International Scholars Journals

International Journal of Histology and Cytology ISSN 2447-9535 Vol. 5 (8), pp. 456-465, December, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

The effect of Erythropoietin versus Estrogen on the renal parenchyma of an animal model of renal ischemia reperfusion plus ovariectomy: Comparative Histomorphological study

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Accepted 07 December, 2018

Renal ischemic / reperfusion injury (IR) remains a major problem in clinical practice. Erythropoietin is evaluated as a nephroprotective agent. However, its nephroprotective effect when it is co-administered with estrogen is doubtful in females. The objective of this study was to compare the effect of erythropoietin and estrogen either alone or combined in renal (IR) ovariectomized rats. Sixty female rats were divided into six groups. Group I: control. Group II: Female rats exposed to IR. Group III: ovariectomized rats exposed to IR. Group IV: ovariectomized rats received estrogen then exposed to IR. Group V: ovariectomized rats received erythropoietin before IR. Group VI: ovariectomized rats received estrogen then received erythropoietin before IR. Serum creatinine and blood urea nitrogen were measured. Kidney specimens were processed for light and electron microscopic examination. Group II and III showed degeneration of cells of the tubular lining cells with tubular dilatation. Electron microscopy showed marked ultrastructural changes in the renal tubules. Group IV, group V showed improvement in light and electron microscopic changes. Combined estrogen and erythropoietin (group VI) led to a significant decrease in erythropoietin protection in renal IR injury. Erythropoietin could protect against IR, however, minimal effect was obtained when combined with estrogen.

Keywords: Renal ischemia–reperfusion, estrogen, erythropoietin, ovariectomy.

ABBREVIATIONS

- IR, ischemia–reperfusion;
- E, estrogen;
- BUN, blood urea nitrogen;
- NO, nitric oxide;

INTRODUCTION

Renal ischemia reperfusion (IR) is a major leading cause of acute kidney failure and is still considered an important clinical issue. It is associated with high morbidity and mortality rates although great medical care was introduced to these patients (Siedlecki, Irish and Brennan, 2011). IR is considered an intra-renal cause of inadequate kidney perfusion occurring on a microvascular scale. It can occur in various clinical condi-

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tions such as shock with subsequent resuscitation and renal transplantation (Chen and Busse, 2017).

There is a great evidence suggesting that sex hormones have an important role in IR-induced inflammatory process in the kidneys, with rapid progression of non-diabetic kidney diseases, in males compared to females (Kang *et al.*, 2014). The full details of the cellular and molecular mechanisms of these differences are still unclear, and they involve genetic and non-genetic background of sex hormones, particularly estrogen (Ostadal *et al.*, 2009).

Inflammation is a key factor of the pathogenesis of renal IR injury (Ferenbach, Kluth and Hughes, 2007). Ischemia interferes with the continuous oxygen supply required for tissue survival and maintenance of their physiological functions (Banaei, Ahmadiasl and Alihemmati, 2016). As well, when reperfusion occurs, additional renal reperfusion-related injury occurs which involves the development of inflammation mediated oxidative stress via the generation of reactive oxygen species such as superoxide anions (O2-) and generation of reactive nitrogen species such as nitric oxide (NO) and peroxynitrite (OONO-) (Masztalerz *et al.*, 2006). The excessive generation of these reactive species induces lipid peroxidation, inactivation of antioxidant enzymes, disruptions of the cellular cytoskeleton, cellular integrity, DNA breakdown, leukocyte activation, endothelial cell damage, with the end result tissue damage (Kim, Jang and Park, 2010).

Estrogen is a female sex hormone that is reported to have renoprotective effects. Estrogen attenuates glomerulosclerosis and tubulo-interstitial fibrosis and other forms of chronic kidney diseases (Petrica, Gluhovschi and Velciov, 2012)

Erythropoietin (EPO) is an essential growth factor of hemopoiesis, having a possible extrahemopoietic therapeutic effects such as antioxidant, anti-apoptotic, pleiotropic, and anti-inflammatory effects (Arcasoy, 2008). Several experimental studies are being researched regarding the protective effect of EPO against myocardial ischemia, liver, and renal injuries (Chatterjee, 2007). Moreover; there is an existing sex difference in the endogenous erythropoietin being higher in males than in females (Prókai *et al.*, 2011).

It is reported that estrogen inhibits production of EPO in the female rat model of cisplatin-induced nephrotoxicity plus ovariectomy (Pezeshki *et al.*, 2012). As a result, the possible protective effect of EPO against IR may be affected when estrogen is co-administered with EPO. Therefore, this study was conducted to compare the effect of erythropoietin and estrogen both alone and in combination on the kidney tissue during renal IR in ovariectomized rats and to confirm the nephroprotective role of EPO in renal IR when estrogen is co-administered.

MATERIALS AND METHODS

- **Animals**

Sixty adult mature female rats weighing about 200 – 250 g were used in this study. Rats were fed on a standard laboratory diet and water ad libitum. For acclimatization purposes, the animals were left for two weeks prior to inclusion in the experiment.

- **Experimental design**

- The female rats were randomly divided into six groups, ten rats each: -

Group I: control sham-operated rats, rats receive only Sesame oil by subcutaneous (sc) route for 3 weeks, then exposed to sham operation (midline laparotomy incision and dissection of renal pedicles without any renal ischemia).

Group II: IR group**,** rats received Sesame oil by sc route for 3 weeks then exposed to bilateral renal IR; ischemia was produced for 45 min, followed by 24 hours reperfusion.

- The remaining 40 rats had undergone bilateral ovariectomy (OVR), and one week after recovery, the rats received Sesame oil (group III), Estrogen (group IV), EPO (group V) and both (group VI) for 3 weeks then exposed to IR. The rats were distributed as follows:

Group III: OVR + IR group, Sesame oil sc.

Group IV: OVR + E + IR group**,** estrogen (25 µg/kg/day; sc) (Yu *et al.*, 2009).

Group V: OVR + EPO+ IR group**,** EPO 5000 U/kg single dose IP was given 20 min before ischemia (Ahmadiasl, Banaei and Alihemmati, 2013).

Group VI: OVR + E + EPO + IR**,** both estrogen & EPO.

- **Drugs & supplements**

Estrogen (FOLONE ampoules), each 1 ml ampoule contains 5 mg estradiol benzoate in oily solution, was purchased from Misr Co., for Pharm. ind. S.A.E. (Cairo, Egypt). Sesame oil was purchased from Indian Co. (Cairo, Egypt). Erythropoietin (ERYPRO SAFE 5000 i.u) was purchased from Biocon (India) Ltd., from Pharma Co. Thiopental sodium was purchased from Eipico Co. (Cairo, Egypt).

- **Preparation of estrogen supplements**

Estrogen, in the form of FOLONE 1ml ampoules (5 mg estradiol benzoate/ml) is an oily solution. The injectable solution was prepared by dissolving 1 ml of estradiol benzoate in 36 ml ethanol-sesame oil to obtain a concentration of 28 µg E in 0.2 ml final solution. All rat groups except that treated with E, only received Sesame oily solution as a vehicle (0.2 ml/kg/day sc).

- **Ovariectomy operation technique**

The animals were anesthetized by thiopental sodium (30 mg/kg, intraperitoneal injection). The anesthetized rat was placed on the operating theatre in dorsal recumbent position with its tail directed toward the surgeon. The ventral aspect of the lumbar region was shaved, cleaned with 75% ethanol, followed by thorough scrubbing with 10% povidone iodine. Longitudinal one cm long ventral midline incision was done above the symphysis pubis by a scalpel blade; the skin edges were laterally retracted,

and the abdominal muscle layer and the peritoneum were incised. Both fallopian tubes were exposed and ligated; the ovaries can usually be seen embedded in a pad of fat in the abdomen; then the ovaries were removed by cutting them with scissors, taking care not to rupture the ovarian capsules. The remaining tissues were replaced into the peritoneal cavity. Finally, the incision was closed using a sterile 2/0 suture. The removed tissue was ensured to be the ovaries by histological sections. The rats were kept in their cages and left for about four weeks waiting for IR (Flores *et al.*, 2008).

- **Induction of renal IR injury**

The animals were anesthetized by thiopental sodium, as mentioned before. A Midline abdominal incision was done and both kidneys were exposed. The renal pedicle was occluded for 45 min by applying non-traumatic vascular clamps to induce ischemia followed by 24 hours reperfusion. Occlusion was confirmed by a significant blanching of the kidney color and a return to a red shade, within one minute upon reperfusion. After the surgical procedures, the midline incision was sutured followed by the local application of povidone iodine solution. At the end of the reperfusion period, the animals were anesthetized again, blood sample was taken, stored at −20 °C until measurement, and then the animals were killed by decapitation. Both kidneys were removed and fixed in 10% formalin solution for histological assessments. The same surgical procedures were done in sham-operated group, but without applying the clamps (Talebi *et al.*, 2016).

- **Mortality and postoperative care**

The animals were left in airy room until recovery from anesthesia, then housed in hygienic cages with suitable temperature, and were allowed free access to water and food. Five rats died during the surgical procedures due to intraoperative bleeding and were replaced by other animals. No post-operative mortality was observed.

- **Evaluation of renal functions**

Blood samples were sent for laboratory assay, for measuring BUN and serum creatinine using the standard procedures and diagnostic kits (Oh and Briefel, 2017).

- **Histological procedures**

For light microscopy, specimens were obtained from both kidneys and fixed in 10% formalin solution and processed for paraffin sections. The tissue sections were stained by hematoxylin and eosin (H & E) to examine the tissue damage based on certain criteria; tubular atrophy, hyaline cast, ischemic necrosis, vacuolization, and debris. The samples were scored as 0–4, according to the damage intensity. 0: means normal (no tissue damage). 1: means low damage (up to 25% of tissue damage), 2: Means mild damage (between 26% and 50% of tissue damage), 3: Means moderate damage (between 51% and 75% of tissue damage) and 4: means severe damage (more than 75% of tissue damage) (Sahu *et al.*, 2014). For electron microscopy, the specimens were collected from both kidneys, processed and semithin sections (0.5–1 µm) were prepared, stained with toluidine blue and examined with a light microscope. Ultrathin sections were then prepared, contrasted with uranyl acetate and lead citrate and then examined with a transmission electron microscope (Dykstra and Reuss, 2011).

- **Statistical analysis**

The values obtained from all groups are expressed as mean ± standard deviation (SD). One-way ANOVA (Analysis of variance) and post hoc LSD test was used (with IBM SPSS software, version 20) to test the difference about mean values of measured parameters among groups. Difference of statistical significance was considered at *P*-value of less than 0.05.

RESULTS

I. Histological results

1. Light microscopic results

Histological examination of H&E stained sections of group I (control group) showed the renal corpuscles, proximal convoluted tubules (PCT), and distal convoluted tubules (DCTs). The renal corpuscle was composed of a glomerulus surrounded by Bowman's capsule with Bowman's space. The glomerulus appeared lobulated with evident glomerular capillaries. The parietal layer of Bowman's capsule was lined by flat squamous cells resting on a thin basement membrane. The PCTs had narrow lumena and were lined by a single layer of pyramidal cells. Their cells had indistinct cell boundaries, an acidophilic granular cytoplasm, rounded basal nuclei, and an apical brush border, whereas the DCTs showed wider lumena and the lining cells were cuboidal, with a faint acidophilic cytoplasm and central rounded nuclei (Fig. 1A).

Group II (IR group) and group III (OVR+IR) showed focal structural changes in the kidney. Some of the renal corpuscles were enlarged, with large glomeruli but most of them appeared small and their glomeruli were small or even atrophic, leaving a wide Bowman's space (Fig. 1B). Renal tubules showed variable degrees of affection. Some of the convoluted tubules (PCTs) appeared with wide lumena. Most of their lining cells showed a vacuolated cytoplasm and a detached apical part (Figs. 1B, 1C). Destruction of their brush borders and cellular

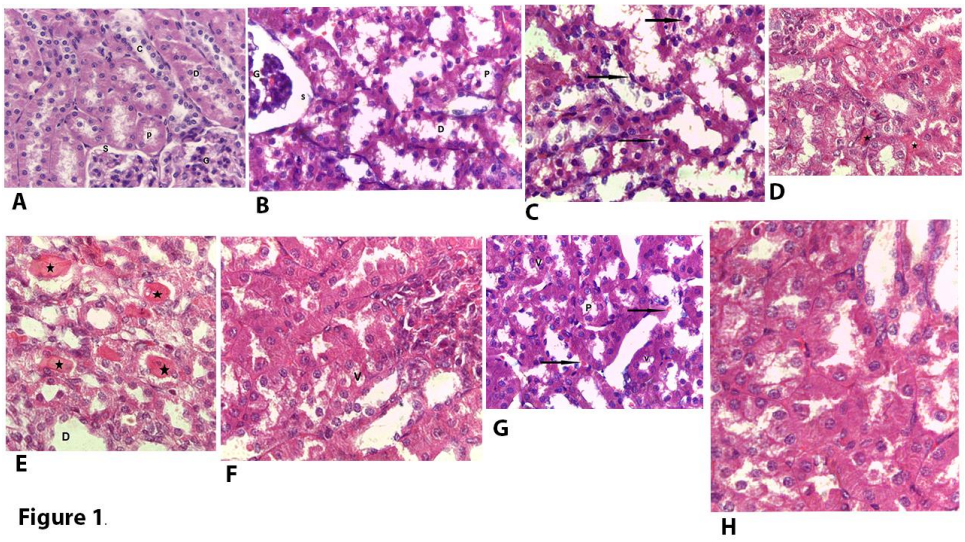

Figure 1.

debris were also observed in the lumena of some tubules. Some of their cells attained pyknotic nuclei (Figs. 1C). Occasional spots of hemorrhage were also observed in between the renal tubules (Figs. 1B, 1C). The cells lining DCTs showed dilatation of their lumena as compared with the control group and contained many sloughed cytoplasmic fragments, pyknotic nuclei and marked cytoplasmic vacuolations (Fig. 1C). The Interstitial spaces and lumena of the proximal convoluted tubules are dilated and contained casts (Figs. 1D, 1E).

In group IV (OVR+E+IR) and group V (OVR+EPO+IR), the kidneys sections relatively retained their normal histological structure. However, the lining cells of few PCTs still showed cytoplasmic vacuolation. Few red blood cells denoting hemorrhage were also observed in between the renal tubules (Figs. 1F, 1G).

While in group VI (OVR+E+EPO+IR), the kidney sections showed mild cytoplasmic vacuolation of the tubular lining cells, sloughing of the proximal & distal convoluted tubules and destruction of their apical brush borders (Fig.1H).

Figure 1: Photomicrographs of H & E stained kidney sections (magnification, X400) representative to the studied groups. In control group (A), renal glomeruli (G) and proximal (P) & distal (D) convoluted tubules are of normal morphology. IR group (B, C) shows atrophic glomeruli (G) and wide Bowman's space (s). Proximal (P) and distal (D) convoluted tubules show wide lumena, with their lining cells exhibiting vacuolated cytoplasm, detached apical part and destroyed brush borders. Most of their nuclei are pyknotic (arrows). OVR + IR group (D, E) shows vacuolated cytoplasm of the cells lining proximal & distal tubules with loss of their brush borders. Casts are seen within renal tubules and in between them (stars). OVR + E + IR group (F) shows partial restoration of the renal tubules & hemorrhagic spots. OVR + EPO + IR group (G) shows more or less restoration of the renal tubules with still vacuolations & hemorrhagic spots (arrows) are seen. OVR + E + EPO + IR group (H) shows vacuolations and sloughing of the lining cells of the renal tubules.

2. Electron microscopic results

Electron microscopic examination of a control rat (group I) revealed the PCTs showing basal euchromatic spherical nuclei. The cytoplasm contained small electron-dense lysosomes. Numerous basal elongated mitochondria are seen alternating with the basal membrane infoldings. The apical surface showed many long microvilli projected toward the lumen (Fig. 2A). The DCTs showed low cuboidal lining epithelium with rounded nuclei and few blunt apical microvilli. Elongated basal mitochondria alternating with numerous basal membrane infoldings were seen (Fig. 2B).

In group II (IR group) & group III (OVR + IR), the PCTs were markedly affected showing multiple cytoplasmic vacuolations. The basal regions showed an apparent decrease in mitochondria which appeared swollen, and irregularly arranged with disrupted basal membrane infoldings. The mitochondria were swollen, rounded. Some cells had short and irregular microvilli on their apical surfaces (Figs 2C, 2E). The DCTs showed loss of their microvilli with extrusion of their apical cytoplasm into the tubular lumen. The mitochondria were small, degenerated, and irregularly arranged. An absence of the basal membrane infoldings and many cytoplasmic vacuoles were also observed (Figs 2D and 2F). The lining cells of both PCTs & DCTs showed some degenerative changes with cytoplasmic rarefaction (Figs. 2D, 2E, 2F).

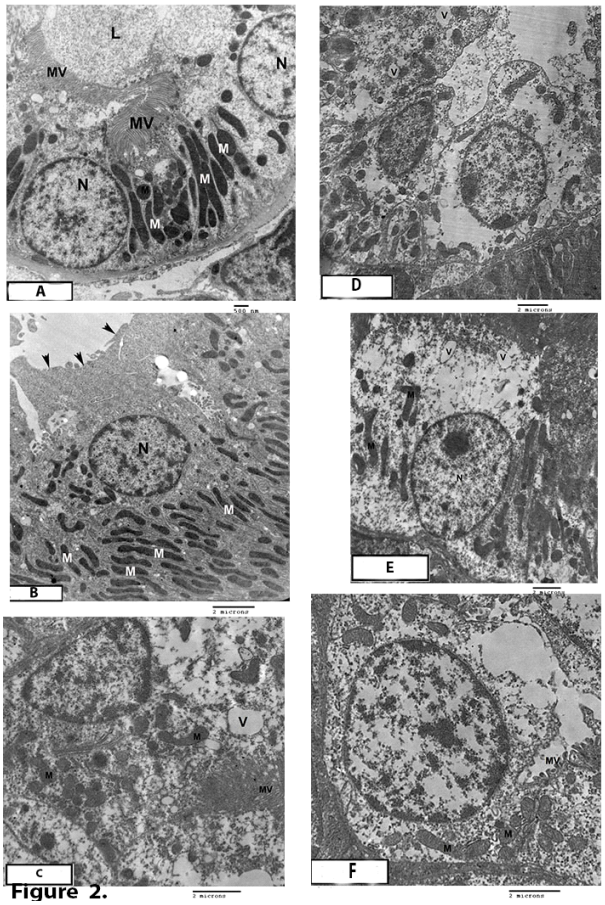

Figure 2.

In group IV (OVR + E + IR) group V (OVR + EPO + IR), the PCTs retained their normal architecture, except for some cytoplasmic vacuoles and numerus lysosomes (Figs. 3A, 3C). The DCTs showed some cytoplasmic vacuoles. The mitochondria were more or less normal but still did not restore their normal arrangement (Figs. 3B, 3D).

In group VI (OVR + E + EPO + IR), the PCTs showed multiple cytoplasmic vacuolations. The basal regions showed an apparent decrease in mitochondria, with disrupted basal membrane infoldings. The mitochondria were swollen, degenerated, and irregularly arranged. (Fig. 3E). The DCTs showed destruction of their apical microvilli. The mitochondria are small and irregularly arranged. Many cytoplasmic vacuoles were also seen as well as irregular shaped nuclei (Fig. 3F).

Figure 2: Photomicrographs of electron microscopic processed kidney sections representative to the first three groups. In control group (A, B; magnification; 4000, 2000 respectively), the renal tubules are of normal morphology. In IR group (C, D; magnification; 2500, 1500 respectively), the proximal convoluted tubules shows short irregular apical microvilli, large cytoplasmic vacuoles and small sized irregularly arranged mitochondria. The distal tubules show destruction of the apical microvilli, cytoplasmic vaculations, in addition to cytoplasmic rarefications. OVR + IR group (E, F; magnification; 1500, 2500 respectively) shows similar changes to that of the last group in addition to, loss of the basal membrane infoldings.

Figure 3: Photomicrographs of electron microscopic processed kidney sections representative to the next three groups. OVR + E + IR group (A, B; magnification; 2500, 1500 respectively) show partial restoration of the tubular microstructure. OVR + EPO + IR group (C, D; magnification; 2000, 1500 respectively) show more or less retained tubular histology. OVR + E + EPO + IR group (E, F; magnification; 2500, 2000 respectively) shows loss of the basal infoldings, disorganization of the mitochondrial arrangement and appearance of many cytoplasmic vacuoles in the proximal convoluted tubules. Similar changes are seen in the distal convoluted tubules in addition to, destruction of the apical microvilli.

II. Serum urea and creatinine studied in different groups

Analysis of the data showed that serum creatinine and BUN were significantly increased ($p < 0.05$) in group II (IR group) and group III (OVR + IR group) compared to control

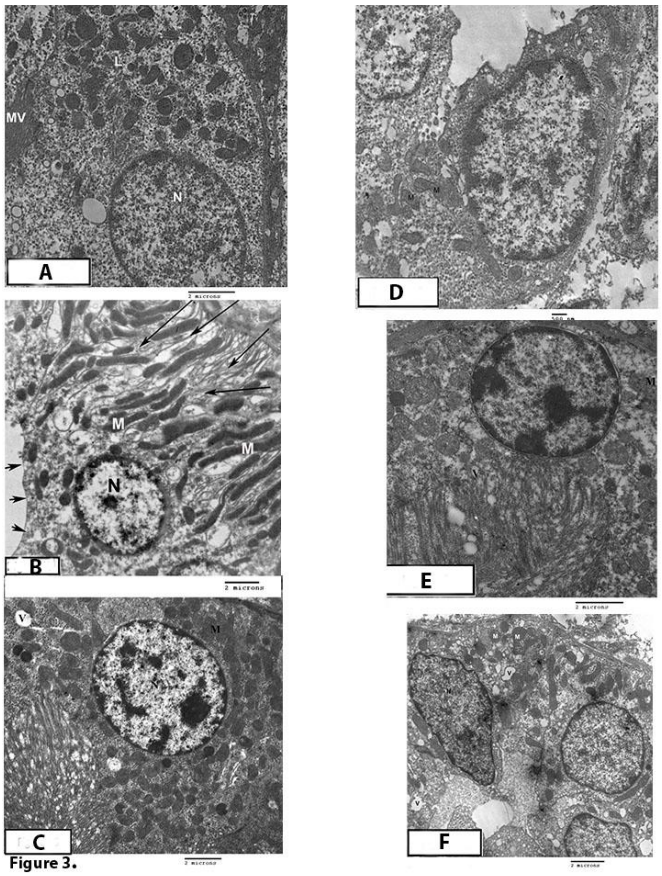

Figure 3.

group. As well, there was significant increase ($p < 0.05$) in serum creatinine and BUN in group III (OVR + IR group) when compared with IR group (group II). Administration of estrogen to ovariectomized rats exposed to IR (group IV) resulted in a significant decrease ($p < 0.05$) in serum creatinine and BUN when compared to OVR + IR group (group III). Administration of EPO to ovariectomized rats exposed to IR (group V) resulted in a significant decrease ($p < 0.05$) in serum creatinine & BUN when compared to OVR + IR group (group III) and OVR + E+ IR group (group IV).
Administration of EPO and estrogen to ovariectomized rats exposed to IR (group VI) resulted in a significant decrease ($p < 0.05$) in serum creatinine & blood BUN when compared to OVR + IR (group III) and when compared to OVR + E + IR group (group IV). Moreover, it resulted in significant increase ($p < 0.05$) in serum creatinine & BUN when compared to OVR + EPO + IR group (group V). (Table 1).

III. Histomorphological evaluation of the renal tissue of ovariectomized rats exposed to renal IR.

The data obtained, in Table 2, showed significant increase ($p < 0.05$) in renal tissue damage in IR (group II) and group III (OVR + IR) when compared to the control group. There was significant decrease ($p < 0.05$) in groups (IV, V) when compared with group III. There was significant decrease in group V when compared with group III ($p < 0.05$). There was significant increase ($p < 0.05$) in renal tissue damage of group VI when compared with group IV & group V ($p < 0.05$).

DISCUSSION

Renal ischemia/reperfusion is a common cause of acute kidney injury, which can be complicated by chronic kidney disease if untreated properly (Ferenbach and Bonventre, 2016). This notion supported the findings of the present study, which demonstrated that renal IR induced serous alterations in the kidney of ovariectomized rats, which were protected against by EPO. However the co-administration of estrogen with EPO abolished the protective effect of the latter, which was confirmed by the histological results as well as the results of the renal functions. Different processes and mechanisms are encountered to disturb the structural and functional integrity of the kidney after IR. The main mechanisms underlying IR induced renal alterations include oxidative stress, microvascular dysfunctions, imba-

Blood urea nitrogen (BUN) (mg/dl)	Serum creatinine (mg/dl)	Groups
37.2 ± 0.38	0.7 ± 0.05	Group I
67.5 ± 1.4 *	2.9 ± 0.20 *	Group II
88.5 ± 1.3 * ©	4.3 ± 0.15 * ©	Group III
60.2 ± 1.3 #	2.8 ± 0.14 #	Group IV
43.9 ± 0.75 $	1.05 ± 0.15 $	Group V
52.7± 0.95 ¤	2 ± 0.15 ¤	Group VI

Table 1. Mean ± SD of serum creatinine and blood urea in different groups.

***** Significant difference when compared to group I ($p < 0.05$).

© Significant difference when compared to group II ($p < 0.05$).

Significant difference when compared to group III ($p < 0.05$).

$ Significant difference when compared to group III & IV ($p < 0.05$).

¤ Significant difference when compared to group III & IV & V ($p < 0.05$).

Table 2. Mean ± SD of the renal damage score in different groups.

Criteria	Group I	Group II	Group III	Group IV	Group V	Group VI
Tubular vacuolization	1.00±0.17	3.00±0.22	3.40±0.22	2.03±0.16	2.02±0.06	3.68±0.24
Cell detachment	0.65±0.12	3.15±0.20	3.05±0.30	2.06±0.09	2.66±0.19	3.26±0.19
Congestion	0.37±0.16	3.20±0.21	3.40±0.31	2.01±0.23	2.0±0.23	3.58±0.27
Hyaline casts	0.75±0.18	1.80±0.10	1.90±0.12	1.80±0.11	1.89±0.11	1.99±0.18
Interstitial edema & hemorrhage	0.85±0.14	2.30±0.22	2.40±0.24	1.64±0.26	1.74±0.28	2.97±0.29
Total score	3.72±0.46	14.11±0.66 *	14.12±0.60 *	10.02±0.35 ©	10.12±0.57 © #	15.48±0.89 $

*Signifcant difference when compared to group I ($p < 0.05$).

© Signifcant difference when compared to group III ($p < 0.05$).

Signifcant difference when compared to group III ($p < 0.05$).

$ Signifcant difference when compared to group IV & V ($p < 0.05$).

lance of vasoactive substances, and local release of inflammatory mediators (Sharfuddin and Molitoris, 2011).

In the present study, induction of renal IR in non-ovariectomized rats as in group II (IR group) resulted in marked injury of the renal parenchyma with significant elevation of the renal damage score and confirmed by significant elevation of the renal functions. Sloughing of the tubular lining cell with detachment of their apical borders and vacuolization of their cytoplasm are important finding denoting damage, in addition to the EM ultrastructural changes such as loss of apical microvilli, loss of cellular polarity and organization, disorganization of the cytoplasmic organelles and loss of the basal membrane infoldings. These results were parallel to the finding of other investigators (Moeini *et al.*, 2013). Severe renal affection leads to apoptotic changes involving the tubular lining cells with more susceptibility to the epithelial lining cells of the PCTs (Havasi and Borkan, 2011).

However, induction of renal IR in ovariectomized rats as in group III (OVR + IR group) leads to significant deterioration of renal functions with significant increase in renal damage score when compared with IR in non-ovariectomized (IR group) rats indicating the protective effect of the endogenous estrogen on renal functions. These results were in accordance with (Barekat, Talebi and Nematbakhsh, 2018) who reported that the renal IR injury was exacerbated by ovariectomy. On the opposite, other studies (Park *et al.*, 2004) demonstrated that depletion of estrogen in female mice by ovariectomy did not affect renal IR induced kidney damage. This could be

explained by the time interval between ovariectomy and renal IR which was 15 days before and not 4 weeks before renal IR as in this study, in addition the difference in animal species might share in this contrast where mice were used, while rats were used in the current study.

The explanation of the renal damage occurring during IR was proposed by (Bonventre and Yang, 2011) who suggested an endothelial dysfunction leading to defective production of NO with the end result arteriolar vasoconstriction and local compromise of the renal microcirculation. In addition, tubular epithelial cells injury was involved. Another explanation for the renal damage during IR was suggested by other authors (Ahmadiasl *et al.*, 2014) who proposed an underlying inflammatory mechanism due to the increase in synthesis of pro-inflammatory cytokine such as TNF-a during renal IR.

In the current study, group IV (OVR + E + IR group) showed better histological results, compared to those of group II (IR group). This finding may reflect that the dose of treated estrogen is higher than that of replacement. In this study, the dose of the used estrogen was 25 µg/kg/day (sc for three weeks), which is higher than the physiological dose used by (Graves *et al.*, 2011) who used 17-β-estradiol-3-benzoate (10 µg in 0.1 ml sesame oil, sc) daily as replacement therapy for 4 days.

The protective effect of estrogen may be related to endothelin- 1(ET-1). (Takaoka *et al.*, 2002) showed that 17- β -estradiol was capable of preventing the renal dysfunction and tissue damage induced by renal IR in male rats by decreasing the renal content of ET-1, a deleterious mediator in the pathogenesis of acute ischemic kidney injury. So, 17- β -estradiol appears to inhibit the enhanced ET-1 production in renal tissues and the consequent renal damage. In contrast, recent studies (Iran-Nejad *et al.*, 2015) reported that estradiol could not protect the male kidney from renal IR injury. As well, other studies encountered NO as an important mediator in renal IR. (Hussien and Emam, 2016) reported severe depletion of NO in kidney tissues after renal IR in ovariectomized rats and its elevation after treatment by estrogen.

In group V (OVR + EPO + IR group), EPO supplementation in ovariectomized rats, at a dose of 5000 U/kg single dose IP, 20 min before ischemia reperfusion showed a significant decrease in the measured renal functions and renal damage score when compared to OVR + IR group or IR group. Our findings are in accordance with numerous investigators (Hu *et al.*, 2012) who reported that administration of EPO protected tissue and organ function in various experimental studies of renal IR.

The protective effect of EPO was suggested by (Sautina *et al.*, 2010) who considered NO as one of the signaling pathways associated with EPO. They reported that EPO stimulates vascular NO production directly or indirectly through stimulation of the endothelial NO synthase and increasing shear stress in endothelial cells.

Other studies (Li and Okusa, 2006) attributed the protective effect of EPO to the ability of EPO to interrupt the complement system and inflammatory pathway activated during the renal IR. Pro-inflammatory cytokine and chemokine production start to increase in damaged tissue. These chemokines attract neutrophils and macrophages to the injured renal tissue and pretreatment with EPO significantly decreases polymorphonuclear leukocyte infiltration. Other study showed that EPO treatment inhibits the inflammatory process in the kidney during renal IR by decreasing the release of inflammatory mediators cytokines (Hu *et al.*, 2012).

Finally, the co-administration of estrogen with EPO in this study revealed significant decrease in the protective effect of EPO when combined with estrogen (OVR + E+ EPO+IR group), compared to the group V that receives EPO only (OVR + EPO+IR group). This indicates that estrogen significantly decreases the renal levels of EPO, with subsequent decrease of its nephroprotective effect. This means that estrogen deficiency induced by ovariectomy lead to increase in renal EPO level, while combination of both estrogen & erythropoietin leads to significant decrease in EPO renal level when compared with EPO + IR + OVR group. These results were parallel to that of other authors (Pezeshki *et al.*, 2012) who reported that estrogen decreases hypoxic induction of plasma EPO, and reduces EPO gene expression in kidneys (Todorov *et al.*, 2000).

CONCLUSION

Estrogen had a protective effect against renal IR induced kidney damage in female rats. Additionally, EPO treatment had a protective role; however, combination of both led to a decrease in the protective effect of EPO less than if administered alone. So, it is recommended to stop or at least decrease the dose of estrogen in postmenopausal females supplemented with EPO in acute renal injury.

ACKNOWLEDGMENT

Great consideration and deep gratitude were expressed to all our colleges in the histology departments, faculties of medicine, Benha and Alfayoum universities for their support and valuable information.

Statement of Ethics

The animal experiments conform to the internationally accepted standards and all ethical issues regarding animal handling and treatment were followed according to the protocol of faculty of veterinary medicine, Benha University, Egypt.

Conflict of interest

The authors have no conflicts of interest to declare.

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