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Cardiac and Renal Protective Role of Erythropoietin in a Rat Model of Acute Myocardial Infarction

Akmal Ahmed Hassan Diab, MD[1]; Khaled Abdelfattah Abulfadle[1], MD; Nourelhuda A. Mohammed[1], MD and Fatma Nabil Hashim[1]
[1]Physiology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Corresponding Author:
Fatma Nabil Hashim
omamfatma@gmail.com
physiology department, faculty of medicine, zagazig university

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ABSTRACT

Background: Acute kidney injury is of a high prevalence rate in acute myocardial infarction (AMI) patients. Erythropoietin (EPO) protects tissues from ischemia.
Aim of the study: To investigate the possible cardiac and renal protective effects of EPO in a rat model of AMI and their mechanisms.
Materials and Methods: 27 male adult albino rats were divided randomly and equally into 3 groups [control, myocardial infarction (MI) and MI pre-treated with EPO]. In control and MI groups, **2 ml saline were injected subcutaneously once every other day for 7 days**. In MI pretreated with EPO group, rats were injected subcutaneously with 1000 IU/kg once every other day for 7 days. **In the 6th and 7th days, rats in MI and MI pretreated with EPO groups were injected subcutaneously with isoproterenol (ISO) hydrochloride (150 mg/kg body weight /day), to induce MI**. 1hr after the 2nd dose of ISO, ECG was recorded. Serum was separated from collected blood at the end of experiment for measurement of creatinine, TNF-α, MDA, LDH, IL-6, GSH and CK-MB. Cardiac and renal immunohistochemical study was done.
Results: In MI group, increased cardiac and renal Bax immunostaining and increased serum level; of LDH, CK-MB, creatinine, IL-6, MDA and TNF-α were noticed, with a significant reduction in serum GSH in comparison with control group. In MI pretreated with EPO group, these changes were ameliorated.
Conclusion: Erythropoietin has cardiac and renal protective effects in a rat model of AMI which **is owed to** its antioxidant, anti-inflammatory and antiapoptotic roles.
Key words: Erythropoietin, Acute kidney injury, Myocardial infarction.

INTRODUCTION

Acute myocardial infarction (AMI) is a disorder that is characterized by presence of myocardial cell necrosis following sustained ischemia [1]. It occurs as an acute manifestation of atherosclerosis of coronary arteries [2]. Isoproterenol (ISO), a □-adrenergic agonist. **At high doses, it increases heart rate and cardiac contractility causing myocardial ischemia due to imbalance between oxygen demand and supply [3]**. Also, many metabolites produced from ISO caused oxidative stress [3]. These cardiac changes with ISO are similar to human MI [4]. Acute kidney injury (AKI) showed a sudden (within hours) reduction in kidney function [5]. A higher mortality rate was detected in AMI patients complicated with AKI [6]. Erythropoietin (EPO) is a glycoprotein hormone that is secreted by the fetal liver and after birth, its production becomes mainly from the kidney [7]. Hypoxia is the main stimulus for EPO production increasing erythropoiesis that improved active tissues oxygen supply [8]. EPO is a tissue-protective hormone that can prevent tissue destruction by signaling through a non-hemopoietic receptor protecting tissues from ischemic injury [9]. As the available data about the role of EPO on kidney function in MI were scarce, this study was done to declare cardiac and renal changes occurred in a rat model of MI, the possible protective role of EPO, and the involved mechanisms.

MATERIALS AND METHODS

Twenty-seven adult healthy male albino rats weighing 100- 150 g, were purchased from Zagazig Faculty of Veterinary Medicine **Animal house**. **In physiology department (postgraduate research lab)**, rats were kept in steel wire cages (50x30x20 cm), under hygienic conditions, 4-5 rats per cage, on

ordinary diet purchased from Zagazig Faculty of Agriculture. All rats were kept at a comfortable temperature (20 to 24 ºC), had free access to water and maintained on normal light-dark cycle. Before starting the study, the animals were acclimated to animal house conditions for 2 weeks. Physiology Department Committee and the Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC) approved the experimental protocol (ZU-IACUC/3/F/67/2018).

Experimental Design:

Twenty-seven male adult albino rats were divided randomly and equally into 3 groups [control, myocardial infarction (MI) and MI pre-treated with EPO]. In control and MI groups, **2 ml normal saline were injected subcutaneously once every other day for seven days**. In the 6th and 7th days of the study, rats in MI group were injected subcutaneously with isoproterenol (ISO) hydrochloride (150 mg/kg body weight /day) [10], dissolved in normal saline, to induce MI. In MI pretreated with EPO group, rats were injected subcutaneously with 1000 IU/kg once every other day [11] for 7 days and MI was induced as in MI group.

One hour after the 2nd dose of ISO injection, electrocardiogram (ECG) was recorded by the use of invasive ECG monitor [Power Lab 4/20 (data acquisition system, AD Instruments Pty Ltd, Australia)]. After intraperitoneal anesthesia using sodium thiopental 60 mg/kg [12], each rat was put on a non-electrically conductive flat surface. To record ECG, 3 bipolar leads were used (negative, positive and reference ECG electrodes were put at the right foreleg, left foreleg, and left thigh, respectively). The results were automatically calculated depending on voltage calibration (millivolts).

24-hour urine collection was done in the 7th day of the study. Each rat was housed with free access to water in a metabolic cage. A funnel of a suitable size was arranged for urine collection at the bottom of the metabolic cage, and to retain fecal matter, a perforated plastic disc is in the funnel. At the bottom of the funnel, the 24-hour urine sample was collected for the rat in a beaker that was arranged, and centrifuged for 10 minutes at 3000 rpm. The supernatants were transported into another set of dry and clean tubes and stored at -20°C until the time of analysis.

From the retro-orbital plexus using microhematocrit tubes, blood samples were collected at the end of the experiment under sodium thiopental anesthesia (60mg/kg intraperitoneally) [13], left to clot at room temperature for 30 minutes and then, centrifuged for 15 minutes at 3000 rpm. The clean, clear sera were separated using automatic pipettes, and stored until analysis in Eppendorf tubes at -20˚C.

At Zagazig Faculty of Medicine Biochemistry Department, chemical analysis was done for estimation of serum tumor necrosis factor-alpha (TNF-α), serum and urine creatinine, serum; malondialdehyde (MDA), reduced glutathione (GSH), interleukin-6 (IL-6), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH).

Creatinine clearance (ml/min) = [Urine creatinine (mg/dl) x Urine volume (ml/day)]/ [Serum creatinine (mg/dl) x Time (min)] [14].

- **Histopathological examination:**

Deparaffinated tissue sections (heart and kidney) were stained with Hematoxylin and Eosin dyes (H&E) and examined for morphological analysis of the kidney and to asses pathological changes. After complete preparation, images were analyzed under optical/light microscope at magnifications of 20 to 40×10 by an experienced investigator pathologist.

- **Immunohistochemical study (Immunohistochemical examination of Bax):**

Immunohistochemical study was done in cooperation with Zagazig Faculty of Medicine Pathology Department. After decapitation, cardiac and renal specimens **were preserved** and prepared for immunohistochemical study. Using serial sections (4 μm thickness) of cardiac and renal paraffin blocks, Bax immunostaining was done.

Kits were purchased from:

ISO was purchased from Sigma-Aldrich (St Louis, USA), (catalog No. I6504)

EPO was purchased from SEDICO. From Sigma-Aldrich (St Louis, USA), commercial kits were purchased for estimation of; serum tumor necrosis factor-

alpha (TNF-α) (catalog No. MBS355371) and both serum and urine creatinine (catalog No. MAK080). Also, from Biodiagnostic-Egypt, kits were purchased for estimation of serum; malondialdehyde (MDA) (catalog No. MBS738685), reduced glutathione (GSH) (catalog No. MBS724319), interleukin-6 (IL-6) (catalog No. MBS355410), creatine kinase-MB (CK-MB) (catalog No. MBS2515061) and lactate dehydrogenase (LDH) (catalog No. MBS269777).

Statistical Analysis: The obtained data were presented as mean ± standard deviation (SD). IBM SPSS Statistics Software (Version 26 for Windows) was used to perform one-way analysis of variance (ANOVA) and Tukey HSD for post hoc multiple comparisons to compare means. With P value ≤ 0.05, significance was considered.

RESULTS

A- R wave amplitude (mV) and ST- segment depression (mV) changes one hour after the second dose of ISO: (Table-1)

ST-segment depression amplitude was significantly increased, while R wave amplitude was significantly reduced in MI group when it is compared with that in control group. These changed were reversed with the use of EPO. **In MI pretreated with EPO group, ST-segment depression amplitude was significantly increased and R wave amplitude was significantly decreased when it is compared with that in control group but when it is compared with that in MI group, ST-segment depression amplitude was significantly decreased and R wave amplitude was significantly increased.**

B- Serum cardiac enzymes changes: (Table-2)

CK-MB and LDH serum levels were significantly increased in MI group when compared with that in the control group and this change was reversed with the use of EPO. **In MI pretreated with EPO, CK-MB and LDH serum levels were significantly increased when compared with that in the control group while they showed significantly decreased when compared with that in MI group.**

C- Kidney function changes: (Table-2)

Serum creatinine was significantly elevated, while urine creatinine and creatinine clearance were significantly diminished in MI group when compared with that in the control group. These changes were reversed with the EPO treatment. **In MI pretreated with EPO group, Serum creatinine was significantly increased, while urine creatinine and creatinine clearance were significantly decreased when compared with that in the control group but when compared with MI group, serum creatinine was significantly decreased, while urine creatinine and creatinine clearance were significantly increased.**

D- Serum inflammatory and oxidative stress markers changes: (Table-2)

TNF-α, IL-6 and MDA serum levels were significantly increased, while serum GSH was significantly reduced in MI group when compared with that in control group. These changes were ameliorated with EPO treatment. **In MI pretreated with EPO group, TNF-α, IL-6 and MDA serum levels were significantly increased, while serum GSH was significantly decreased when compared with that in control group. In other hand, when compared with MI group, TNF-α, IL-6 and MDA serum levels were significantly decreased, while serum GSH was significantly increased.**

E-Immunohistochemical study:

- Cardiac immunohistochemical study declared minimal Bax immunostaining in control group (Fig. 1a), enhanced Bax immunostaining in MI group (Fig. 1b) and a decrease in Bax immunostaining in MI pretreated with EPO group (Fig. 1c).
- Renal immunohistochemical study declared minimal Bax immunostaining in control group (Fig. 2a), enhanced Bax immunostaining in MI group (Fig. 2b) and a decrease in Bax immunostaining in MI pretreated with EPO group (Fig. 2c).

F- Histopathological study:

- **Cardiac histopathological study in control group (Fig. 3a) revealed normal architecture of cardiac wall with branching and anastomosing myofibers bounded with endomysium, cardiomyocytes have central oval, euchromatic nuclei (black arrow) and**

flat nuclei of fibroblasts (green arrow) (H&E x400), Photomicrographs of MI group (Fig. 3b) showing pyknotic (apoptotic) nuclei (black arrow), vascular changes (green arrow), inflammation (yellow arrow) (H&E x400), and Photomicrographs of MI pretreated with erythropoietin group (Fig. 3c) showing pyknotic (apoptotic) nuclei (green arrow) and moderated inflammation (yellow arrow) (H&E x400).

- **Renal histopathological study revealed normal glomeruli and tubules (H&E x400) in control group (Fig. 4a), Photomicrograph of MI group (Fig. 4b) revealed tubular necrosis (yellow arrow) and thyroidization (green arrow) (H&E x100), and in MI pretreated with erythropoietin group (Fig. 4c) photomicrograph showed mild tubular necrosis (yellow arrow) (H&E x400).**

Table-1: R wave amplitude (mV) and ST- segment depression (mV) changes one hour after the second dose of ISO (9 rats/group)

Groups Parameters	Control	MI	MI pretreated with erythropoietin
ST- segment depression (mV)	0.03±0.002	0.16±0.03[a]	0.07±0.003[a&b]
R- wave amplitude (mV)	0.73±0.03	0.38±0.011[a]	0.56±0.04[a&b]

- Data were expressed as mean ± SD. [a]P<0.05 in comparison with control group. [b]P<0.05 in comparison with MI group. MI, myocardial infarction.

- **Table-2: Biochemical changes in the different groups** (9 rats/group)

Groups Parameters	Control	MI	MI pretreated with erythropoietin
Serum CK-MB (ng/ml)	2.43±0.71	61.74±4.43[a]	36.86±3.62[a&b]
Serum LDH (IU/L)	189.42±6.09	275.13±2.79[a]	252.65±2.89[a&b]
Serum creatinine (mg/dl)	0.81±0.07	5.54±0.41[a]	2.01±0.27[a&b]
Urine creatinine (mg/dl)	56.61±2.25	22.11±1.96[a]	44.35±2.03[a&b]
Creatinine clearance (ml/min)	0.28±0.049	0.0048±0.0012[a]	0.058±0.009[a&b]
Serum MDA (ng/ml)	10.39±1.95	34.78±3.60[a]	25.50±4.41[a&b]
Serum GSH (ng/ml)	20.39±1.95	2.06±0.24[a]	4.62±0.74[a&b]
Serum IL-6 (pg/ml)	1.37±0.28	14±2.73[a]	4.24±1.02[a&b]
Serum TNF-α (pg/ml)	1.58±0.32	20.88±5.06[a]	8.21±1.92[a&b]

- Data were expressed as mean ± SD. [a]P<0.05 in comparison with control group. [b]P<0.05 in comparison with MI group. CK-MB, creatine kinase myoglobin binding; MI, myocardial infarction; MDA, malondialdehyde; GSH, reduced glutathione; LDH, lactate dehydrogenase; TNF-α, tumor necrosis factor alpha; IL-6, interlukin-6.

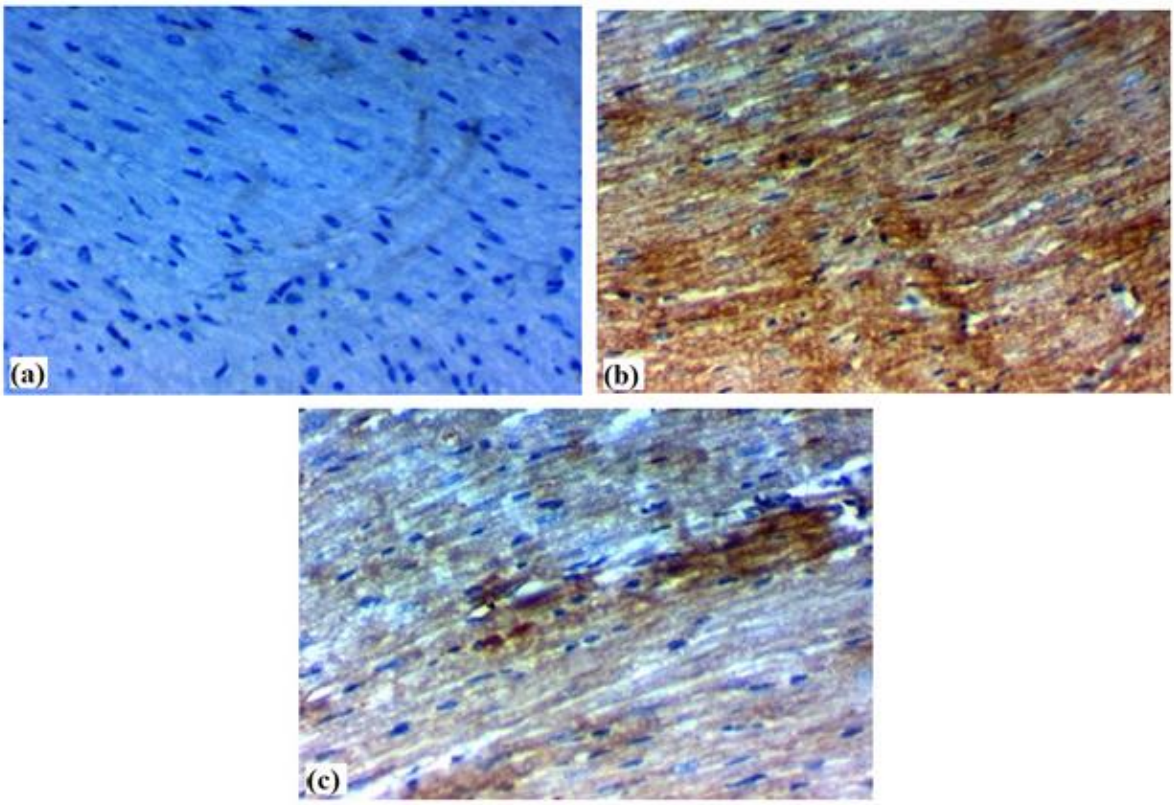

Fig. 1 Cardiac immunohistochemical study (IHC x400). (a) Control group with minimal Bax immunostaining. (b) MI group with enhanced Bax immunostaining. (c) MI pretreated with EPO group declared a decrease in Bax immunostaining.

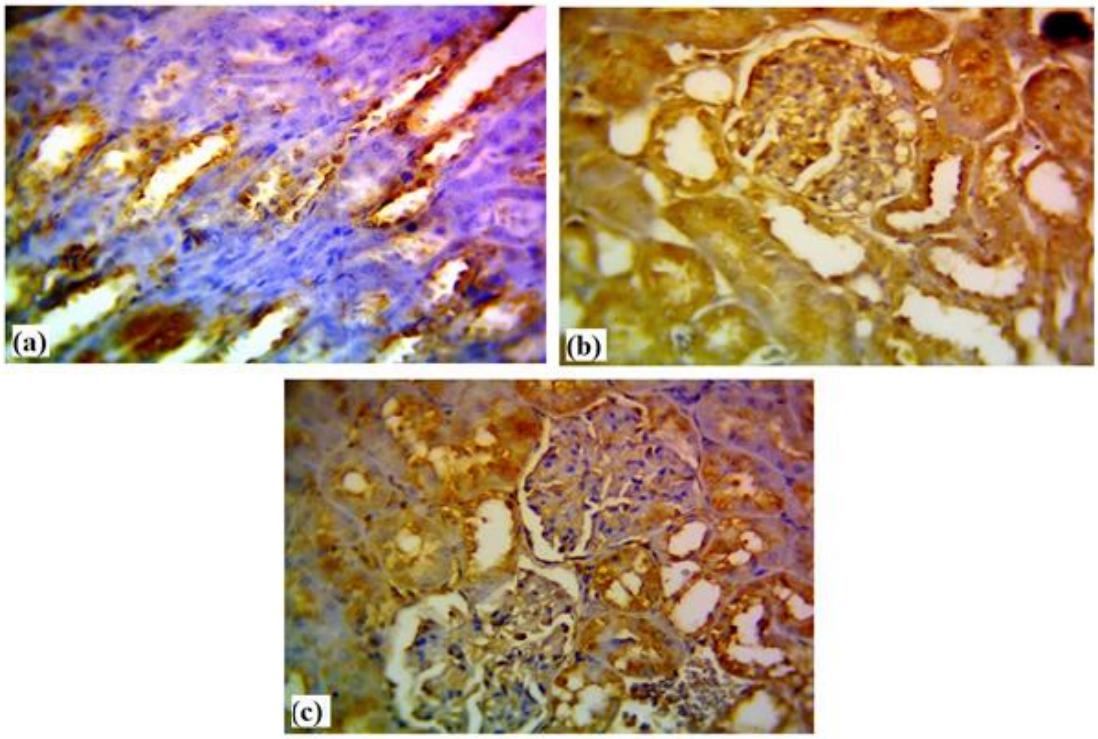

Fig. 2 Renal immunohistochemical study (IHC x400). (a) Control group with minimal Bax immunostaining. (b) MI group with enhanced cytoplasmic Bax immunostaining (c) MI pretreated with EPO group declared a decrease in cytoplasmic Bax immunostaining.

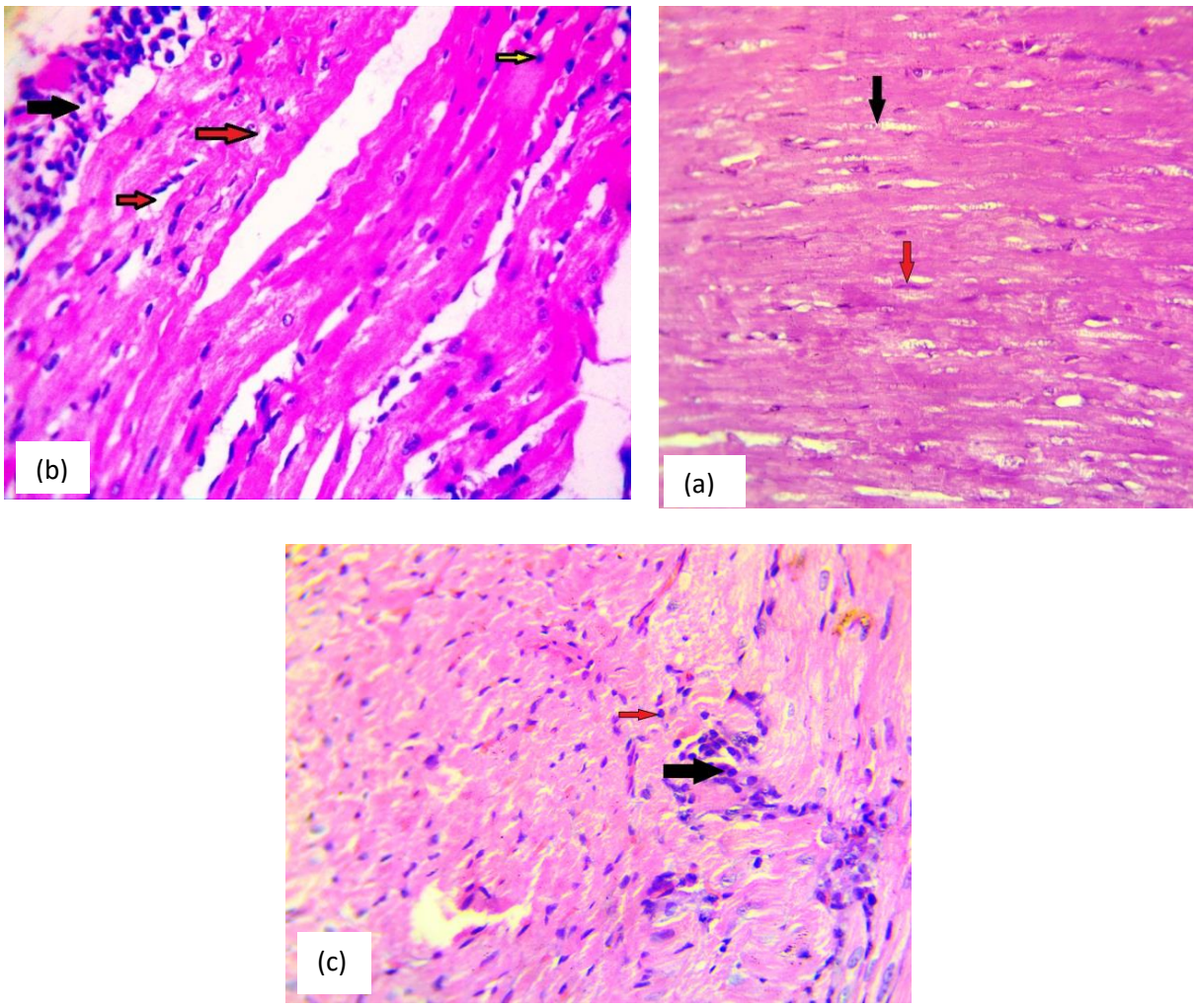

Figure-3: Photomicrographs of histopathological examination of the heart in different studied groups in experiment-1. (a) Control group; (b) MI group; (c) MI pretreated with erythropoietin group (H&E X400).

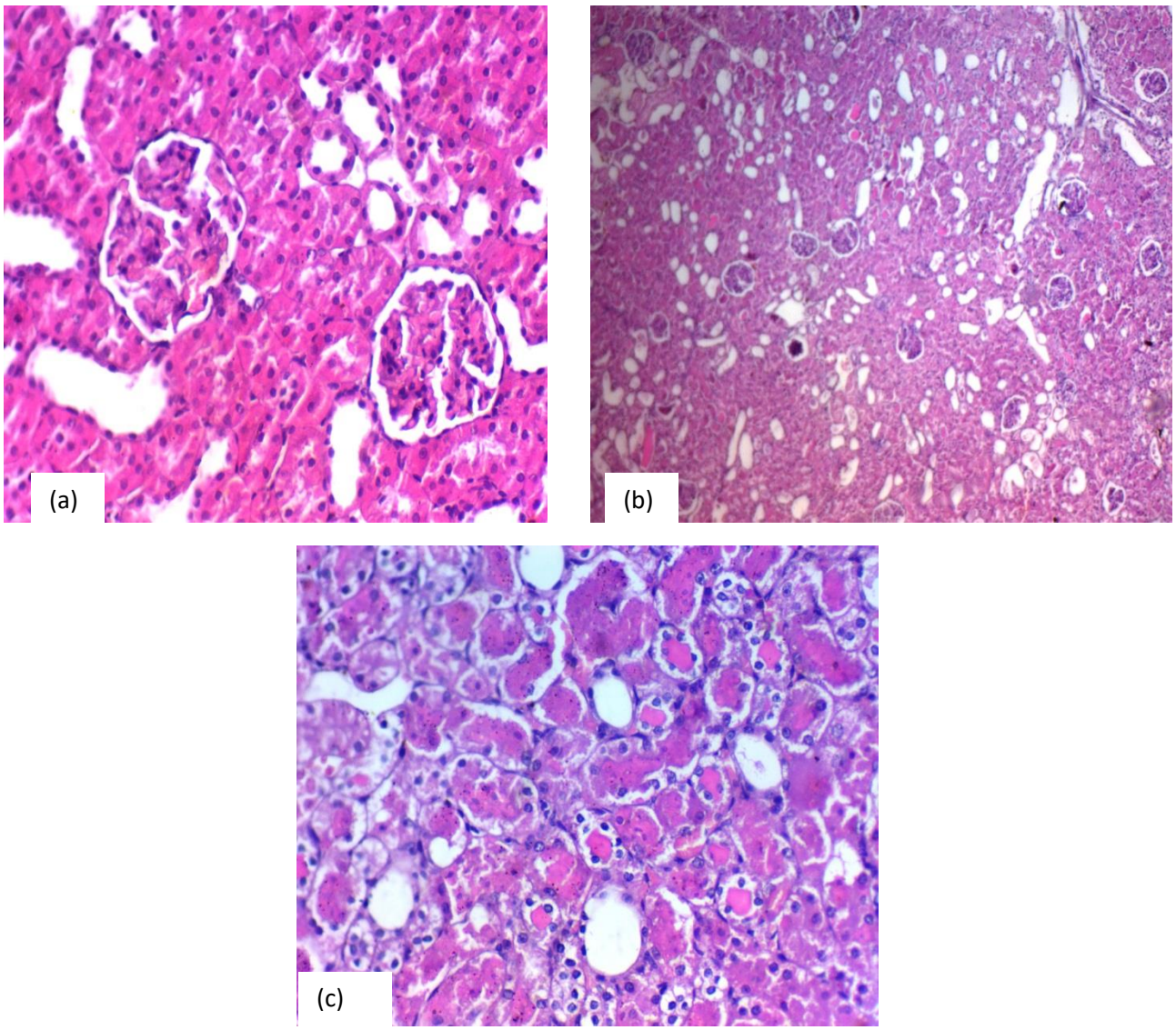

Figure-4: Photomicrograph of histopathological examination of the kidney in different studied groups in experiment-1. (a) Control group; (b) MI group; (c) MI pretreated erythropoietin group.

DISCUSSION

This study was planned to evaluate the possible protective role of erythropoietin on cardiac and renal function changes in a rat model of myocardial infarction. The results of this study declared that erythropoietin had a protective role on cardiac and renal function in myocardial infarction induced by isoproterenol in rats possibly through its anti-inflammatory, antioxidant and antiapoptotic actions.

The induction of myocardial infarction in MI group was confirmed by ECG changes (increased depression in S-T segment and decreased R wave amplitude) and elevated serum cardiac enzymes (CK-MB and LDH) which was in line with Awada et al. [15], Yang et al [16] and Allijn et al. [17]. Awada et al. [18] declared that S-T segment depression was due to electrical potential difference between ischemic and normal areas which causes current flow from depolarized ischemic to normal regions.

Yang et al. [19] reported that depression of R wave amplitude was owed to the myocardial edema induced by myocardial infarction and they correlate the R wave amplitude with the infarct size. Allijn et al. **[20]** confirmed that cardiac enzymes (CK-MB and LDH) leaked out from the part of cardiac muscle which was damaged.

The current study declared occurrence of acute kidney injury with myocardial infarction as there was a significant increase in serum creatinine with a significant reduction in urine creatinine and creatinine clearance in MI group which was in agreement with Bruetto et al. [21]. The possible mechanisms involved in renal function changes with myocardial infarction could include inflammation and oxidative stress as evidenced by the significant elevation in serum pro-inflammatory cytokines (TNF-α and IL-6), presence of oxidative stress (elevated serum MDA and reduced serum GSH), **histopathological examination of heart showed vascular and inflammatory changes this was in line with Tian et al [22]. , also renal tissue in MI group showed tubular necrosis as supported by Ozbilgin et al [23],** and increased Bax immunostaining in MI group which was in line with Del Vecchio et al. [24] and Cases et al. [25].

Del Vecchio et al. [26] found that IL-6 and TNF-α were produced by cardiomyocytes and local myocardial mononuclear macrophages activated by myocardial ischemic tissue in the infarcted area. Cases et al. [27] confirmed that oxidative stress was due to action of ISO which enhanced generation of oxygen free radicals and cell membrane lipid peroxidation causing increased serum MDA and decreased GSH that was incorporated in cellular protection against damaging effects of oxygen free radicals.

With erythropoietin myocardial infarction pretreatment, cardiac and renal changes were ameliorated with concomitant improvement of inflammation, oxidative stress, **decreased inflammation in histopathological study** and Bax expression in immunohistochemical study as evidenced by significant changes in inflammatory and oxidative stress markers and reduction in Bax immunostaining which confirmed the possible mechanism of action of EPO as anti-inflammatory, anti-oxidant and antiapoptotic hormone which was in agreement with Elliott and Sinclair [28] and Hand and Brines [29].

Limitations include that the results may be different from a study on human, small sized sample and the short duration of EPO pretreatment.

CONCLUSION

Erythropoietin has cardiac and renal protective effects in a rat model of acute myocardial infarction which could be owed to its antioxidant, anti-inflammatory and antiapoptotic roles.

RECOMMENDATION

This study was done on rats; thus, we hope to be conducted on human. Also, the number of rats in each group was small and need to be increased. Furthermore, this study was of short duration and long-term therapy was not considered so in future studies long- term therapy with longer duration would be considered.

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