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Intestinal barrier dysfunction in liver cirrhosis assessed by iohexol test

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Abstract. – OBJECTIVE: To study gut barrier function in patients with liver cirrhosis (LC) by evaluating the intestinal permeability (IP) and its relationship with the severity and etiology of the disease.

PATIENTS AND METHODS: The study included 31 patients with LC and 25 healthy controls. Child-Pugh score was used for evaluation of the LC severity. IP was assessed by the rise in levels of iohexol, which was administered orally (25 mL, 350 mg/mL) 2 h after breakfast. Three and six hours later serum (SIC mg/L) and urine (UIC g/mol) iohexol concentrations were determined by a validated HPLC-UV technique.

RESULTS: Patients with LC had significantly higher mean SIC value compared with control group at 3 h (2.05 ± 1.67 *vs.* 1.25 ± 1.41 mg/L, *p*=0.021, as well as at 6 h (2.20 ± 2.65 *vs.* 1.11 ± 1.06 mg/L, *p*=0.001) after ingestion. No significant difference was found in mean SIC value of patients at 3 and 6 h. 23% of the patients had an increased IP. The mean iohexol urine recovery of patients was similar to that of the controls both at 3 h and at 6 h. Mean SIC values were significantly higher in patients with advanced Child C class than in healthy controls or the subgroup with Child B class, both at 3 h (2.54 ± 1.95 mg/L *vs.* 1.11 ± 1.06 mg/L, *p*=0.007) or (2.57 ± 1.85 mg/L *vs.* 1.35±1.32 mg/L, *p*=0.005) and at 6 h (2.57 ± 1.85 mg/L *vs.* 1.25 ± 1.40 mg/L, *p*=0.002) or 2.54 ± 1.95 mg/L *vs.* 1.07 ± 0.35 mg/L, *p*=0.02). Cirrhotic patients with ascites had significantly higher SIC in comparison with the controls, both at 3 h (2.31 ± 1.74 *vs.* 1.25 ± 1.41 mg/, *p*=0.009) and at 6 h (2.20 ± 1.87 *vs.* 1.11 ± 1.06 mg/l, *p*=0.007). In the subgroup of patients with alcoholic LC, the mean SIC values at 3 and 6 h (2.29 ± 1.80, 2.33 ± 1.85 mg/L, respectively) were significantly higher (*p*= 0.016, *p*=0.003) compared to the control group (1.25 ± 1.41, 1.11 ± 1.06 mg/L, respectively).

CONCLUSIONS: Increased IP is found in 23% of cirrhotic patients. Permeability alterations are significantly more pronounced in patients with advanced LC with the presence of ascites and in those with alcoholic etiology.

Key Words:
Intestinal permeability, Iohexol test, Liver cirrhosis, Ascites, Alcohol.

Introduction

Intestinal lumen is colonized by a broad range of highly diverse microbiota that promotes metabolism and digestion in symbiotic relationship with the host[1]. Intestinal mucosal barrier provides the first defensive line against the luminal environment and is crucial in the maintenance of the host intestinal homeostasis. It is a selectively permeable barrier since it allows the passage of water, electrolytes, and nutrients, but prevents the invasion of foreign antigens, microorganisms, and their toxins[2,3]. Intestinal epithelial cells are tightly connected by intercellular junctional complexes. Tight junctions (TJs) seal the intraepithelial space and are the key regulator of the paracellular permeability[4].

There is a close interplay between the gut and the liver, named "gut-liver axis"[5]. Due to the unique anatomy and vascular system of gut-liver axis the liver receives approximately 70% of its blood supply from the intestine via the portal vein[6,7]. Being the first organ in the body exposed to gut-derived substances, including not only absorbed nutrients, but also bacteria and their products such as lipopolysaccharide (LPS, endotoxin)[8], the liver serves as an initial site of filtration of all dangerous substances. The

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normal balance in the gut-liver axis is maintained by integrity of the gut barrier, the composition of the microbiota, and the immune defense in the gut and liver[9]. Disruption of the mucosal barrier leads to increased intestinal permeability (IP) and translocation of microbial products and viable bacteria from the intestinal lumen that contributes to liver disease by inducing hepatic inflammation[7,10,11]. Translocation of bacterial products will also lead to an activation of the mucosal immune system and secretion of inflammatory mediators, which in turn might increase the dysfunction of the mucosal barrier[3,12]. Furthermore, evidence[13-16] suggests that the bacterial translocation is a contributing factor in the pathogenesis of chronic liver diseases and the development of complications in liver cirrhosis (LC).

Intestinal permeability in LC has been investigated in numerous studies with inconsistent results. Studies differ with respect to the methods for assessing permeability, both in the administration procedures of different probes, and to outcome measures[12,17-19]. In a previous study[20] we have demonstrated that the water-soluble contrast medium iohexol is a suitable marker for assessing the intestinal barrier function and its increase in patients with active inflammatory bowel diseases is related to the disease activity. Moreover, measurement of serum concentration of iohexol following its ingestion improves the convenience of the permeability testing.

The aim of this study was to investigate gut barrier function in patients with LC, by evaluating the IP, and to establish the relation between changed IP, and severity of the liver dysfunction and etiology of the LC.

Patients and Methods

Patients

The study included 31 patients with LC (25 males and 6 females; mean age 48.9 years, range 36-69), hospitalized in the Clinical Centre of Gastroenterology. Liver cirrhosis was diagnosed by the clinical examination, biochemical data (liver function test, coagulation profile), serology, abdominal ultrasound, and upper gastrointestinal endoscopy to assess further complications of portal hypertension. Child-Pugh score was used for evaluation of the disease severity. Patients with impaired renal function, urinary tract infection, uncontrolled type 1 diabetes mellitus, malignancy, major abdominal surgery, hepatic encephalopathy, extra hepatic biliary obstruction, inflammatory bowel diseases, celiac disease, autoimmune conditions, and pregnant women were not enrolled.

Healthy Controls

Twenty-five healthy volunteers (18 males, 7 females, mean age 40.6 years: range 25-68) were recruited as control group. None of them had signs or symptoms of gastrointestinal, liver or renal diseases.

None of the investigated patients and healthy subjects had used alcohol, non-steroidal anti-inflammatory drugs, acid-suppressive drugs, probiotics or prokinetics in the two weeks preceding the study. Renal function of all investigated subjects was assessed by serum creatinine levels and calculation of the glomerular filtration rate (GFR).

Assessment of the Intestinal Permeability

Iohexol (Omnipaque™, Nycomed-General Electric, Oslo, Norway) was administered orally (25 mL of 350 mg/mL injection solution) in the morning, 2 h after breakfast, immediately after voiding. Drinking was forbidden for the next 3 h. Food was permitted after 5 h. Blood and urine samples were collected at 3 and 6 h after the iohexol ingestion. Serum was separated within 45 min after blood withdrawal by centrifugation at 1500 g for 10 min. Serum and urine were kept frozen at –20°C until analysis. Concentrations of iohexol were determined by a validated HPLC-UV technique as previously described[20]. Briefly, sample preparation consisted of protein precipitation and dilution for urine samples, separation was performed on a C8 analytical column, with a mobile phase of 5% aqueous acetonitrile at isocratic flow rate of 1.2 ml/min. Column effluent was monitored at 240-245 nm and run time was about 7 min. Calculation was based on external standardization using six-point calibration curves. Selectivity was confirmed by comparing the signal in blank and spiked samples at the lower limit of quantification from 12 different individual sources of human serum and urine. Accuracy and precision (within-run and between-runs) were all within 12%. Extraction recovery was over 90%; linearity range was 0.25 ÷ 10.00 mg/L for serum samples and 2.50 ÷ 700.00 mg/L for urine samples; $R2>0.998$. Freeze-thaw stability was determined for three cycles, each lasting 24 h, post-preparative stability was documented for

12 h at 8°C, short-term stability was proven for 8 h in the dark at room temperature, and for 4 h at daylight. Urine creatinine was also determined, and urine results were assessed as absolute concentrations and as IOH/creatinine (g/mol) ratios. Results of the control group (mean ± 2SD) were used as the cut-off level for increased IP at 95% confidence interval (CI). Values exceeding mean ± 2SD were considered as abnormal.

Ethical Consideration

The study protocol was approved by the Ethics Committee of University Hospital Queen Joanna, Sofia. Written informed consent was obtained from all patients and control subjects.

Statistical Analysis

All values were expressed as mean ± standard deviation (SD) or proportions. Data processing was performed using descriptive statistics, nonparametric Mann-Whitney U test for overall testing the difference between subject groups, Wilcoxon matched paired test for the difference between pairs of groups, and Chi-square for comparing proportions. Each hypothesis was tested at a significance level of $p < 0.05$.

Results

The mean duration of LC for the patients was 2.7 years (ranging from 1 to 7 years). Alcohol was identified as a cause of LC by a history of drinking at least 80 g alcohol daily for more than five years. All patients with alcoholic LC were negative for hepatitis B surface antigen and antibody against hepatitis C. The mean Child-Pugh score of the patients was 7.6 ± 0.63 in Child B class and 12.06 ± 1.12 in Child C class of LC.

Table I. Characteristics of patients with liver cirrhosis (n=31).

Variables	No. of patients
Sex (M/F)	25/6
Age (years) mean (range)	48.9 (36-69)
Etiology	
Alcohol	19
Virus (HBV/HCV)	12
Child stage	
A/B/C	
0/15/16	
Ascites	
Yes	20
No	11

Glomerular filtration rate was normal in all investigated subjects. The baseline demographic and clinical characteristics of patients with LC are shown in Table I.

Intestinal Permeability

All permeability tests were well tolerated and no side effects were reported. Based on the results obtained from healthy subjects in the previous study[20], values of SIC over 4.0 and 3.3 mg/L at 3 and 6 h after ingestion respectively, were considered abnormal. Using those cut-offs, one of the healthy controls had an increased IP at 3 h post iohexol ingestion, and respectively two - at 6 h post iohexol ingestion.

IP in LC and Healthy Controls

Patients with LC have significantly higher mean SIC value compared with control group at 3 h (2.05 ± 1.67 *vs.* 1.25 ± 1.41 mg/L, *p=0.021,* Mann-Whitney U test), as well as at 6 h (2.20 ± 2.65 *vs.* 1.11 ± 1.06 mg/L, *p=0.001*) after ingestion (Figure 1). No significant difference was found in mean SIC value of patients at 3 and 6 h *(p=0.501)* (Wilcoxon matched paired test). Seven out of 31 (23%) of patients were with increased IP in the two periods studied. The mean value of iohexol urine recovery of cirrhotic patients was similar to that of the controls both at 3 h (14.59 ± 16.58 *vs.* 14.64 ± 10.44 g/mol, *p=0.434*) and at 6 h (20.64 ± 10.78 *vs.* 20.78 ± 11.67 g/mol, *p=0.934*).

IP and Severity of LC

Analysis of IP relating to the Child-Pugh class of LC showed that permeability alteration, as-

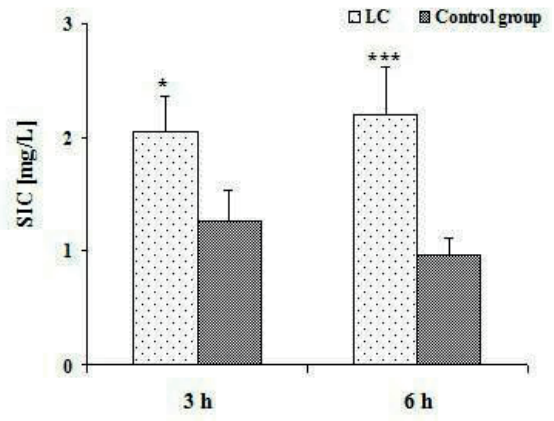

Figure 1. SIC (mean ± SE) at 3 and 6 h post ingestion in patients with LC and healthy controls. With asterisk are noted significant difference between patients and control group (* - $p < 0.05$, *** - $p < 0.001$).

sessed by iohexol resorption was significantly higher in patients with advanced Child C class than in healthy controls or the subgroup with Child B class, both at 3 h (2.54 ± 1.95 mg/L *vs.* 1.11 ± 1.06 mg/L, *p=0.007*) or (2.57 ± 1.85 mg/L *vs.* 1.35±1.32 mg/L, *p=0.005*) and at 6 h (2.57±1.85 mg/L *vs.* 1.25±1.40 mg/L, *p=0.002*) or 2.54 ± 1.95 mg/L *vs.* 1.07 ± 0.35 mg/L, *p=0.02*). The mean value of SIC between the patients of subgroup Child B class and control subjects were similar at 3 h (*p=0.634*), as well as at 6 h (*p=0.262*) after iohexol ingestion (Figure 2A).

Abnormal IP was determined in 37.5% of Child C class of cirrhosis and only in 6.7% of patients from Child B class subgroup. Cirrhotic patients with ascites (n=20) had significantly higher SIC in comparison with the control group, both at 3 h (2.31 ± 1.74 *vs.* 1.25 ± 1.41 mg/, *p=0.009*) and at 6 h (2.20 ± 1.87 *vs.* 1.11 ± 1.06 mg/l, *p=0.007*) (Figure 2B). With increased IP were 30.0% of patients with ascites and 9.1% of patients without this complication.

IP and Etiology of LC

In the subgroup of patients with alcoholic LC, the mean SIC values at 3 and 6 h (2.29±1.80, 2.33±1.85 mg/L, respectively) were significantly higher (*p=0.016, p=0.003*) compared to the control group (1.25±1.41, 1.11±1.06 mg/L, respectively). No significantly different mean SIC values were found between alcoholic and viral LC subgroups of patients (Figure 2C). However, the permeability disturbances, assessed by iohexol test, were almost 4-fold more frequent in the subgroup with alcoholic LC (31.6%) in comparison to those of the viral LC (8.3%).

Discussion

The intestinal barrier function in LC has been investigated in several studies. Comparative interpretation of the results is difficult due to the use of different marker substrates and different study protocols for assessing IP[12,17]. In some of those studies, increased IP was reported in 17-49% of cirrhotic patients[21-27]. However, normal IP in LC patients was found by others[28-31]. In our study, by measuring the serum levels and urine recovery of iohexol following oral administration, increased IP was found in 23% of LC patients. Pathophysiology of gut barrier dysfunction in LC is multifactorial[32]. It can be a result of both direct and indirect effects of etiological factors, as well as by characteristics of the cirrhosis itself[18]. Indeed, in LC there are structural and functional abnormalities in the intestinal mucosa associated with portal hypertention or the toxic effects of alcohol[15,19], malnutrition[33],and impaired liver function[34]. In experimental model of acute portal

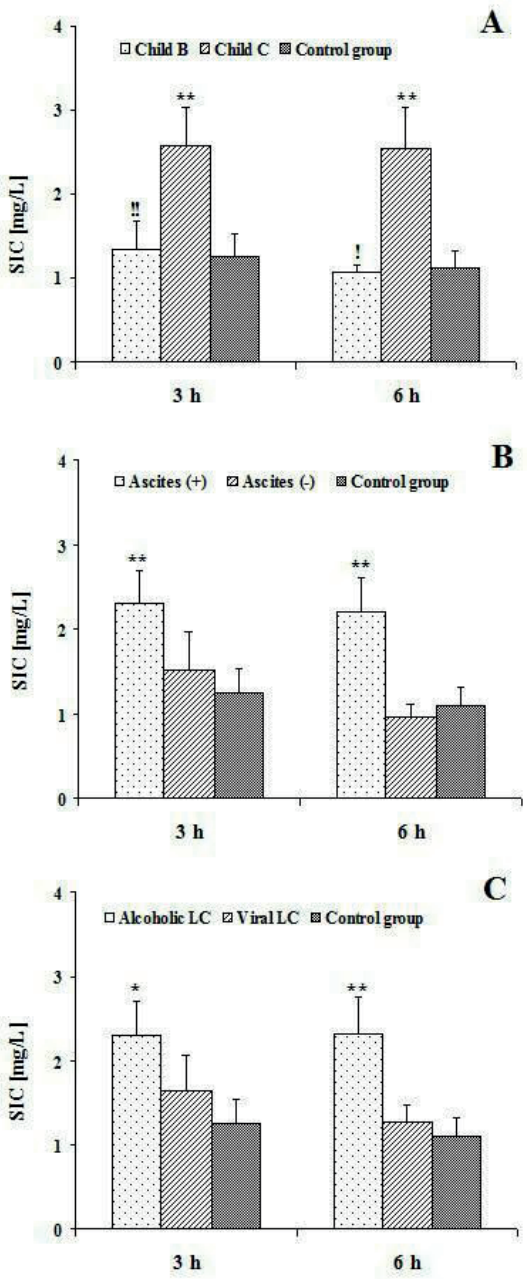

Figure 2. SIC (mean ± SE) at 3 and 6 h post ingestion in healthy controls and LC patients divided into severity (Child class) **(A)**, presence of ascites **(B)** and etiology **(C)**. With asterisk are noted significant difference between patients and control group (* - $p < 0.05$, ** - $p < 0.01$), and with ! - $p < 0.05$, !! - $p < 0.01$ - between two subgroups of patients.

hypertension, congestion and edema, significantly decreased brush border enzyme activities, increased IP, and bacterial translocation through the intestinal wall, were revealed[35]. In addition, dilated extracellular space between adjacent intestinal epithelial cells, and reduced number of shorter and wider microvilli in the duodenal mucosa of cirrhotic patients were observed[36]. Furthermore, in experimental and clinical studies, it was determined that in LC there is an increased oxidative stress[37-40], generation of reactive oxygen radicals in the intestinal mucosa[37,38], and imbalance of cell proliferation and apoptosis[40]. Thus, in addition to the vascular congestion, induced by the oxidative stress damage of enterocytes can also contribute to the barrier disturbances[38,40]. However, in another study no signs for oxidative stress and low-grade intestinal inflammation in cirrhotic patients with compensated disease were found[41]. Recently, the reduced expression of TJ proteins occludin and claudin-1[42] and increased expression of pore-forming claudin-2[43] have been shown as an important mechanism contributing to gut barrier dysfunction in LC patients. Notably, these alterations were significantly more pronounced in decompensated patients[42,43] and were correlated with the levels of endotoxemia[42]. The increased production of tumor necrosis factor alpha (TNF-α)[44], as well as increased inducible nitric oxide syntase (iNOS)-dependent NO production[45], gut-derived endotoxin and alcohol have been suggested as leading factors for altered TJ proteins expression[10,15].

While serum iohexol concentrations are elevated in patients with LC, its urinary recovery was similar to that of controls. In interpreting these results some methodological issues should be taken into account[18]. The urine recovery of the substrate may be influenced by non-intestinal factors[18], such as gastric evacuation and intestinal transit that are delayed in LC[32], as well as a renal function and tissue distribution. Thus, the "normal" results may be due to impaired renal perfusion as part of a spectrum of hemodynamic alterations in LC. Although the baseline serum concentrations and clearance of creatinine in the studied patients were in normal range, probably the impaired renal perfusion and dysfunction play a significant role in the iohexol excretion. The distribution of iohexol in ascites fluid may be another possible reason for the low urinary recovery and therefore gut permeability alteration in LC patients with ascites is not well appreciated. In this study we did not test iohexol concentration in ascitic fluid. However, some authors[28,46] did not find the presence of permeability substrates in ascites, and exclude the possibility of its low urine excretion to be a result from its redistribution in ascites.

In this study we found out that patients with advanced LC have significantly more pronounced permeability alterations compared to the control group. Increased IP was observed in 7/31 patients; in 6 of them LC is in advanced Child C stage. Available data in the literature concerning the relationship between the gut barrier dysfunction and severity of LC are contradictory mostly due to the methodology and/or selection of patients[12,18]. Some scholars[18,22,23,26,46] reported a connection between the increased IP and severity of LC, while others[24,27,29] failed to reproduce these results. More advanced liver diseases have more severe course, due to complications such as portal hypertension, ascites, hypoproteinaemia, encephalopathy, infection, etc. Moreover, as mentioned above[42,43], it has been found out that TJ proteins expression and redistribution are more pronounced in decompensated patients, compared to the compensated ones, with a correlation between permeability disturbances and the levels of endotoxemia[42]. Indeed, in patients with cirrhosis, the plasma endotoxin levels progressively increased in relation to the severity of liver dysfunction[47].

The permeability derangements in this study were more pronounced in patients with advanced liver stage, which possess ascites. Ascites was found in 20 patients, 16 of whom with Child class C. The mean SIC values of patients with ascites were significantly higher, compared with the healthy controls. Similar results for significantly increased IP in LC patients with ascites, but not in patients without ascites compared to healthy subjects were reported in other studies[18,46,48]. It has been documented that alcohol consumption causes the gut leakiness, resulting in significant increase in permeability for macromolecules[49,50-55]. We found that patients with alcoholic, but not with viral etiology of LC have an increased IP compared to healthy controls. Several mechanisms, underlying the ethanol-induced barrier disruption have been proposed, including direct damage to epithelial cells, loss of integrity of TJs, and changes in intestinal microbiota[50,51,56]. Indeed, chronic alcohol abuse results in quantitative and qualitative changes of the intestinal microbiota composition[57]. Moreover, alcohol can also promote bacterial growth and accumulation of endotoxin in the intestinal lumen[57].

In addition, alcohol metabolism by the Gram negative bacteria and intestinal epithelial cells can result in accumulation of acetaldehyde, which in turn can increase IP to endotoxin[50,51,58]. Increasing evidence suggests the crucial role of acetaldehyde in barrier impairment by redistribution of TJ proteins, as well as by increasing tyrosine phosphorylation of TJs and adherens junction proteins[15,59]. Notably, alcohol-induced dysbiosis might trigger intestinal inflammation, resulting in disruption of the intestinal barrier integrity and bacterial translocation[10]. In addition, alcohol-induced production of iNOS may contribute to increased IP of endotoxin[60]. Thus, it is clinically important that the leaky gut is considered to be one of the underlying mechanisms of alcohol-mediated endotoxemia in patients with alcoholic liver disease[15,19,54,55]. However, whether increased IP in alcoholic LD is due to the effects of alcohol on the intestinal barrier or the barrier is influenced by LC is still a matter of debate. In a study on IP for macromolecules and endotoxemia in patients with chronic alcohol consumption at different stages of alcoholic liver disease, Parlesac et al[49] support the hypothesis that the increased permeability is mainly due to the damaging effects of alcohol. They found the same or even higher increase of IP in patients with initial hepatic impairment, in comparison to that in patients with formed LC. Keshavarzian et al[55] also found that IP was increased only in alcoholics with cirrhosis compared to alcoholics without liver disease, and to non-alcoholics with liver disease, suggesting that the gut leakiness may be the critical link between chronic alcohol consumption and liver damage.

Conclusions

We demonstrate that IP is increased in 23% of patients with LC. Permeability alterations are significantly more pronounced in patients with advanced LC with the presence of ascites and in those with alcoholic etiology. Measurement of a single serum concentration of the water-soluble contrast medium iohexol 3 h after its oral administration not only provides an acceptable information for intestinal barrier derangements in these patients, but is also clinically convenient and simple to use. Future studies are needed to better understand the underlying mechanisms and the exact role of intestinal barrier dysfunction in LC, which would highlight the new therapeutic targets and strategies for these patients.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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