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THE ROLE OF ERYTHROPOIETIN IN A RAT MODEL OF RENAL ISCHEMIA/ REPERFUSION INJURY

RENAL İSKEMİ/REPERFÜZYON HASARLI SIÇAN MODELİNDE ERİTROPOETİNİN ROLÜ

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ABSTRACT

Objective: A powerful activator of erythroid progenitor cells, erythropoietin (EPO) is markedly elevated during hypoxia. A major cause of renal cell death is renal ischemia caused by artery blockage or organ transplantation, and reperfusion exacerbates the damage. The study aimed to investigate the effect of EPO treatment on renal injury following ischemia and reperfusion (I/R).

Method: Thirty rats assigned to five groups of six rats each as control, EPO, ischemia, ischemia/reperfusion (I/R) and I/R+EPO.The renal tissue samples were evaluated in terms of hematoxylin-eosin (H&E) staining for histopathological changes, immunoexpression of Beclin-1 for autophagy, and the TUNEL assay for apoptosis.

Results: The H&E staining showed the impairment in the tubular epithelium, glomerular and peritubular hemorrhage in the renal tissues of I/R group. Less histopathological changes were observed in I/R + EPO group. Renal tissue Beclin-1 immunoexpression and TUNEL positive cells were significantly increased in the I/R group compared with the others ($p<0.05$). Treatment with EPO decreased the number of the TUNEL positive cells and Beclin-1 expression.

Conclusion: The data showed that EPO treatment could be effective in reducing renal injury following renal I/R and alleviate histomorphological damage.

Key Words: Apoptosis, Autophagy, Erythropoietin, Histolopathology, Renal ischemia/reperfusion

ÖZ

Amaç: Eritroid progenitör hücrelerinin güçlü bir aktivatörü olan eritropoietin (EPO), hipoksi sırasında belirgin şekilde yükselmektedir. Arteriyel blokaj veya organ naklinin neden olabileceği renal iskemi böbrek dokusundaki hücre ölümünün başlıca sebebi olmakla birlikte reperfüzyon oluşan bu hasarı arttırmaktadır. Bu çalışmada eritropoietin uygulanmasının iskemi/reperfüzyon (I/R) sonrasında böbreklerde oluşan hasar üzerindeki etkisini araştırmayı amaçladık.

Yöntem: Otuz adet rat her grupta altı adet olacak şekilde kontrol, EPO, iskemi, iskemi/reperfüzyon (I/R) ve I/R+EPO gruplarına ayrıldı. Böbrek doku örnekleri histopatolojik incelemeler için hematoksilin-eozin (H&E) ile boyandı, otofajiyi tespit edebilmek için Beclin-1 proteininin immünoekspresyonu ve apoptoz için TUNEL yöntemi kullanılarak değerlendirildi.

Bulgular: H&E boyama sonucunda I/R grubuna ait böbrek dokularında böbrek tübüllerinin epitelinde bozulma, glomerüler ve peritübüler kanama olduğu tespit edildi.I/R+EPO grubunda histopatolojik değişikliklerin azaldığı gözlendi.Renal Beclin-1 immünoekspresyonu ve TUNEL pozitif hücrelerin I/R grubunda diğer gruplara kıyasla istatistiksel olarak anlamlı düzeyde arttığı ($p<0.05$) saptandı. EPO uygulamasının Beclin-1 ekspresyonunu ve TUNEL pozitif hücre sayısını azalttığı tespit edildi.

Sonuç: Bu çalışma ile elde ettiğimiz veriler doğrultusunda eritropoietin tedavisinin renal iskemi/reperfüzyon sonrası böbrek hasarını azaltmada ve histomorfolojik hasarı hafifletmede etkili olabileceğini düşünmekteyiz.

Anahtar Kelimeler: Apoptoz, Otofaji, Eritropoietin, Histolopatoloji, Renal iskemi/reperfüzyon

INTRODUCTION

The balance of salt and water, as well as blood pressure, are crucial functions of the kidneys. Ischemia/reperfusion (I/R) injury which usually follows sepsis, infarction, or organ transplantation, is occured by limited blood flow and oxygen to tissues It causes induction of cytokines, chemokines, and reactive oxygen species, which lead to tissue damage [1].

Acute renal failure, a prevalent clinical disease associated with high mortality and morbidity despite major advances in supportive care, is primarily caused by I/R damage [2].

Renal tubular cells' morphologic response, which includes loss of cell polarity, cell death, dedifferentiation of viable cells, proliferation, differentiation, and restoration of a normal epithelium, is dependent on the degree and intensity of ischemia [3,4].

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Numerous research have studies on different triggers to slow the progression of injury processes because acute ischemia injury causes excessive apoptotic cell death. Except for supportive therapy, there isn't an efficient therapy right now. Therefore, it is necessary to find effective therapeutic intervention options for renal I/R injury.

Erythropoietin (EPO) a key protein in the production of erythrocytes is a hypoxia-inducible hematopoietic hormone. It primarily promotes angiogenesis and neovascularization, which improve blood flow.In response to hypoxia, the kidney and the fetal liver both create EPO. Erythropoietin's biological effects are mediated via its binding to erythropoietin receptors (EPOR), and the fact that EPOR is present in renal mesangial, glomerular, and tubular epithelial cells suggests that the potential role of EPO in the kidney [5]. EPO has a variety of protective actions, including anti-inflammatory, anti-oxidant, and anti-apoptotic [6].

Erythropoietin also exhibits powerful tissue-protective effects against I/R injury in a wide range of organs including, the heart [7], liver [8], and central nervous system [9].

The cellular process of removing useless and unhealthy components is known as autophagy. Autophagy makes easier for these unnecessary cellular components to be recycled and destroyed by lysosomes [10]. Autophagy is a mechanism for preserving cellular energy and acts as an adaptive response to negative stress in certain disease processes. This process is necessary to encourage cell survival and inhibit disease progression [11].

Indeed, due to the molecular interactions between autophagy and cell death mechanisms including apoptosis and necrosis, autophagy can both prevent and aid in cell death, depending on the nature and duration of the stress [12,13]. This autophagy paradox-destroys on one hand, protects on the other-indicates that the dynamics and role of autophagy during renal IR injury are not well understood [14].

The renal I/R injury's pathophysiology is complex, multifaceted, and controlled by numerous, interrelated molecular pathways and signaling cascades. The current study's objective was to examine how EPO administration affected cell apoptosis, autophagy, and renal tissue damage in the rat I/R injury model.

METHOD

Experimental Design

We used 30 female Wistar albino rats (200-220g at 8 to 10 weeks old) for this study. Rats were maintained in laboratory under controlled environmental conditions, ad libitum access to water and standard pellet food, and housed according to a 12 h light–dark cycle.

The animals were dividev into five groups (n=6).

Group I (control): The control group rats were sacrificed and their kidney tissues were removed under anesthesia without any procedure.

Group II (EPO): In this group, rats were intraperitoneally (i.p) injected with only of human recombinant EPO (Eprex; Janssen-Cilag AG Sihlbruggstrasse, Switzerland) (1500 IU/kg/day) [15] without occlusion.

Group III (Ischemic group): Right nephrectomy applied quickly under anesthesia. After that, ischemia was induced by utilizing an atraumatic microvascular clamp to occlude the left renal artery and vein for 30 minutes.

Group IV(I/R group): The rats were subjected to clamping-induced ischemia for 30 minutes in the I/R group, after the clamping was relaxed, it underwent reperfusion for 3 hours [16].

Group V (I/R+ EPO group): In the I/R+ EPO group, the rats were subjected to clamping-induced ischemic injury for 30 min; then 1500 IU/kg/day of EPO were i.p. injected to the rats prior to reperfusion.

Surgical Prosedure

Each rat was anesthetized with intramuscular ketamine hydrochloride at 50 mg/kg body weight (Keta-Control, Cat No: 210034, Doğa İlaç, Turkey) and xylazine hydrochloride at 10 mg/kg body weight (Xylazinbio, Cat No. 825827A Bioveta, Türkiye).

Renal I/R damage was performed according to previous study briefly; right nephrectomy was completed quickly under anesthesia. After that, ischemia was induced by utilizing an atraumatic microvascular clamp to occlude the left renal artery and vein for 30 minutes. After the clamping was relaxed, it underwent reperfusion, and the change in kidney color was used to validate blood flow. The abdomen is closed in two layers with 2-0 sutures.

After 3-hours of reperfusion the rats were sacrificed by using servical dislocation method under anesthesia and immediately the kidney tissues were removed.

Histological Evaluation

Renal tissues were fixed in a 10% formalin solution for 24–48 hr for histological examination. The tissues were washed under tap water for 6 hours, then pass through a gradually increased alcohol for 1 hour, then put in xylene for 15 minutes. After that, samples of renal tissue were scut into 5 µm sections using a microtome (Leica RM2245, Germany), after being embedded in paraffin blocks. Then the sections were subjected to deparaffinization and dehydration, pass through decreased alcohol series. Then the slides were stained with hematoxylin (Mayer's hematoxylin, Merck) in 30 seconds and eosin in 15 seconds.The slides were photographed under a light microscope (LeicaDM4000, Wetzlar, Germany).

Sections were examined in groups and graded according to the extent of cortical involvement on a scale of 0 to 4 (0=normal; 0.5=small damage within focal areas; 1=<10% damage in the cortex; 2=10 to 25% damage in the cortex; 3=25 to 75% damage in the cortical area; and 4=>75% damage in the cortical area [17]. All scores were added up and displayed on a graph as avarage values.

Immunohistochemical Analysis

Slides were deparaffinized, placed in xylene, then rehydrated using ethanol in increasing concentrations. Antigen retrieval was carried out using citrate buffer in a microwave and the tissues were then blocked in blocking serum (UltraV Block, ScyTek Laboratories, Utah, USA). The slides were incubated with Beclin1 (anti-Beclin1 antibody, ab13847, Abcam, Cambridge, UK) primary antibody, 1:50 dilution with PBS, overnight. The primary antibody was incubated on tissues for five minutes before washed twice with PBS., and then, incubated with biotinylated anti-mouse seconder antibody (BA-9200; Vector Laboratories, Burlingame, CA) for 10 minutes at room temperature. Following the application of the secondary antibody, the tissues were rinsed three times in five minutes with PBS and then incubated for ten minutes with streptavidin peroxidase and then taken into PBS. DAB substrate kit solution was applied for reaction. The slides were counterstained with hematoxylin (Merck, ScyTek Laboratories) and covered with entellan.

The slides were examined and photographed under the light microscope (LeicaDM4000, Wetzlar, Germany) As previously mentioned, H-SCORE analyses were utilized to evaluate the immunohistochemistry results [18].

TUNEL assay

Apoptotic cells were stained by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique using an apoptosis detection kit (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany). Deparaffinized paraffin sections were rehydrated and treated in a microwave with 10 mM citrate buffer twice for 5 min each, allowed to cool for 20 min. Endogenous peroxidase activity was suppressed with 3% hydrogen peroxide following three

PBS washings. Following equilibration buffer incubation for 10–15 s, the sections underwent TdT enzymatic labeling of nuclear DNA strand breaks in a humidified chamber at 37 °C for 60 min. The typical labels were revealed by adding an alkaline phosphatase-converter with subsequent staining with the chromogenic substrate. Counterstaining was performed with Mayer's hematoxylin (Merck, ScyTek Laboratories). TUNEL-positive cells' distribution was counted and reported as earlier [19].

Ethical Approval

The study was approved (Approval code:672) on 04.07.2023 by the animal ethics committee of Kobay Company (Türkiye).

Statistical Analysis

Statistical analysis was performed by one-way ANOVA followed by Dunnett's test and the Mann Whitney U test by using Sigma Plot 12 (Jandel Scientific Corp., San Rafael, CA). $P<0.05$ was used to indicate that the data were statistically significant. The mean and standard error (SEM) of three independent experiments are used to represent all data.

RESULTS

Histomorphological Changes

The histological changes of renal tissue samples from each group of rats were seen (Figure 1). The renal sections in the control revealed a healthy histological structure with glomerular structure and tubular cells (Figure 1A). The EPO group exhibited no histological changes in renal morphology (Figure 1B). Peritubular and glomerular hemorrhage was observed in the ischemia and I/R groups (Figure 1C and D). Furtermore, renal sections of the I/R group showed extensive tubular degeneration which included tubular dilatation, tubular necrosis, vacuolization of tubular epithelial cells, and loss of brush borders compared to control and EPO groups. The I/R+EPO group had less histopathological alterations in renal tissue (Figure 1E) when compared with ischemia and I/R groups. Renal tissue damage was significantly increased in I/R group and decreased with the EPO treatment (Figure 1F).

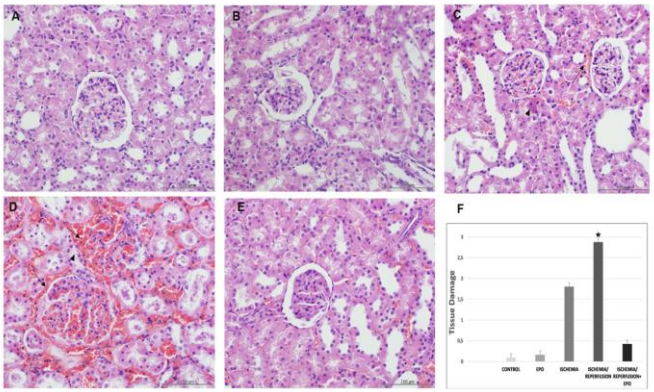

Figure 1. Histomorphological evaluation of renal tissues of rats induced I/R injury. A: Control group; the normal renal tissue structure. B: EPO group; exhibited no changes in renal morphology. C: Ischemic group; peritubular hemorrhage (arrow) and vacuolization of the tubular epithelium (arrowhead) was observed. D: I/R group; showed extensive peritubular and glomerular hemorrhage (arrow), tubular dilation, and loss of brush border (arrowhead). E:I/R+EPO group; histomorphological changes were significantly attenuated treated with EPO. (Stained with H&E; scale bar, 100 µm.; magnification, 400x) F: Quantitative analysis of tissue damage data are means±SEM. *$p<0.05$.

TUNEL Assay Results

The apoptosis in renal I/R damage was evaluated using TUNEL labeling. In the renal tissues from the control and EPO groups, there were no detectable TUNEL positive cells (Figure 2A and B). On the other hand, several TUNEL labeled cells were observed in ischemia and I/R groups (Figure 2C and D) compared to control (Figure 2A) and EPO (Figure 2B) groups. The proportion of TUNEL-positive cells was much lower in the I/R+ EPO group (Figure 2E) compared to ischemia (Figure 2C) and I/R (Figure 2D) groups. In the I/R group, the number of TUNEL positive cells was significantly increased ($p<0.005$) compare to the other groups (Figure 2F).

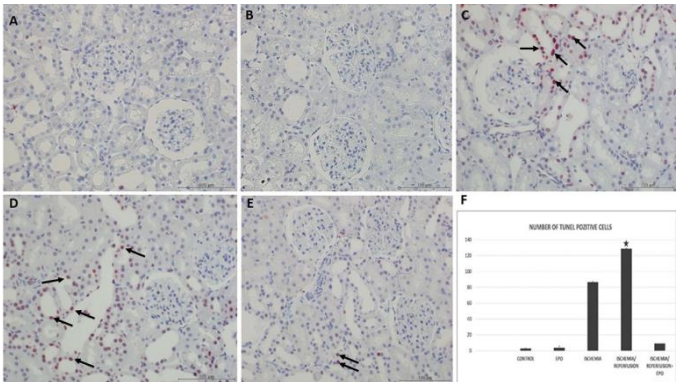

Figure 2. TUNEL staining in renal tissue samples of rats induced I/R injury. No TUNEL positive cells in Control (A) and EPO group (B). Several TUNEL positive cells were detected in in renal tissue samples of rats induced ischemia (C) and I/R injury group (D). A few TUNEL positive cells (arrowa) were detected in the renal tissue samples of rats from I/R+EPO group (E). (Scale bar, 100 µm;magnification 40X). F : Quantitative analysis of TUNEL-positive cells. The number of the TUNEL positive cells was significantly increased in the I/R group compare to other groups. Data are means±SEM. *$p<0.05$.

Immunohistochemistry Results

To determine how autophagy influences renal I/R injury we investigated the autophagic protein Beclin 1 expression using immunohistochemistry. There was not any specific immunostaining of Beclin 1 in rat kidney tissues from control and EPO groups (Figure 3A and B).The immunoexpression of Beclin 1 was observed in the ischemic rat kidney (Figure 3C). Additionally, the immunostaining of Beclin 1 was higher in I/R group (Figure 3D) compared to other groups. The immunohistochemical images, which revealed that pretreatment with EPO decreased the expression levels of Beclin1 in the renal tissue samples of I/R+ EPO (Figure 3E) when compared to those of ischemia (Figure 3C) and I/R groups (Figure 3D). The ımmunoexpression level of Beclin 1 was significantly increased ($p<0.005$) in the I/R group compare to others (Figure 3F).

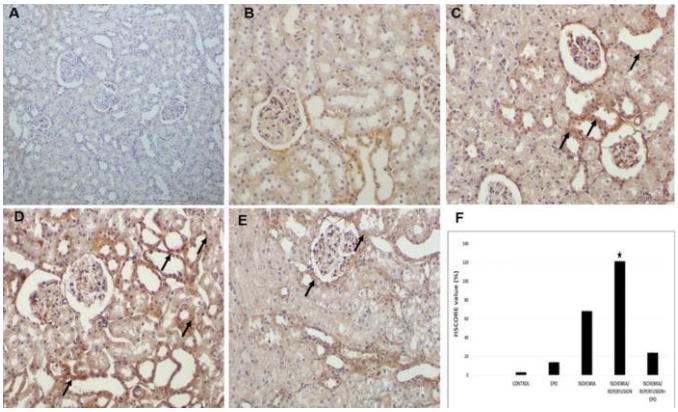

Figure 3. The immunoexpression of Beclin 1 in renal tissue samples of rats induced I/R injury. A:Control group: here was not any staining. B:EPO group. C:Ischemia group: Modarate staining of Beclin 1was observed. D:I/R group showed strong immunostaining of Beclin 1. E:I/R+EPO group: Low Beclin 1 staining was observed. (scale bar, 100 µm, magnification, x400); F:H-SCORE values of Beclin1. The expression levels of Beclin were significantly increased in the I/R

group and decreased in the I/R+EPO group. Data are expressed as SEM. *p<0.05.

DISCUSSION

In the current study we investigated the effects of EPO on the I/R injury in the rat experimental model. Although there has been studies in the literature about this issue we focused on firstly to figure out the connection between apoptosis and the autophagy in the renal I/R injury. Secondly we aimed to determine the effects of EPO on this pathway. Therefore we investigated the histomorphological changes, the apoptotic cells and the autofagic pathway.

The findings of the current study releaved that renal I/R damaged affect the histologic architecture of the kidney. When comparing the I/R group to the control and ischemic groups , there was a significant increase in apoptotic TUNEL positive cells and autofagic marker Beclin-1 expression in the renal tissue. Both the quantity of TUNEL positive cells and the expression of Beclin-1 were reduced by EPO treatment. Although ischemia already causes damage, reperfusion may result in a wide range of issues. It could result in patients life-threatening situations when related to systemic issues. The oxygen reactions following reperfusion causes tissue injury and initiates a chain reaction of damaging cell results in inflammation, cell death, and finally organ insufficiency [20].

The ability to maintain intracellular calcium homeostasis and the ability to regenerate ATP, following reperfusion are the factors that ischemia reversibility [21].

Several experimental I/R models have demonstrated the protective effects of EPO, through a number of pathways, including the control of microvascular damage, the reduction of tubulointerstitial damage, anti-inflammatory and anti-apoptotic actions, and reduced fibrocyte accumulation [22].

The possible renoprotective effects of EPO on renal I/R model rats were examined in the current study. The histomorphological results revealed that I/R caused severe damage to renal tissue particularly diffuse congestion and tubular injury that was ameliorated with the EPO treatment. In the kidney with IRI, tubular necrosis and interstitial infiltration of inflammatory cells are typical clinical features. Inflammatory responses to I/R injury include neutrophil and macrophage infiltration, oxidative stress, and increased production of inflammatory cytokines [23].

According to reports, EPO protected the kidney from I/R by reducing the amount of tissue congestion and inflammatory cell infiltration [24]. The results of the current study are analogous to those of an earlier investigation into the underlying molecular mechanisms of renal I/R established in EPO-treated rat models.

The generation of red blood cells is regulated by the glycoprotein cytokine erythropoietin, which is mostly produced by the kidney. Apart from its well acknowledged impact, numerous investigations have demonstrated that EPO functions as a factor that protects tissue [25-28].

EPO has been used not only in renal I/R but also in the recovery of I/R injury in other tissues. Guneli and co-workers [29] have investigated the possible protective effects of EPO against intestinal I/R injury in rats . TUNEL labeling was used to identify apoptotic cells and the histological evaluation was performed to determine the level of the injury. Intestinal tissue was damaged by I/R compared to the sham demonstrated by an increase in TUNEL-positive cells.

One type of cell death that helps remove dying cells from groups of proliferating or developing cells is called apoptosis, sometimes referred to as programmed cell death [30]. On the other hand, in pathological conditions, apoptosis activation leads to extensive and fast cell death, which causes tissue malfunction.

Some researchers has prefered to use EPO combination with other subjects for example Banaei et al. [31] have investigated the anti-apoptotic effect of EPO and melatonin on renal I/R injury in rats. The study's findings demonstrated that I/R enhanced the number of TUNEL-positive cells. The tubular epithelium's renal impairment was corroborated by the IR group's histopathological findings. TUNEL positive cells and histological alterations were reduced by EPO and melatonin treatment.

In hospitalized patients, acute kidney injury (AKI) is the most prevalent ailment. An efficient treatment strategy for ischemia/reperfusion-induced AKI (IR-AKI) is essential because it plays a significant role in end-stage disease. A strong erythroid progenitor cell stimulant, EPO is markedly elevated in hypoxic environments. Using an IR-AKI male C57BL/6 mice model, Kwak et al. [32] have investigated the renoprotective properties of EPO. The study's findings demonstrated that EPO considerably reduced tissue damage and renal dysfunction brought on by IR-AKI. In mice given EPO, apoptotic cell death and oxidative stress were considerably decreased.

In the current study we can cleary say that the I/R injury in renal tissue results in the increasing of apoptotic cells which were confirmed by the the increase number of TUNEL positive cells.This findings is consistent with other research showing that renal I/R starts a convoluted chain of events that ultimately cause damage and the necrotic and apoptotic death of renal cells [33].Apoptosis during renal I/R markedly increased and EPO treatment before reperfusion reduces I/R-induced apoptosis in renal tubular cells. Based on TUNEL labeling, our findings showed that EPO administration protects renal tissue against I/R-induced apoptosis.

To maintain intracellular homeostasis, damaged organelles and macromolecules are degraded and recycled by the conserved multistep process known as autophagy. The autophagic pathway is comprised of four distinct sections: (i) initiation; (ii) engulfment of mature structures by a double-membrane structure, resulting in the generation of autophagosomes; (iii) autolysosome formation through the fusion of autophagosome and lysosome; and (iv) the ultimate process, which involves the degradation and recycling of the engulfed structures [34]. Beclin 1, p62, and microtubule-associated protein light chain 3B (LC 3B) have all been shown to be essential components of the autophagy process [35].

Under stressful circumstances such as hypoxia, nutrition, and growth-factor deficiency, the autophagy pathway is activated. Recent research has shown that basal autophagy in the kidney is essential for maintaining the proximal tubules'homeostasis. Renal function was impaired by the deletion of important autophagy proteins, which also raised p62 levels and oxidative stress. Autophagy deletion in the proximal tubules increased tubular damage and renal function in AKI mice, emphasizing the renoprotective effects of autophagy in AKI [10].

In both healthy and damaged tissue, autophagy is a self-degradative process that involves the turnover and recycling of cytoplasmic components. In the early phases of programmed cell death, autophagy has been demonstrated to be protective, but in some circumstances, it can potentially encourage apoptosis.Bendix and collages have examined erythropoietin's impact on autophagy signaling in a developing rat brain using an in vivo oxygen toxicity model. The study showed that in the developing brain, high oxygen levels cause up-regulation of Beclin 1 indicators for autophagic cell death and these alterations are neutralized by EPO treatment [36].

Beclin-1 starts the production of phagophores, which are the progenitors of autophagosomes, when autophagy is started [37]. Zhang et al. have focused on how autophagy affects renal I/R damage. Kidney tissue samples were graded histopathologically. Light chain 3 (LC3), Beclin-1, p62, and the quantity of autophagic vacuoles were indicators of autophagy. TUNEL was used to induce apoptosis, and caspase-3

was expression was assed by immunohstochemically. Following renal I/R damage, autophagy was triggered. Unlike our findings; the study show that autophagy may be triggered during I/R injury and may even be beneficial in cases of kidney injury [38]. Due to the close reliance of their constituent cells on the preservation of normal oxidative metabolism, neurologic, renal, and cardiovascular tissue are especially vulnerable to I/R injury [39].

In rats that have undergone cerebral I/R, Huang et al. have examined the impact of post-conditioning ischemia on the expressions of the hippocampus neuron autophagy-related proteins LC3-II and Beclin-1. The findings of the investigation showed that cerebral ischemia post-conditioning increased the expressions of Beclin-1 and LC3-II, two proteins linked to autophagy [40].

Another investigation of ischemic myocardium has been published in the literature. Autophagy seems to help with survival in this situation, but it can also cause cell death during reperfusion. The length and severity of stress, as well as the degree of autophagy in cardiac tissue, appear to have an impact on autophagy's overall function. Ventricular fibrillation (VF) is one of the main side effects of reperfusion intervention. The study have examined for any potential relationships between VF and autophagy. Researchers have demonstrated that the fibrillated myocardium had increased levels of Beclin-1 and LC3B-II/LC3B-I ratio, two autophagy indicators, compared to tissue from nonfibrillated hearts [41].

Autophagy is most frequently used by cells and is mainly regulated by Beclin-1 and ubiquitin [42,43]. Actually, a known biomarker for the presence of autophagy in biological samples is the Beclin-1 protein [44].

To learn more about function of autophagy we investigated the renal expression of Beclin-1. The immunoexpression of Beclin-1 was increased remarkably in the I/R group compare to other groups. Similary some studies in the literature we also found the increase expression of Beclin 1 in ischmia and ischemi reperfusion groups. Additionally Beclin 1 expression was decreased with the treatment of EPO compare to ischemia and I/R groups. Therefore we can cleary say that I/R injury trigger the autophagy. Although recent studies reported that autophagy has renoprotective effects, ınterestingly, our results showed autophagy activation following I/R was suppressed by EPO treatment ,based on the immunohistochemistry staining of decreased Beclin-1 levels.

Limitations

This study has potential methodology limitations including sample size, surgery process.

CONCLUSION

Histological analysis performed in our work demonstrated that I/R led to tubular epithelial cell enlargement, pyknotic nuclei, and congestion. Dilated Bowman's space and glomerular atrophy were additional effects of renal I/R. Treatment with EPO reduced the histological alterations linked to renal IR injury.

Additionally;in the current study we determined the correlation between the apoptosis and the autophagy depends on the findings of TUNEL and immunohistochemitry assay results. The proliferation of TUNEL positive cells in the ischemia and I/R groups, compared to other groups, indicates that an increase in apoptosis and EPO showed its anti-apoptotic effects depends on the decreased the TUNEL positive cells with the treatment of EPO in I/R+EPO group.

As well as an excessive elevation in autophagy due to the higher immunoexpression of autophagic marker Beclin 1 following I/R injury. According to immunohistochemical labeling of reduced Beclin-1 levels, EPO therapy prevented higher autophagic activation after I/R.We thought that EPO supressed the exsessive activation of autophagy.

In conclusion, EPO treatment ameliorated the severe damage of renal tissue caused by I/R injury and also enhanced kidney histomorphology at cellular level, lowered apoptosis, and suppressed the excessive autophagic activation.Taken to gether the results of the current study may contribute to the literature with different aspects.

Ethical Approval: *2023/672 Animals Ethics Committee of Kobay Company (Türkiye)*

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