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Protective Effect of Erythropoietin on post-MI Liver Tissue

Meltem İÇKİN GÜLEN [1], Aysel GÜVEN BAĞLA [1], Özlem Tuğçe ÇİLİNGİR KAYA [2], Feriha ERCAN [2]

ABSTRACT

Aim: Cardiac hepatopathy arises due to heart failure and influences has effects on heart recovery after myocardial infarction (MI).The aim of this study was to investigate the protective effect of Erythropoietin (EPO) on liver tissue exposed to ischemia due to MI.

Material and Methods: Experimental MI was established by left anterior descending coronary artery ligation (CAL) and EPO or saline was injected immediately after CAL to five groups of rats, which groups are Control, Saline, EPO 5000, EPO 10000, CAL+1h. CAL+1h group was sacrificed one hour after CAL without any treatment. Other groups were sacrificed six hours after the operation. Liver tissues were examined histopathologically by Hematoxylin Eosin (HE) staining and electron microscopy.

Results: Degenerative changes in liver tissue such as vacuolization, sinusoidal dilatation, hepatocyte pyknosis, Kuppfer cell activation were observed. Vacuolization, and sinusoidal dilatation increased in the Saline group compared to the control group (p=0.010 for both). Degenerated hepatocytes with pyknotic nuclei as well as activated Kuppfer cells were decreased in the EPO 10000 group compared to the Saline group (p=0.009), and activated Kupfer cells were decreased compared to the Saline and CAL+1h groups (p=0.035 and p=0.019, respectively).

Conclusion: EPO protected liver tissue from histopathological damages regardless of dose, when given at the time of MI. EPO, when given immediately after MI, protected liver tissue from histopathological damage regardless of dose. Considering the mutual interaction of liver and heart, applying EPO to MI patients at first sight may prevent post-MI liver damage and contribute to the recovery of the heart.

Keywords: Cardiac hepatopathy; erythropoietin; ischemia, liver; myocardial infarction.

Eritropoietinin MI Sonrası Karaciğer Dokusu Üzerinde Koruyucu Etkisi

ÖZ

Amaç: Kardiyak hepatopati, kalp yetmezliğine bağlı olarak ortaya çıkar ve miyokard infarktüsü (MI) sonrası kalbin iyileşmesini etkiler. kalp dokusu iyileşmesi üzerinde etkileri bulunmaktadır. Bu çalışmanın amacı, MI nedeniyle iskemiye maruz kalan karaciğer dokusu üzerinde Eritropoetinin (EPO) koruyucu etkisinin araştırılmasıdır.

Gereç ve Yöntemler: Sol ön inen koroner arter ligasyonu (KAL) ile deneysel MI oluşturuldu ve Kontrol, SF (serum fizyolojik), EPO 5000, EPO 10000, KAL+1s. olmak üzere beş grup sıçana KAL'dan hemen sonra EPO veya SF enjekte edildi: KAL+1s grubu KAL'dan bir saat sonra herhangi bir tedavi uygulanmadan sakrifiye edildi. Diğer gruplar, operasyondan altı saat sonra sakrifiye edildi. Karaciğer dokuları Hematoksilen Eozin (HE) boyama ve elektron mikroskobu ile histopatolojik olarak incelendi.

Bulgular: Karaciğer dokusunda vakuolizasyon, sinüzoidal dilatasyon, hepatosit piknozu, Kuppfer hücre aktivasyonu gibi dejeneratif değişiklikler gözlendi. SF grubunda vakuolizasyon ve sinüzoidal dilatasyon Kontrol grubuna göre arttı (her ikisi için p=0,010). EPO 10000 grubunda piknotik çekirdekli dejenere hepatositler SF grubuna göre azalırken (p=0,009), ve aktive Kuppfer hücreleri SF ve KAL+1s gruplarına göre azaldı (sırasıyla p=0,035 ve p=0,019).

1 Canakkale Onsekiz Mart University, School of Medicine, Department of Histology and Embryology, Canakkale, Turkey
2 Marmara University, School of Medicine, Department of Histology and Embryology, Istanbul, Turkey

Sorumlu Yazar / Corresponding Author: Meltem İÇKİN GÜLEN , e-mail: meltemickin@hotmail.com
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Sonuç: EPO, MI sırasında meydana gelmesinden hemen sonra verildiğinde, dozdan bağımsız olarak karaciğer dokusunu histopatolojik hasarlanmadan korumuştur. Karaciğer ve kalbin karşılıklı etkileşimi göz önüne alındığında, MI hastalarına ilk görüşte EPO uygulanması, MI sonrası karaciğer hasarını önleyebilir ve kalbin iyileşmesine katkıda bulunabilir.

Anahtar Kelimeler: Kardiyak hepatopati; eritropoietin; iskemi; karaciğer; miyokard infarktüsü.

INTRODUCTION

Ischemic hepatitis occurs as a result of acute hypoperfusion (1). Cardiogenic shock is one of the conditions that cause ischemic hepatitis which appears with a sudden increase in serum hepatic transaminases indicating severe hepatocellular necrosis (2). Heart failure causes liver damage because the liver receives 25% of the blood pumped by the heart (2), but liver dysfunction in heart failure has rarely been studied (3).

In cardiac hepatopathy due to heart failure, decreased arterial perfusion and passive congestion lead to cardiogenic hypoxic hepatitis. In this situation, liver function may be impaired and it is important to maintain perfusion of vital organs as well as to treat primary heart disease. Impaired liver function worsens cardiac dysfunction (2) and liver-derived cytokines have been shown to be effective in heart recovery after myocardial infarction (MI) (4). The negative effects of MI on liver tissue and the potential of various agents to prevent these negative effects have rarely been studied. In studies investigating post-MI liver tissue, the effects of betaine (an essential organic osmolyte) (3) and tribulus terrestris (a fruit) (5) have been examined.

Previous studies on ischemic liver mostly focused on cold ischemia and reperfusion during liver transplantation (6-11) or hemorrhagic shock (12-14). Studies investigating the effects of erythropoietin (EPO), which is the main hemopoietic hormone, for the prevention of cold ischemia in the liver that occur during transplantation started with Yilmaz (11). Similar to this study, blood vessels were occluded in order to induce ischemic hepatitis in subsequent studies (6-8). EPO is secreted by the kidney as well as the fetal liver for erythropoiesis in mammals (15). Production and secretion of EPO and its receptor (EPO-R) are regulated by the oxygenation of the tissue. EPO and EPO-R are expressed in many tissues and also have various effects on nonhemopoietic cells. It shows antiapoptotic, neuroprotective and cardioprotective effects in ischemia. It takes a role in angiogenesis, neurogenesis and immune response. It has a protective effect on metabolic changes, neuronal and vascular degenerations and inflammatory cell reactivation. Apart from kidney and liver, EPO and EPO-R are also expressed in the brain, cardiovascular, digestive, respiratory, endocrine and reproductive systems. EPO mobilizes bone marrow-derived endothelial progenitor cells in blood vessels and enables them to participate in the circulation (15). The protective effects of EPO on heart (16,17) and kidney tissues (18-20) have been demonstrated in several studies. We also previously demonstrated the protective effect of EPO administered at the time of experimental MI on both heart and kidney tissues (21,22). EPO has been used exogenously in the treatment of chronic renal failure (CRF) anemia for years, but there are different opinions about determining the lowest possible dose of EPO due to its side effects (23). Use of high doses of recombinant human EPO (rhEPO) may increase the risk of thrombosis (24). It has been observed that early treatment of anemia in chronic renal failure slows down the progression of kidney disease. It was thought that this may be due to the potential renal and cardiovascular protection of the drugs used in the treatment of anemia, not the correction of anemia (23). Considering the effect of the liver tissue on the post MI heart tissue recovery, we examined the histopathological effects of two different doses of EPO on the liver tissue in an experimental MI model.

MATERIAL AND METHODS

Animal rights were protected in accordance with the principles of the Guide for the Care and Use of Laboratory Animals and animal protocols were approved by the Gazi University Animal Experiments Local Ethics Committee (project number GUET-08.059). We studied the liver tissues of rats that we performed experimental MI and worked on heart and kidney tissues before (21,22). Additional approval for the liver tissues was received from the ethics committee.

All interventions on animals were performed at Gazi University Laboratory Animal Care and Experimental Research Center, Ankara. Male Wistar rats (250-300 g) were randomly allocated into five groups, which are: control group (n=3), rats sacrificed 6h after sham operation; saline group (n=7), rats given intraperitoneal (i.p.) injection of saline immediately after CAL and sacrificed 6h after surgey; EPO 5000 group (n= 9), rats given i.p. injection of EPO 5000 U/kg (rhEPO-α, Eprex 4,000 IU/0.4 ml pre-filled syringe; Janssen Cilag AG, Schaffhausen, Switzerland) (standard dose) immediately after CAL and sacrificed 6h after surgey; EPO 10000 group (n= 9), rats given i.p. injection of EPO 10000 U/kg (high dose) immediately after CAL and sacrificed 6h after surgey; CAL+1h group (n=8), rats sacrificed 1h after CAL without treatment. The dose of EPO was determined according to previous reports (25).

AMI was generated by left anterior descending CAL. Rats were anesthetized with 45 mg/kg ketamine (Alfamine 10%; Alfasan International BV, Woerden, The Netherlands) and 5 mg/kg xylazine (Alfazyne 2%; Alfasan International BV) administered i.p. Saline, standard dose EPO or high dose EPO was administered i.p. to groups saline, EPO 5000 and EPO 10000 groups, respectively immediately after CAL. Rats were allowed to awaken and transferred to their cages where they stayed until the end of the experiment. They were fully anesthetized and liver tissues were obtained for light and electron microscopic investigations. The rats were then sacrificed by cervical dislocation.

Light microscopy

Half cm thick pieces of liver tissue were fixed in 10% formalin solution, subjected to gradient dehydration through a series of ethanol, immersed twice in xylol and embedded in paraffin. Four µm thick sections were cut and deparaffinized in xylol, subjected to gradient rehydration through a series of ethanol and bidistilled water. Sections were stained with hematoxylin and eosin (H&E) (Harris Hematoxylin and 1% Alcoholic Eosin, BESLAB, Turkey) , and examined using ZEN blue

edition software and Zeiss Scope A1 microscope and photographed with Axiocam 503 color camera.

Nonoverlapping ten fields (20X) were selected randomly from the sections from the middle of the liver tissues for light microscopic evaluation. Liver sections were scored using a semiquantitative scale to evaluate the degree of vacuolization of hepatocytes, hepatocellular degeneration with pyknotic nuclei, sinusoidal dilatation and activation of Kuppfer cells. Histological damage was scored according to the previous reports as 0: no damage; 1: damage in <10% of all hepatocytes/10 HPF; 2: damage in 10%-30% of all hepatocytes/10 HPF; damage in >30% of all hepatocytes/10 HPF (26).

Electron microscopy

Liver samples (1mm3) were fixed four hours with 2.5 % glutaraldehyde (pH: 7.3) in 0.1 M phosphate-buffered saline (PBS) at 4 ºC, then post-fixed in 2 % OsO4 (0.1 M), dehydrated in a graded series of ethanol, and embedded in epoxy resin. Semithin sections were stained with toluidin blue. Ultrathin sections (70 nm) were stained with uranyl acetate andlead citrate. They were finally examined and photographed under a transmission electronmicroscope (1200 SX TEM; JEOL, Tokyo, Japan).

Two researchers blinded to sample grouping performed the histopathologic analysis of specimens by light microscopy and electron microscopy.

Statistical analysis

Statistical analysis was performed using the SPSS version 19.0 (SPSS Inc., Chicago, IL). Results were expressed as mean ± SD, median, minimum, maximum. All data were evaluated with the Kolmogorov–Smirnov test and the Shapiro–Wilk test for determining normal distribution. Values for $p \leq 0.05$ were considered statistically significant. Nonparametric tests were performed for histopathological examinations, comparison between groups was done using Kruskal-Wallis was performed. A p-value of < 0.05 was considered statistically significant. Resulting p-values were corrected according to the Bonferroni method ($p<0.017$). Data were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons.

RESULTS

Light microscopy

Saline group showed increase in vacuolization and sinusoidal dilatation compared with the control group (p=0.010 for both, Figure 1A, 1C, 2 and Table 1). EPO 10000 group showed a decrease in degenerated hepatocytes with pyknotic nuclei in the EPO 10000 group compared to the Saline group (p=0.009). Activated Kupfer cells were decreased compared to the Saline and CAL+1h groups (p=0.035 and p=0.019, respectively, Figure 1C, 1E, 2 and Table 1). Number of activated Kuppfer cells in EPO 10000 group were also lower compared with CAL+1h group (Figure 1B, 1E and 2).

Degenerated hepatocytes with pyknotic nuclei were decreased in the EPO 10000 group compared to the SF group (p=0.009), and activated Kupfer cells were decreased compared to the SF and CAL+1h groups (p=0.035 and p=0.019, respectively).

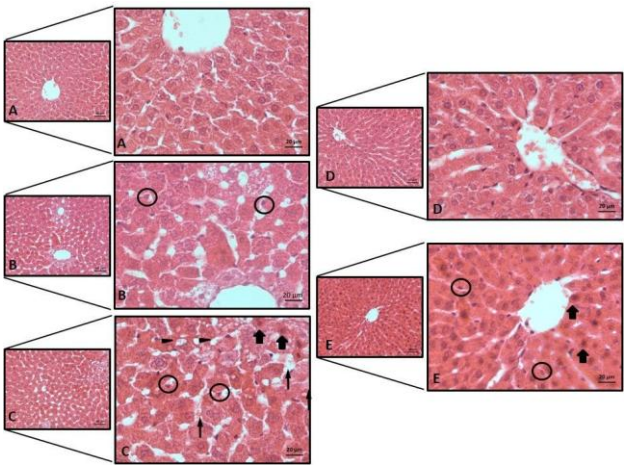

Figure 1. Light micrographs of experimental groups. **A.** Control group showed regular liver morphology. B. CAL+1h group showed increase in number of activated Kuppfer cells (circles). C. Saline group showed increase in vacuolization (arrow heads), sinusoidal dilatation (thin arrows), number of activated Kuppfer cells and degenerated hepatocytes with pyknotic nuclei (thick arrows). D. EPO 5000 group. E. EPO 10000 group showed a decrease in number of degenerated hepatocytes with pyknotic nuclei and activated Kuppfer cells. HE staining. Scale bars= 50 µm, 20 µm.

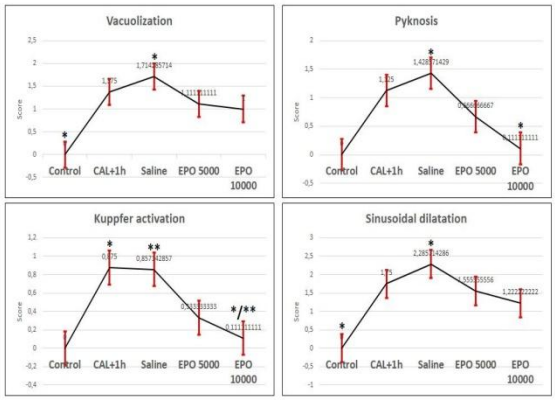

Figure 2. Histopathological results.

Vacuolization: *Saline group showed an increase in scores compared with control group (p=0.010).

Pyknosis: *EPO 10000 group showed a decrease in scores compared with saline group (p=0.009).

Kuppfer activation: */**EPO 10000 group showed a decrease in scores compared with *CAL+1h group (p=0.019) and with **saline group (p=0.035).

Sinusoidal dilatation: *Saline group showed increase in scores compared with control group (p=0.010).

Electron microscopy

Regular liver parenchyme with hepatocytes and sinusoids were observed in control group (Figure 3A and 3B). A few number of increase in degenerated hepatocytes with vacuole formation and degenerated bile canaliculi with loss of microvillar formation in some region were observed in CAL+1h group (Figure 3C and 3D). Severe increase in degenerated hepatocytes with organelle loss

Table 1. Comparison of histological parameters between groups

Groups	Vacuolization	Pyknosis	Kuppfer activation	Sinusoidal dilatation
Control (n=3)				
m±SD	0.0	0.0	0.0	0.0
median (min-max)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
Saline (n=7)				
m±SD	1.7±0.5*	1.4±0.5	0.9±0.4	2.3±0.9##
median (min-max)	2.0 (1.0-2.0)	1.0 (1.0-2.0)	1.0 (0.0-1.0)	3.0 (1.0-3.0)
EPO 5000 (n=9)				
m±SD	1.1±0.6	0.7±1.1	0.3±0.5	1.6±0.9
median (min-max)	1.0 (0.0-2.0)	0.0 (0.0-3.0)	0.0 (0.0-1.0)	1.0 (1.0-3.0)
EPO 10000 (n=9)				
m±SD	1.0±1.0	0.1±0.3**	0.1±0.3#	1.2±0.8
median (min-max)	1.0 (0.0-3.0)	0.0 (0.0-1.0)	0.0 (0.0-1.0)	1.0 (0.0-2.0)
CAL+1h (n=8)				
m±SD	1.4±0.7	1.1±0.9	0.9±0.4	1.8±0.9
median (min-max)	1.0 (1.0-3.0)	1.0 (0.0-3.0)	1.0 (0.0-1.0)	1.5 (1.0-3.0)
p	0.017	0.004	0.002	0.018

All results are presented as mean ± standard deviation (m ± SD) and median (min-max), $p<0.05$: Kruskal Wallis Test.

*$p<0.017$ vs. Control group, **$p<0.17$ vs. Saline group, #$p<0.017$ vs. Saline and CAL+1h groups, ## $p<0.017$ vs. Control group

and bile canalliculi degeneration (arrowhead, Figure 3F) were observed in saline group (Figure 3E and 3F). These degenerated hepatocytes with organelle loss and bile canaliculi microvillar loss were decreased in rats in EPO 5000 and EPO 10000 groups (Figure 3G and 3H).

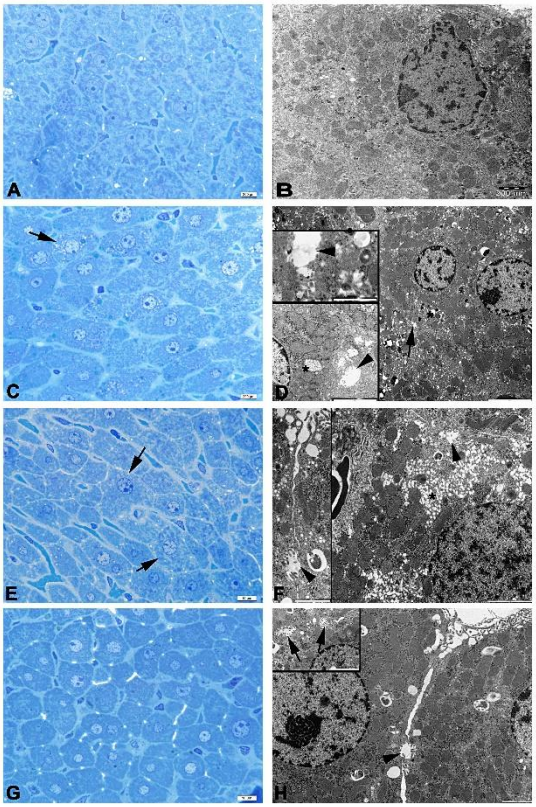

Figure 3. Representative light (A, C, E and G) and electron (B, D, F and H) micrographs of experimental groups. Regular liver parenchyme with hepatocytes and sinusoids were observed in control group (A and B). Moderate increase of degenerated hepatocytes (arrow, C) with organelle loss (*, D), regular (arrow) and degenerated bile (arrowhead) canaliculi with loss of microvillar formation (D) were observed in CAL+1h group (C and D). Severe increase in degenerated hepatocytes (arrow, E) with organelle loss (*, F) and degenerated canalliculi with loss of microvilli (arrowhead) were observed in saline group (E and F). Quite regular hepatocytes and bile canaliculi (arrow), and some degereated bile canaliculi (arrowhead) with microvillar loss were decreased in EPO 5000 and EPO 10000 groups (G and H). A, C, E and G: TB staining, B, D, F and H: UA and LC staining. Scale bars= 200 µm, 2 µm.

DISCUSSION

The adverse effect of hypoxia in the liver is a problem encountered in many clinical situations. Liver functions are significantly impaired in many conditions that lead to impaired blood flow in the liver such as hemorrhagic shock (HS) (14,27,28), liver transplantation (24,29) and heart failure (30).

Effects of some nutrients have been studied on liver damage induced by experimental heart failure. Betaine, which is known to have protective effects against tetrachloride carbon, alcohol and I/R damage on liver, was shown to reduce apoptosis, mononuclear cell infiltration, portal and central and congestion, and sinusoidal dilatation (3). Tribulus terrestris fruit aqueous extract was shown to decrease sinusoidal dilatation, central vein congestion and hepatocyte vacuolation in liver of rats with isoproterenol induced MI (5). Both nutrients revealed decrease hepatic enzymes indicating liver injury.

Researchers who studied ischemia reperfusion injury (I/R) in the liver mostly used the method of Sepodes et al. who created ischemia by ligating hepatic artery and portal vein (6). Protective effects of different antioxidant and anti-inflammatory substances have been shown on hepatic I/R created by this method; such as Genistein, a natural compound (31) or Melatonin, a hormone secreted by the pituitary gland (32). Administration of 1000u/kg rhEPO before ischemia, decreased histopathological damage and caspase-3 activity in the liver as well as the increase in ALT and AST, enzymes showing liver function and MDA, indicating oxidative stress (6).Hepatoprotective effect of rhEPO was shown also by Luo et al. who administered 1000 u/kg EPO 24 hours before creating hepatic ischemia with Sepodes' method and showed that EPO reduced hepatic I/R injury. They observed an increase in the amount of hemoxygenase-1 (HO-1) in the liver which is one of the most important cytoprotective mechanisms activated during cellular stress caused by hypoxia. Hepatocyte necrosis and inflammatory cell infiltration were reduced and hepatic lobule structures were preserved with rhEPO in their study (7). In these studies, it was suggested that EPO reduces oxidative damage by showing a decrease in MDA or an increase in HO-1 (6,7). In both studies, researchers thought that the protective effect of EPO arose through receptor-mediated signaling, since hemoglobin levels did not change with rhEPO treatment.

EPO has an antiapoptotic effect (15). In liver I/R, rhEPO has been shown to reduce liver caspase-3 and caspase-9 activities (6,8). We also observed hepatocyte vacuolization and pyknotic nuclei, which are accepted as apoptosis indicators (30). Moreover, these findings decreased with rhEPO treatment. We observed that the effects of EPO we applied in two different doses (5000 and 10000 u/kg) was the same. In a previous study to examine the effect of different doses of EPO (1000 and 5000 u/kg), it has been shown that intraportal administration of rhEPO injection before ischemia is advantageous in decreasing caspase-9 activity and histopathological damage and low dose is more effective in histopathological improvement than high dose. It was observed that rhEPO given at the beginning of reperfusion after ischemia was not as effective as rhEPO given before ischemia occurred. Regardless of the dose, preconditioning has been shown to be more effective than postconditioning (9).

In studies examining liver I/R damage in HS, rhEPO was shown to prevent liver damage. Comparision of effects of intravenous EPO given 60 minutes before and 30 minutes after experimental HS in rats in a study revealed that EPO is more effective in reducing liver damage if it is given before HS occurs (12). In a later experimental HS study, it was shown that EPO given three hours before the induction of HS significantly attenuated renal, hepatic

and neuromuscular injury and dysfunction caused by HS (13).

In our study, we performed rhEPO treatment immediately after CAL. In fact, we aimed to investigate the effect of rhEPO on liver tissue, when given a patient with MI at first sight; because regular function of liver tissue is necessary for the recovery of heart disease (2). Thus, we administered rhEPO just before expected liver ischemia and demonstrated that this has a protective effect. Although patients survive with early treatments after MI, they have to live with heart failure. Different treatment methods should be investigated in order to provide ventricular remodeling after MI. Cardiomyokines and hepatokines play a role in the communication between heart and liver. Exosomes and migrasomes, non-secretory gene-related signals also play a role in this communication (33). Liver-derived cytokines have been shown to be effective in heart recovery after MI. Tang et al. reported IL-22, an important cytokine regulating inflammation and tissue repair, is involved in cardiac repair after experimental MI (4). Liu et al. suggested macrophage-derived IL-22 production prevented ethanol-induced hepatocyte apoptosis, after demonstrating IL-22 is produced by mouse primary macrophages (34). In our study, larger and more activated Kuppfer cells in saline and CAL+1h groups may indicate these cells' role in post-MI cardiac recovery. The decrease in this activity in the EPO 10000 group may indicate that EPO contributes to this healing and that no more cytokines need to be released from Kuppfer cells.

The limitation of this study is that we did not study hepatic enzymes biochemically. This may have helped understanding the functional recovery of liver.

CONCLUSION

We demonstrated the protective effect of rhEPO on degenerative changes in liver tissues of rats with experimental MI, when it is applied immediately after CAL. EPO may have shown this protective effect because it was given before the onset of ischemia in liver tissue. We showed that the protective effects of two different doses of EPO, when injected immediately after CAL, were not different histopathologically. The importance of the protective effect is that there is a mutual interaction between the function of the heart and the liver. Administration of EPO to MI patients at first sight may contribute to the recovery of the heart by preventing ischemic damage of the liver.

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