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EFFECTS OF ERYTHROPOIETIN ON THE SERUM AND LIVER TISSUE LEVELS OF COPPER AND ZINC IN RATS WITH OBSTRUCTIVE JAUNDICE

EFEKTI ERITROPOETINA NA NIVOE BAKRA I CINKA U SERUMU I TKIVIMA JETRE KOD PACOVA SA OPSTRUKTIVNOM ŽUTICOM

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Summary

Background: Erythropoietin is an anti-apoptotic, anti-inflammatory, angiogenetic cytokine and has protective properties against oxidative stress. In this study we investigated the effects of erythropoietin on the levels (serum and liver tissue) of copper and zinc in cholestatic rats.
Methods: Thirty-two Wistar albino rats used in the study were divided into four groups – Group I: Sham; Group II: Erythropoietin; Group III: Obstructive Jaundice; Group IV: Obstructive Jaundice+Erythropoietin. After the first operation, rats were followed up for seven days and then operated for the second time. Rats were sacrificed by intracardiac blood taking, and the liver tissue samples were obtained immediately.
Results: Erythropoietin reduces copper, and increases zinc levels in serum and liver tissues after obstructive jaundice ($p<0.05$). Furthermore, it has been shown that the levels of alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transferase, alkaline phosphatase and total bilirubin/direct bilirubin were significantly lower in Obstructive Jaundice+Erythropoietin group than Obstructive Jaundice group.
Conclusions: Erythropoietin affects the changes in copper and zinc levels, thus decreasing the liver damage biochemically in rats with obstructive jaundice. However, further investigations are needed to discover how erythropoietin therapy might reduce target organ damage in cholestatic liver cases by affecting copper and zinc levels.

Keywords: obstructive jaundice, erythropoietin, liver damage, copper, zinc

Kratak sadržaj

Uvod: Eritropoetin je citokin sa antiapoptotskim, antiinflamatornim i angiogenetskim svojstvima koji deluje protektivno u odnosu na oksidativni stres. U ovoj studiji istraživali smo efekte eritropoetina na nivoe (u serumu i tkivima jetre) bakra i cinka kod pacova sa holestazom.
Metode: Trideset dva albino pacova soja Wistar korišćena u studiji podeljena su u četiri grupe – Grupa 1: kontrola; Grupa 2: eritropoetin; Grupa 3: opstruktivna žutica; Grupa 4: opstruktivna žutica+eritropoetin. Posle prve operacije pacovi su praćeni sedam dana a zatim operisani drugi put. Pacovi su žrtvovani intrakardijalnim vađenjem krvi i odmah su uzeti uzorci tkiva jetre.
Rezultati: Eritropoetin snižava nivoe bakra, a podiže nivoe cinka u serumu i tkivima jetre posle opstruktivne žutice ($p<0,05$). Pored toga, pokazano je da su nivoi alanin aminotransferaze, aspartat aminotransferaze, gama-glutamil transferaze, alkalne fosfataze kao i nivoi ukupnog/direktnog bilirubina bili značajno niži u Grupi 4 nego u grupi sa opstruktivnom žuticom.
Zaključak: Eritropoetin utiče na promene u nivoima bakra i cinka i na taj način biohemijski umanjuje oštećenja jetre kod pacova sa opstruktivnom žuticom. Međutim, potrebna su dalja istraživanja kako bi se otkrilo na koji način terapija eritropoetinom, kroz uticaj na nivoe bakra i cinka, može umanjiti oštećenja ciljnih organa u slučajevima holestaze jetre.

Ključne reči: opstruktivna žutica, eritropoetin, oštećenja jetre, bakar, cink

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Introduction

Obstructive jaundice (OJ) is a frequent situation in surgery clinics resulting in higher mortality and morbidity. OJ is a clinical condition which may be caused by bile stones, cessation of bile flow due to tumors and strictures, and accumulation of bile acids in the liver (1). Its etiopathogeny is not clear yet. The release of free oxygen radicals, due to bile acid damage, results in interference with Kupffer cells and neutrophils, and this clinical picture may not be limited to the liver, but its systemic effects could cause damage of various organs (2). OJ causes dilatation in hepatocytes and bile ducts, and due to high concentrations of hydrofobic bile acids it causes oxidative damage and inflammation (3). Mediators liberated as a result of oxidative damage, by increasing the quantity of free radicals, cause activation of the coagulation cascade, impairment of microcirculation and clinical pictures which may lead to multiple organ failure (4). In diseases such as primary biliary cirrhosis, alcoholic cirrhosis and OJ, it has been shown that zinc (Zn) levels decrease in the liver, while copper (Cu) and mangenese levels increase (5). Zn is one of the basic trace elements which plays a catalytic and structural role in many enzymes (6). Besides its anti-inflammatory and anti-apoptotic effects, Zn has important antioxidant properties and affects the processes such as growth and development, cancer formation and aging (7). While Zn is necessary for liver function, liver is important for zinc hemostasis (8). In experimental animal models, although not understood exactly, it has been seen that zinc has hepatoprotective properties in terms of acute and chronic liver damage (8). Furthermore, it has been shown that zinc supplementation leads to reduction in blood ammonia (9). Cu is a trace element which takes its place as a co-factor in a number of enzymatic reactions (amine oxidase, Cu-dependent superoxide dismutase, cytochrome oxidase and tyrosinase) (10). In primary biliary cirrhosis, alcoholic cirrhosis and other cholestatic syndromes, accumulation of excessive Cu in the liver is reported (7). Erythropoietin (EPO) has an effect in reducing inflammatory response via reducing the levels of pro-inflammatory cytokines and apoptosis induced by cytokines (11). Recent data show that EPO supports angiogenesis, reduces oxidative stress and accelerates wound healing (12, 13). Our aim in this study is to investigate the effects of EPO on the levels of Cu and Zn which are both basic trace elements, and its effects on the liver tissue damage in rats with OJ.

Materials and Methods

Chemical

Erythropoietin was purchased from Sigma (E5627–Erythropoietin human recombinant, expressed in Chinese hamster ovary cells, lyophilized powder, cell culture tested, ~ 100,000 units/mg protein) and dissolved in phosphate buffer saline.

Animals

Thirty-two female Wistar albino rats, each weighing 200–250 g, were included into the study at the Dicle University Health Sciences Application and Research Center. The study was conducted in accordance with the rules of the National Institute of Health Guide for the Care and Use of Laboratory Animals, following approval from the Ethics Committee. Rats were housed under standard conditions in an air-conditioned room with 12 h light and dark cycles, at a constant temperature (22 ± 2 °C). The rats were housed in cages, and allowed free access to standard rat chow and water before the experiments. The animals were fasted overnight the day before surgery, but had access to water.

Experimental design

Thirty-two Wistar albino rats were divided into four groups (n=8):

Group 1 (Sham, S); only the common hepatic duct was dissected and followed up for 7 days,

Group 2 (Erythropoietin, EPO); the common hepatic duct was dissected, and EPO was given at a dose of 500 IU/kg daily and followed up for 7 days,

Group 3 (Obstructive Jaundice, OJ); the common hepatic duct was dissected and ligated; followed up for 7 days,

Group 4 (Obstructive Jaundice + Erythropoietin, OJ+EPO); the common hepatic duct was dissected and ligated, EPO was given at a dose of 500 IU/kg daily and followed for 7 days.

Surgical procedure

Anesthesia was obtained by giving 50 mg/kg Ketamine hydrochloride (Ketalar®; Parke Davis, Pfizer, Istanbul, Turkey) and 10 mg/kg Xylazine (Rompun®; Bayer AG, Leverkusen, Germany) to the rats via intramuscular injection. For skin antisepsis, povidone iodine was applied and middle line incision was preferred. After laparotomy, the common bile duct was ligated with 4/0 silk and the incision was closed as a double layer after 4 mL physiologic solution was given to the peritoneal area. After 7 days, rats were given standard rat chow and water. The rats were anesthetised again by administering 50 mg/kg Ketamine hydrochloride (Ketalar®; Parke Davis, Pfizer, Istanbul, Turkey) and 10 mg/kg Xylazine (Rompun®; Bayer AG, Leverkusen, Germany) i.m., and then sacrificed by taking intracardiac blood. Liver tissue samples were taken out for analysis by the thoracoabdominal incision.

Biochemical analyses

In the blood samples, alanine transaminase (ALT) (IU/L), aspartate transaminase (AST) (IU/L), alkaline phosphatase (ALP) (IU/L), gamma glutamyl transferase (GGT) (IU/L), total bilirubin (TB) (mmol/L), direct bilirubin (DB) (mmol/L), Zn (ppm) and Cu (ppm) analyses were performed. Also, Zn (μg/protein) and Cu (μg/protein) measurements were performed in the liver tissue.

AST, ALT, ALP, GGT, TB and DB were measured in serum by a spectrophotometric method using an Architect® c16000 autoanalyzer (Abbott Laboratories, Abbott Park, IL, USA).

Weighed tissue samples were taken into heat-resistant glass tubes and 2.5 mL 65% nitric acid was added and incubated at room temperature for 1 h, then incubated at 100–120 °C for 2 h. After cooling at room temperature, 0.5 mL 65% perchloric acid was added and incubated at 150–180 °C for 2 h. After cooling, a vehicle solution was added to obtain the final 5 mL solution for measurement (12). Zn and Cu were determined by a Shimadzu 6401S atomic absorption/emission spectrometer. The acetylene flow rate and the burner height were adjusted in order to obtain the maximum absorbance signal with a slit of 0.5 nm, at a wavelength of 213.9 nm for Zn and 324.8 nm for Cu. The radiation sources were hollow cathode lamps (Shimadzu, Japan). Operating conditions were those recommended by the manufacturer (Operation Manual-Atomic Absorption Spectrophotometer AA-6800, SHIMADZU, 2000).

Statistical analysis

Statistical analysis was performed using SPSS for Windows 11.5 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation for biochemical values. Groups were compared by using the nonparametric Kruskal-Wallis test. Mann-Whitney U test was used for binary comparisons. P value of less than 0.05 was considered as significant.

Results

All animals survived throughout the experimental procedures. Liver functions and bilirubin levels of all the groups are listed in *Table I*, and the serum and liver tissue levels of Cu and Zn in *Table II*. Comparison of the groups in terms of Cu and Zn levels in the serum and liver tissue is shown in *Table II*.

In Group III, the serum values of TB, DB, ALT, AST, GGT and ALP were found significantly increased

Table I BALT, AST, ALP, GGT, TB, DB levels in serum.

Groups	S (n=8)	EPO (n=8)	OJ (n=8)	OJ+EPO (n=8)	p
ALT, IU/L	48.3±18.2	41.8±15.7	105.0±9.7§ ¶	77.1±15.1¥ ¶ ~	<0.001
AST, IU/L	271.8±80.5	222.7±64.8	510.7±108.5§ ¶	389.7±88.2€¶ $	<0.001
ALP, IU/L	52.3±10.9	51.37±11.0	113.1±18.1§ ¶	89.3±11.2 ¶*	<0.001
GGT, IU/L	33.8±9.7	33.37±9.5	120.5±11.5§ ¶	103.7±5.9 ¶*	<0.001
TB	0.3±0.04	0.3±0.1	10.8±2.6§ ¶	6.4±1.5 ¶*	<0.001
DB	0.1±0.0	0.1±0.0	4.2±1.1§ ¶	2.1±0.9 ¶*	<0.001

§ Significantly different when compared to Sham group ($p<0.001$), ¥ significantly different when compared to Sham group ($p<0.01$), $ significantly different when compared to Sham group ($p<0.05$), ¶ significantly different when compared to EPO group ($p<0.001$), ~ significantly different when compared to OJ group ($p<0.001$), * significantly different when compared to OJ group (p 0.01), $ significantly different when compared to OJ group ($p\leq0.05$).

Table II Cu (μg/protein) and Zn (μg/protein) levels in serum and liver tissue.

Groups	S (n=8)	EPO (n=8)	OJ (n=8)	OJ+EPO (n=8)	p
Serum Cu	1.13±0.14	1.1±0.2	3.85±0.43§¶	2.68±0.91¥¶*	<0.001
Serum Zn	0.75±0.14	1.2±0.3§	0.54±0.13¥¶	0.71±0.14¶$	<0.001
Liver Cu	2.99±0.71	2.6±0.8	4.49±0.86§†	3.37±0.94*	0.003
Liver Zn	24.72±4.97	26.7±5.0	16.23±2.40¥¶	21.47±3.97£*	<0.001

§ Significantly different when compared to Sham group ($p<0.001$), ¥ significantly different when compared to Sham group ($p\leq0.01$), ¶ significantly different when compared to EPO group ($p<0.001$), † significantly different when compared to EPO group ($p<0.01$), £ significantly different when compared to EPO group ($p<0.05$), * significantly different when compared to OJ group ($p\leq0.01$), $ significantly different when compared to OJ group ($p\leq0.05$).

when compared to the other groups ($p<0.05$). Zn levels in serum were higher in Group II ($p<0.001$) compared to Group I, while the other parameters did not show any significant differences ($p>0.05$) in these two groups. When Group II was compared to Group III, all the parameters were significantly different ($p<0.001$). Comparison of Group II and Group IV showed no significant difference in terms of Cu levels in liver tissue ($p>0.05$), while Zn levels were found to be significantly different ($p=0.028$). All the other serum parameters of these two groups were significantly different ($p<0.05$). Cu and Zn levels in the liver tissue of Group III and Group IV were significantly different ($p=0.010$ and $p=0.003$, respectively), and all the serum parameters of these two groups were also significantly different ($p<0.05$).

Discussion

Diseases which result in liver damage may change the levels of trace elements like Fe, Zn, Cu and Mn in the liver tissue, and these changes play a key role in the liver fibrosis process. D'Uscio et al. (14) found that treatment with EPO increased the vascular expression of SOD1 (superoxide dismutase). Although modulatory effects of EPO on Cu and Zn levels in certain liver and kidney diseases have been reported in some studies, its effects in OJ have not been clarified (3, 15). In general, the increase of Cu and decrease of Zn levels in the liver tissue and serum may be the characteristics of chronic diseases, such as severe cholestasis, biliary atresia and cirrhosis (16, 17). In cirrhotic patients, low levels of Zn and high levels of Cu are associated with the severity of liver fibrosis (5). In our study it was also established that ligation of the main bile ducts of rats leads to an increase in Cu levels and a decrease in Zn levels in serum and liver tissue. In ischemia-reperfusion models of experimental animal studies increased Cu and reduced Zn levels have been shown to be associated with oxidative stres (18). Devipriya et al. (13) reported increased Cu levels and decreased Zn levels in alcohol given rats. In cholestasis associated with impaired bile flow, Cu accumulates in the liver and Cu metabolism is impaired. The finding of increased levels of Cu in OJ rats in our study was supported by the results of Devipriya et al. (13). Excess cumulation of Cu in rats triggers oxidative damage of the kidney and liver tissue DNAs and ultimately contributes to many degenerative disorders (19). In this study we observed that OJ group had higher levels of serum and liver Cu, respectively ($p<0.001$ and $p=0.003$), than other groups. Also, ALT and AST levels were significantly increased in OJ group compared to Sham and EPO groups ($p<0.001$). These results support the finding that the accumulation of Cu in serum and liver leads to organ damage. In a study by Rodriguez et al. (5) a control group was compared to patients with alcoholic cirrhosis and it was found out that the liver Zn concentration is lower in cirrhotic patients. Furthermore, it was shown that Zn treatment prevented liver damage induced by ethanol, in both acute and chronic exposure to alcohol (20). We also established that Zn levels were significantly reduced in OJ and OJ+EPO groups compared to the non-obstructed groups. When rats in OJ and OJ+EPO groups were compared, in OJ+EPO group Zn levels in serum and liver tissue were higher ($p=0.038$ and $p=0.003$, respectively), and ALT ($p<0.001$), AST ($p=0.05$), TB and DB ($p=0.02$), GGT ($p=0.03$), and ALP ($p=0.007$) levels were lower in serum samples. These results support the view that EPO treatment may increase the levels of Zn in serum and liver tissue, and reduce liver damage.

EPO is an anti-apoptotic, anti-inflammatory, angiogenetic cytokine that has protective properties against oxidative stress (21, 22). Nishiya et al. (23) reported that EPO improves ventricular function after myocardial infarction, by increasing angiogenesis and reducing apoptosis. Johnson et al. (24) noted that EPO shows renoprotective effects by reducing acute renal damage. Liu et al. (25) showed that EPO implementation is cardioprotective due to suppression of the inflammatory response in myocardial ischemia-reperfusion damage. When given at early stages, EPO has been reported to have positive effects on liver ischemia-reperfusion damage by reducing oxidative stress and kaspase-3 activation (26). In our study an increase in serum Zn levels was observed ($p<0.001$) compared to Sham group, but there were no significant differences in other parameters. When OJ and OJ+EPO groups were compared, Cu levels in serum and liver tissue were lower ($p\leq0.01$), and Zn levels in serum and liver tissue were significantly higher in OJ+EPO group. Furthermore, in OJ+EPO group, GGT, ALP, TB, DB, ALT and AST ($p<0.05$) levels were lower compared with OJ group.

Conclusion

It is a well-known fact that the levels of Cu are increased, and the levels of Zn are decreased in the liver tissue of cirrhotic patients, and the severity of liver fibrosis is associated with these levels. It has been shown in this study that erythropoietin has regulatory effects on the serum and liver tissue levels of Cu and Zn, by decreasing Cu levels and increasing Zn levels. These results suggest that EPO can be used as an effective chemoprotective agent in OJ cases for regulating Cu and Zn levels. However, further investigation is needed to support our findings, and to explain the mechanism of how EPO treatment reduces target organ damage in cholestatic liver damage by affecting Cu and Zn levels.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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