Rifaximin, but not growth factor 1, reduces brain edema in cirrhotic rats

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Abstract

AIM: To compare rifaximin and insulin-like growth factor (IGF)-1 treatment of hyperammonemia and brain edema in cirrhotic rats with portal occlusion.

METHODS: Rats with CCl₄-induced cirrhosis with ascites plus portal vein occlusion and controls were randomized into six groups: Cirrhosis; Cirrhosis + IGF-1; Cirrhosis + rifaximin; Controls; Controls + IGF-1; and Controls + rifaximin. An oral glutamine-challenge test was performed, and plasma and cerebral ammonia, glucose, bilirubin, transaminases, endotoxemia, brain water content and ileocecal cultures were measured and liver histology was assessed.

RESULTS: Rifaximin treatment significantly reduced bacterial overgrowth and endotoxemia compared with cirrhosis groups, and improved some liver function parameters (bilirubin, alanine aminotransferase and aspartate aminotransferase). These effects were associated with a significant reduction in cerebral water content. Blood and cerebral ammonia levels, and area-under-the-curve values for oral glutamine-challenge tests were similar in rifaximin-treated cirrhotic rats and control group animals. By contrast, IGF-1 administration failed to improve most alterations observed in cirrhosis.

CONCLUSION: By reducing gut bacterial overgrowth, only rifaximin was capable of normalizing plasma and brain ammonia and thereby abolishing low-grade brain edema, alterations associated with hepatic encephalopathy.

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Key words: Hyperammonemia; Low-grade brain edema; Hepatic encephalopathy; Rifaximin; Insulin-like growth factor 1; Cirrhosis

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INTRODUCTION

Hepatic encephalopathy (HE) is a complication of advanced hepatic insufficiency characterized by a wide range of neurological and neuropsychiatric symptoms, ranging from subclinical manifestations to hepatic coma[1]. When cirrhotic patients develop HE, their survival prognosis considerably worsens[2], and liver transplantation has to be considered[3]

It is well known that high plasma ammonia levels play a central role in the multifactorial network of mechanisms leading to HE[4-6]. In fact, ammonia reaches the liver via the portal vein from the intestine as a result of bacterial degradation of nitrogenous compounds and as a consequence of the metabolism of glutamine by the enzyme glutaminase[7]. In addition, urea cycle activity in cirrhotic patients is decreased due to the reduction of liver cell mass[8]. Both the presence of portosystemic shunts and the loss of parenchymal cells in the liver of cirrhotic patients lead to an increase in plasma ammonia levels and are key factors in the development of HE in these patients[9]. A traditional therapeutic approach to HE is to decrease plasma ammonia levels by decreasing ammoniagenic substrates, as well as inhibiting ammonia generation, reducing its intestinal absorption, and facilitating its elimination[10].

Non-absorbable antibiotics and/or non-absorbable disaccharides have been used as a standard treatment of HE in human cirrhosis[10-14]. Recent studies have demonstrated that rifaximin reduces the risk of hospitalization involving HE without producing side effects[15,16]. Rifaximin is a non-absorbable rifamycin derivative with activity against aerobic and anaerobic microorganisms, which are an important source of ammonia[17,18]. Furthermore, rifaximin is not absorbed by the gut, thereby allowing the antibiotic to reach high concentrations in the intestinal tract and to remain in the feces in its active form[19-21].

Insulin-like growth factor (IGF)-1 is a powerful anabolic hormone that exerts anabolic and trophic effects in many tissues, acting in an endocrine, paracrine and autocrine manner[22]. Levels of IGF-1 are markedly decreased in liver cirrhosis. Several studies have shown that the administration of low doses of IGF-1 (i.e., 4 µg/100 g body weight per day) reduces liver fibrosis, improves liver function, increases intestinal absorption of nutrients and corrects osteopenia and hypogonadism in experimental liver cirrhosis[19,20]. Previous work from our laboratory has demonstrated that IGF-1 therapy enhances intestinal barrier function, and reduces endotoxemia and bacterial translocation in cirrhotic rats[23]. Most of these alterations are considered to be precipitating factors leading to HE, therefore, the administration of IGF-1 could be a novel therapeutic approach for this condition.

The aim of this study was to compare the efficacy of rifaximin and IGF-1 in the treatment of HE using a combined model of intrahepatic hypertension (CCl₄-induced cirrhosis plus ascites) and extrahepatic hypertension generated through portal vein occlusion - a proven new animal model of hyperammonemia and brain edema related to decompensated advanced liver cirrhosis, recently described in our laboratory[24], which exhibits most of the alterations present in type C HE.

MATERIALS AND METHODS

Male Sprague-Dawley OFA rats weighing about 100 g were included in the study. All animals were caged individually at a constant room temperature of 21 °C, exposed to a 12/12-h light/dark cycle and provided free access to a standard rodent chow (A04; Harlan Ibérica S.A, Barcelona, Spain). Rats received 1.5 mmol/L phenobarbital, an inducer of cytochrome P450 enzymatic activity, in their drinking water. The study was conducted according to guidelines established by the Guide for the Care and Use of Laboratory Animals and was approved by the Ethical and Research Committee of our research institute.

Experimental design

Six groups of rats were studied. (1) Cirrhosis (group 1; n = 9): rats with CCl₄-induced liver cirrhosis with ascites plus portal vein occlusion treated with placebo (saline); (2) Control (group 2; n = 10): sham-operated control rats treated with placebo; (3) Cirrhosis + IGF-1 (group 3; n = 9): rats with CCl₄-induced liver cirrhosis with ascites plus portal vein occlusion treated with IGF-1 (2 µg/100 g s.c. twice daily for 14 d); (4) Control + IGF-1 (group 4; n = 9): sham-operated control rats treated with IGF-1 (2 µg/100 g s.c. twice daily for 14 d); (5) Cirrhosis + R (group 5; n = 9): rats with CCl₄-induced liver cirrhosis with ascites plus portal vein occlusion treated with rifaximin (50 mg/kg daily by gavage for 14 d); and (6) Control + R (group 6; n = 9): sham-operated control rats treated with rifaximin (50 mg/kg daily for 14 d).

Animal procedures

Ascitic cirrhotic rats with portal occlusion were assessed as previously described[25]. Briefly, when animals reached a body weight of 200 g, cirrhosis was induced by intragastric administration of CCl₄ through an orogastric stainless steel tube (Poper and Sons, New Hyde Park, NY, United States). The initial dose was 20 µL, and subsequent doses were adjusted based on changes in body weight[26]. Six weeks after starting cirrhosis induction, animals underwent partial portal vein occlusion (> 0.9 mm portal diameter) achieved by ligating around a 20 G needle, followed by complete portal vein occlusion 48 h later[27]. Surgical procedures were performed under strict aseptic conditions; animals were anesthetized for surgery using ketamine, diazepam and atropine, and were subsequently administered 30 µg (s.c.) buprenorphine (Buprex; Schering-Plough, Madrid, Spain) for 3 d. Cirrhosis induction was continued (CCl₄ administration) until ascites developed. When ascites was diagnosed (by abdominal paracentesis), animals were randomized to receive the corresponding treatment (placebo, rifaximin or IGF-1) for 14 d. Control rats were subjected to sham operation and were also randomized in parallel. Twelve hours after
finishing treatment, rats underwent an oral glutamine-challenge test, and immediately afterward were sacrificed by bilateral thoracotomy. Peripheral and portal blood, cecum fecal content, and solid tissue (brain and liver) were obtained.

**Oral glutamine-challenge test**
A load of 100 mg/kg of L-glutamine (SHS S.A., Barcelona, Spain) was administered through an orogastric stainless steel tube. Venous blood samples (150 µL) from the femoral vein were drawn preload (baseline) and every 30 min for 4 h for ammonia determination. Body temperature was monitored and maintained between 36 °C and 38 °C using an infrared lamp. Samples were centrifuged in situ for 10 min at 2000 × g, and plasma was stored at -80 °C until analysis. The area under the curve (AUC) of the ammonemia response was also calculated using Graph Pad Prism for Windows version 5.01 (La Jolla CA, United States).

**Biochemical characterization**
Blood samples were obtained during sacrifice. Biochemical determinations [aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and glucose] were made using an autoanalyzer (Dimension Clinical Chemistry System, Dade Behring-Siemens, Madrid, Spain).

**Endotoxin levels**
Endotoxemia was quantified in all groups of rats using a Limulus amebocyte lysate kinetic test (Endosafe Charles River, L’Abresle Cedex, France). Briefly, plasma samples were diluted 1:5 with endotoxin-free water and then heated to 70 °C for 5 min. Afterwards, samples were further diluted (final dilution, 1:50) and assessed.

**Cecal bacterial content**
During the course of laparotomy and after harvesting all other samples, the cecal region was identified and 1 mL of content was obtained by cecal puncture. Cecal bacterial content was measured by culturing serially diluted samples (1/8000 and 1/160 000) on non-selective blood agar plates. All samples were cultured in triplicate. After an incubation period of 48 h, the number of colony-forming units (CFU) was counted. The composition of isolated flora was determined using standard bacteriological identification techniques. The results were expressed as CFU/mL of cecal content. Cecal bacterial overgrowth was defined as a stoal bacterial count greater than the mean of healthy control rats plus two standard deviations[29].

**Determination of plasma and brain ammonia**
Ammonia was measured in plasma and cerebral cortex. Briefly, blood (150 µL) was drawn from the femoral vein and centrifuged in heparinized tubes. The resulting plasma samples were stored at -80 °C until analysis. Brain samples were weighed, homogenized, and deproteinized by adding five volumes of cold perchloric acid (6%) and then centrifugation at 12 000 × g for 20 min. After neutralization with KHCO3 (25% w/v), samples were stored at -80 °C until analysis, which was performed using a commercial enzymatic Ammonia Assay Kit (Sigma-Aldrich, Madrid, Spain).

**Low-grade brain edema**
Low-grade brain edema was measured as brain water content. Briefly, a frontal left hemisphere brain sample from each rat was excised, weighed, and heated to 90 °C for 48 h in a drying oven to evaporate all water content. Then, dried samples were weighed again. The difference between initial and final weight was considered as the water content[29].

**Hepatic histology**
Liver samples for histological examination were collected in 4% formaldehyde, subsequently embedded in paraffin wax, sliced into 5-µm sections, and stained with hematoxylin and eosin. Liver samples were evaluated using the Scheuer scoring system[29].

**Statistical analysis**
Unless otherwise indicated, results are expressed as mean ± SE or proportions, as appropriate. Comparisons of means among groups were performed using one-way analysis of variance or corresponding non-parametric (Kruskal-Wallis) tests; post hoc comparisons to identify pairs of groups significantly different at the 0.05 level were made using the Duncan test or the Mann-Whitney U test, respectively. Differences in proportions among groups were compared using the χ2 test. Statistical analysis were performed with SPSS for Windows version 13.0 (Chicago, IL, United States).

**RESULTS**

**General features**
No differences in any of the parameters studied, except for fecal bacterial count (as expected), were observed among the three sham-operated control groups. In contrast, all parameters were significantly altered in cirrhosis plus portal vein occlusion groups compared to control groups.

**Body weight and ascites development**
Body weight at sacrifice was similar in cirrhotic groups and was significantly lower than in controls (overall P = 0.017). No differences in the time elapsed between the first CCl4 dose and ascites development were observed among the groups (range: 8-15 wk). None of the ascitic rats showed any signs of infection or sepsis.

**Biochemical characterization**
Liver function and liver damage parameters are summarized in Table 1. Liver cirrhosis plus portal vein occlusion resulted in a significant increase in serum AST, ALT and bilirubin, and a decrease in serum glucose con...
centrations. However, in rifaximin-treated cirrhotic rats, these alterations tended to be less marked. In fact, no differences in bilirubin or transaminases were observed in this group compared to controls. By contrast, all of these biochemical parameters remained significantly altered in the IGF-1-treated group compared to control groups, indicating that IGF-1 treatment was unable to improve liver function.

Endotoxin levels
Portal blood endotoxin levels were significantly increased only in placebo-treated cirrhotic rats (0.582 ± 0.069 μg/L vs 0.374 ± 0.037; *P = 0.044). By contrast, both IGF-1 and rifaximin treatments normalized endotoxia levels, producing similar values relative to their respective controls (Table 1).

Blood and brain ammonia levels and oral glutamine-challenge test
Liver cirrhosis plus portal vein occlusion resulted in hyperammonemia. Blood ammonia levels were increased in placebo-treated cirrhotic rats compared to placebo-treated controls (284 ± 29 μmol/L vs 144 ± 47 μmol/L; *P = 0.007). Rifaximin treatment improved ammonemia in cirrhotic rats, reducing ammonia to levels similar to those observed in rifaximin-treated controls (205 ± 47 μmol/L vs 128 ± 37 μmol/L; *P = 0.122). By contrast, IFIG-1 treatment failed to reduce plasma ammonia levels, which remained significantly increased compared to those observed in controls (323 ± 58 μmol/L vs 142 ± 35 μmol/L; *P = 0.004; Figure 1).

Similarly to ammonemia, brain ammonia levels were significantly higher in placebo-treated cirrhotic rats than in placebo-treated controls (0.38 ± 0.03 mmol/kg vs 0.22 ± 0.01 mmol/kg; *P = 0.006). Rifaximin treatment normalized brain ammonia levels in cirrhosis, yielding values similar to those observed in rifaximin-treated control rats (0.27 ± 0.03 mmol/kg vs 0.24 ± 0.03 mmol/kg; *P = 0.429). Again, IFIG-1 treatment was ineffective; brain ammonia levels in IFIG-1-treated cirrhotic rats were significantly higher than those in controls (0.35 ± 0.04 mmol/kg vs 0.25 ± 0.02 mmol/kg; *P = 0.039; Figure 2).

Further analysis of these results (Figure 3) showed that AUCs after oral glutamine-challenge tests were significantly increased in placebo and IFIG-1 treatments in cirrhotic rats compared with each control group (3770 ± 501 vs 2419 ± 501; *P = 0.043 and 5216 ± 1144 vs 2120 ± 428; *P = 0.021). By contrast, the AUC in rifaximin-treated cirrhotic rats was similar to that observed in controls (3310 ± 870 vs 2525 ± 302; *P = 0.240).

Cecal bacterial content
Cecal bacterial content was significantly increased in

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### Table 1 Biochemical features of groups studied

|                | Endotoxin (EU) | Glucose (mmol/L) | Bilirubin (μmol/L) | ALT (UI/L) | AST (UI/L) |
|----------------|----------------|------------------|-------------------|------------|------------|
| Cirrhosis      | 0.582 ± 0.069a | 9.41 ± 2.17b     | 22.0 ± 6.71c      | 181.2 ± 16.1d | 333.2 ± 84.2e |
| Controls       | 0.374 ± 0.037  | 27.63 ± 2.39     | 2.9 ± 0.15        | 68.9 ± 9.9  | 228.3 ± 24.6 |
| Cirrhosis + IGF-1 | 0.432 ± 0.033 | 9.43 ± 0.70    | 10.2 ± 2.96e      | 210.3 ± 58.6f | 482.3 ± 121.8g |
| Controls + IGF-1 | 0.363 ± 0.032 | 25.17 ± 2.42     | 3.2 ± 0.10        | 94.4 ± 22.1 | 179.2 ± 30.1 |
| Cirrhosis + R   | 0.410 ± 0.041  | 17.75 ± 3.06g    | 5.0 ± 1.23        | 121.4 ± 18.4 | 262.1 ± 49.1 |
| Controls + R    | 0.368 ± 0.027  | 29.39 ± 2.08     | 3.1 ± 0.20        | 97.7 ± 23.4 | 236.2 ± 43.2 |

*p < 0.05 vs all control groups; ^P < 0.05 vs cirrhosis + R; &P < 0.05 vs control + IFIG; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

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**Figure 1** Blood ammonia levels. Comparison of the concentrations of basal plasma ammonia in cirrhotic groups (closed bars) and control groups (open bars). Cirrhosis plus portal vein occlusion resulted in a significant increase in basal ammonia levels. Rifaximin-treated cirrhotic rats showed plasma ammonia levels similar to those observed in controls; by contrast, insulin-like growth factor (IGF)-1 treatment was unable to normalize these values. *P < 0.05 vs each control group. Cirrhosis; Cont; Control; R: Rifaximin.

**Figure 2** Brain ammonia levels. Comparison of the concentrations of brain ammonia in cirrhotic groups (closed bars) and control groups (open bars). Liver cirrhosis plus portal vein occlusion resulted in an increase in brain ammonia levels compared to controls, whereas in rifaximin-treated cirrhotic rats, these levels remained similar to those in controls. Insulin-like growth factor (IGF)-1 did not significantly decrease these values compared to the respective controls. *P < 0.05 vs each control group. Cirrhosis; Cont; Control; R: Rifaximin.
Comparison of cecal bacterial content

Low-grade brain edema was determined by measuring the percentage brain water content in cirrhotic groups (closed bars) and control groups (open bars). Placebo-treated cirrhotic rats showed the presence of low-grade brain edema. Brain water content in the cirrhosis + rifaximin group was similar to that observed in cirrhosis + placebo group. P < 0.05 vs all control groups; *P < 0.05 vs cirrhosis + R. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

Both the placebo-treated cirrhotic group and cirrhotic rats treated with IGF-1 compared with their respective controls (18.6 ± 3.6 × 10^6 CFU/mL vs 8.4 ± 1.3 × 10^6 CFU/mL; P = 0.042 and 20.4 ± 4.6 × 10^6 CFU/mL vs 8.9 ± 1.4 × 10^6 CFU/mL; P = 0.047). Only rifaximin treatment reduced bacterial content in cirrhotic rats; the values in rifaximin-treated cirrhotic rats were similar to those observed in the rifaximin-treated control group (4.4 ± 1.4 × 10^6 CFU/mL vs 4.4 ± 1.3 × 10^6 CFU/mL; P = 0.931), and were significantly lower than those in the placebo-treated cirrhosis group (P = 0.003) and cirrhosis + IGF-1 group (P = 0.002). Moreover, as shown in Figure 4, rifaximin eliminated bacterial overgrowth (threshold value, 17.64 ± 10^6 CFU/mL) in cirrhotic rats, whereas almost 50% of rats in the placebo-treated cirrhosis group (4/9 rats) and cirrhosis + IGF-1 group (5/9 rats) showed bacterial overgrowth (overall P < 0.05 vs each control and cirrhosis + rifaximin groups).

**Brain water content**

Lever cirrhosis plus portal vein occlusion resulted in a significant increase in brain water content compared to placebo-treated controls (79.17% ± 0.22% vs 78.26% ± 0.04%; P = 0.002). Rifaximin treatment was accompanied by a significant reduction in low-grade brain edema, as demonstrated by the fact that brain water content in this group was similar to that measured in rifaximin-treated control rats (78.61% ± 0.31% vs 78.24% ± 0.19%; P = 0.233) and significantly lower than that observed in placebo-treated cirrhotic rats (P = 0.046). IGF-1 treatment did not diminish brain edema; brain water content in the cirrhosis + IGF-1 group was similar to that observed in the cirrhosis + placebo group. P < 0.05 vs all control groups; *P < 0.05 vs cirrhosis + R. Cirr: Cirrhosis; Cont: Control; R: Rifaximin.

**Histology**

All ascitic cirrhotic rats with portal vein occlusion developed liver cirrhosis with regeneration nodules, necrosis, and steatosis regardless of treatment received. All CC1-induced animals scored F4 with the Scheuer system. As expected, all control groups showed normal hepatic histology (Figure 6).

**DISCUSSION**

In this study, we demonstrated the effectiveness of rifaximin in normalizing both ammonemia and brain ammonia levels, and consequently averting the appearance of low-grade brain edema in ascitic cirrhotic rats with portal vein occlusion; an experimental model of hyperammonemia related to decompensated advanced cirrhosis. These alterations play a central role in the multifactorial mechanisms leading to HE as a result of chronic liver disease.

Although administration of low doses of IGF-1 has been proposed as a promising therapy for cirrhotic patients on the basis of preclinical data showing that this hormone displays hepatoprotective and antifibrogenic
activities⁷,⁹,¹¹, we observed virtually no positive effect of IGF-1 on most of the alterations that lead to HE in this experimental model.

Given that the major precipitating factor leading to HE in cirrhosis is the presence of large amounts of ammonia, not only in the bloodstream but especially in the brain, and further considering that the main source of this ammonia is production by enteric bacteria, the two key factors that warrant particular attention are: (1) Deregulated function of the liver, which introduces ammonia into the urea cycle and is the main ammonia-detoxifying organ; and (2) The presence of bacterial overgrowth related to disturbed intestinal transit.

In this context, rifaximin treatment was accompanied by a slight, but significant, improvement of some parameters of liver function, such as glucose, bilirubin and ALT. This improvement cannot be mainly attributed to a decrease in endotoxin levels, which is known to promote activation and release of proinflammatory cytokines such as tumor necrosis factor (TNF-α)⁰,¹¹, because these parameters were also diminished in the IGF-1 treated group (group 3) without producing any positive effect on liver function.

Notwithstanding these observations, a recent study has observed a direct effect of norfloxacin, another “non-absorbable” antibiotic widely used for selective intestinal bacterial decontamination, in cirrhotic patients. Norfloxacin actively accumulates in polymorphonuclear cells, leading to a decrease in plasma TNF-α and interferon-γ levels, and a reduction in oxidative stress.⁴¹ Although these mechanisms were not explored in the present study, we cannot rule out the possibility that a similar action of rifaximin could explain our results. Further studies are needed to examine this possibility.

However, we did not observe the hepatoprotective effects of IGF-1 administration in experimental cirrhosis that have been reported by others. In our study, hepatic function (glucose, bilirubin, AST, ALT) in IGF-1 treated cirrhotic rats was similar to that observed in untreated cirrhotic rats. We attribute these differences to the fact that, in our study, all animals presented with well-established cirrhosis plus ascitic decompensation.

As mentioned previously, cirrhotic patients present several alterations in gut motility that could lead to an increase in gut bacterial content.⁴² A close cause and effect relationship between bacterial overgrowth and plasma ammonia levels has been reported, reflecting the fact that enteric bacterial fermentation is the main source...
of ammonia. In our study, cirrhotic groups treated with placebo or IGF-1 (groups 1 and 2) showed a significant increase inecal bacterial content compared with control groups. By contrast, rifaximin treatment dramatically reducedecal bacterial content, not only in cirrhotic rats but also in control rats (groups 3 and 6), as reported in other studies. As a consequence of this reduction in bacterial content, plasma ammonia levels in rifaximin-treated cirrhotic rats (group 3) remained similar to those observed in controls (groups 4-6). Similarly, brain ammonia levels were normalized in rifaximin-treated rats. In keeping with this, low-grade brain edema was absent in this group of rats. Again, consistent with its inability to modify cecal bacterial content, IGF-1 failed to improve any of these parameters.

In conclusion, our data indicate that, by reducing gut bacterial overgrowth and improving liver function, rifaximin may be useful in the treatment of most alterations associated with HE in experimental cirrhosis, whereas the administration of low doses of IGF-1 is not indicated in this condition.

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