hemorrhage near the epicardium.

Staining of the heart tissues from rats of (neph + EPO) group 16 weeks after nephrectomy have reported that some muscle bundles show signs of hypertrophy (increase in muscle fiber size and decrease in intermuscular space while preservation of normal cigar shaped nucleus) while others show degenerative changes with slight fibrous tissue proliferation.

Coronary vessels appear dilated and congested with some areas of hemorrhage but it is notable that there is increase in the number of sprouting capillaries recommended for trichrome stain. To confirm presence of fibrosis and to enumerate the number of new capillaries in order to compare between both renal groups and sham group we have further done trichrome staining.

Pathological examination of sham group showed that the cardiac muscle fibers appear normal in size with normal average intermuscular space with little fibrous connective tissue cells in between, normal cigar shaped nucleus, normal coronary vessels appeared normal with no congestion or dilatation, the epicardium was normally thin.

- Cyan coloured arrows points to decreased intermuscular space.
- Black coloured arrows points to normal cigar shaped nucleus, dark stained and centrally located.
- Yellow coloured arrows points to preserved normal muscular striations.
- Red coloured arrows points to degenerated muscle fiber with brightly eosinophilic cytoplasm, small bright nucleus may be pushed a side and lost normal striations.

**Results of trichrome stain:**

We used trichrome stain for renal and sham groups to illustrate the proliferation of fibrous connective tissue cells and for numbering of sprouting capillaries. The (neph + EPO) group showed significant decrease in proliferation of fibrous connective tissue compared with untreated (neph) but did not show any significant difference with sham. While as regard capillaries numbering, (neph + EPO) group showed significant increase compared with untreated (neph) and sham groups.
Table (1): Echocardiographic data for DM group (D), (D + EPO) group and control group (C) at baseline, 4, 8, and 12 weeks of the study.

<table>
<thead>
<tr>
<th>Value</th>
<th>Group</th>
<th>DM group (D)</th>
<th>(D + EPO) group</th>
<th>Control group (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF (%) base line</td>
<td>86.61±11.75</td>
<td>76.30±5.40</td>
<td>86.83±5.34</td>
<td></td>
</tr>
<tr>
<td>FS (%) base line</td>
<td>53.98±14.56</td>
<td>39.28±4.31</td>
<td>49.67±7.00</td>
<td></td>
</tr>
<tr>
<td>EF (%) 4 weeks</td>
<td>67.27±7.09*</td>
<td>68.50±6.41*</td>
<td>84.17±4.17</td>
<td></td>
</tr>
<tr>
<td>FS (%) 4 weeks</td>
<td>31.95±6.02*</td>
<td>33.83±5.78*</td>
<td>45.33±5.09</td>
<td></td>
</tr>
<tr>
<td>EF (%) 8 weeks</td>
<td>44.53±8.03*</td>
<td>44.921±7.33*</td>
<td>78.67±8.07</td>
<td></td>
</tr>
<tr>
<td>FS (%) 8 weeks</td>
<td>18.56±4.08*</td>
<td>16.901±6.73*</td>
<td>40.50±6.28</td>
<td></td>
</tr>
<tr>
<td>EF (%) 12 weeks</td>
<td>25.00±3.54*</td>
<td>23.881±4.75*</td>
<td>78.17±8.30</td>
<td></td>
</tr>
<tr>
<td>FS (%) 12 weeks</td>
<td>11.40±1.97*</td>
<td>9.831±1.35*</td>
<td>39.67±5.82</td>
<td></td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.
* : Statistically significant compared to corresponding value in DM control (C) group (p<0.05).

Table (2): Echocardiographic data for nephrectomy untreated group, nephrectomy treated and sham groups at baseline, 4, 8, 12 and 16 weeks of the study.

<table>
<thead>
<tr>
<th>Value</th>
<th>Group</th>
<th>(neph) group</th>
<th>(neph + EPO) group</th>
<th>(sh) group</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF (%) base line</td>
<td>89.32±10.32</td>
<td>83.69±13.17</td>
<td>81.58±9.70</td>
<td></td>
</tr>
<tr>
<td>FS (%) base line</td>
<td>56.56±15.23</td>
<td>51.84±14.27</td>
<td>49.74±19.83</td>
<td></td>
</tr>
<tr>
<td>EF (%) 4 weeks</td>
<td>75.67±18.40</td>
<td>78.71±9.41</td>
<td>82.75±9.64</td>
<td></td>
</tr>
<tr>
<td>FS (%) 4 weeks</td>
<td>38.00±12.44</td>
<td>41.00±9.09</td>
<td>40.50±4.80</td>
<td></td>
</tr>
<tr>
<td>EF (%) 8 weeks</td>
<td>87.75±7.41</td>
<td>87.75±4.99</td>
<td>79.75±11.95</td>
<td></td>
</tr>
<tr>
<td>FS (%) 8 weeks</td>
<td>52.00±9.83</td>
<td>51.00±6.16</td>
<td>40.00±6.38</td>
<td></td>
</tr>
<tr>
<td>EF (%) 12 weeks</td>
<td>44.59±3.21*</td>
<td>74.74±5.22@</td>
<td>80.75±5.32</td>
<td></td>
</tr>
<tr>
<td>FS (%) 12 weeks</td>
<td>19.35±3.32*</td>
<td>38.26±4.87@</td>
<td>39.50±4.65</td>
<td></td>
</tr>
<tr>
<td>EF (%) 12 weeks</td>
<td>41.84±6.35*</td>
<td>56.81±1.89*@</td>
<td>76.56±1.37</td>
<td></td>
</tr>
<tr>
<td>FS (%) 12 weeks</td>
<td>17.47±2.89*</td>
<td>21.95±8.84*</td>
<td>39.57±1.65</td>
<td></td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.
* : Statistically significant compared to corresponding value in (sh) group (p<0.05).
@: Statistically significant compared to corresponding value in nephrectomy group (p<0.05).

Table (3): Body weight recorded at the beginning and at the end of the study for all groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>DM group (D)</th>
<th>(D + EPO) group</th>
<th>Control group (Neph)</th>
<th>(neph + EPO) group</th>
<th>(sh) group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal body weight</td>
<td>173.50±6.12</td>
<td>176.67±6.06</td>
<td>171.50±5.24</td>
<td>175.86±5.18</td>
<td>173.75±4.43</td>
<td>175.00±5.77</td>
</tr>
<tr>
<td>Final body weight</td>
<td>106.67±4.08*</td>
<td>106.50±5.96*</td>
<td>243.33±5.16</td>
<td>191.14±6.64@</td>
<td>190.00±5.35@</td>
<td>243.75±4.79</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.
* : Statistically significant compared to corresponding value in DM control group (p<0.05).
@: Statistically significant compared to corresponding value in sham operated group (p<0.05).

Table (4): Comparison between capillary numbering and area % fibrosis between nephrectomy, nephrectomy + Epo. Group and sham operated group at 16th week.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>(Neph) group</th>
<th>(Neph + EPO) group</th>
<th>Sham (sh) group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP N</td>
<td>23.80±7.79</td>
<td>76.33±9.48*</td>
<td>18.00±1.79</td>
<td></td>
</tr>
<tr>
<td>area % fibrosis</td>
<td>1.16±0.75</td>
<td>0.36±0.28@</td>
<td>0.68±0.37</td>
<td></td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD.
* : Statistically significant compared to corresponding value in sham operated group (p<0.05).
@: Statistically significant compared to corresponding value in nephrectomy group (p<0.05).
Fig. (1): Changes in ejection fraction over time in all groups.

Fig. (2): Changes in fraction of shortening over time in all groups.

Fig. (3): Serum creatinine level (mg/dl) at the end of 4 weeks from the beginning of the study in all the study groups.

Values are represented as mean ± SD.
*: Statistically significant compared to corresponding value in DM control or sham operated group ($p<0.05$).
#: Statistically significant compared to corresponding value in DM group ($p<0.05$).
@: Statistically significant difference between (neph + Epo) group and (neph) group ($p<0.05$).
$: Statistically significant difference between (neph + Epo) group and (DM+Epo) group ($p<0.05$).

Fig (4): Fasting blood sugar level (mg/dl) at the end of 4 weeks from the beginning of the study in all the study groups.

Values are represented as values are represented as mean ± SD.
*: Statistically significant compared to corresponding value in DM control or sham operated group ($p<0.05$).
#: Statistically significant compared to corresponding value in DM group ($p<0.05$).
@: Statistically significant difference between (neph + Epo) group and (neph) group ($p<0.05$).
$: Statistically significant difference between (neph + Epo) group and (DM+Epo) group ($p<0.05$).

Fig (5): Comparison between pathological finding in heart tissue H & E between (neph), (neph + EPO) and (sham) operated groups.
Discussion

The present study was designed to investigate and compare the effect of long term administration of Erythropoietin (EPO) treatment on experimental models of cardiomyopathy; diabetic and uremic and whether it could prevent or delay development of cardiac dysfunction or has no effect. Also, the study aims to investigate whether this role differs according to the cause of cardiomyopathy and what possible molecular alterations involved in both models and their modulation by EPO.

Echocardiographic results indicate that in experimental model of renal failure there was a significant deterioration of cardiac function (as represented by the significant decrease of EF% and FS%) and development of heart failure by the 12th week after nephrectomy. It also showed that long term treatment with EPO resulted in significant preservation of cardiac function and prevent the development of heart failure. However, only at the 16th week EF% was reduced compared with control however it is still within the normal range and it is significantly higher than that of non-treated nephrectomy group.

In addition, echocardiographic results of diabetic cardiomyopathy of experimental model of STZ-induced diabetes show significant deterioration of cardiac function (as represented by the significant decrease of EF% and FS%) and development of heart failure by the 8th week after STZ injection. It also, shows that long term treatment with EPO did not cause any significant difference as the EPO treated rats show significant deterioration of cardiac function and heart failure development when compared with control group and this deterioration was similar to that had shown with non-treated rats.

Many animal and human studies proved that factors related to the uremic state itself provoke the development of LVH and further cardiac dysfunction, regardless of pressure and volume overload. The correction of hypertension in rats with renal injury does not prevent the development of cardiac hypertrophy [16]. In humans, LVH develops in high-risk populations with kidney disease, despite the effective control of hypertension. In diabetic patients with known diabetic nephropathy, a blood pressure-independent LVH occurs [17].

Clinical studies have shown that administering EPO to partially or fully correct the anemia associated with CKD induces a regression of LVH [18]. A reduction in LV mass has been associated with improved survival and reduced hospitalizations in uremic patients [19]. Interestingly, recent cardiac studies have shown that EPO has direct cardioprotective actions, independent from its hematopoietic effects, including decreasing apoptosis [20], promoting neovascularization [21] and reducing oxidative stress [22]. Thus, the decreased EPO production during CKD may render uremic hearts more susceptible to injury.

In the current study, echocardiographic findings showed that EF% and FS% in uremic group treated with EPO up to the 12th week were significantly higher than that of non-treated uremic group. They also show no significant difference compared with sham operated group. Only at 16th week after nephrectomy EPO treated group begin to show significant decrease in EF% & FS% compared with control group even the values were within normal range. However, compared with non-treated group EF% and FS% were significantly higher. These results reflect the role of EPO administration in delaying the deterioration of cardiac function after nephrectomy and its role in prevention of heart failure development. This role was performed away from its effect on anemia as the dose used did not affect the hematocrit level.

Standing with this data Ogino and his colleagues [23] have examined the effect of asialoerythropoietin (asialoEPO), a nonerythrogenic derivative of Erythropoietin (EPO), on renal dysfunction-associated heart failure. They also compared between asialoEPO and rhEPO regarding its effect on anemia and cardiac functions. Eight weeks later, when renal dysfunction was established, anemia and cardiac dysfunction and remodeling were apparent. They have reported that although only rhEPO relieved the nephrectomy-induced anemia, both rhEPO and asialoEPO significantly and similarly mitigated left ventricular dilation and dysfunction. The hearts of rhEPO-or asialo EPO-treated mice showed less hypertrophy, reflecting decreases in cardiomyocyte hypertrophy and degenerative subcellular changes, as well as significant attenuation of fibrosis, leukocyte infiltration, and oxidative deoxyribonucleic acid damage. They also reported that these changes were accompanied by restored expression of GATA-4, sarcomeric proteins, and vascular endothelial growth factor and decreased inflammatory cytokines and lipid peroxidation. They conclude that EPO receptor signaling exerts direct cardio protection in an animal model of renal dysfunction-associated heart failure, probably by mitigating degenerative, profibrosis, inflammatory, and oxidative processes but not through relief of anemia.
This results regarding pathological examination of heart tissues from renal untreated group (neph) have revealed infiltration of fibrous connective cells with characteristic spindle shape nucleus. Some cardiac muscle fibers show signs of hypertrophy such as increase of muscle cell size with decreased intermuscular spaces while preservation of normal cigar shaped nucleus. Some muscle bundles show vacuolar degeneration with hyalinosis in some bundles. Hypertrophy and fibrous infiltration cope with the criteria of uremic cardiomyopathy while degenerative changes and vascular congestion might have resulted from the relative ischemia as it was not obvious with EPO treated group.

In addition, hypertrophy can be detected in cardiac tissue of renal treated group (neph + epo) with some areas of degeneration but the matter of interest is presence of numbers of sprouting capillaries with vascular dilatation. Their numbers were significantly higher than untreated and sham operated groups and this was demonstrated through trichrome staining of cardiac tissue of these groups. Trichrome stain also shows little infiltration of fibrous connective tissue in EPO treated group compared with untreated one reflecting the antifibrotic effect of EPO. These increased number of capillaries compared with untreated group (neph) might explain how could Epo preserve the cardiac function of (neph) group up to 12 th week as it increases blood supply to hypertrophied muscles with improved oxygen and nutrient supply.

This is in accordance with Jaquet et al. [24] who have reported the ability of EPO to induce neovascularization even similar to vascular endothelial growth factor VEGF. Also, they have reported the obvious role of EPO in angiogenesis and new capillary formation through activation of endothelial progenitor cells and stimulation its migration from bone marrow. This also may play a role in EPO cardiac protection as formation of new vessels will increase oxygen and nutrients supply to hypertrophied myocardium so increasing its ability to compensate volume and pressure overload accompanied with renal failure.

On the other hand; Katie et al., [25] have reported that EPO treatment had no significant impact on in vivo cardiac function in uremia. In controls, EPO increased LVDP and cardiac contractility, in keeping with the observed increase in blood pressure. They also reported that dysfunction was not evident in uremic hearts; therefore, the protective role of EPO (in terms of cardiac function) during uremia could not be established. While in our study the cardiac dysfunction in the form of decreased ejection fraction was significant. Also, the maintained cardiac function in uremic groups treated with EPO was preserved until 16 weeks when it began to deteriorate.

These different results may be due to their shorter duration of uremia which persists for 12 weeks while in our study the duration of uremia persists for 16 weeks. In addition, it might be depending on the onset of EPO administration. We began one week after surgery by a dose of 1000 iu/kg (8.4 µg/kg twice a week) for the whole 16 weeks of the study. While with Katie et al., [25] study they began EPO administration 2 weeks before sacrifice with a dose of 30 ggm/kg twice a week. It may be also due to different severity of uremic condition or different animal strains.

• Diabetic cardiomyopathy model:

The current study results indicate that STZ diabetic group displayed a significant decrease in EF% and FS% compared with control group after 4 weeks of induction of diabetes. By time, the echocardiographic data showed marked progression of the deterioration in the systolic function at 8 th week, which developed to frank heart failure by 12 th week.

Standing with our data Joffe and colleagues [26] who have reported that diabetic animals were characterized by impaired systolic performance. This was further proven by in vitro study of isolated perfused hearts of the same study; they reported a lower peak developed pressure dP/dt. Endocardial and mid-wall shortening did not differ between diabetic and control animals, despite a lower wall stress in the diabetic animals suggesting reduced intrinsic myocardial contractility. Impaired LV relaxation indicative of diastolic dysfunction was also recorded.

In addition, Sun and his Colleagues [27] have reported the development of both systolic and diastolic dysfunction through echocardiographic assessment by 12 th week after STZ injection. There was a significant decrease in Left Ventricular Ejection Fraction (LVEF) and increase in both LVEDV and LVESV.

On the other hand, Iltis et al., [28] have stated that MRI analyses of cardiac function in 8 weeks STZ-diabetic rats, showed no significant difference in the LV end-diastolic volume, end-systolic volume, stroke volume and ejection fraction from age matched controls. In addition, in a study done by Al-Shafei and colleagues [29], no detectable changes
in both LVEDV and LVESV normalized to body surface area was observed. These results may reflect the difference in the strain of rats, since this factor has been shown to clearly influence DCM in the STZ model of type I diabetes. Strain differences exist in their susceptibility to DCM with STZ-induced diabetes in rodent models even though the diabetic cardiovascular complications closely imitate the human condition.

Erythropoietin was found to be a cardioprotective agent when used in ischemic reperfusion injury. Its protective functions have been reported with both acute and chronic administration. Chronic administration of low-dose EPO improved function in an experimental model of post-MI heart failure, without modifying hematocrit. Moreover, administration of EPO reduced ventricular dysfunction, improved ejection fraction and ameliorated apoptosis after induction of MI in rats.

However, our study results showed that, when STZ diabetic animals were injected with EPO 1000 IU/kg for 12 weeks there was no significant effect on cardiac function compared with non-treated diabetic group. In addition, on pathological examination of cardiac tissues of both diabetic groups there was multiple focal areas of coagulative necrosis with marked vacuolation in muscle bundles and blood vessels referring to marked degenerative changes which could explain the marked functional deterioration of the rats of these group on echocardiographic examination.

Standing with our result, Ghaboura et al. have studied the effect of underlying diabetes on the ability of EPO in decreasing the infarct size after ischemia reperfusion injury. They studied that in both types of diabetes 1 & 2 and compare them with non-diabetic control group. They have reported that in hearts from healthy controls, EPO decreased infarct area and increased phosphorylated forms of Akt, ERK 1/2, and their downstream target GSK-3. This suggests that STZ-induced diabetes abolished EPO-induced cardioprotection against I/R injury through a disruption of upstream signaling of GSK-3 the matter which does not occur with insulin resistance. They suggested that insulin deficiency specifically disrupts EPO protective signaling. They have also concluded that direct inhibition of GSK-3 may provide an alternative strategy to protect diabetic hearts against I/R injury.

It is worth to note that EPO was reported by Masahiro and his colleagues to protect against ischemia reperfusion injury through phosphorylation of GSK-3 at the time of reperfusion by AKT-dependent and independent mechanisms.

In this study the previously reported role of EPO as a cardioprotective agent was not detected with diabetic state as with EPO administration for 12 weeks there was no any improvement in cardiac function of diabetic rats (D-EPO group). This could be explained by increased endoplasmic stress in diabetes so further investigation should be done in order to measure the level of ER stress markers which also known as ER chaperones such as glucose-regulated protein 78 also known as BiP and glucose-regulated protein 94.

Not only through ER stress but also GSK-3 seems to have an important role in pathophysiology of a DCM and so could cut off the beneficial role of EPO on the diabetic myocardium. Insulin or hyper insulinemia may carry the answer. It is noted that in DM there is decreased AKT activity which in turn leads to decrease the phosphorylated form of GSK-30 leading to different pathological changes that end with cardiomyopathy.

In conclusion, the present study proved that EPO is significantly effective in improving cardiac dysfunction in experimental uremic cardiomyopathy. However, in diabetic cardiomyopathy it was significantly ineffective in preventing cardiac dysfunction associated with insulin deficiency. This differential effects of EPO most probably lie in the diverse molecular signaling pathways being activated differentially according to the underlying systemic disease. More studies are needed to verify the specific pathways counteracting EPO cardioprotective signaling to maximize the effect of EPO as a broad spectrum anti-heart failure drug.

References


