Combination Anti-Apoptotic Effect of Erythropoietin and Melatonin on Ischemia Reperfusion-Induced Renal Injury in Rats

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Received:* 22 *Nov. 2015; Accepted: 17 Apr. 2016

Abstract- Renal ischemia-reperfusion (IR) contributes to the development of acute renal failure (ARF). Oxygen free radicals are considered to be principal components involved in the pathophysiological tissue alterations observed during renal IR. The purpose of this study was to investigate the combination effect of melatonin (MEL) and erythropoietin (EPO), which are a potent antioxidant and anti-apoptotic agents, in IR-induced renal injury in rats. Wistar Albino rats were unilaterally nephrectomized and subjected to 45 min of renal pedicle occlusion followed by 24 h reperfusion. MEL (10 mg/kg, *i.p*) and EPO (5000 U/kg, *i.p*) were administered prior to ischemia. After 24 h reperfusion, following decapitation, blood samples were collected for the determination of superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA) levels. Also, renal samples were taken for histological evaluation and apoptosis assay. Ischemia-reperfusion increased SOD, GPx, MDA levels, and TUNEL positive cells. Histopathological findings of the IR group confirmed that there was renal impairment in the tubular epithelium. Treatment with EPO and MEL decreased SOD, GPx, and MDA levels, histopathological changes, and TUNEL positive cells. These results indicated that the combination of MEL and EPO could not exert more nephroprotective and anti-apoptotic effects than MEL treatment in renal ischemia-reperfusion injury.

Acta Med Iran, 2016;54(10):624-630.

Keywords: Renal ischemia reperfusion; Apoptosis; Melatonin; Erythropoietin; Lipid peroxidation

Introduction

Renal ischemia-reperfusion injury (IRI), which occurs during kidney transplantation, partial nephrectomy, and elective urological operations, is a common cause of acute renal failure (ARF). Ischemia insult, during renal transplantation, is responsible for primary graft dysfunction (1). Reperfusion (re-establishing blood flow) of ischemic renal tissue is highly damaging and initiates a series of cellular events that lead to necrotic and apoptotic cell death. Several mechanisms contributed to the pathophysiology of ischemia-reperfusion injury, such as reactive oxygen species (ROS), ATP depletion and increased neutrophil infiltration (2). Therefore, increased generation of inflammatory cytokines and ROS in the reperfusion phase is believed to play a pivotal role. The excessive production of reactive oxygen and nitrogen species (RNS) after reperfusion results in the expression of genes for pro-inflammatory mediators, the lipid peroxidation of the cellular membranes and oxidative DNA damage, with the subsequent generation of toxic metabolites, causing apoptotic cell death (3).

Lipid peroxidation is related to IR injury-induced tissue damage, and malondialdehyde (MDA) is an indicator of the rate of lipid peroxidation (4). Several anti-inflammatory and antioxidant agents have been explored to be effective in reducing renal ischemia-reperfusion injury (5,6).

Erythropoietin (EPO) is a hypoxia-inducible hematopoietic factor, a key protein in red blood cell production, which is predominantly expressed in the kidney. EPO has multiple protective effects, including antioxidant, anti-inflammatory, and anti-apoptotic effects (7). The biological effects of erythropoietin are mediated by binding to its specific cell surface receptor (EPOR), and the presence of functional EPOR in renal mesangial and tubular epithelial cells has pointed to a

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potential role for erythropoietin in the kidney (8). One important effect of erythropoietin is the reduction in apoptosis and oxidative stress (9). It is also revealed that renal EPO level lowered after renal ischemia-reperfusion (10).

Melatonin (N-acetyl-5-methoxytryptamine) is the major product of the pineal gland that functions as a regulator of sleep, circadian rhythm, and immune function (11). Melatonin (MEL) and its metabolites have potent antioxidant/anti-inflammatory properties and have been proved to be highly effective in a variety of disorders linked to inflammation and oxidative stress (12). MEL not only neutralizes RNS and ROS species but also acts through stimulation of several antioxidative systems and stabilizing cell membranes (13). It modulates the gene expression of several protective enzymes and reduces apoptosis and lipid peroxidation (14).

Therefore, ROS have been shown to contribute to the cellular damage induced by ischemia-reperfusion. The aim of the present study was to examine the anti-apoptotic effect of EPO and MEL on renal IR injury using biochemical, histological parameters, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

Materials and Methods

Animals

In this study, 50 male Wistar Albino rats (weighing 200-300 g) were obtained from the experimental animal research center, Medical Faculty, Tabriz University, Iran. The rats were housed in a temperature (21±2 C) and humidity (60±5%) controlled room in which a 12-12 h light-dark cycle was maintained. They had free access to standard water and food. The study was approved by the University Ethics Committee.

Surgery and experimental protocol

Under anesthesia (75 mg/kg ketamine hydrochloride and 8 mg/kg xylazine, intraperitoneal injection), right nephrectomy was performed and then, the left renal pedicle (artery and vein) was occluded by placing a microvascular clamp for 45 min to induce ischemia and then subjected to reperfusion for 24 h.

The animals were divided into five groups of 10 animals each (n=10):

The sham group of animals underwent the only nephrectomy without occlusion

IR group (ischemic control)

MEL+IR group

EPO+IR group

EPO+MEL+IR group

MEL (10 mg/kg; *i.p*) or vehicle (1% alcohol in saline) was administered 10 min prior to ischemia. MEL (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol and then diluted in saline to give a final alcohol concentration of 1% ethanol.

EPO (Neorecormon, Roche, Mannheim, Germany) was administered as a 5000 U/kg single dose, intraperitoneally 30 min before ischemia.

Biochemical analysis

Blood samples and left kidneys were obtained after 24 h of reperfusion in each group. The SOD and GPx activities in whole blood and plasma levels of MDA, an end product of lipid peroxidation, were measured. The blood samples were centrifuged at approximately 4000 g for 10 min at 4 C. The uric acid level in the serum was determined using the Autoanalyser (Alcyon 300 USA).

Malondialdehyde assessment

Plasma MDA levels were measured using the thiobarbituric acid reactive substances (TBARS) method (15).

Glutathione peroxidase and superoxide dismutase assessment

Whole blood glutathione peroxidase activity was determined using a commercial kit (Ransel; Randox) and expressed as unit per gram of Hb. This method is based on Paglia and Valentine (16). Superoxide dismutase activity was measured in blood samples using the commercially available kit (Ransod; Randox Laboratories Crumlin U.K) (17).

Histological evaluation

The left renal tissues were fixed in 10% buffered formalin solution, dehydrated in ascending grades of alcohol and embedded in paraffin. Sections of 5 μm were taken, stained with hematoxylin-eosin (H&E), and examined under a light microscope (Olympus BH-2, Tokyo, Japan) in a blinded manner by a pathologist. Renal tissues were evaluated in terms of tubular epithelial cell swelling, dilated Bowman's space, glomerular atrophy, congestion, and tubular epithelial pyknotic nuclei. Histological changes were scored on a 4-point scale: (-) none, (+) mild, (++) moderate, and (+++) severe damage (18).

TUNEL assay

TUNEL staining was performed using an In Situ Cell

Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, the sections were de-paraffinized, hydrated by successive series of alcohol, washed with distilled water followed by phosphate-buffered saline (PBS) and de-proteinized by proteinase K (20 µg/ml) for 30 min at 37° C. Then the sections were rinsed and incubated with the TUNEL reaction mixture. The sections were rinsed and visualized using converter-POD with 0.02% 3, 3′-diaminobenzidine (DAB). The sections were counter-stained with hematoxylin. Ten fields were randomly chosen for each slide, then, TUNEL positive cells were counted. All counting procedures were performed blindly (19).

Statistical analysis

All the data are presented as mean ± standard deviation (M±SD). Significance testing between groups was performed using one-way analysis of variance (ANOVA) with SPSS Version 19 and multiple comparison post hoc tests to determine significant differences between groups. A *P*-value of less than 0.05 was considered statistically significant.

Results

The effect of EPO and MEL on renal ischemia reperfusion injury was investigated in 45 minutes of renal ischemia followed by 24-hour reperfusion. Biochemical analysis results are outlined in Table 1, and the results of histological evaluation and TUNEL assay are shown in Tables 2,3.

Table 1. Biochemical measurements after 24 h of reperfusion

	Sham group	IR group	MEL+IR group	EPO+IR group	EPO+MEL+IR group
MDA (nmol/ml)	2.19±0.69	2.64±1.35	2.01±0.61	2.46±0.55	2.22±1.15
SOD (U/g Hb)	1139.05±84.33	1251.44±203.9[a]	1139.82±121.42[b]	1030.13±78.38[b]	1132.65±73.51[b]
GPx (U/g Hb)	36.65±1.62	41.09±4.46[a]	36.54±3.70[b]	35.70±1.98[b]	37.28±1.82[b]
Uric acid (mg/dl)	1.36±0.74	1.31±0.80	1.30±0.73	1.17±0.40	0.70±0.27[b]

[a]Significantly increased when compared with sham group, P<0.05.
[b]Significantly decreased when compared with IR group, P<0.05.
MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; EPO, erythropoietin; MEL, melatonin; IR, ischemia-reperfusion.

Table 2. Tubulointerstitial changes in the kidney after 24 h reperfusion (H&E)

Groups	Tubular epithelial cell swelling	Dilated Bowman's space	Glomerular atrophy	Congestion	Tubular epithelial pyknotic nuclei
Sham	-	-	-	-	-
IR	+++	+++	+++	+++	+++
MEL	-	+	+	-	-
EPO	+	++	+	++	+
EPO+MEL	-	+	+	-	+

A minimum of 10 fields for each kidney slide was examined and assigned for severity of changes using scores on a scale of: (–) none, (+) mild, (++) moderate and (+++) severe damage. (n=7 for each group)

Table 3. Evaluation of TUNEL Positive Cells in the kidney after 24 h reperfusion

Groups	Sham	IR	MEL	EPO	EPO+MEL
TUNEL Positive Cells	**-/Rarely +**	**+++**	**+**	**++**	**+**

A minimum of 10 fields for each kidney slide were examined and assigned for severity of changes using scores on a scale of: TUNEL Positive Cells:
None (-) = no apoptotic cells/Rarely+,
Mild (+) = a few apoptotic cells, Moderate (++) = more apoptotic cells, Severe (+++) = widespread apoptotic cells.

Effects of ischemia-reperfusion

The level of uric acid in the IR group was lower than that in the sham group, but the difference was statistically insignificant (*P*>0.05). The level of MDA in the IR group was higher than that in the sham group, but the difference was not statistically significant (*P*>0.05).

The levels of SOD and GPx in the IR group were significantly higher than those in the sham group ($P<0.05$).

Histological examination of the kidneys showed that there were no histological changes in the sham group (Figure 1A). In the IR group, tubular epithelial cell swelling, dilated Bowman's space, glomerular atrophy, congestion and tubular epithelial pyknotic nuclei were higher than those in the sham group (Figure 1B). A prominent augmentation in the number of TUNEL positive cells was observed in rats undergoing IR (Figure 2B).

Effects of melatonin on renal ischemia reperfusion

Serum uric acid level in the MEL+IR group was lower than that in the IR group, but the difference was not statistically significant ($P>0.05$). The level of MDA in the MEL+IR group was lower than that in the IR group, but the difference was not statistically significant ($P>0.05$). The levels of SOD and GPx in the MEL+IR group were significantly lower than those in the IR group ($P<0.05$).

Melatonin pretreatment resulted in marked attenuation of dilated Bowman's space and glomerular atrophy, with the absence of tubular epithelial cell swelling, congestion and tubular epithelial pyknotic nuclei induced by ischemia-reperfusion (Figure 1C). The MEL treatment led to a marked reduction in the number of TUNEL positive cells compared with the IR group (Figure 2C).

Effects of erythropoietin on renal ischemia reperfusion

Serum uric acid level in the EPO+IR group was lower than that in the IR group, but the difference was not statistically significant ($P>0.05$). The level of MDA in the EPO+IR group was lower than that in the IR group, but the difference was not statistically significant ($P>0.05$). The levels of SOD and GPx in the EPO+IR group were significantly lower than those in the IR group ($P<0.001$).

Erythropoietin pretreatment resulted in mild to moderate tubular changes (Figure 1D). EPO group showed a moderate reduction of TUNEL positive cells compared with the IR group, a number of TUNEL positive cells was higher in EPO group comparing to MEL group (Figure 2D).

Effects of erythropoietin and melatonin on renal ischemia reperfusion

In the EPO+MEL+IR group, the serum level of uric acid was significantly lower than that in the IR group ($P<0.05$). In the EPO+MEL+IR group, the level of MDA was lower than that in the IR group, but the difference was not statistically significant ($P>0.05$). The levels of SOD and GPx in the EPO+MEL+IR group were significantly lower than those in the IR group ($P<0.05$).

EPO and MEL combination treatment resulted in marked attenuation of dilated Bowman's space, glomerular atrophy, and pyknotic nuclei with the absence of tubular epithelial cell swelling and congestion induced by ischemia-reperfusion. Therefore, combination therapy appears to have similar histological results to the melatonin treatment (Figure 1E). A few apoptotic cells were observed in the group receiving both EPO and MEL. Therefore, evaluation of TUNEL positive cells in the EPO+MEL group showed similar results to the MEL group (Figure 2E).

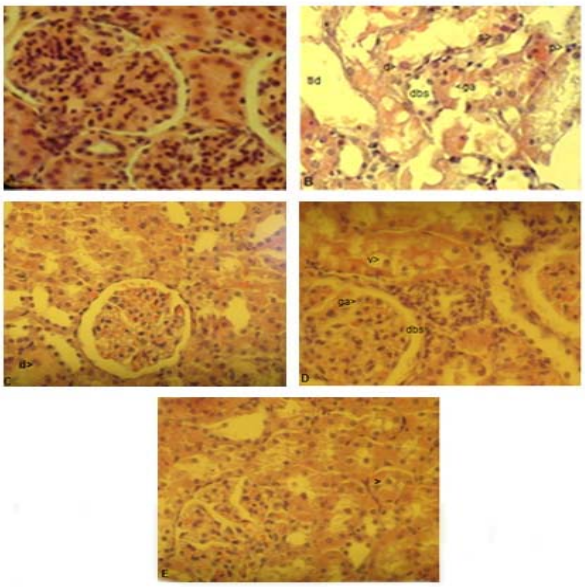

Figure1. Histopathological evaluation of rat kidneys after 45 min ischemia followed by 24 h reperfusion. Kidney sections are stained with hematoxylin and eosin (H&E) and examined by a light microscope (A). The normal renal tissue structure in the sham group. Healthy appearance of glomerular and tubular cells (40×HE). (B) Tubular epithelial cell swelling (s), degeneration (d), dilated Bowman's space (dbs), glomerular atrophy (ga), tubular lumen dilation (tld) and pyknotic nuclei (p) in the IR group (40×HE). (C) The lesser degree of degeneration (d) in the MEL group (40×HE). (D) Vacuolization (v), dilated Bowman's space (dbs) and glomerular atrophy (ga) in the EPO group (40×HE). (E) The lesser degree of vacuolization in the EPO+MEL group (40×HE).

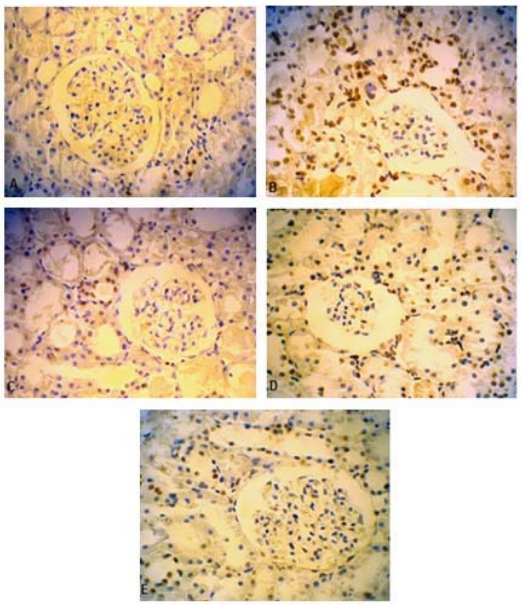

Figure 2. Evaluation of TUNEL positive cells in renal after 45 min ischemia followed by 24 h reperfusion. Apoptosis was evaluated by TUNEL staining of kidney sections. (A) Apoptotic cells were rarely observed in the sham group. (B) Server increased a number of TUNEL positive cells was observed in IR group. (C) A few apoptotic cells were observed in MEL group. (D) Moderate apoptotic cells were observed in EPO group. (E) A few apoptotic cells were observed in EPO+MEL group. Magnification 40.

Discussion

Renal IR is a common result of clinical procedures such as organ procurement, vascular surgery, or renal transplantation. Furthermore, renal IR injury is a leading cause of ARF, which is associated with high mortality rates. ARF is characterized by increased vascular resistance in the kidney, a low rate of filtration through the glomeruli, and tubular necrosis. These deleterious effects have been attributed to ROS generation during renal reperfusion (20). ROS contributes to lethal cell damage. IR injury has been attributed to ROS-mediated lipid peroxidation (21).

Lipid peroxidation, as a free radical generating system, has been proposed to be closely related to IR-induced tissue injury, and MDA is a good indicator of the degree of lipid peroxidation. In the present experiment, the levels of MDA are increased by IR, which reflects increased lipid peroxidation due to increased oxidative stress. Erythropoietin decreased the level of MDA, which shows that it decreased the amount of oxidative stress and subsequently lipid peroxidation. Consistent with our findings, Ates *et al*., (22) demonstrated that EPO decreased the level of MDA after right nephrectomy, clamping of the left renal pedicle, and reperfusion in rats. Our results show that melatonin causes a reduction in MDA production, indicating a reduction in lipid peroxidation and cellular damage. This protective effect of MEL may be in part by scavenging the very reactive $ONOO^-$ and OH (23). We found that the plasma level of MDA was decreased by EPO+MEL, which indicates that EPO and MEL combination treatment decreased the magnitude of oxidative stress and lipid peroxidation.

We have found that renal IR significantly increased the levels of SOD and GPx. Increased antioxidant enzyme levels show a cellular defensive response to overproduction of reactive oxygen species after renal IR. Increased levels of SOD and GPx also have been reported in renal and intestinal injury induced by ischemia-reperfusion (24-26). Also, increased levels of SOD have been reported in IR-induced liver injury (27). Increased level of SOD may be explained by that increased superoxide production due to renal IR causing an increase in the level of this endogenous scavenging antioxidant enzyme. Gulmen *et al*., (28) think that the level of antioxidant enzymes during IR injury is determined by several factors including the magnitude of IR injury, the duration of IR periods, and the specific organ subjected to IR injury. We have also found that erythropoietin and melatonin significantly reduced the levels of SOD and GPx. Thus, EPO and MEL might have reduced the renal IR-induced oxidative stress by their antioxidant properties. Consistent with this finding, Kiris *et al*., (18) found that EPO significantly reduced the level of SOD after aortic IR. Our results indicate that the combination of EPO and MEL (EPO+MEL) significantly reduced the levels of SOD and GPx. This finding may indicate that EPO and MEL combination treatment decreases the renal IR-induced oxidative stress.

We explored that the single application of EPO and MEL and their combination reduced the serum level of uric acid. Therefore, it seems that combination therapy has beneficial effects on IR-induced renal injury as indicated by lower levels of uric acid.

In our study, histological evaluation showed that IR caused changes in tubules as shown by tubular epithelial cell swelling, pyknotic nuclei, and congestion. Renal IR also caused dilated Bowman's space and glomerular atrophy. EPO treatment attenuated the histopathological changes associated with renal IR injury. On the other hand, attenuating effect of EPO on the morphological changes in renal tissue caused by IR injury has been reported (29). Sener *et al*., (30) reported that melatonin

has protective effects on IR-induced renal injury, and the histopathological changes are reversed by MEL treatment. Also, they proposed that melatonin appears to play a cytoprotective role in the kidney insulted by ischemia-reperfusion. Supporting this proposal, we have found that MEL has protective effects on tubular function. MEL severely attenuated the histopathological changes; nearly the normal renal tissue structure was preserved by melatonin pretreatment. This cytoprotective effect of MEL may be due to its powerful antioxidant properties. Also, EPO and MEL combination treatment reduced the histopathological changes in renal tissue caused by IR injury. Therefore, histological evaluation indicated that combination therapy appears to have similar histological results to the MEL group.

The present study demonstrated that IR-induced a severe increase in cell death (apoptosis). This finding is in agreement with previous studies reporting that renal IR initiates a complex cascade of events that eventually result in injury and subsequently in necrotic and apoptotic death of renal cells (31). The administration of either EPO or MEL (especially) reduced apoptotic cell death. This effect of EPO and MEL may be due to their anti-apoptotic properties. Spandou *et al*., (32) demonstrated that EPO inhibits apoptotic cell death so that inhibition of apoptosis is one of the most potential protective mechanisms of EPO. On the other hand, Taghizadeh *et al*., (33) indicated that the MEL treatment led to a significant decrease in the number of TUNEL positive cells after liver IR. Also, EPO+MEL group similar to MEL severely reduced TUNEL positive cells, this indicates that combination therapy decreases apoptotic cell death, but it did not have a better result than MEL (34,35).

In conclusion, ROS are considered to be principal components involved in the pathophysiological tissue alterations observed during renal IR. Antioxidant defense systems prevent ROS formation and scavenge ROS. The administration of EPO and MEL, which are potent antioxidant and anti-apoptotic agents, appears to have beneficial effects on IR-induced renal injury as indicated by lower degrees of histopathological changes and apoptotic cell death. However, EPO and MEL combination treatment exerted more nephroprotective effects than EPO treatment, and almost had protective effects similar to the single application of MEL. Therefore, combination therapy may be an alternative option for therapeutic procedures with a single application of EPO, but it seems that MEL, with its potent antioxidant properties, merits consideration as a potential therapeutic agent in renal IR injury without any need to combination therapy. However, further studies are required to clarify the exact mechanisms mediating the effect of EPO and MEL combination therapy in renal IR injury.

Acknowledgment

This study was financially supported by Drug Applied Research Center of Tabriz University of Medical Sciences.

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