

Gut-liver axis improves with meloxicam treatment after cirrhotic liver resection

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Received: February 22, 2014 Revised: June 7, 2014

Accepted: June 25, 2014

Published online: October 28, 2014

Abstract

AIM: To investigate the effect of meloxicam on the gut-liver axis after cirrhotic liver resection.

METHODS: Forty-four male Wistar rats were assigned to three groups: (1) control group (CG); (2) bile duct ligation with meloxicam treatment (BDL + M); and (3) bile duct ligation without meloxicam treatment (BDL). Secondary biliary liver cirrhosis was induced *via* ligation

of the bile duct in the BDL + M and BDL groups. After 2 wk, the animals underwent a 50% hepatectomy. In the BDL + M group 15 min prior to the hepatectomy, one single dose of meloxicam was administered. Parameters measured included: microcirculation of the liver and small bowel; portal venous flow (PVF); gastrointestinal (GI) transit; alanine aminotransferase (ALT); malondialdehyde; interleukin 6 (IL-6), transforming growth factor beta 1 (TGF- β 1) and hypoxia-inducible factor 1 alpha (HIF-1 α) levels; mRNA expression of cyclooxygenase-2 (COX-2), IL-6 and TGF- β 1; liver and small bowel histology; immunohistochemical evaluation of hepatocyte and enterocyte proliferation with Ki-67 and COX-2 liver expression.

RESULTS: Proliferative activity of hepatocytes after liver resection, liver flow and PVF were significantly higher in CG *vs* BDL + M and CG *vs* BDL group ($P < 0.05$), whereas one single dose of meloxicam ameliorated liver flow and proliferative activity of hepatocytes in BDL + M *vs* BDL group. COX-2 liver expression at 24 h observation time (OT), IL-6 concentration and mRNA IL-6 expression in the liver especially at 3 h OT, were significantly higher in BDL group when compared with the BDL + M and CG groups ($P < 0.01$, $P < 0.001$, $P < 0.01$, respectively). Liver and small bowel histology, according to a semi quantitative scoring system, showed better integrity in BDL + M and CG as compared to BDL group. ALT release and HIF-1 α levels at 1 h OT were significantly higher in BDL + M compared to CG and BDL group ($P < 0.001$ and $P < 0.01$, respectively). Moreover, ALT release levels at 3 and 24 h OT were significantly higher in BDL group compared to CG, $P < 0.01$. GI transit, enterocyte proliferative activity and number of goblet cells were in favor of meloxicam treatment *vs* BDL group ($P < 0.05$, $P < 0.001$, $P < 0.01$, respectively). Additionally, villus length were higher in BDL + M as compared to BDL group.

CONCLUSION: One single dose of meloxicam admin-

istered after cirrhotic liver resection was able to cause better function and integrity of the remaining liver and small bowel.

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Key words: Liver cirrhosis; Liver resection; Gut-liver axis; Meloxicam; Cyclooxygenase-2; Microcirculation

Core tip: Cyclooxygenase-2 (COX-2) inhibitors have been used in liver cirrhosis (LC), suppressing inflammation and liver fibrosis. However, the effects of COX-2 inhibition on the gut-liver axis after cirrhotic liver resection was not investigated previously. For the first time it can be concluded that one single dose of meloxicam administered 15 min prior to a 50% hepatectomy in LC, alleviates impairment of many functions of the gut-liver axis such as microcirculation, hepatocyte and enterocyte regeneration rate, and gastrointestinal transit, enabling better function and integrity of the remaining liver and the small bowel.

Hamza AR, Krasniqi AS, Srinivasan PK, Afify M, Bleilevens C, Klinge U, Tolba RH. Gut-liver axis improves with meloxicam treatment after cirrhotic liver resection. *World J Gastroenterol* 2014; 20(40): 14841-14854 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i40/14841.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i40.14841>

INTRODUCTION

Liver cirrhosis (LC) represents the final common pathophysiological end stage for a broad spectrum of chronic liver diseases. It is characterized by the replacement of normal tissue with fibrous tissue, caused by different noxae (*e.g.*, viruses, alcohol, metabolic disorders *etc.*), and loss of normal liver function.

LC has become a considerable public health problem with high morbidity and mortality. Previously published data show that LC affects up to 4.5%-9.5% of the global population in reference to autopsy studies^[1,2]. Cirrhosis *per se*, carries very high risk factor of altering into a hepatocellular carcinoma (HCC), because up to 85%-95% of HCCs arise in cirrhotic livers^[3-5]. Surgical resection is the only potentially curative treatment for HCC in noncirrhotic patients and in cirrhotics with preserved liver function^[6]. However, the recurrence rate after resection with up to 70% remains high, especially in patients with cirrhosis^[6]. The up-regulation of cyclooxygenase-2 (COX-2) has been shown in human LC as a result of active inflammation, even higher in comparison to patients with HCC^[7,8].

COX is the key enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid; also COX represents the main pharmacological target for non-steroidal anti-inflammatory drugs (NSAIDs). There are three COX isoforms which have been identified: First,

COX-1 which is expressed constitutively in many tissues and plays an important role for various physiological functions^[9]; Second, in contrast, COX-2 is dramatically up-regulated in response to cytokines, hormones, and mitogenic or inflammatory stimuli^[10]; Third, and latest detected COX-3, which is especially sensitive to acetaminophen and related compounds, as a variant of COX-1, which is abundantly expressed in cerebral cortex and heart^[11]. PGs produced by the action of COX are important mediators of systemic vasodilatation and inflammation in LC^[9]. Furthermore, prostacyclin I₂ has been shown to play a role in hyperdynamic circulation in portal hypertension (PHT)^[12-14].

PHT, as a consequence of cirrhosis leads to hemodynamic disturbances throughout the gastrointestinal (GI) tract causing venous and capillary ectasia in the mucosa and submucosa with associated inflammatory activity^[15]. Many clinical and experimental studies have reported that LC causes impairment of the intestinal microcirculation and mucosal barrier. This affects the motor activity of the gut and contributes to an increased intestinal permeability and bacterial translocation^[16-20]. It is well known that patients with LC exhibit PHT which causes various pathological changes in the entire GI tract (from esophagus to anus)^[21,22]. Indeed, these changes are termed congestive gastroenteropathy and they are attributed to alterations in the GI microcirculation^[23-27]. Due to the drastic reduction within the microvascular bed after major hepatectomy, the remaining liver is exposed to higher portal venous pressure (PVP), known to induce irreversible sinusoidal endothelium injury as described in small-for-size liver grafts (SFSGs)^[28-31]. On the other hand, many surgical centers have reported that SFSGs were successfully treated with surgical manipulations, by reducing PVP and portal venous flow (PVF) with different portocaval shunts or splenectomy^[29,32-35]. Yagi *et al.*^[32] found that the intestinal mucosa was severely damaged with PHT following SFSG liver transplantation in a porcine model. Moreover, to our knowledge there is still a lack of quantitative data that describe the gut-liver axis within an experimental setting after liver resection. Therefore, the aim of this study was to elucidate the mechanisms involved in disturbed function of the gut-liver axis after cirrhotic liver resection and possible pharmacological interventions with a preferential COX-2 inhibitor in a rat model.

MATERIALS AND METHODS

Experimental protocols and animals

All experiments were conducted in accordance with German Federal Law regarding the protection of animals and the British Government's proposals for the transposition of European Directive 2010/63/EU on the protection of animals used for scientific purposes. The Guide for the care and use of laboratory animals (8th edition, National Institutes of Health Publication, 2011, United States) were also followed. Forty-four male Wistar albino rats with a mean body weight (BW) = 300 g were ob-

tained from Charles River Laboratories GmbH (Sulzfeld, Germany). The animals were housed in Type 2000 rat filter top cages (Tecniplast, Hohenpreisenberg, Germany) under specific pathogen free (SPF)-conditions according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines (www.felasa.eu) in a temperature and humidity controlled environment with a 12-h light/dark cycle and allowed food (standard rat diet, Sniff-Spezial Diäten GmbH, Soest, Germany) and water ad libitum.

Experimental groups and operative procedures

The animals were divided into three groups: (1) control group (CG): no bile duct ligation ($n = 15$); (2) bile duct ligation group with meloxicam treatment 10 mg/kg BW (BDL + M) ($n = 15$); (3) bile duct ligation group without meloxicam treatment (BDL) ($n = 14$). Each group was divided into three subgroups according to the time points of observation time (OT); 1, 3 and 24 h OT post liver resection ($n = 5$ at each time point in each group, except $n = 4$ at 3 h OT in the BDL group). After induction of general anesthesia by inhalation of 5 vol% isoflurane and 5 L/min of oxygen (Abbott GmbH and Co. KG, Wiesbaden, Germany), for about 2 min, anesthesia was then maintained by reducing the isoflurane to 2 vol%, 2 L/min of oxygen. Preoperative prophylactic antibiotic treatment and analgesia were achieved by subcutaneous injection of cefuroxime sodium (16 mg/kg per 24 h) (Cefuroxim Fresenius; Fresenius Kabi Deutschland GmbH, Bad Hamburg, Germany), and of buprenorphine (0.1 mg/kg per 24 h) (Temgesic; Essex Pharma, München, Germany) for 24 h after surgery. The abdomen was opened by a midline laparotomy. In the second and third groups (BDL + M and BDL), a secondary biliary LC was induced *via* ligation of the bile duct. After laparotomy, the duodenum was reached through the incision in order to place the common bile duct under tension. The common bile duct was isolated and ligated twice using PDS-6.0 (Ethicon, Johnson and Johnson, Lenneke Marelaan, Belgium). The abdomen wall was closed in two-layers, the first layer with a continuous suture and the skin with interrupted suture. The animals were allowed to recover in a special intensive care unit (Vetario; Brinsea Products Ltd., North Somerset, United Kingdom) with warmed air (30–35 °C) for at least 15 min. After 2 wk the animals underwent a partial liver resection of a 50% of the liver mass [left liver lobe (LLL), anterior caudate (AC) and posterior caudate (PC), liver lobe]. In the BDL + M group, the animals were treated with a single dose of the preferential COX-2 inhibitor meloxicam at a dose of 10 mg/kg body weight *iv via* penile vein (Metacam® Boehringer Ingelheim Vet-medica GmbH Ingelheim/Rhein-Deutschland), 15 min before resection. All surgical procedures were performed under aseptic conditions. In the first group (CG) the same surgical procedure was performed without prior BDL and meloxicam administration. This group served as the control group.

Microcirculation of the liver, small bowel and portal venous flow measurements

The microcirculation of the liver and small bowel was assessed by a combined laser Doppler flowmetry and near infrared spectrophotometry device (O2C: oxygen to see; LEA Medizintechnik GmbH, Giessen, Germany). The portal venous flow was evaluated with a transit-time perivascular flowmeter (T403; Transonic Systems, Inc., Ithaca, NY).

Gastrointestinal transit evaluation

The GI was investigated 24 h after operation using oral administration of indocyanine green (ICG) (Pulsion Medical System, Munich, Germany). For this purpose, the animals were briefly anaesthetized (isoflurane 2 vol%, 2 L/min of oxygen); thereafter, they received 0.2 mL of ICG (5 mg/mL) *via* gastric tube 22 h post operatively. Two hours after administration of the ICG, the GI transit in the small bowel was visualised *ex vivo* using the IC-View System (Pulsion Medical System, Munich, Germany). The non-invasive IC-VIEW system contains of an infrared source and a digital video camera with the special ability to detect near-infrared light in a special mode with a filter. Excitation light is provided by an IC-VIEW camera mounted near-infrared light source (NIR-light, laser class 3B, energy 0.16 W, wave length 780 nm). ICG-derived fluorescence is detected by the digital IC-VIEW Video Camera and can be viewed in real-time. Results are recorded on the camera tape and the digital data is then transferred to a PC (Sony Vaio Laptop, PCG-3192; Sony Corp., Tokyo, Japan), stored and analyzed. Details are described previously^[36].

Hepatocellular damage

Hepatocellular damage was evaluated by the increase of liver parenchymal enzyme, alanine aminotransferase (ALT), in serum, after liver resection according to the different time points at 1, 3 and 24 h.

Histopathology and immunohistochemistry analysis

Tissue samples of the liver and intestine (jejunum) were collected at the time when the rats were euthanized. The samples were immediately fixed in 4% neutral buffered formalin (Roti®-Histofix 4%, Roth, Karlsruhe-Germany), then were shaken overnight on a vortex shaker (Lab net, International Inc. United States). The specimens were processed in grading series of alcohol and xylene, embedded in paraffin and sectioned at 4–6 µm thin slices using a microtome and were stained with hematoxylin and eosin (HE). Tissue sections of the liver and the small bowel were all examined in a blinded fashion by two independent investigators including a senior pathologist (Afify M). A semiquantitative scoring system was used, which was modified from the previous scoring system of Neil and Hubscher^[37], regarding the histological features of hepatic parenchyma (neutrophil and macrophage infiltration, bile duct hyperplasia, regenerative activities of hepatocytes, and necrosis). Examinations of the whole

slides were performed using a Leica DM 2500 optic microscope (Wetzlar, Germany) under $\times 400$ magnification. Lesions were graded on a scale from 1 to 4 (1 = no changes or negligible, lesions affecting 0%-10% of the field; 2 = mild, lesions affecting 10%-40% of the field; 3 = moderate, lesions affecting 40%-70% of the field; 4 = severe, lesions affecting > 70% of the field). Samples of the small intestine were also examined using HE stained sections. Ischemic mucosal lesions of the small bowel was graded using a semiquantitative scoring system according to Chiu *et al.*^[38]. The villus length and the number of goblet cells were measured in 10 villi of each sample.

Assessment of liver fibrosis

To quantify hepatic fibrosis histopathologically, liver specimens were stained with picosirius red (PSR) and sectioned as previously prescribed^[39]. The sections were analyzed using a microscope (Olympus BX51, Hamburg, Germany), equipped with filters to provide circularly polarized illumination. Five photos at $\times 400$ magnification were taken for each specimen ($n = 5$). Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, United States) photo analysis software was employed for quantification of collagen I (red) and III (green) by calculating color area (μm^2). In addition, for each biopsy sample stained with PSR, there was a histological evaluation of the BDL-induced liver damage. For this purpose, the Ishak scoring system was adopted, which scores cirrhotic alterations of the liver from (0 to 6 grade) according to the fibrous expansion from portal to portal and portal to central area of the liver^[40].

Immunohistochemistry assessment of the liver and small bowel regeneration with Ki-67 and COX-2 liver expression

Tissue sections of the liver and small bowel were subjected to immunohistochemical testing using the avidin-biotin-complex method and diaminobenzidine. Antibodies used in this study were monoclonal mouse Ki-67 (MIB5, 1:10; DAKO, Glostrup, Denmark) for assessing cell proliferation rate. The Ki-67 labeling index (LI) represented the percentage of hepatocytes or enterocytes with Ki-67-positive nuclei relative to the total number of hepatocytes or enterocytes in randomly selected sections. For liver, ten fields avoiding periportal area, while for the gut, ten crypts (under $\times 400$ magnification) were chosen in each animal ($n = 5$), with a total of fifty fields for each group. For the immunohistochemistry of the COX-2 expression, we used polyclonal rabbit antimurine COX-2 serum (Cayman Chemical, Ann Arbor, MI, United States) diluted to 2.5 mg/mL. Immunoreactive complexes were visualized with the peroxidase substrate 3-amino-9-ethyl-carbazole. The same quantifying method was followed as for Ki-67.

Malondialdehyde

In order to assess the impact of oxygen free radicals after cirrhotic liver resection, we measured malondialdehyde

(MDA) concentrations in the serum. One hundred microliters of plasma were mixed with 750 μL of 0.44 $\text{MH-}\alpha\text{PO}_4$, 250 μL of 42 mmol/L aqueous solution of thiobarbituric acid (TBA), and 500 μL H_2O . Samples were incubated in boiling water for 60 min and then chilled on ice. Fluorescent LPO-TBA adduct were measured using a fluorescence spectrophotometer Tecan Infinite (Tecan Deutschland GmbH, Crailsheim, Germany). Different dilutions of tetraethoxypropane were used as the external standard. Further details were described previously^[41].

Enzyme-linked immunosorbent assay

Serum levels of pro-inflammatory cytokine, interleukin 6 (IL-6) (R&D Systems, Minneapolis, MN, # R6000B), transforming growth factor beta 1 (TGF- β 1) (R&D Systems, Minneapolis, MN, # MB100B) and hypoxia-inducible factor 1 alpha (HIF-1 α) (MyBioSource.com, San Diego, California, United States, # MBS727993), were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Real-time reverse transcription polymerase chain reaction

Liver samples and 5-cm portions of the jejunum (retrieved mucosa from muscularis/serosa), were immediately snap frozen in liquid nitrogen and kept at -80°C until analysis. Total RNA was extracted using a commercially available RNA/protein extraction kit (NucleoSpin[®] RNA/Protein, Machery-Nagel, Düren/Germany). Using a high capacity reverse transcription kit (Applied Biosystems[®], Carlsbad, California, United States), 250 ng of total RNA was reverse transcribed to cDNA. A polymerase chain reaction (PCR) reaction was performed using 50 ng of cDNA, a Taqman[®] Gene Expression Master Mix (Applied Biosystems[®]) and specific TaqMan[®] probes for IL-6 (Rn01410330_m1), TGF- β 1 (Rn00572010_m1), COX-2 (Rn01483828_m1) and the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Rn01775763_g1) on a StepOne-Plus[®] Cyclor (Applied Biosystems[®]). The gene expression levels of the cytokines were calculated as a relative quantity (RQ) value according to the $\Delta\Delta\text{Ct}$ method, which reflects the differences in threshold for each target gene relative to GAPDH and the control group.

Statistical analysis

The results are expressed as the mean \pm SE for each group. Levels of ALT, MDA, IL-6, HIF-1 α and TGF- β 1 in the serum, microcirculation of the liver, small bowel and portal venous flow measurements between groups at each time point were tested using 2-way analysis of variance (ANOVA) and Bonferroni's post-hoc test. Whereas, quantifications of the goblet cells, villus length, Ki-67, COX-2 liver expression between groups at 24 h OT were tested using one-way ANOVA and Tukey's Multiple Comparison Test. A non-parametric method (the Dunn procedure under the Kruskal-Wallis test) was used for

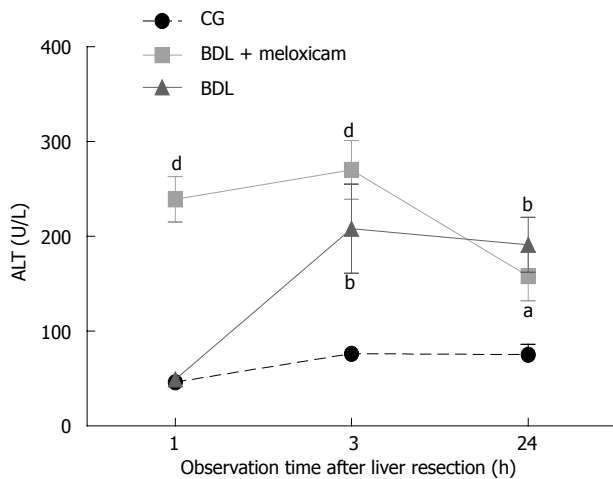


Figure 1 Release of alanine aminotransferase in the serum after liver resection at three different time points 1, 3 and 24 h for three groups: Control group; bile duct ligation + meloxicam; and bile duct ligation. Values are given as mean \pm SE. 2-way ANOVA. Bonferroni's post-test: ^a $P < 0.05$, ^b $P < 0.01$ vs CG; ^c $P < 0.01$ vs CG and BDL ($n = 15$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; ALT: Alanine aminotransferase.

Table 1 The microcirculation of the liver, small bowel, and portal venous flow measurements after liver resection for three groups

	CG	BDL + M	BDL	P value
Liver flow (AU)	232 \pm 11	207 \pm 10	188 \pm 11 ^a	< 0.05 ^a
Small bowel flow (AU)	323 \pm 10	280 \pm 21	289 \pm 21	NS
PVF (mL/min)	27 \pm 3	17 \pm 2 ^a	17 \pm 3 ^a	< 0.05 ^a

Values are given as mean \pm SE; 2-way ANOVA. Bonferroni's post-test: $n = 15$, ^a $P < 0.05$ vs CG. CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; NS: Not significant; AU: Arbitrary unit; PVF: Portal venous flow.

real-time reverse transcription PCR (RT-PCR) results in liver and small bowel for multiple comparisons between the groups. A P -value of < 0.05 was considered statistically significant. Calculations were made with the help of Prism 5 Windows software (GraphPad Software Inc., San Diego, CA).

RESULTS

Enzyme release

ALT release was measured as general parameter of hepatocellular damage. In our study, we observed significantly higher levels of parenchymal enzyme release in BDL + M at 1 h OT compared to CG and BDL, $P < 0.01$. At two other time points 3 and 24 h, ALT release was high in all three groups, however it was much higher in BDL + M and BDL compared to CG ($P < 0.01$, $P < 0.05$) (Figure 1).

Microcirculation of the liver, small bowel and portal venous flow measurements

The microcirculation of the liver and small bowel, as an important factor in maintenance of their function, was

assessed by an O2C-device after liver resection at three different time points. Liver flow was significantly higher in CG vs BDL ($P < 0.05$). Although meloxicam treatment maintained liver flow in BDL + M compared to the BDL group, this difference did not reach statistical significance (Table 1). Regarding the microcirculation of the small bowel no statistical difference were observed between the three groups (Table 1). PVF was evaluated with a transit-time perivascular flowmeter on the portal vein after liver resection according to three different time points. In CG, PVF was significantly higher compared to BDL + M and BDL groups, $P < 0.05$ (Table 1).

GI transit evaluation

In order to evaluate GI transit after liver resection, ICG was administered orally through a gastric tube 22 h post-operatively in the 24 h group. After laparotomy, the entire small bowel was resected and visualized with the IC-VIEW System *ex vivo*. In the CG and BDL + M animals, homogenous ICG signal could be detected in nearly the entire small bowel up to the caecum (77 and 82 cm respectively); however in the BDL group the ICG signal was observed in a much shorter length of the bowel (52 cm) (Figure 2A). The histogram (Figure 2B) shows ICG transit of small bowel in centimeter (cm). In BDL vs BDL + M, the ICG signal was seen in a significantly shorter length of bowel (52 cm vs 82 cm of the small bowel length, $P < 0.05$).

Histopathological and immunohistochemical assessment of the liver

Histopathological changes of the liver (HE staining), at 24 h OT after liver resection were analyzed using a semiquantitative scoring system^[37]. The liver tissue in CG showed negligible lesions (score 1) (Figure 3A). There was mild periportal neutrophil and mononuclear cell infiltration with expressed regenerative activities of hepatocytes (megakaryocytes and megalocytosis). On the other hand, liver tissue in the BDL group showed moderate to severe lesions (mean; score 4) (Figure 3C). The microscopy showed biliary proliferation in different locations involving multiple lobules, spreading from portal to portal space, with a tendency to reach the central vein. Moreover, Kupffer cell proliferation, some necrotic areas, and inflammatory cell infiltration in periportal areas were seen in this group. On the contrary, in the substrate meloxicam treatment group (BDL + M) the lesions were of moderate degree (mean; score 3) (Figure 3B). In this group there was seen moderate proliferation of biliary epithelium (reserve cells) in different locations extending in moderate degree, not reaching another portal area.

Hepatocyte proliferation was assessed with immunohistochemistry Ki-67-positive nuclei. Note the higher number of Ki-67-positive hepatocytes in CG (Figure 3D) and BDL + M (Figure 3E) compared to the BDL group (Figure 3F). Whereas, COX-2 expression in the liver assessed with immunohistochemistry was much more expressed in the BDL group compared to CG and

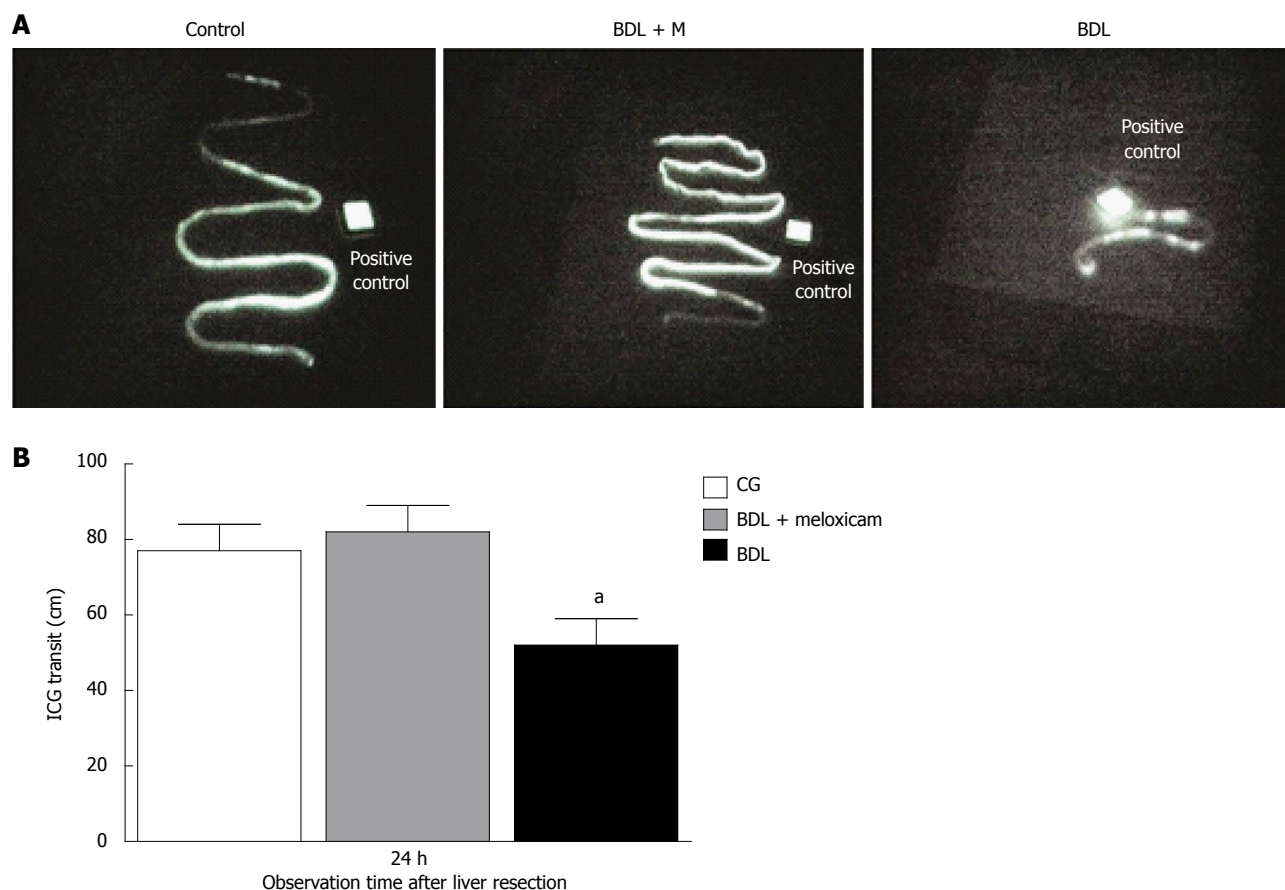


Figure 2 Indocyanine green transit evaluation into the gastrointestinal tract with IC-View System in a 24 h observation time of three groups: Control group; bile duct ligation + meloxicam; and bile duct ligation. A: Images of ICG transit in small bowel viewed by IC-View System; B: Histogram of the quantified gastrointestinal transit. Values are given as means \pm SE. One-way ANOVA. Tukey's Multiple Comparison Test: ^a $P < 0.05$ vs BDL + M ($n = 5$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; ICG: Indocyanine green.

BDL + M groups (Figure 3G-I). Table 2 summarizes the quantified immunohistochemical findings of hepatocyte proliferation with Ki-67-positive nuclei and COX-2 expression after liver resection of three groups at 24 h OT. Ki-67-positive hepatocytes was significantly higher in CG *vs* BDL group, $P < 0.05$. Although the number of Ki-67-positive hepatocytes was higher in BDL + M *vs* BDL, the difference did not reach statistical significance (Table 2). Hepatic COX-2 positive cells were significantly attenuated with meloxicam treatment in BDL + M *vs* BDL group, $P < 0.01$; also the BDL group showed higher expression of COX-2 *vs* CG, $P < 0.01$ (Table 2).

Histopathological and immunohistochemical assessment of the small bowel

The semiquantitative scoring system according to Chiu *et al.*^[38] for scoring mucosal lesions of the small bowel was chosen because we thought that it is most appropriate for these type of lesions. Although, in CG the ischemic lesions with development of Gruenhagen space at the apex of the villus were seen (grade 1) (Figure 4A), and in the BDL group with extension of sub-epithelial space with moderate lifting of the epithelial layer from the lamina propria (grade 2) (Figure 4C), these changes were not significantly different in the BDL + M group (Figure 4B

and C) (Table 3). In nearly all sections there was a variable degree of leukocyte infiltration, a sign of previous ischemic and degenerative changes that indicates a degree of proliferative activity of the epithelial lining and the duration, which allowed such regeneration. Moreover, proliferation of enterocytes was assessed with immunohistochemistry for Ki-67-positive enterocytes. Note the higher number of Ki-67-positive enterocytes in CG (Figure 4D) and BDL + M (Figure 4E) compared to the BDL group (Figure 4F). Table 3 summarizes the quantified immunohistochemical findings of enterocyte proliferation with Ki-67-positive nuclei in ten randomly selected crypts of the gut ($\times 400$). In addition, in this table are shown the quantified number of goblet cells and the length of the villus in ten randomly picked villi of the small intestine ($\times 400$).

In the gut, the number of Ki-67-positive enterocytes was significantly higher in CG compared to BDL + M [91 ± 2 arbitrary unit (AU) *vs* 77 ± 1 AU, $P < 0.001$] and CG compared to BDL group (91 ± 2 AU *vs* 50 ± 1 AU, $P < 0.001$). Additionally, the number of Ki-67-positive enterocytes was also significantly higher in BDL + M *vs* BDL group (77 ± 1 AU *vs* 50 ± 1 AU, $P < 0.001$) (Table 3). The number of goblet cells was significantly higher in the BDL group compared to BDL + M and CG, $P < 0.01$

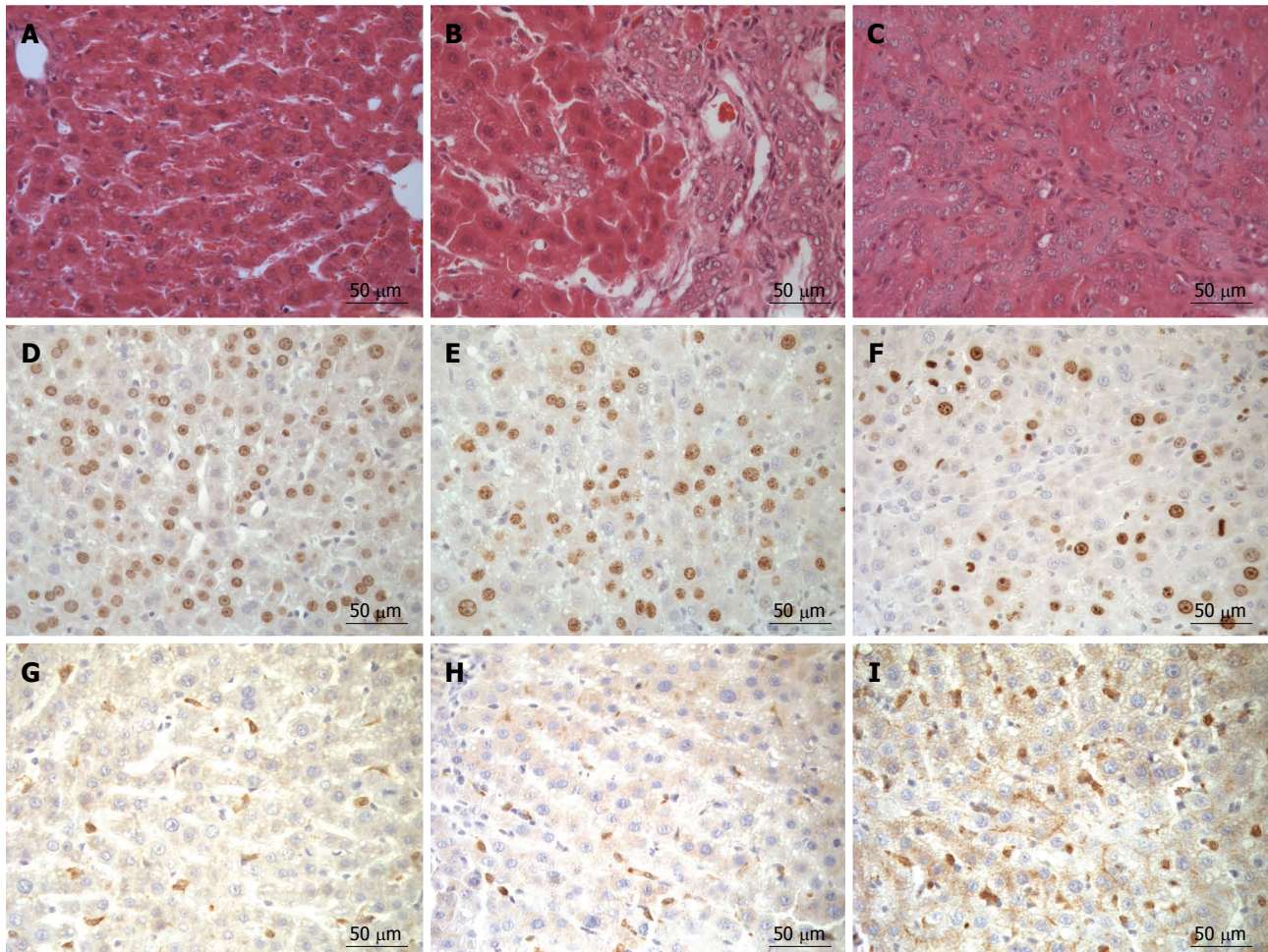


Figure 3 Histopathological findings. HE staining of liver tissue at 24 h after liver resection of three groups: CG (A), BDL + M (B) and BDL (C); Immunohistochemical analysis of hepatocyte proliferation with Ki-67-positive nuclei, CG (D), BDL + M (E) and BDL (F), and COX-2 expression after liver resection, CG (G), BDL + M (H) and BDL (I) ($\times 400$) ($n = 5$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; HE: Hematoxylin and eosin; COX-2: Cyclooxygenase-2.

Table 2 Quantifying of Ki-67 positive cells, cyclooxygenase-2 expression, and fibrotic scoring system of the liver according to Ishak *et al.*^[40]

	CG	BDL + M	BDL	P value
Ki-67 LI (AU)	88 \pm 13	54 \pm 11	38 \pm 9 ^a	< 0.05
COX-2 expression (AU)	29 \pm 4 ^d	23 \pm 8 ^d	57 \pm 7	< 0.01
Ishak score	0 (0-1)	5 (3-6)	5 (4-6)	NS

Values are given as mean \pm SE; 1-way ANOVA. Tukey's Multiple Comparison Test: $n = 5$; ^a $P < 0.05$ vs CG; ^d $P < 0.01$ vs BDL. CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; NS: Not significant; AU: Arbitrary unit; LI: Labeling index; COX-2: Cyclooxygenase-2.

(Table 3). Villus length was significantly longer in CG compared to BDL group, $P < 0.05$. Although length of the villus was longer in BDL + M compared to BDL, the difference did not reach statistical significance (Table 3).

Assessment of liver fibrosis

Collagen I and III quantification with the help of Image-Pro Plus 4.5 software did not show any difference between the BDL + M and BDL groups, while it was significantly higher compared to CG (Figure 5). In order

to evaluate BDL-induced liver fibrosis, the Ishak scoring system was adopted. We were able to see different grades of fibrosis even within the same group of BDL and BDL + M. In the BDL group, according to fibrous expansion portal-to-portal (P-P) and portal-to-central (P-C) linkage with fibrotic bands, liver samples were scored from grade 4; fibrous expansion of portal areas with marked bridging P-P as well as P-C, up to grade 6; definite cirrhosis, with the mean value grade 5; marked bridging P-P and P-C with occasional nodules (incomplete cirrhosis) (Figure 5C). In BDL + M, even though some slides have shown lower grade, the mean value was the same grade 5 (Figure 5B). In CG, there was no fibrosis grade 0 (Figure 5A).

ELISA

Proinflammatory cytokine IL-6, HIF-1 α and TGF- β 1 were evaluated with ELISA. The data showed that IL-6 concentration was significantly higher in BDL especially at 3 h OT, compared to BDL + M (151 \pm 101.7 pg/mL vs 52.4 \pm 23.6 pg/mL, $P < 0.001$) and BDL group vs CG (151 \pm 101.7 pg/mL vs 12.4 \pm 6.2 pg/mL, $P < 0.001$) (Figure 6A). HIF-1 α level was found to be significantly higher in BDL + M especially at 1 h OT, compared to BDL group (3.1 \pm 0.5 pg/mL vs 2.2 \pm 0.4 ng/mL, $P <$

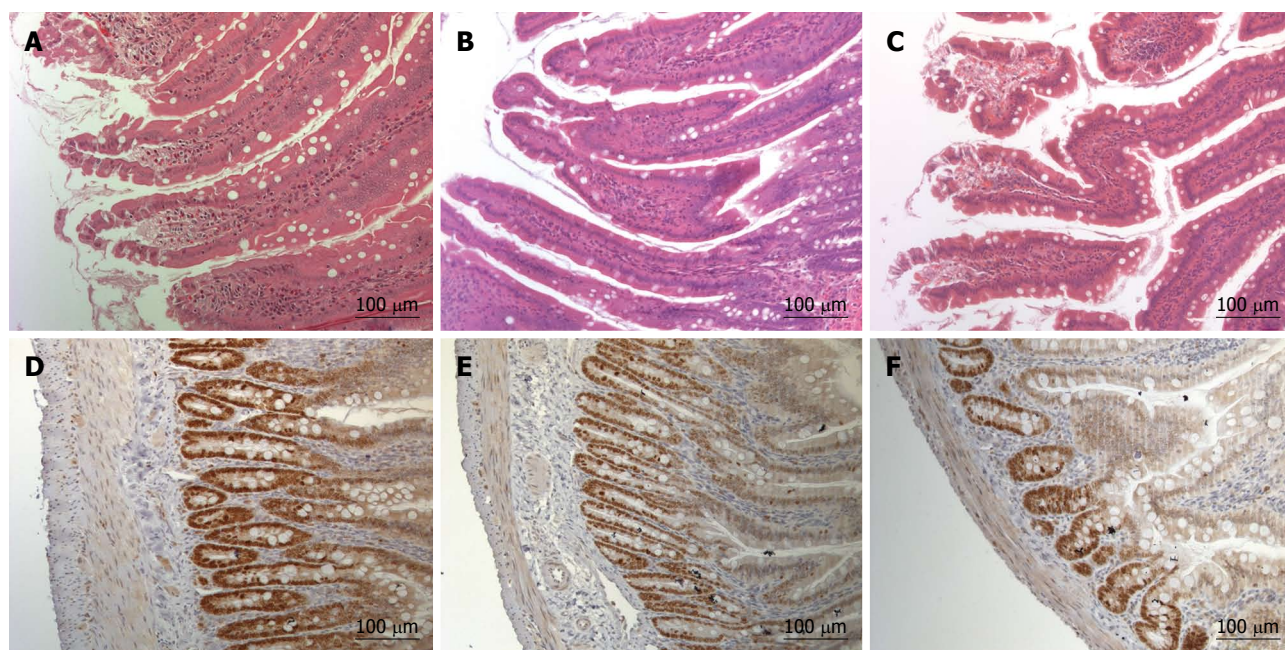


Figure 4 Histopathological findings. HE staining of the small bowel at 24 h after liver resection of three groups: CG (A), BDL + M (B) and BDL (C) ($\times 200$); Immunohistochemical analysis of enterocyte proliferation with Ki-67-positive nuclei, CG (D), BDL + M (E) and BDL (F), of three groups ($\times 400$) ($n = 5$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; HE: Hematoxylin and eosin.

Table 3 Quantifying of Ki-67, goblet cells, villus length and ischemic lesions of the small bowel according to Chiu *et al.*^[38]

	CG	BDL + M	BDL	P value
Ki-67 LI (AU)	91 \pm 2	77 \pm 1 ^b	50 \pm 1 ^b	< 0.01
Goblet cells (AU)	31 \pm 2 ^d	32 \pm 3 ^d	55 \pm 5	< 0.01
Length of the villi (μ m)	617 \pm 24	562 \pm 30	494 \pm 22 ^a	< 0.05
Chiu score	1 (0-2)	0 (0-1)	1 (0-2)	NS

Values are given as mean \pm SE; 1-way ANOVA. Tukey's Multiple Comparison Test: $n = 5$; ^a $P < 0.05$, ^b $P < 0.01$ vs CG; ^d $P < 0.01$ vs BDL. CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; NS: Not significant; AU: Arbitrary unit; LI: Labeling index.

0.01) and CG, BDL + M *vs* CG (3.1 ± 0.5 pg/mL *vs* 2.1 ± 0.4 ng/mL, $P < 0.01$) (Figure 6B). Regarding TGF- β 1 there was no statistical difference between BDL and BDL + M groups (192.6 ± 32.1 pg/mL *vs* 212.2 ± 46.04 pg/mL), but was significantly higher in BDL + M compared to CG (212.2 ± 46.04 pg/mL *vs* 136.9 ± 12.3 pg/mL, $P < 0.05$), only at 24 h OT (Figure 6C).

Lipid peroxidation

The MDA levels in serum were assessed to measure the impact of oxygen free radicals on lipid peroxidation (Figure 7). We could observe significantly higher levels of MDA in the BDL and BDL + M groups compared to the CG group: BDL *vs* CG (21.14 ± 3 nmol/mL *vs* 11.01 ± 0.6 nmol/mL, $P < 0.001$), and BDL + M *vs* CG (19.49 ± 2.77 nmol/mL *vs* 11.01 ± 0.6 nmol/mL, $P < 0.01$).

mRNA expression of IL-6, TGF- β 1, and COX-2 in the liver and small bowel

The expression of messenger RNA (mRNA) for IL-6,

TGF- β 1, and COX-2 in the liver and mucosa/serosa of the small bowel were analyzed by RT-PCR. Similar to the ELISA results, IL-6 expression in the liver was up-regulated significantly in the BDL group, especially at 3 h OT compared to BDL + M and CG, $P < 0.01$. However, it was significantly down-regulated at 24 h OT in both groups compared to CG, $P < 0.05$ (Figure 8A).

In mucosa, IL-6 was undetectable; while in muscularis/serosa was down-regulated significantly in BDL + M compared to BDL and CG especially at 3 h OT, $P < 0.01$ (Figure 8A). There was no statistical difference between the BDL + M and BDL groups in TGF- β 1 mRNA expression in liver and small bowel. Whereas, statistical difference was observed in comparison with CG: in the liver at 24 h OT, BDL *vs* CG, $P < 0.05$; in mucosa at 3 h OT, BDL + M and BDL *vs* CG, $P < 0.001$; and in muscularis/serosa at 3 h OT, BDL *vs* CG, $P < 0.01$ (Figure 8B). In the liver, meloxicam treatment, as we assessed with immunohistochemistry, down-regulated significantly COX-2 mRNA expression in BDL + M at 3 h OT *vs* BDL at 24 h OT, $P < 0.05$. In mucosa and muscularis/serosa of the small bowel there was no statistical difference in COX-2 mRNA expression between the BDL + M and BDL groups. While, statistical difference was observed in comparison with CG; BDL + M and BDL groups *vs* CG at 3 h OT, $P < 0.01$, and BDL + M *vs* CG at 24 h OT, $P < 0.05$ (Figure 8C).

DISCUSSION

LC as a final pathophysiological pathway of many chronic liver diseases, has become a considerable public health problem because its incidence in western countries

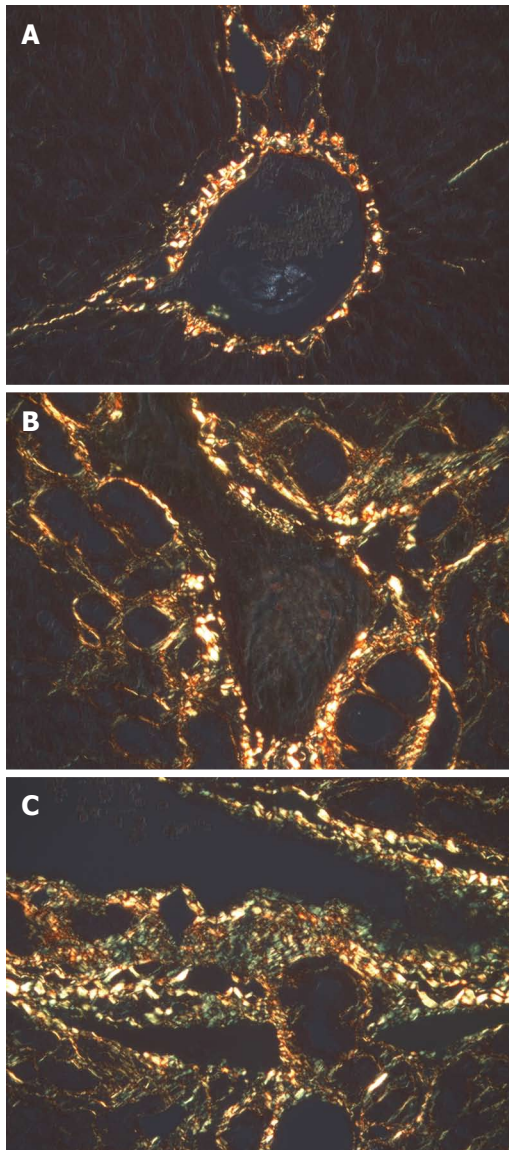


Figure 5 Quantification of collagen I (red) and collagen III (green) at 24 h after liver resection of three groups: Control group (A); bile duct ligation + meloxicam (B); and bile duct ligation (C). Ishak scoring system was adopted to score cirrhotic alterations in the liver of three groups ($n = 5$).

is rising^[16]. Several noxae (*e.g.*, viruses, alcohol, metabolic disorders, *etc.*), trigger an inflammatory response within the liver and, if the causes persist, the pathophysiological process leads to end-stage liver disease such as LC. In this inflammatory milieu, many studies have confirmed that COX-2 is up-regulated, regardless of the cause either by cholestasis, viruses or toxins^[7,9]. COX-2 is an inducible enzyme originally found to be induced by various stimuli, such as cytokines, mitogens and growth factors^[8]. It has been previously shown that the administration of selective COX-2 inhibitors from the beginning of acute inflammation in LC induced either by BDL or toxins could suppress inflammation and fibrogenesis^[7,42]. Contrary to others, in the present study we administered a single dose of meloxicam in a LC rat model, when fibrosis was already established for 2 wk.

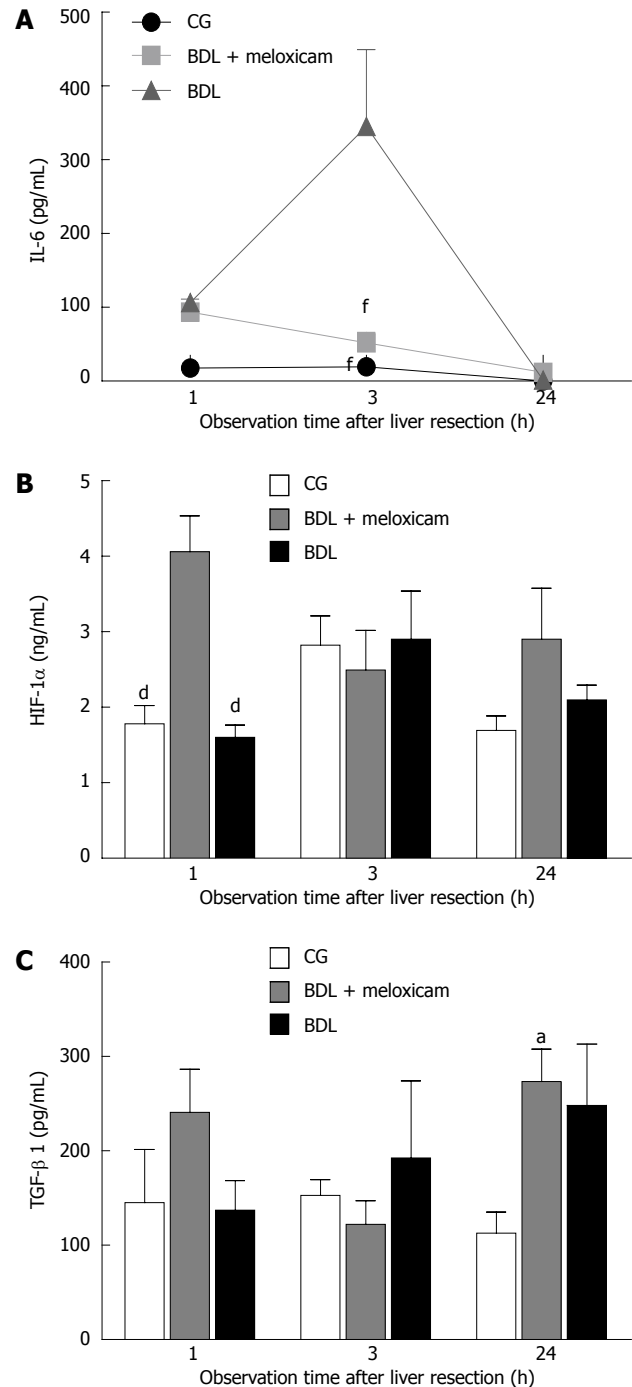


Figure 6 Release of interleukin 6 (A), hypoxia-inducible factor 1 alpha (B) and transforming growth factor beta 1 (C) in the serum after liver resection in three different groups at 1, 3 and 24 h. Values are given as mean \pm SE. 2-way ANOVA. Bonferroni's post-test: ^a $P < 0.05$ vs CG; ^b $P < 0.01$ vs BDL + M; ^c $P < 0.01$ vs BDL ($n = 15$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; IL-6: Interleukin 6; HIF-1α: Hypoxia-inducible factor 1 alpha; TGF-β1: Transforming growth factor beta 1.

Thereafter, about 50% of liver volume was resected. Our aim was to investigate gut-liver axis disturbances after liver resection and, possible pharmacological interventions with selective COX-2 inhibition in a rat model. In this study, we administered meloxicam, an oxycam derivative of NSAIDs, which preferentially inhibits

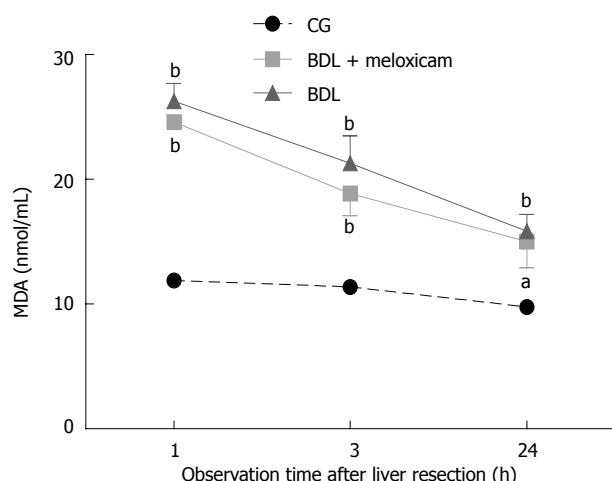


Figure 7 Release of malondialdehyde in the serum after liver resection at three different time points 1, 3 and 24 h for three groups: Control group; bile duct ligation + meloxicam; and bile duct ligation. Values are given as mean \pm SE. 2-way ANOVA. Bonferroni's post-test: ^a $P < 0.05$, ^b $P < 0.01$ vs CG ($n = 15$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; MDA: Malondialdehyde.

COX-2 over COX-1. It is a preferred drug for rheumatoid arthritis and osteoarthritis due to lower cardiac and gastrointestinal side effects compare to other selective COX-2 inhibitors^[43-46]. In the present study, one single dose of meloxicam was able to ameliorate the ischemia/reperfusion injury of the liver and small bowel after liver resection in the BDL + M group in comparison to the BDL group. Due to the inflammatory process in the liver induced by bile duct ligation and surgical stress, many functions of the liver were disturbed. However, suppression of inflammation with meloxicam preserved many of them. Microcirculation of the liver, as an important parameter in maintenance of liver function, was better maintained with meloxicam treatment compared to the BDL group. Although there was no difference in PVF between the BDL + M and BDL groups, whereas in CG was significantly higher, suppression of inflammation with meloxicam was connected with better liver regeneration in comparison with the BDL group. This was in line with down-regulation of COX-2 expression and up-regulation of Ki-67 in the liver assessed by immunohistochemistry. Moreover, attenuation of IL-6 release into the serum as a proinflammatory cytokine with meloxicam, assessed by ELISA and RT-PCR, showed better regeneration and integrity of the liver in comparison with the BDL group.

We were surprised that elevation of ALT in the serum after liver resection was significantly higher in BDL + M compared to CG and only after 1 h OT compared to the BDL group. This rise of ALT in BDL + M group may be attributed to meloxicam metabolism and elimination. It was reported that cholestasis produced by BDL has been associated with decreased concentrations of hepatic microsomal cytochrome P450 and decreased hepatic microsomal oxidative drug metabolism^[47,48]. Thus, the half-life of meloxicam may be extended. This could probably

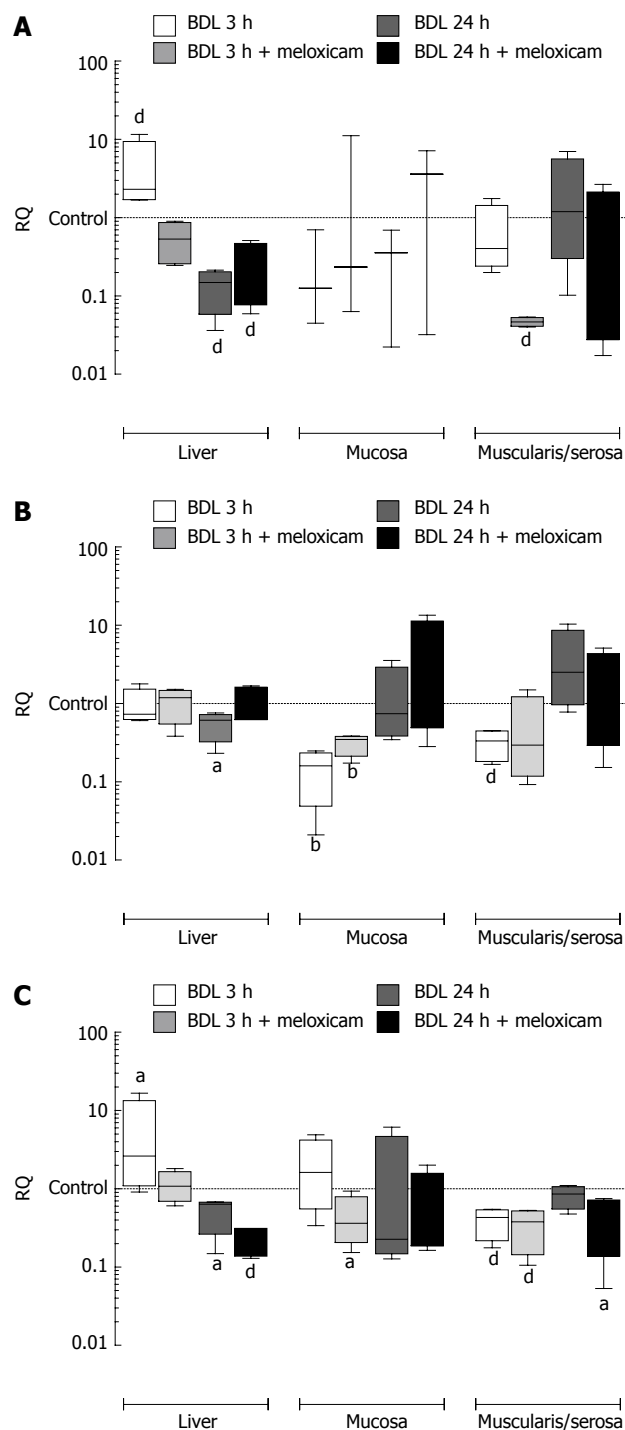


Figure 8 Hepatic and the small bowel (mucosa and muscularis/serosa) messenger RNA (mRNA) expression for interleukin 6 (A), transforming growth factor beta 1 (B), and cyclooxygenase-2 (C), after liver resection at two time points 3 and 24 h. Values are given as mean \pm SE. Multiple comparisons between groups under the Kruskal-Wallis test were performed. ^a $P < 0.05$, ^b $P < 0.01$ vs BDL + M and BDL; ^d $P < 0.01$ vs BDL + M and CG ($n = 15$). CG: Control group; BDL: Bile duct ligation; BDL + M: BDL + meloxicam; IL-6: Interleukin 6; TGF- β 1: Transforming growth factor beta 1; COX-2: Cyclooxygenase-2.

lead to a concentration-dependent adverse effect. This minorside effect of meloxicam was attenuated at 24 h OT after liver resection. Indeed, liver histology of 24 h OT in the BDL group also showed moderate to severe

changes in liver tissue according to the semiquantitative scoring system with biliary proliferation in different locations involving multiple lobules, inflammatory cells infiltration, and some areas of necrosis. Meloxicam treatment attenuated these changes to a moderate degree. Interestingly, HIF-1 α was significantly higher in BDL + M compared to BDL and CG, especially at 1 h OT. Its role in liver regeneration and fibrosis is controversial. Tajima *et al*^[49] showed that HIF-1 α found in hepatocytes after liver resection was required for the maintenance of normal blood glucose levels by regulating gluconeogenesis and glycogen synthesis during liver regeneration. Research with HIF-1 α KO mice in hepatocytes corroborated these results. On the other hand, Moon *et al*^[50] reported that liver fibrosis was reduced in HIF-1 α -deficient mice after 3 wk of BDL compared to the wild mice type. The extent of LC determined by the Ishak scoring system and collagen I and III quantification in the BDL + M group was not different compared to the BDL only group. Since, in our rat model, in the treatment group after liver resection HIF-1 α was up-regulated, this may be very helpful to overcome the stress after surgery by regulating glucose utilization during liver regeneration. Yamamoto *et al*^[51] showed that HIF-1 α was up-regulated in liver and intestine during liver regeneration after hepatectomy, which is in line with our results.

Cholestasis induced by BDL may cause mitochondrial dysfunction, biochemical, and molecular changes related to oxidative stress in the liver. In order to evaluate the oxygen free radical activity we measured MDA levels in serum. This activity was higher in the BDL group; however meloxicam could not suppress this activity significantly. Huang *et al*^[52] reported high levels of MDA in serum after two, even higher after 4 wk post-BDL, suggesting that the extent of lipid peroxidation increased with progressive fibrosis. Nevertheless, previously it was documented that COX-2 inhibitors administered from the beginning of acute inflammation could suppress inflammation, fibrosis, and oxidative stress by lowering MDA levels and profibrogenic factors in LC induced by CCl₄ treatment^[42]. In our study due to the severe oxidative stress, establishment of fibrosis, and adding surgical stress by the hepatectomy, the single dose of meloxicam did not lower MDA levels significantly.

Earlier it was reported that administration of COX-2 inhibitors from the beginning of the inflammation process is able to suppress fibrosis in LC caused by BDL and toxins, through down-regulation of TGF- β 1^[7,42]. Similar to liver fibrosis and collagen quantification, TGF- β 1 as a profibrogenic factor assessed with ELISA, and TGF- β 1 mRNA expression in liver and small bowel assessed with RT-PCR, showed no significant difference between BDL + M and BDL group. Williams and Iredale^[53] reported that liver regeneration is characterized not only by hepatocyte proliferation but also by increased TGF- β 1 expression. Thus, hepatocytes proliferate despite the presence of a strong antiproliferative stimulus.

The small bowel, as an integral part of GI tract, plays an important role in many functions of the body, while

in LC many of these are compromised. It was shown previously that the GI transit is altered in patients with LC^[20]. In the present study, meloxicam treatment showed significant acceleration of GI transit compared to the BDL group. This is in agreement with the other results published previously. Josephs *et al*^[54] reported that COX-2 inhibition improved early postoperative GI transit, whereas Schwarz *et al*^[55] have shown that PGs, produced by the action of COX-2, are major participants in rodent postoperative ileus induced by intestinal manipulation. Moreover, they showed that due to the molecular up-regulation of COX-2, jejunal circular muscle contractility and GI transit was decreased. Experiments in COX-2 KO mice have confirmed these findings. Same results were shown earlier by our group, that intestinal manipulation caused GI transit delay and an impairment in bowel wall perfusion and microcirculation^[36]. Similarly, in our study, COX-2 mRNA expression in the gut was attenuated by meloxicam treatment. Thus, although PGs are both inhibitory and excitatory on intestinal smooth muscle cells, the data in this study showed predominantly inhibitory effects on small bowel circular smooth muscle cells. However, a single *iv* administration of meloxicam caused no difference on microcirculation of the small bowel between groups; however, histological and immunohistochemical analysis showed that meloxicam treatment attenuated ischemic lesions in comparison with the BDL group. Proliferative activity, assessed by the number of Ki-67-positive nuclei enterocytes, the number of goblet cells quantified, and the length of the villi were in favor of treatment with meloxicam. These results are in agreement with other results previously published. Gulo *et al*^[56] have shown that the number of goblet cells was increased in mucosa of the small bowel under chronic ischemia. Additionally, ischemic intestinal injury has been shown to be reflected by a lowering of villus length of the gut^[56,57].

In summary, it can be concluded that even one single dose of meloxicam administered after cirrhotic liver resection could improve the impairment of many functions of the gut-liver axis, enabling better function and integrity of the remaining liver and small bowel. In future, further studies are warranted to have better insight about the pharmacokinetics of meloxicam in established cholestatic LC, in order to avoid some side effects in LC. Perhaps, by reducing meloxicam dosage and using it in a repetitive manner we would be able to gain better results in gut-liver axis after cirrhotic liver resection.

ACKNOWLEDGMENTS

The authors thank Pascal Paschenda and Mareike Schulz, for their skillful technical assistance.

COMMENTS

Background

Liver cirrhosis (LC), represent end-stage of wide variety of chronic liver diseases which leads to hemodynamic disturbances throughout the gastrointestinal (GI) tract associated with inflammatory activity. Cyclooxygenase-2 (COX-2) is

up-regulated in this milieu, and administration of selective COX-2 inhibitors from the beginning of acute inflammation in LC could suppress inflammation and fibrogenesis. Whereas, influence of meloxicam on gut-liver axis after cirrhotic liver resection was not studied previously.

Research frontiers

The aim of this study was to elucidate the mechanisms involved in disturbed function of the gut-liver axis after cirrhotic liver resection and possible pharmacological interventions with the COX-2 inhibitor meloxicam in a rat model, recognizing reports of up-regulation of COX-2 in human liver cirrhosis as a result of active inflammation. Involvement of different pathophysiological mechanisms on microcirculation of small bowel after cirrhotic liver resection in rats is not yet clarified.

Innovations and breakthroughs

The related research will help to understand the basic mechanism about the initiation of fibrosis as well as regeneration and the systemic consequences for the small bowel function. This is the first study to report that one single dose of meloxicam administered 15 min. Before cirrhotic liver resection could improve the impairment of many functions of the gut-liver axis, enabling better function and integrity of the remaining liver and small bowel such as microcirculation, liver and small bowel regeneration and GI transit *etc.*

Applications

The study indicates that meloxicam may serve as a novel therapeutic target for improving gut-liver axis functions after cirrhotic liver resection in the future.

Terminology

Meloxicam, an oxycam derivative of non-steroidal anti-inflammatory drugs, which preferentially inhibits COX-2 over COX-1. It is a preferred drug for rheumatoid arthritis and osteoarthritis due to lower cardiac and gastrointestinal side effects compare to other selective COX-2 inhibitors.

Peer review

The manuscript is excellently written and addresses an important topic. The aim of this work is of interest, the methodology is appropriate and the results are enlightening. This work could help to clarify if one single dose of meloxicam administered after cirrhotic liver resection could improve the impairment of some functions of the enterohepatic circuit.

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P-Reviewer: Castiella A, Mattner J, Romero MR
S-Editor: Nan J **L-Editor:** A **E-Editor:** Zhang DN





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