

INTESTINAL PERMEABILITY ASSESSED BY ⁵¹Cr-EDTA IN RATS WITH CCl₄-INDUCED CIRRHOSIS

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ABSTRACT – *Context* - The straight relationship between cirrhosis and impaired intestinal barrier has not been elucidated yet. *Objectives* - To verify ⁵¹Cr-EDTA-intestinal permeability in rats with CCl₄-induced cirrhosis and controls. *Method* - Fifty male Wistar rats weighing 150-180 g were separated in three groups: 25 animals received CCl₄ 0.25 mL/kg with olive oil by gavage with 12 g/rat/day food restriction for 10 weeks (CCl₄-induced cirrhosis); 12 received the same food restriction for 10 weeks (CCl₄-non exposed). Other 13 rats received indomethacin 15 mg/kg by gavage as positive control of intestinal inflammation. *Results* - The median (25-75 interquartile range) ⁵¹Cr-EDTA-IP values of cirrhotic and CCl₄-non exposed rats were 0.90% (0.63-1.79) and 0.90% (0.60-1.52) respectively, without significant difference ($P = 0.65$). Animals from indomethacin group showed ⁵¹Cr-EDTA-IP, median 7.3% (5.1-14.7), significantly higher than cirrhotic and CCl₄-non exposed rats ($P < 0.001$). *Conclusion* - This study showed the lack of difference between ⁵¹Cr-EDTA-intestinal permeability in rats with and without cirrhosis. Further studies are necessary to better clarify the relationship between intestinal permeability and cirrhosis.

HEADINGS - Intestinal absorption. Edetic acid. Liver cirrhosis, experimental. Rats.

INTRODUCTION

The gastrointestinal system is the largest site of exposure to the outside environment in the human body. The intestinal epithelium works as a selective barrier, allowing the entrance of nutritive substances, electrolytes and water, but blocking the entrance of potentially noxious elements, such as antigens, toxins or bacteria^(9,10), a function called intestinal barrier (IB). Intestinal permeability (IP), on the other hand, can be defined as the facility the intestinal mucosa surface can be penetrated by unmediated diffusion of specific solutes in a given period of time⁽⁵⁾. The integrity of the IB can be evaluated by IP to specific markers, such as some sugars (lactulose, mannitol, rhamnose), polyethylene glycols and radioactive substances, such as ^{99m}Tc-labelled-diethylenetriaminepentaacetic acid (^{99m}Tc-DTPA), and ⁵¹Cr-labelled-ethylenediaminetetraacetate (⁵¹Cr-EDTA).

IB can be disturbed in patients with cirrhosis, and this can be one of the factors leading to bacterial translocation (BT), and consequently, spontaneous bacterial peritonitis (SBP), a complication of cirrhosis associated with high morbidity and mortality. However, the straight relationship between cirrhosis and impaired IB has not been elucidated yet. Some structural or

functional alterations in IB, probably as a consequence of vascular stasis due to portal hypertension, could lead to increased IP to intraluminal microorganisms. Congestion in intestinal mucosa has been shown by cirrhotic patients, which has been related to a widening of intercellular spaces⁽²⁴⁾, and consequently, a reduction in the absorptive surface, suggested as the main mechanism involved in abnormal IP in cirrhotic patients⁽⁷⁾.

Some studies suggest that alterations in IP may be involved in cirrhosis, as shown in clinical and experimental settings using lactulose/mannitol^(7, 18, 25) and ^{99m}Tc-DTPA^(20, 24).

In order to contribute to a better understanding of the relationship between alterations of IB and cirrhosis, this study evaluated IP of ⁵¹Cr-EDTA, a simple probe that requires low radioactivity load, in rats with carbon tetrachloride (CCl₄)-induced cirrhosis compared to CCl₄-non exposed controls.

METHODS

This study was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil, and followed international guidelines for the care and use of laboratory animals⁽¹⁴⁾.

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Fifty male Wistar rats with initial weights of 150-180 g were used in the study. The animals were caged in plastic boxes with 5 animals/cage in 12-hour dark-light cycles and at constant temperature of 18-22°C. The animals received standard rat chow (Nuvilab CR1® Nuvital S.A., Colombo, PR, Brazil) with dietary restriction of 12 g/rat daily (60 g each cage) and water ad libitum⁽¹³⁾. The animals were separated in two groups:

CCl₄-induced cirrhosis group: 25 animals received CCl₄ 0.25 mL/kg diluted in olive oil until a final volume of 1 mL once a week for 10 weeks. CCl₄ was administered intragastrically by gavage, using a 6F-polyethylene catheter (MarkMed Ltda., São Paulo, SP, Brazil) without sedation. The experimental model was adapted from Proctor and Chatamra⁽²¹⁾ and Rosa et al.⁽²³⁾, with the modification of dietary restriction and phenobarbital was added to the drinking water (350 mg/L) 1 week before starting CCl₄ administration. At the end of CCl₄-induced cirrhosis period, 21 animals showed cirrhosis on liver histology and these were the animals that participated in the study.

CCl₄-non exposed group: 12 animals received the same dietary restriction for 10 weeks. They did not receive CCl₄, but had the same volume of olive oil as the CCl₄-induced cirrhosis group, and had phenobarbital in the drinking water.

In order to obtain a positive control group of abnormal IP, we used a model of intestinal inflammation due to indomethacin. Thirteen male Wistar rats weighting 200-230 g received a single dose of indomethacin 15 mg/kg diluted in a 1.25% sodium-bicarbonate solution by gavage⁽²⁾. This group is referred as indomethacin group.

⁵¹Cr-EDTA intestinal permeability (⁵¹Cr-EDTA-IP)

Intestinal permeability was assessed by measuring the 5-hour urinary excretion of ⁵¹Cr-EDTA after 10 weeks in CCl₄-induced cirrhosis and CCl₄-non exposed groups; and after 48 hours in indomethacin group. Animals were kept individually in metabolic cages 24 hours before the ⁵¹Cr-EDTA trials for adaptation in the new environment. The rats received ⁵¹Cr-EDTA 2.5 µCi diluted in 1 mL of distilled water by gavage. After that, each animal had free access to tap water for the entire 5-hour urine collection. This time period was chosen in order to avoid fecal contamination⁽⁵⁾. The rats were sacrificed by intraperitoneal injection of ketamine (150 mg/kg) and xilasine (60 mg/kg). Laparotomy was performed and the bladder was emptied by puncture, with a 5-mL syringe. The urine obtained by this procedure was added to that collected in the metabolic cage. The total volumes were standardized to 4 mL and 3 aliquots of 1 mL were measured in a gamma counter (Autogamma Cobra 5002, Packard, USA). Data were expressed as⁽⁴⁾:

$$PI (\%) = \frac{\text{(radioactivity of 5-hour urine collected)}}{\text{(radioactivity of ingested dose)}} \times 100$$

Histological analysis

Liver and spleen of each animal were weighed and the relative weights (weight of the organ/body weight) were

recorded from CCl₄-induced cirrhosis and CCl₄-non exposed animals. Liver specimens were fixed in buffered 10% formalin, embedded in paraffin and stained with hematoxylin-eosin and picrosirius to evaluate the extent of liver fibrosis by an experienced pathologist who was unaware of the experimental groups. Tissue slides were classified as normal (grade 0), fibrosis (grades 1-3) or cirrhosis (grade 4) as follows: grade 0 = no fibrosis; 1 = stellate enlargement of portal tract but without septa formation; 2 = enlargement of portal tract with rare septa formation; 3 = numerous septa without cirrhosis; 4 = cirrhosis (architectural distortion, numerous septa and regeneration nodules⁽³⁾).

Histological analysis of small intestine and colon were performed in approximately 50% of the three groups (11/21 from CCl₄-induced cirrhosis group, 6/12 from CCl₄-non exposed group and 7/13 from indomethacin group). Tissue samples were fixed in buffered 10% formalin, embedded in paraffin and stained with hematoxylin-eosin. Acute inflammation of small bowel and colon was considered when paucity of goblet cells, neutrophil exudation, ulceration, or erosion of the mucosa were observed.

Statistical analysis

Data were analysed by SPSS® version 12.0. The percentual values of IP were expressed in median and 25-75 interquartile range. IP values among CCl₄-induced cirrhosis and CCl₄-non exposed were compared by using Mann-Whitney test and IP values among CCl₄-induced cirrhosis, CCl₄-non exposed and indomethacin groups were compared by using Kruskal-Wallis test. Statistically significant values were considered when *P*<0.05.

RESULTS

After 10 weeks of CCl₄ administration, 21/25 animals (84%) from CCl₄-exposed group showed liver cirrhosis upon histological examination. In addition, 4 of them had ascites. All CCl₄-non exposed animals had normal liver histology (Figure 1).

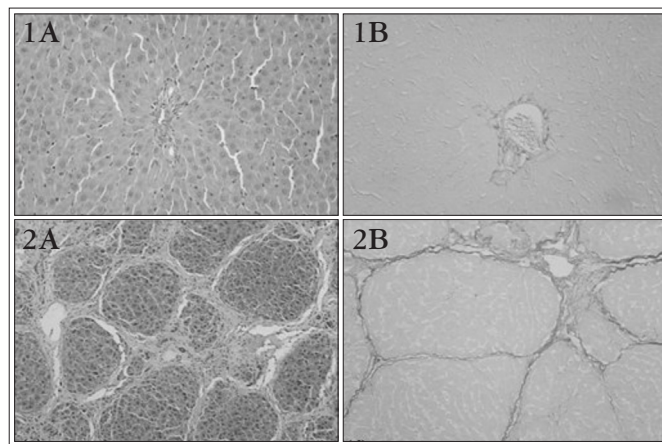


FIGURE 1. Photomicrographs of liver sections from CCl₄-non exposed (1) and cirrhotic rats (2) stained with H-E (A) and picrosirius (B) (100x)

As an indirect estimation of portal hypertension, we compared the relative weights of liver and spleen in both groups. The relative weights of liver of CCl₄-induced cirrhosis animals (mean 4.43 g ± 0.61) were not statistically different from CCl₄-non exposed group (4.47 g ± 0.57) ($P = 0,861$). The relative weights of spleen of CCl₄-induced cirrhosis animals (mean 0.49 g ± 0.11) were significantly greater than CCl₄-non exposed group (mean 0.30 g ± 0.04) ($P < 0.001$).

The histological analysis of liver showed cirrhosis in 21/25 animals (84%) from CCl₄-exposed group, and 4 of them had ascites. All CCl₄-non exposed animals had normal liver histology (Figure 1).

The median (25-75 interquartile range) ⁵¹Cr-EDTA-IP values of CCl₄-induced cirrhosis and CCl₄-non exposed rats were 0.90% (0.63-1.79) and 0.90% (0.60-1.52) respectively, without significant difference ($P = 0.65$). Animals from indomethacin group showed ⁵¹Cr-EDTA-IP significantly higher than CCl₄-induced cirrhosis and CCl₄-non exposed rats (median 7.3% (5.1-14.7)) ($P < 0.001$) (Figure 2).

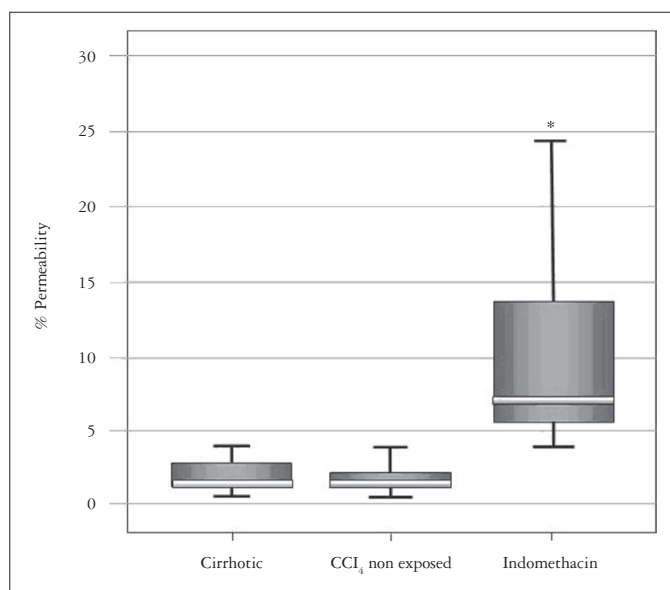


FIGURE 2. Values of percentage of intestinal permeability, presented as median (25-75 interquartile range), to ⁵¹Cr-EDTA in cirrhotic, CCl₄ non-exposed and indomethacin group rats. Intestinal permeability values were significantly higher in indomethacin group (*) than cirrhotic and CCl₄ non-exposed rats (Kruskal-Wallis test, $P < 0.001$)

The histological analysis of small bowel and colon from the CCl₄-induced cirrhosis group showed no abnormalities except for superficial ischemic necrosis in the top of the villi in three rats. No abnormalities were also observed in the CCl₄ non exposed group. Histological analyses of small bowel and colon were performed in rats from indomethacin group. In the indomethacin group, 10 rats showed acute inflammation with paucity of goblet cells, erosions and/or mucosa ulcerations in the small bowel and colon. One of them showed severe transmural ulceration in the small bowel

and the other 3 animals showed no abnormalities, despite increased ⁵¹Cr-EDTA-IP values (Figure 1).

DISCUSSION

In this study, IP was assessed by using ⁵¹Cr-EDTA in male Wistar rats with CCl₄-induced cirrhosis and CCl₄-non exposed group. There was no significant difference in IP values between the two groups. In addition, both groups had significantly lower IP values compared to rats with intestinal inflammation induced by indomethacin.

Disturbances in IP are thought to be involved in BT, and consequently in SBP, a major complication of cirrhosis⁽¹²⁾. It has been described a dilated extra-cellular space between enterocytes and a reduced number of microvilli in cirrhotic patients⁽²⁴⁾. An elongated and straight microvilli in jejunum was seen in animals with compensated cirrhosis, but also short and irregular microvilli in animals with decompensated cirrhosis⁽⁸⁾, that was associated with reduced transport of oligosaccharides and aminoacids. These abnormalities could be due to a deficiency of insulin-like growth factor (IGF-1), an anabolic peptide produced mainly in the liver and known to stimulate gastrointestinal growth and function under normal condition. IGF-1 might display a direct anti-inflammatory activity in the gut that contributes to the protection of IB since a reduction in intestinal TNF- α (tumor necrosis factor alfa) levels was found in IGF-1 treated rats⁽¹⁶⁾. Furthermore, an increased intestinal oxidative stress^(1, 17, 22) was demonstrated, including disturbed enterocyte mitochondrial function and increased lipid peroxidation of brush border membranes⁽¹⁷⁾. Cirrhosis also has been directly linked with elevation of proinflammatory cytokines, nitric oxide and oxidative stress in serum and hepatic tissue⁽¹¹⁾.

The lack of difference in IP values in this present study could be explained by some factors such as: the animals lacked severe cirrhosis represented by ascites, and they did not have a marked intestinal mucosa damage. From all animals with cirrhosis in this study only four had ascites and this might indicate that the majority of animals had less severe cirrhosis although they might had portal hypertension, as suggested by the increased relative weight of spleen. It has been seen that IP depends on severe cirrhosis as patients with decompensated cirrhosis with ascites and/or encephalopathy had shown differences in IP when compared to patients without decompensated cirrhosis and controls^(18, 24, 26).

Histological analysis of small intestine and colon showed no marked abnormalities in cirrhotic rats, except for superficial ischemic necrosis on the top of villi seen in three animals. This is in accordance to other studies that have shown mild abnormalities in cirrhotic animals seen by electronic microscopy^(22, 24). In this study, intestinal inflammation was induced by indomethacin in order to have a positive control for ⁵¹Cr-EDTA permeability. Intestinal inflammation can disrupt intestinal mucosa integrity in a way that can be detected

by ⁵¹Cr-EDTA-IP, as has already been assessed in intestinal disorders with gross mucosal abnormalities⁽¹⁹⁾.

Comparing IP results in patients with and without cirrhosis, controversial results were found, ranging from negative results to higher IP values in the ascites group than controls and cirrhotic without ascites⁽¹⁵⁾. Regarding IP studies in animals, we are not aware of other studies with ⁵¹Cr-EDTA in the literature. However, two studies used ^{99m}Tc-DTPA to evaluate IP, which was increased in rats with cirrhosis and ascites compared to controls^(20, 25). This difference of IP between ⁵¹Cr-EDTA and ^{99m}Tc-DTPA is intriguing as theoretically both markers share physicochemical properties and use the same permeation route⁽⁶⁾.

Comparing all the different IP probes, lactulose-mannitol and lactulose-rhamnose seemed to be the most sensitive in evaluating IP. This fact could be expected as those probes can evaluate the common permeation route through tight junctions plus a minor route. Mannitol passes through the intestinal villi by both the transcellular and paracellular pathways. In contrast, lactulose passes across the larger pores located at the tight junctions, between crypt cells⁽⁷⁾, following the same route as ⁵¹Cr-EDTA and ^{99m}Tc-DTPA⁽⁶⁾.

Although the high sensitivity of lactulose-mannitol and lactulose-rhamnose in detecting IP, its real role on assessment of BT has been discussed. An increased IP to those probes may not necessarily mean that BT would also be increased since the bacteria have a much larger dimension than lactulose⁽²⁶⁾. Even though the use of two different probes can be more accurate to assess IP than just one, this study with ⁵¹Cr-EDTA is the first to evaluate a simpler, low-cost method to assess IP in cirrhotic rats comparing with a positive control of intestinal damage induced by indomethacin.

In conclusion, we showed a lack of difference between ⁵¹Cr-EDTA-intestinal permeability in rats with and without cirrhosis. Further studies are necessary to better clarify the relationship between intestinal permeation and cirrhosis.

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Ramos ARL, Matte U, Goldani HAS, Oliveira OLM, Vieira SMG, Silveira TR. Permeabilidade intestinal ao ⁵¹Cr-EDTA em ratos com cirrose induzida por CCl₄. Arq Gastroenterol. 2010;42(2):188-92.

RESUMO - Contexto - A relação direta entre cirrose e alterações na barreira intestinal ainda não foi devidamente esclarecida. **Objetivo** - Verificar a permeabilidade intestinal ao ⁵¹Cr-EDTA em ratos com cirrose induzida por tetracloreto de carbono (CCl₄) e controles. **Método** - Cinquenta ratos Wistar machos pesando 150-180 g foram separados em três grupos: 25 animais receberam CCl₄ 0,25 mL/kg diluído em óleo de oliva por gavagem com restrição dietética de 12 g/rato/dia por 10 semanas (grupo cirrose induzida por CCl₄); 12 receberam a mesma restrição dietética por 10 semanas (grupo não exposto ao CCl₄). Outros 13 ratos receberam indometacina 15 mg/kg por gavagem como controle positivo de inflamação intestinal. **Resultados** - A mediana (intervalo interquartil 25-75) dos valores de permeabilidade intestinal ao ⁵¹Cr-EDTA dos grupos cirrose induzida por CCl₄ e não exposto ao CCl₄ foram 0,90% (0,63-1,79) e 0,90% (0,60-1,52), respectivamente, sem significância estatística ($P = 0,65$). Os animais do grupo indometacina apresentaram uma mediana de permeabilidade intestinal ao ⁵¹Cr-EDTA de 7,3% (5,1-14,7), sendo significativamente maior do que os grupos cirrose induzida por CCl₄ e não exposto ao CCl₄ ($P < 0,001$). **Conclusão** - Este estudo não demonstrou diferenças entre a permeabilidade intestinal ao ⁵¹Cr-EDTA em ratos com e sem cirrose. Mais estudos são necessários para melhor esclarecer a relação entre a permeabilidade intestinal e cirrose.

DESCRIPTORES - Absorção intestinal. Ácido edético. Cirrose hepática experimental. Ratos.

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