The effects of erythropoietin on bacterial translocation and inflammation in rats with obstructive jaundice

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INTRODUCTION: *Obstruction of the common bile duct is associated with hepatic paranchymal damage and increased susceptibility to subsequent bacterial infections. Erythropoietin has antiinflammatory and cytoprotective effects and it induces antiinflammatory cytokines and suppresses the production of proinflammatory cytokines. In this study, we aimed to investigate the effect of Erythropoietin on bacterial translocation, inflammation and tissue damage in rats with obstructive jaundice.*
MATERIALS AND METHODS: *Thirty-two Wistar albino rats (200-250 g) were divided into 4 groups as follows: Group 1 (Sham); only hepatoduodenal ligament dissection, Group 2 (Erythropoietin); hepatoduodenal ligament dissection and given 500 IU/kg Erythropoietin subcutaneously, Group 3 (Obstructive jaundice); complete hepatoduodenal ligament ligation, Group 4 (Obstructive jaundice + Erythropoietin); complete hepatoduodenal ligament ligation and given 500 IU/kg Erythropoietin subcutaneously. After 7 days, the rats were sacrificed by taking blood from the heart for biochemical analyses. Peritoneal swab culture, liver, mesenteric lymph nodes, spleen and ileum were collected for microbiological and histopathological examinations.*
RESULTS: *Erythropoietin reduced the secretion of inflammatory cytokines, oxidative damage and bacterial translocation, prevent the formation of inflammatory changes in intestine and liver after obstructive jaundice.*
Conclusion: The treatment of EPO in rats with OJ reduces bacterial translocation, inflammation and tissue damage.

KEY WORDS: Bacterial translocation, Erythropoietin, Inflammation, Obstructive jaundice.

Introduction

Obstruction of the common bile duct is associated with hepatic paranchymal damage and increased susceptibility to subsequent bacterial infections. The mechanisms of injury caused by cholestasis are unclear. The relationship between oxidative stress as well as lipid peroxidation and cholestatic liver injury has been reported [1]. Also, decreased antioxidative capacity and increased lipid peroxide levels have been reported in hepatic mitochondria in common bile duct-ligated rats [2]. It has been shown that inflammation contributes cholestatic liver injury by increasing production of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and IL-6 [3].
Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide syntheses inhibitor, is synthesized by protein-arginine-methyltransferases on arginine residues of nuclear proteins. Most of free circulating ADMA is primarily metabolized by dimethylarginine dimethylamino-

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hydrolase (DDAH) enzyme which is widely distributed in various tissues, including the liver [4]. ADMA levels are increased in adult rats with bile duct ligation (BDL) [5], It has suggested that reduced NO production by ADMA induces impaired vasorelaxation, resulting in portal hypertension and tissue damage in adult BDL rats [6].
Bacterial translocation occurs in obstructive jaundice due to alterations in both intestinal barrier and reticuloendothelial system. These alterations may be mucosal damage in the intestinal lumen due to lack of bile, apoptosis, bacterial overgrowth, motility disorder associated with oxidative stress [7].
Erythropoietin (EPO) is a member of the type I cytokine super family, which consists of several cytokines such as interleukin IL-6, IL-11, IL-12 and others. The pleiotropic effects of EPO apart from its hematopoietic effects, as tissue protective effects in different organs via paracrine pathways are well known [8]. EPO receptors are expressed on many different cells such as endothelial cells, cells of the central nervous system, uterine cells and liver cells [9]. Moreover, EPO has antiinflammatory and cytoprotective effects and it induces antiinflammatory cytokines and suppresses the production of proinflammatory cytokines like TNF-α and IL-6 [10].
The aim of the study is to investigate the effect of EPO on bacterial translocation, inflammation and tissue damage in rats with OJ.

Materials and Metods

Chemical

Erythropoietin was purchased from Sigma (E5627-Erythropoietin human recombinant, expressed in CHO cells, lyophilized powder, and cell culture tested and ~ 100,000 units/mg protein).

Animals

Thirty-two albino rats, each weighing 200-250 g. were included into study at Dicle University Health Sciences Application and Research Center. The experimental study was approved by the Committee of Experimental Animals of Dicle University. All experimental procedures complied with the guide for the Care and Use of Laboratory Animals. Rats were housed under standard conditions in an air-conditioned room with 12 h light and dark cycles, with 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.
Thirty-two Wistar Albino rats were divided into four groups (n=8);
Group 1 (**Sham, S**); only hepatoduodenal ligament dissection was performed.
Group 2 (**Erythropoietin, EPO**); Hepatoduodenal ligament dissection was performed and 500 IU/kg EPO was given daily via subcutaneous route.
Group 3 (**Obstructive jaundice, OJ**); Hepatoduodenal ligament dissection with common hepatic duct ligation.
Group 4 (**Obstructive jaundice + EPO, OJ+EPO**); Hepatoduodenal ligament dissection with common hepatic duct ligation and 500IU/kg EPO was given daily via subcutaneous route for 7 days.

Surgical Procedure

Rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar ®, Parke Davis, Eczacibasi, Istanbul, Turkey) and 10 mg kg xylazine (Rompun ®, Bayer AG, Leverkusen, Germany) via intramuscular injection for all surgical procedures. For laparotomy, a midline incision was performed under sterile conditions and hepatoduodenal ligament was dissected. After 2 mL saline was given into peritoneal area, abdominal wall was closed in one layer in groups S and EPO. In groups OJ and OJ+EPO, after laparotomy and midline incision, hepatoduodenal ligament was dissected, and common hepatic duct was placed in the middle and ligated with 3-0 silk suture, obstructing the passage. Then 2 mL saline was given into peritoneal area and abdominal wall was closed in one layer [11].
After a period of 7 days, the rats were anaesthetized and sacrificed by taking blood from the heart for biochemical analyses. Under complete sterile conditions, immediately, a thoracoabdominal midline incision was performed. After opening the abdomen, peritoneal swab culture was taken for microbiological analyses and a 1 mL blood sample was drawn from the inferior vena cava. Liver, mesenteric lymph node (MLNs) and spleen were collected for microbiological. And also liver, MLNs and ileum were resected for histopathological examinations. Serum was obtained from the centrifugation of the blood was stored at -80 °C until analyses. The tissues for histopathological evaluation were put into plastic containers with 10% formaldehyde solution after washing with saline for removing foreign tissue residues and blood.

Microbiological assay

Blood samples were obtained from the heart and cultured aerobically and anaerobically using the BacTec™ Peds battles (Becton-Dickinson Diagnostic Inc., Sparks, MD, USA). Identification was realized, by the BD-Phoenix 100 TM system. Peritoneal swab and positive cultures were plated out on blood agar, eosin methylene blue (EMB) agar, chocolate agar and Sabouraud-dextrose agar. At the same time, MLNs, spleen and liver were removed and placed in sterile glass bottles containing sterile brain-heart infusion media. The bottles were reweighed and tissue homogenates were prepared in 2 mL brain-heart infusion using a sterile mortar and pestle. A

portion (0.1 ml) of each homogenate was cultured on blood agar, EMB agar, and chocolate agar and Sabouraud-dextrose agar. All agar plates were examined after 24 h and 48 h of incubation at 37°C. The incidence of bacterial translocation was calculated by determining the number of rats with positive bacterial culture divided by the total number of rats studied.

Biochemical analyses

AlanineTransaminase (ALT), Aspartate Transaminase (AST), Total Bilirubin (TBil), Direct Bilirubin (DBil), Alkaline phosphatase (ALP), Gama-glutamil transferase (GGT), Total oxidant activity (TOA), Total antioxidant capacity (TAC), Paraxonase (PONX), Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-1 beta (IL-1β), C-reactive protein (CRP) and Asymmetric dimethyl arginine (ADMA) analyses were performed in blood samples.

Measurement of liver enzymes and bilirubin.
ALT, AST, ALP, GGT, Total and direct bilirubin were measured by spectrophotometric method in serum using an Architect® c16000 auto analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Measurement of TOA
TOA of supernatant fractions was determined using a novel automated measurement method, developed by Erel [12]. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of mol H_2O_2 Equiv./L.

Measurement of the TAC
TAC of supernatant fractions was determined using a novel automated measurement method developed by Erel [13]. In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in Reagent 1, is mixed with hydrogen peroxide, which is present in Reagent 2. The sequential produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, antioxidative effect of the sample against the potent-free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The results are expressed as mmol Trolox Eq./L.

Measurement of the PONX:
Serum PONX levels were measured spectrophotometrically by modified Eckerson method. Initial rates of hydrolysis of paraoxon (0.0-diethyl-0-p-nitrophenylphosphate; Sigma Chemical Co. London, UK) were determined by measuring liberated- p-nitrophenol at 405 nm at 37°C. The results are expressed as U/L (14).

Measurement of the TNF-α, IL-6, IL-1β, CRP and ADMA
TNF-α, IL-6 and IL-1β (Diasource; Nivelles, Belgium), ADMA (Immundiagnostic; Bensheim, Germany) and CRP (DRG; NJ, USA) levels were measured using commercially available ELISA kits.

Histopathological assessment

Ileal segment, MLNs and liver tissues were put into the 10% formalin solution in paraffin blocks and it is prepared by slicing 4-μm sections. Tissues stained with hematoxylin-eosin and standard protocols were applied. Liver, MLNs and ileal segment samples were examined for inflammatory cell infiltrate grading, and also ileal segments were examined under light microscopy for ileal

Table I - *The results of cultures according to the groups.*

	S (n=8)	EPO (n=8)	OJ (n=8)	OJ + EPO (n=8)
Blood culture (a/b)	0/8	1/8	7/8 ŦB	2/8
Liver culture (a/b)	0/8	1/8	7/8 ŦB	2/8
Splenic culture (a/b	1/8	1/8	3/8	1/8
Mesenteric culture (a/b)	2/8	1/8	5/8	2/8
Peritoneal culture (a/b)	1/8	2/8	4/8	2/8

The data are given as a/b= The number of rats with positive culture/total number of rats;
Groups are as follows: S=Sham, E=Erythropoietin, OJ=Obtructive Jaundice;
OJ+EP= Obstructive Jaundice+Erythropoietin;
Ŧ Significantly different when compared with S group (p=0.002);
β Significantly different when compared with EPO group (p=0.01).

mucosal injury. The changes were graded as follows; Grade 0, no changes; Grade 1, mild changes; Grade 2, moderate changes; Grade 3, severe changes.

Statistical analysis

Statistical analysis was performed by SPSS for Windows 11.5 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation values for biochemical values and histopathological scores. 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 significant.

Results

Microbiological assay

The number of positive cultures in blood and tissues according to the groups are summarized in Table I. The number of positive blood and liver cultures were higher in OJ group than the others. The other parameters (spleen, mesenteric and peritoneal cultures) were similar among the groups. The most frequently detected microorganism in all positive cultures was Escherichia coli. The other microorganisms according to frequency were Klebsiella and Pseudomonas, respectively.

Biochemical analysis

The biochemical results according to the groups are shown in Table II.

Bilirubin and Liver Enzyme Levels

Serum TBil and DBil levels, ALT, AST, ALP and GGT values were found to be significantly higher in the OJ and OJ+EPO groups as compared with the S and EPO groups ($p<0.001$). And also, the differences were found to be significant in chemical values between OJ and OJ+EPO groups ($p<0.001$).

PONX, TAC and TOA

OJ is significantly associated with oxidative stress. TOA levels were higher in OJ and OJ+EPO groups than S and EPO groups. However, TAC and PONX levels were increased ($p=0.005$ and $p=0.028$, respectively) and TOA level were decreased ($p=0.028$) with the treatment of EPO. The treatment with EPO significantly prevented the development of oxidative stress.

TNF-α, IL-6, IL-1β, CRP and ADMA

The inflammatory cytokines TNF-α, IL-6, IL-1β and CRP were increased after OJ. In OJ+EPO group, all these cytokines were significantly decreased when compared with OJ group (Table II). The ADMA levels were significantly higher in the OJ and OJ+EPO groups than S and EPO groups, but there was no difference between the OJ and OJ+ EPO groups.

Histopathological assessment

The results of histopathological evaluation are shown in Table III.

The inflammation of both liver and intestine ($p<0.001$), and the intestinal damage scores ($p=0.004$) were significantly higher in OJ groups than S group. Also, the

Table II - *The results of biochemical analysis according to the groups.*

Groups	S (n=8)	EPO (n=8)	OJ (n=8)	OJ + EPO (n=8)
ALT (U/L)	48±8	43±24	125±11 $^{\mathrm{Tβ}}$	101±18 $^{\mathrm{Tβ\,θμ}}$
AST (U/L)	214±39	256±35	401±103 $^{\mathrm{Tβ}}$	279±91$^{\theta}$
Total Bilirubin (gr/dL)	0,3±0,0	0,4±0,1	10±2 $^{\mathrm{Tβ}}$	7,2±2,8 $^{\mathrm{Tβμ}}$
Direct Bilirubin (gr/dL)	0,1±0,0	0,1±0,0	4,2±1,1 $^{\mathrm{Tβ}}$	2,2±0,9 $^{\mathrm{Tβ\,θμ}}$
ALP (U/L)	52±10	46±9	124±17 $^{\mathrm{Tβ}}$	97±13 $^{\mathrm{Tβ\,θμ}}$
GGT (U/L)	33±9	33±9	120±11 $^{\mathrm{Tβ}}$	103±6 $^{\mathrm{Tβ\,θμ}}$
PONX (U/L)	35±9	71±14 $^{\mathrm{T}}$	83±10 $^{\mathrm{Tβ}}$	108±20 $^{\mathrm{Tμ}}$
TAC (mmol Trolox Eq./L)	0,7±0,1	1,5±0,3	0,8±0,1 $^{\mathrm{Tβ}}$	1,4±0,4 $^{\mathrm{Tθ}}$
TOA (μmol H_2O_2 Equiv./L)	12,1±1,4	14±5	57±7 $^{\mathrm{Tβ}}$	22±7 $^{\mathrm{θμ}}$
ADMA (μmol /L)	2,4±0,9	3,3±1,5	0,5±0,2 $^{\mathrm{Tβ}}$	0,6±0,2 $^{\mathrm{Tβμ}}$
TNF-α (pg/mL)	2,0±1,0	1,9±1,4	3,4±0,7 $^{\chi}$	2,1±0,4 $^{\theta}$
IL-6 (pg/mL)	31±9	26±6	55±22	27±5
IL-1β (pg/mL)	0,5±0,1	0,4±0,1	0,8±0,1 $^{\mathrm{Tβ}}$	0,5±0,1 $^{\theta}$
CRP (mg/L)	30±5	28±4	103±21 $^{\mathrm{Tβ}}$	77±17 $^{\mathrm{Tβ\,μ}}$

The data were given as Mean±Standart deviation. S=Sham, E=Erythropoietin, OJ=Obtructive Jaundice, OJ+EP=Obstructive Jaundice+Erythropoietin. $^{\mathrm{T}}$ Significantly different when compared with S group ($p<0.001$), $^{\chi}$ Significantly different when compared with S group ($p<0.008$), $^{\beta}$ Significantly different when compared with EPO group ($p<0.001$), $^{\mu}$ Significantly different when compared with EPO group ($p<0.001$), $^{\theta}$ Significantly different when compared with OJ Group ($p<0.008$).

TABLE III - *The results of histopathological evaluation according to the groups.*

Groups	S (n=8)	EPO (n=8)	OJ (n=8)	OJ + EPO (n=8)
Liver	0,1±0,4	0,1±0,4	2,5±0,5 ŦΜ	1,6±0,7 ŦΜ
MLN diameter (mm)	0,26±0,09	0,26±0,05	0,34±0,08	0,25±0,06
Intestine	1,1±0,4	1,0±0,0	1,9±0,4 ŦΜ	1,1±0,4 β

The data were given as Mean±Standart deviation. S= Sham, E= Erythropoietin, OJ= Obtructive Jaundice, OJ+EP= Obstructive Jaundice+Erythropoietin. ŦSignificantly different than S group (p<0.008), ΜSignificantly different than EPO group (p<0.008), βSignificantly different than OJ group (p=0.004).

diameters of MLNs were greater in OJ groups. The treatment of EPO was significantly decreased the negative histopathological changes; especially the decline in intestinal damage scores was significant (p=0.004) (Figg. 1, 2).

Discussion

The main findings of this study were as follows; (i) the levels of liver function tests, TOA levels, ADMA levels, inflammatory cytokines as TNF-α, IL-6, IL-1β and CRP were increased after OJ showing oxidative stress and inflammatory response, (ii) EPO treatment prevented the development of oxidative stress, reduced the inflammation and bacterial translocation.

In this study, the serum TBil levels and AST, ALT, ALP and GGT activities were significantly higher in the OJ group [15] than in the sham and OJ+EPO groups, suggesting that EPO treatment counteracted the effects of ligation on liver function. In concordance with previous studies, common bile duct ligation (CBDL) increased oxidative stress with high TOA levels [16]. Demirbilek and et al. [17] has reported higher levels of oxidative stress indicators as total nitrite and nitrate, malondihaldehyde (MDA), and lower concentrations of antioxidant enzymes as glutathione (GSH), superoxide dismutase, and catalase in the liver tissue. Also Wang and et al. [18] showed that altered hepatic and intestinal damages in cholestatic rats were associated with increased MDA levels and decreased GSH in both hepatic and intestinal tissues. In addition to the oxidative stress, CBDL caused significantly inflammation with high levels of TNF-α, IL-6, IL-1β and CRP similar to the previous study that reported by Meng and et al [3].

ADMA were found higher after CBDL supporting previous studies that have done on rats with CBDL [19]. ADMA is primarily metabolized in liver and Tain and et al. [20] has reported that their study findings implied that oxidative stress cause ADMA accumulation mainly by the reduction of ADMA metabolism by dimethylarginine dimethyaminohydrolase (DDAH) inhibition which is the major enzyme for ADMA metabolism. Also in this study, we found ADMA levels were increased together with TOA levels in OJ group.

Bacterial translocation is the transition of bacteria or endotoxins from the gastrointestinal tract to extra intestinal spaces, such as mesenteric lymph nodes, liver, spleen, and/or bloodstream. Gut originated bacteremia and sepsis do not occur because of multiple host defense mechanisms that prevent the bacteria and their products from crossing the mucosal barrier in a healthy individual. Bacterial translocation results when this mucosal barrier is impaired [21]. In this study, positive cultures were higher especially in liver and blood cultures in OJ group however there were no difference among groups of

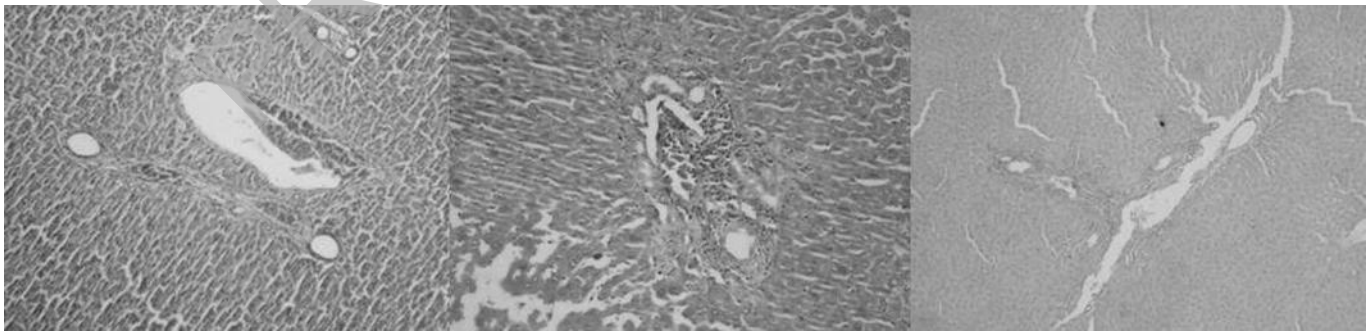

Fig. 1: The effects of EPO on the liver inflammation after OJ. A: Sham group. normal hepatic structure in the liver parenchima without inflammation (H&E stain, x100); B: OJ group. Moderate portal inflammation and edema in the liver (H&E stain, x200); C: OJ + EPO group. Mild portal inflammation and edema in the liver are seen (H&E stain, x200).

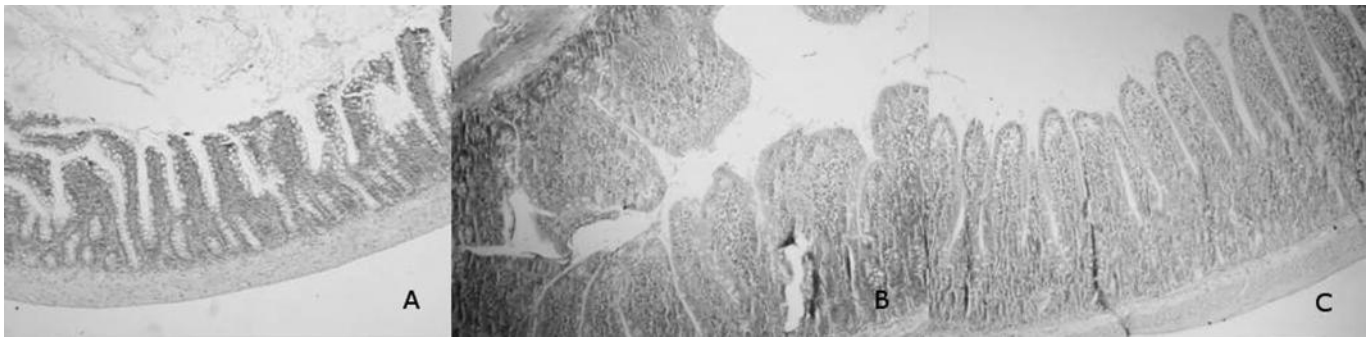

Fig. 2: Microscopic appearance of the effects of EPO on the ileal inflammation and mucosal injury after OJ. A: Sham group. normal intestinal structure (H&E stain,x100); B: OJ group. Subtotal villous atrophy and epithelial degenerative changes in the intestinal mucosa with severe inflammation and edema (H&E stain, x100); C: OJ + EPO group. mild inflammation are seen (H&E stain, x100).

spleen, MLN and peritoneal culture. After EPO treatment these positive cultures decreased. It has believed that bile and bile salts in the intestinal lumen have protective effects against bacterial translocation via regulatory effects on the intestinal flora and a direct detergent effect on endotoxins [22].

EPO has multiple protective effects including antioxidant, antiapoptotic, angiogenic and neuroprotective effects in different organs via paracrine pathways in addition to its hematopoietic effects [8,23]. Moran and et al. studied the effects of EPO on the rats with OJ. They gave 500 IU EPO for two different periods as 3 days and 7 days. Furthermore, they concluded that the EPO attenuates oxidative injury or increases antioxidant production. Also, the longer duration of EPO treatment was more effective for the preservation of antioxidant levels [23]. Similar to this study, EPO treatment for 7 days, reduced the oxidative stress and supported the antioxidant status. Histopathological findings were in line with these findings with low damage scores in the OJ+EPO group.

Conclusion

The treatment of EPO in rats with OJ reduces bacterial translocation, inflammation and tissue damage. The results of our study suggest that EPO may use in preventing the development of bacterial translocation, inflammation, tissue damage and related sepsis in patients with OJ.

Riassunto

L'occlusione dell'epatocoledoco si associa con danno epatico parenchimale ed aumentata suscettibilità alle infezioni batteriche. L'eritropoietina ha effetti antiinfiammatori e citoprotettivi, e induce la produzione di citochine antiinfiammatori sopprimendo quella delle citochine pro-infiammatorie. Lo scopo di questo studio è quello di indagare l'effetto dell'eritropoietina sulla traslocazione batterica, sull'infiammazione e sul danno tissutale in ratti con ittero ostruttivo.

Per questo scopo sono stati utilizzati 32 ratti Wistar albini (200-250g) divisi nei quattro gruppi seguenti: Gruppo 1 (di controllo): semplice dissezione del legamento epatoduodenale; Gruppo 2 (eritropoietina): dissezione del legamento epatoduodenale e somministrazione sottocutanea di 500 IU/kg di eritropoietina; Gruppo 3 (ittero ostruttivo): allacciatura completa del legamento epatoduodenale; Gruppo 4 (ittero ostruttivo + eritropoietina): allacciatura completa del legamento epatoduodenale e somministrazione sottocutanea di 500 IU/kg di eritropoietina. Dopo 7 giorni i ratti sono stati sacrificati prelevando sangue dal cuore per analisi biochimiche. Sono stati quindi raccolti strisci peritoneali per colture, fegato, linfonodi mesenterici, milza ed ileo per esami microbiologici ed istopatologici.

I risultati hanno dimostrato che l'eritropoietina riduce la increzione di citochine infiammatori, il danno ossidativo e la traslocazione batterica, e previene l'insorgenza di alterazioni infiammatorie nell'intestino e nel fegato conseguenti all'ittero ostruttivo.

In conclusione il trattamento con eritropoietina in ratti con ittero ostruttivo riduce la traslocazione batterica, l'infiammazione ed il danno tissutale.

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