

## Assessing Cardioprotective Effect of Erythropoietin in Uremic Cardiomyopathy through Modulation of Nuclear Factor Kappa-b and Associated Proinflammatory Cytokines

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### **Abstract:**

**Background:** Chronic kidney disease is a global public health problem that shortens lifespan by increasing the risk of cardiovascular disease. **Aim:** This study aimed to investigate the cardioprotective effect of erythropoietin (EPO) on uremic cardiomyopathy (UCM) and targeting the possible role of  $\text{NF}\kappa\beta$ . **Methods:** UCM was induced surgically in rats via a one-step 5/6th nephrectomy procedure. Serum level of urea and creatinine were measured to assess renal function. Also, EF%, FS%, LVDd & LVSd were estimated to assess the cardiac function. Moreover, tissue level of  $\text{NF}\kappa\beta$ , IL1, IL6 and  $\text{TNF}\alpha$  were measured to assess the possible mechanism. **Results:** UCM caused a significant increase in urea, creatinine, IL1, IL6,  $\text{TNF}\alpha$ ,  $\text{NF}\kappa\beta$ , LVDd & LVSd with a significant decrease in EF% & FS%. Administration of EPO caused a significant improvement of the aforementioned UCM effects. **Conclusion,** EPO has cardioprotective effect in UCM targeting  $\text{NF}\kappa\beta$ .

**Key words:** CKD, EPO, UCM & nuclear factor kappa beta ( $\text{NF}\kappa\beta$ ).

**Abbreviations:** CKD; Chronic kidney disease, EPO; Erythropoietin, UCM; Uremic cardiomyopathy,  $\text{NF}\kappa\beta$ ; Nuclear factor kappa beta, IL 1; Interleukin 1, IL 6; interleukin 6,  $\text{TNF}\alpha$ ; tumor necrosis factor alpha, EF; ejection fraction, FS; Fractional shortening, LVDd; Left ventricular diastolic diameter, LVSd; Left ventricular systolic diameter.

### **Introduction:**

Patients with end stage renal failure (ESRF) or chronic kidney disease (CKD) have an increased risk of premature cardiovascular events and left ventricular (LV)

abnormalities. It is called Cardio-Renal Syndrome (CRS) that creates a cardiac phenotype unique to the uremic heart, described as UCM (1).

Erythropoietin (EPO) is a glycoprotein hormone that controls erythropoiesis. It is a cytokine for erythrocyte precursors in the bone marrow. It is produced by interstitial fibroblasts in the kidney in close association with peritubular capillary and tubular epithelial tubule. It is also produced in perisinusoidal cells in the liver (2).

Beyond erythropoiesis, EPO was found to act as a cardioprotective agent by preventing 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 (3).

NF- $\kappa$ B is a transcription factor that is composed of five subunits and can regulate downstream gene expression (4). NF- $\kappa$ B, is an essential regulator involved in the productions of several inflammatory cytokines, such as TNF- $\alpha$ , IL-6, and IL-1b. In UCM there is activation of NF- $\kappa$ B signaling and apoptosis marker levels (5).

EPO can modulate many downstream inflammatory cytokines, such as TNF- $\alpha$ , IL-

1b, and IL-6, regulating oxidative stress, apoptosis, inflammatory damage, and regeneration though inhibition of NF- $\kappa$ B (6).

So, the aim of the current study was to investigate the cardioprotective effect of EPO on UCM by targeting the level of NF- $\kappa$ B and inflammatory mediators.

## **Material and methods:**

### **Animals:**

This prospective study was conducted on 32 adult Wistar albino male rats, 6-8 weeks old, weighing between 180 and 220 g. They were obtained from the Experimental Animal Unit of Moshtohor faculty of Agriculture Benha University. The animals were acclimatized to the laboratory conditions for 2 weeks prior to the initiation of the experiment. They had free access to food and water. The experimental treatments were conducted in accordance with the general guidelines for ethical conduct in the care and use of animals in research. The experimental protocol was approved by the Animal Research Ethics Committee of the Faculty of Medicine, Benha University, Egypt. The duration of this experiment was from November 2019 to February 2020.

### **Experimental design:**

- The animals used were randomly divided into 4 main groups each group contain

8 rats. **Group I (control group):** sham-operated rats received saline 1 ml by intra Peritoneal (i.p) injection twice weekly for 16 weeks. **Group (II) (Sham+EPO group):** animals in this group underwent sham operation and received EPO (1000IU/kg) i.p twice weekly for 16 weeks. **Group (III) (nephrectomy group) :** animals in this group underwent 5/6 subtotal nephrectomy and then housed after operation with saline i.p. injection twice weekly for 16 weeks. **Group (IV) (nephrectomy + EPO group):** animals in this group underwent 5/6 subtotal nephrectomy and received EPO (1000IU/kg) i.p twice weekly for 16 weeks (*I*).

#### **Procedure of the experiment:**

Uremia was induced surgically via a one-step 5/6th nephrectomy procedure (*I*). Briefly, animals were anaesthetized with ketamine (35mg/kg of body weight). An incision was made over the left kidney which is exposed and decapsulated. Approximately two-thirds of the kidney was burnt using electro-cautery. Subsequently, another incision was made on the right kidney and was located and decapsulated. The renal vessels were ligated using a non-absorbable suture and the whole kidney removed.

Few sterile saline drops were applied over the abdominal viscera .Abdominal musculature

and skin layers were closed using an absorbable suture. Antibiotics (Benzyl penicillin 1000,000 I.U. once daily for 5 days) were given after surgery to avoid post-operative infection. In control animals, a sham procedure was performed whereby kidneys were decapsulated and replaced intact without intervention. The incisions were closed as described above.

**Echocardiography :** After the end of the treatment period, cardiac function was evaluated by Echocardiography.

Echocardiography was done in rats under ketamine (40 mg/kg i.p.) anesthesia, using My lab 30TM VET Gold (Esoate Co, Italy) equipped with a high-frequency 4–8 MHz phased array transducer. The rats were placed in the proper posture (semi-left lateral position with upright tilt) after the thoracic walls were shaved clean. Ultrasound gel was placed on the thorax to optimize visibility. Doppler, two dimensional (2-D) guided M-mode images were recorded from parasternal long-axis and parasternal short-axis and apical four-chamber views. LVDD and LVSD were measured. EF% and FS% were calculated using the following formulas:

$$FS \% = (LVDD - LVSD) / LVDD \times 100$$
$$EF \% = (LVDD_3 - LVSD_3) / LVDD_3 \times 100$$
All parameters were depended on the mean values of three cardiac cycles (*7*).

**Chemicals used :**

Normal saline (0.9%NaCl) bottles (Nile Co, Egypt, Lot NO: S12231).Epoetin Vial: 1ml containing 2000 IU alpha recombinant human erythropoietin (SEDICO pharmaceutical Co, Egypt, Lot NO: P001091) .

**Biochemical analysis:**

Estimation of urea and creatinine were done by using respective kits from Biodiagnostic, Egypt (Lot NO: ab65340). About 100 mg of heart tissues were homogenized to estimate IL 1 β, IL 6, TNFα, NF-κβ which were done by using respective kits from sigma-Aldrich Co., St Louis, MO, USA. (Lot NO: RAB0278-1KT),(Lot NO: RAB0311-1KT),(Lot NO: RAB0476) and (Lot NO:MBS703405) respectively.

**Statistical analysis:**

The data were analyzed using the computer program SPSS (Statistical package for social science) version 16. The significance of difference was tested using ANOVA (analysis of variance) to compare between more than two groups of numerical (parametric) data, for continuous non- parametric data, Kruskal -Wallis test was used for inter-group analysis, post hoc test (LSD) was used to compare between every two groups, *P* value <0.05 was considered statistically significant & *P* value >0.05 was considered statistically insignificant.

**Results:**

**Table 1:** Serum urea (mg/dl),serum creatinine (mg/dl), tissue IL 1 level (pg/gm tissue), tissue IL6 level (pg/gm tissue),tissue TNFα level (pg/gm tissue) and tissue NF-κβ level (pg/gm tissue) in different experimental groups:

	Group I		Group II (Sham+EPO)		Group III (nephrectomy)		Group IV (nephrectomy+EPO)		ANOVA test	P value
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD		
<b>Urea</b>	19.0	1.69	19.75	1.44	61.0 <sup>ab</sup>	3.04	20.38 <sup>c</sup>	1.3	861.8	<0.001**
<b>Creatinine</b>	0.68	0.126	0.67	0.129	3.99 <sup>ab</sup>	0.58	0.80 <sup>c</sup>	0.12	224.24	<0.001**
<b>IL-1</b>	85.0	4.21	86.0	5.45	175.75 <sup>ab</sup>	10.46	89.13 <sup>c</sup>	5.38	341.97	<0.001**
<b>IL-6</b>	60.5	3.85	58.0	2.51	104.0 <sup>ab</sup>	7.17	58.13 <sup>c</sup>	2.1	212.16	<0.001**
<b>TNF</b>	38.88	2.64	38.88	5.64	53.63 <sup>ab</sup>	3.02	39.75 <sup>c</sup>	3.73	27.12	<0.001**
<b>NFKB</b>	1.595	0.085	1.594	0.09	5.15 <sup>ab</sup>	0.30	1.63 <sup>c</sup>	0.058	915.7	<0.001**

Data are represented as Mean ±SD : group I:control group ; group II: sham+EPO group;; group III :nephrectomy ; group IV:nephrectomy +EPO group; <sup>(a)</sup>P<0.05 vs Group I ; <sup>(b)</sup>P<0.05 vs Group II; <sup>(c)</sup>P<0.05 vs Group III; \*\* is considered statistically highly significant

- There was non-significant increase ( $P>0.05$ ) in urea, creatinine, IL1, IL 6, TNF $\alpha$  and NF- $\kappa\beta$  level in group II when compared with that of group I.
- While there was a significant increase ( $p<0.05$ ) in urea, creatinine, IL1, IL 6, TNF $\alpha$  and NF- $\kappa\beta$  level in group III (when compared with that of group I and group II).
- Moreover, there was non-significant increase ( $P>0.05$ ) in urea , creatinine , IL1 , IL 6 , TNF $\alpha$  and NF- $\kappa\beta$  level in group IV when compared with that of group I and group II, on the contrary there was a significant decrease ( $P<0.05$ ) in urea, creatinine, IL1, tissue IL 6, TNF $\alpha$  and NF- $\kappa\beta$  level in group IV when compared with that of group III

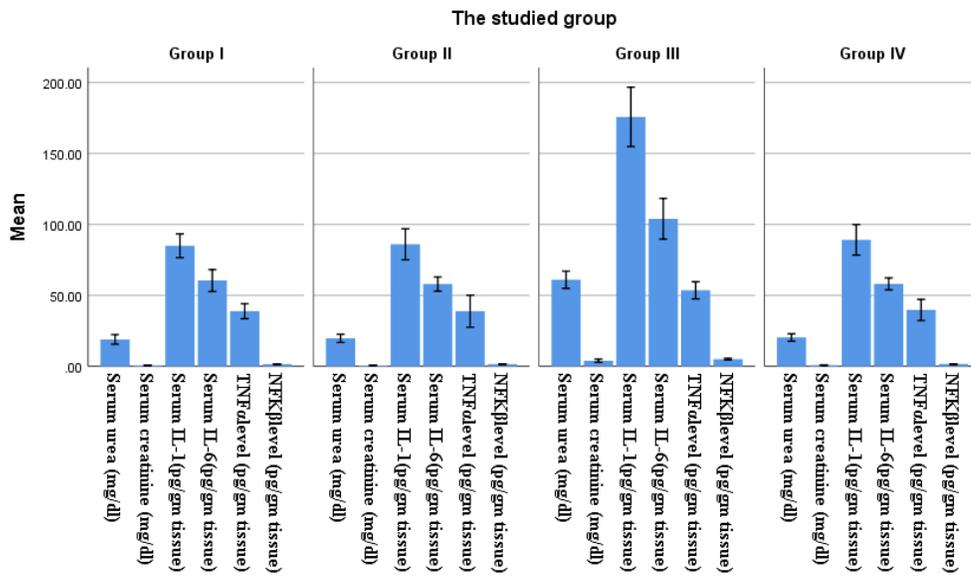


Figure (1) mean serum Urea, creatinine, IL1, IL6, TNF $\alpha$  &NF- $\kappa\beta$  levels in studied groups (Mean  $\pm$ SD).

**Table 2:** Ejection fraction (EF (%)), Left ventricular diastolic diameter (LVDd (mm)) , Left ventricular systolic diameter (LVSd (mm )) and fractional shortening (FS (%)) in different experimental groups:

	Group I		Group II		Group III		Group IV		ANOVA test	P value
	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD	Mean	$\pm$ SD		
<b>EF</b>	81.63	3.78	81.0	4.99	48.13 <sup>ab</sup>	1.13	78.75 <sup>c</sup>	3.01	170.07	<0.001**
<b>FS</b>	44.75	3.69	44.25	5.8	20.63 <sup>ab</sup>	1.3	42.0 <sup>c</sup>	1.31	84.68	<0.001**
<b>LVDd</b>	6.04	0.61	6.36	0.76	8.93 <sup>ab</sup>	0.63	6.49 <sup>c</sup>	0.16	41.42	<0.001**
<b>LVSd</b>	3.36	0.29	3.55	0.61	7.09 <sup>ab</sup>	0.40	3.6 <sup>c</sup>	0.2	158.6	<0.001**

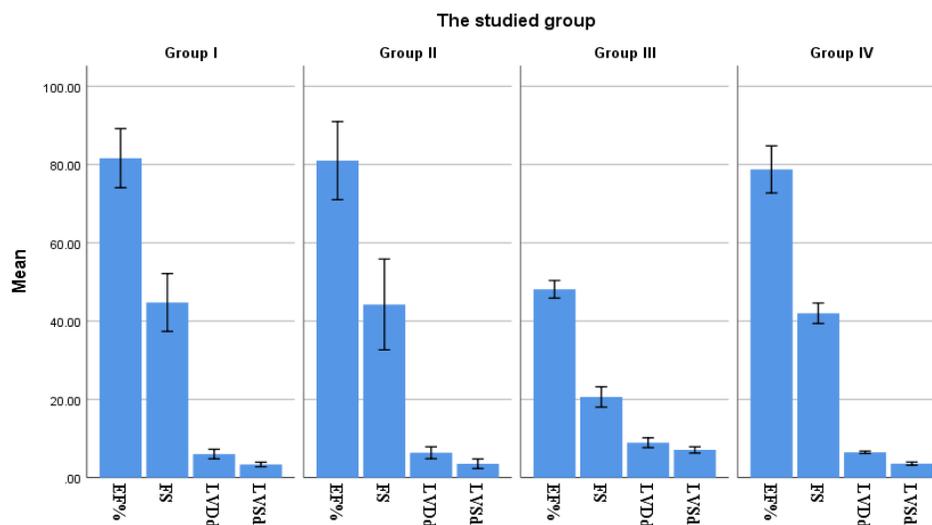
Data are represented as Mean  $\pm$ SD : group I:control group ; group II: sham+EPO group;; group III :nephrectomy ; group IV: nephrectomy +EPO group; <sup>(a)</sup> $p < 0.05$  vs Group I ; <sup>(b)</sup> $p < 0.05$  vs Group II; <sup>(c)</sup> $p < 0.05$  vs Group III; \*\* is considered statistically highly significant

In group II: There was non-significant increase ( $P>0.05$ ) in EF% & FS% in group II when compared with that of group I .

- On the other hand there was a significant decrease ( $p<0.05$ ) in EF% & FS% in group III when compared with that of group I and group II.
- There was non-significant increase ( $P>0.05$ ) in EF% & FS% in group IV when compared with that of group I and group II , on the contrary there was a significant increase ( $P<0.05$ ) in EF% & FS% level in group IV when compared with that of group III.
- In group II: there was non-significant decrease ( $P>0.05$ ) in LVDD & LVSD

level in group II when compared with that of group I .

- On the other hand there was a significant increase ( $p<0.05$ ) in LVDD & LVSD level in group III when compared with that of group I and group II .
- Moreover, there was non-significant decrease ( $P>0.05$ ) in LVDD & LVSD level in group IV when compared with that of group I and group II, on the contrary there was a significant decrease ( $P<0.05$ ) in LVDD & LVSD level in group IV when compared with group III.



▪ Figure (2) mean EF%, FS%, LVDD & LVSD levels in studied groups (Mean  $\pm$  SD)

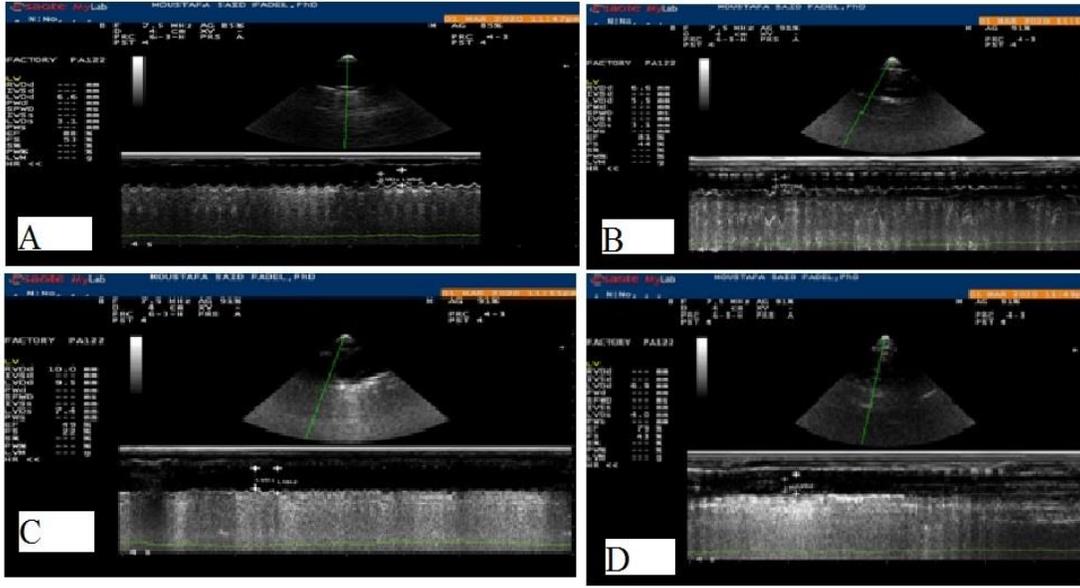
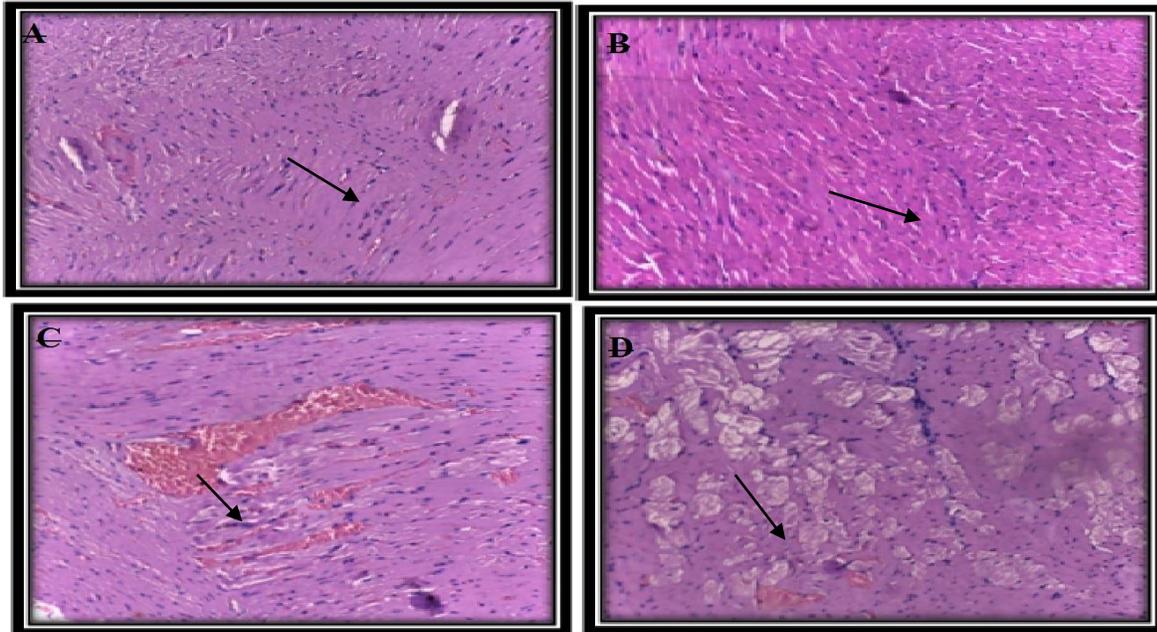


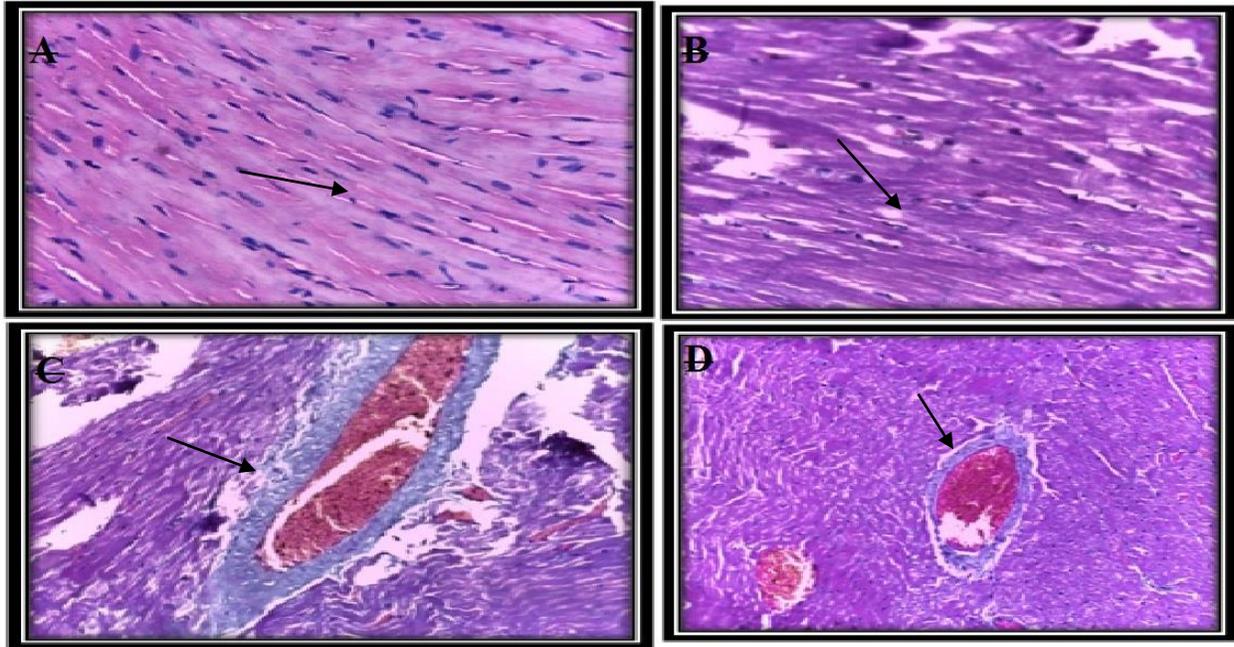
Figure (3) shows the echocardiography as a demonstration.(A) from group I,(B) from group II,(C) from group III ,(D)from group IV

**A-Haematoxylin and Eosin results:**



**Figure (4):** Heart specimen A: group I showing normal histological structure of cardiac tissue (H & E X 400);(B)group II showing preserved normal cardiac structures (H & E X 400);(C) group III showing hypertrophy of cardiac muscle cells& fibrosis (H & E X 400);(D) group IV showing little fibrous c.t. and normal cigar shaped nucleus (H & E X 400).

### B-Masson trichrome results :



**Figure (5):** (A) Masson trichrome stain of cardiac specimen of group I showing normal fibrous c.t. (B): Masson trichrome stain of cardiac specimen of group II showing normal fibrous c.t. (C): Masson trichrome stain of cardiac specimen of group III showing excessive fibrosis. (D): Masson trichrome stain of cardiac specimen of group VI showing decreased proliferation of fibrous tissue.

### Discussion:

In the current study, uremia induced surgically via a one-step 5/6th nephrectomy showed significant increase in serum levels of urea and creatinine when compared with the sham operated groups. These results were in agreement with a previous study (8) who stated that elevated serum urea and creatinine are strongly associated with and have been used as markers of uremia. Subtotal nephrectomy increases renal microvascular permeability which in turn induces interstitial edema that contribute to decrease renal blood flow leading to

deterioration of renal function with elevated serum levels of urea and creatinine (9).

EPO administration twice/week for 16 weeks to uremic rats, caused significant decrease in serum urea and creatinine when compared to group III as seen in table (1) and figure (1).

These results were in agreement with previous reports (10,11). They stated that, administration of EPO caused improvement in renal function by prevention of endothelial injury, improvement of vascular

damage and reduction of sclerosis in the glomeruli, as well as preservation of the architecture of the renal interstitium and peritubular capillaries in uremic rats.

To investigate the effect of subtotal nephrectomy on cardiac function we performed echocardiography after 16 weeks and the results indicated that the untreated uremic group showed a significant decrease of (EF% & FS%) with significant increase in LVDd & LVSd indicating the development of UCM by the 16th week after subtotal nephrectomy when compared with the sham operated groups. These results were in agreement with a previous study (*1*) who observed that CRF induced by 5/6 surgical nephrectomy have diminished contraction force and prolonged both contraction and relaxation time in the left ventricle papillary muscle after 16 weeks.

Interestingly, EPO administration twice weekly for 16 weeks resulted in a significant increase in (EF % & FS%) and significant decrease in (LVDd & LVSd) when compared to the untreated uremic group. These results were in agreement with other studies (*1,2&12*) that stated that EF% & FS% in uremic group treated with EPO was significantly higher than that of non treated uremic group. These results reflect the role

of EPO administration in delaying the deterioration of cardiac function after nephrectomy.

To investigate the mechanism by which EPO ameliorates the manifestations of UCM, we measured the cardiac tissue level of NF- $\kappa$ B and the proinflammatory cytokines (IL 1, IL6 & TNF $\alpha$ ). The nephrectomy group (group III) showed significant increase in tissue levels of N-F $\kappa$ B, IL1, IL6 & TNF $\alpha$  when compared with the sham groups.

These results go along with another study (*13*) who reported that NF- $\kappa$ B is a transcription factor that has increased activity in many cardiac diseases. NF- $\kappa$ B, is an essential regulator involved in the productions of several inflammatory cytokines, such as TNF- $\alpha$ , IL-6, and IL-1b in UCM (*14*). Moreover, inflammatory cytokines are secreted not only by leucocytes, but by most cells, including kidney, cardiac and parenchymal cells that secrete cytokines especially in response to stress (*15*).

The results of the current study are also in agreement with previous reports (*16,17*) that found a significant elevation of proinflammatory mediators in the UCM models suggesting that the severity of organ damage and dysfunction is positively

associated with the level of proinflammatory mediators.

On the other hand, after EPO administration, there was a significant decrease in tissue levels of NF- $\kappa$ B and (IL1, IL6 & TNF $\alpha$ ) in EPO treated uremic rats when compared to untreated uremic group. The mechanism by which EPO can protect against this pathway, is binding of EPO to EPOR, first activates Janus tyrosine kinase 2 (Jak2). Phosphorylation and activation of Jak2 may directly activate inhibitor of NF- $\kappa$ B (I $\kappa$ B kinase or IKK) (18).

Also, these results were in agreement with a previous study (19), which mentioned that anti-inflammatory effect of EPO may be explained by activation of EPOR by EPO which increased PI3K/Akt signaling activation which in turn, lead to inhibition of inflammatory mediator production and release protecting cardiomyocytes from TNF- $\alpha$  apoptosis both in vitro and in vivo.

Histopathological examination supported our results as there was hypertrophy with loss of striations of some muscle fibers. Other muscle fibers showed eosinophilic cytoplasm with pyknotic nuclei and there was coronary congestion and hemorrhage in untreated uremic group when compared with those of sham operated groups. These results

were in agreement with another study (1), who documented that pathological examination of heart tissues from uremic untreated group revealed signs of hypertrophy, vacuolar degeneration with hyalinosis in some bundles

Cardio protective role of EPO was also confirmed by the histopathological examination of cardiac sections. As, EPO attenuated the morphological changes accompanied UCM. Sections from rats which received EPO treatment in group IV showed some muscle fibers showed signs of hypertrophy with preservation of normal cigar shaped nucleus while others showed degenerative changes with slight fibrous tissue proliferations. These results were in accordance with the results of a previous report (1).

The current study also examined fibrosis as a causing mechanism of cardiac dysfunction in UCM. Masson trichrome stain showed numbers of sprouting capillaries with vascular dilatation and showed little infiltration of fibrous ct. While, trichrome stain after EPO administration showed little infiltration of fibrous CT in EPO treated group compared with untreated one reflecting the anti-fibrotic effect of EPO.

These results were in agreement with another study (1) *that* clarified that trichrome stain showed little infiltration of fibrous connective tissue in EPO treated group compared with the untreated one reflecting the anti-fibrotic effect of EPO

Previous study (20) explained the c.t. infiltration in UCM by a close relationship between transforming growth factor beta (TGF $\beta$ ) and NF- $\kappa$  $\beta$  signaling. NF- $\kappa$  $\beta$  can also interact with Activator protein 1 (AP-1) to over express collagens, fibronectin and TGF $\beta$ , enhancing the extra cellular matrix (ECM)accumulation leading to myocardial fibrosis that depends on the balance between ECM proteins and ECM-degradation enzymes. So, we can state that the decrease in fibrous c.t. in rats treated with EPO can be explained by the decrease in the expression level of NF- $\kappa$  $\beta$  in cardiac tissue.

### **Conclusion:**

In conclusion, EPO is significantly effective in improving cardiac dysfunction in experimental uremic cardiomyopathy by decreasing the level of NF-  $\kappa$  $\beta$  with subsequent decrease in IL 1  $\beta$ , IL 6, TNF $\alpha$  levels in cardiac tissue.

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