Helicobacter pylori infection induces hyperammonaemia in Mongolian gerbils with liver cirrhosis

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Abstract
Background and aims—We previously reported the effect of Helicobacter pylori eradication on hyperammonaemia in patients with liver cirrhosis. However, the role of H pylori as a cause of hyperammonaemia is controversial. We developed an animal model with liver cirrhosis and investigated the effect of H pylori infection on hyperammonaemia.

Materials and methods—Five week old male Mongolian gerbils were inoculated orally with broth culture of H pylori. Forty eight gerbils were divided into four groups. Gerbils not inoculated with H pylori were fed a commercial rodent diet (group A) or a choline deficient diet (group C). Gerbils inoculated with H pylori were fed the commercial rodent diet (group B) or the choline deficient diet (group D). Blood ammonia levels of the femoral vein and portal vein were measured 30 weeks later.

Results—All gerbils fed the choline deficient diet developed liver cirrhosis with fatty metamorphosis. The survival rate of group D was significantly lower than that of the other groups. Systemic and portal blood ammonia levels in group D were significantly higher than those in the other groups.

Conclusions—H pylori infection induces hyperammonaemia in gerbils with liver cirrhosis.

Keywords: Helicobacter pylori; hyperammonaemia; liver cirrhosis; Mongolian gerbil; hepatic encephalopathy; choline deficient diet

Hyperammonaemia in patients with liver cirrhosis must be treated rapidly as it is one of the fatal complications of liver failure. It is thought to be caused by bacterial urease in the large intestine. Since the isolation of Helicobacter pylori from the human stomach, it has generally become accepted that H pylori infection is implicated in the pathogenesis of gastroduodenal diseases. H pylori is also known to produce copious amounts of ammonia because of its strong urease activity, which is many times greater than that of urease positive enterobacteria. Previously, we reported the effect of H pylori eradication on hyperammonaemia in two patients with liver cirrhosis. Subsequently, we found that eradication of H pylori reduced systemic ammonia levels in patients with hyperammonaemia with diffuse H pylori infection in the stomach. Some investigators also reported that H pylori infection was likely to be associated with hyperammonaemia with cirrhosis. However, other workers have questioned whether or not the effect of H pylori eradication therapy on hyperammonaemia is due to eradication of H pylori or to the non-specific effect of antibiotic therapy on ammonia producing gut flora.

The role of H pylori as a cause of hyperammonaemia in patients with cirrhosis is thus controversial. There have been no reports on the effect of long term H pylori infection in animals with liver cirrhosis.

Recently, Hirayama et al reported a technique for inoculation of H pylori into the stomach of Mongolian gerbils. In the gerbil model, H pylori can form long term stable colonies in the stomach, which mimics the situation in humans in diseases such as chronic active gastritis, intestinal metaplasia, gastric ulcer, and gastric cancer. Therefore, using this model, we induced liver cirrhosis by feeding a choline deficient diet to gerbils and investigated the effect of long term H pylori infection on hyperammonaemia associated with liver cirrhosis.

Methods
Fifty eight specific pathogen free, five week old male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Ltd, Fukuoka, Japan) were housed in an air conditioned biohazard room for the purposes of H pylori infection, with a 12 hour light/12 hour dark cycle. Animals were fed either a commercial rodent diet (CE-2; Clea Japan, Tokyo, Japan) or a choline deficient diet (CA-6; Clea Japan) and allowed free access to water.

H pylori (ATCC43504; American Type Culture Collection) was grown in brucella broth containing 10% v/v horse serum for 48 hours at 37°C under microaerobic conditions (15% CO2) and high humidity with shaking (120 rpm). The broth culture (500 μl) of H pylori (4x10^8 CFU/ml) was inoculated orally, using a feeding needle, after the gerbils had been fasted for 24 hours.

Forty eight gerbils were divided into four groups (12 in each group). Gerbils not inoculated with H pylori were fed a commercial rodent diet (group A) or a choline deficient diet (group C). Gerbils inoculated with H pylori were fed the commercial rodent diet (group B) or the choline deficient diet (group D). All gerbils were observed for 30 weeks to investigate...
survival. After this, six gerbils each from groups A, B, and C and the five surviving gerbils from group D were sacrificed under anaesthesia with pentobarbiturate for examination of ammonia levels.

Blood (50 µl) was obtained from the right femoral vein and portal vein, and blood ammonia levels were quantitated using an Amicheckmeter (AA-4120; Kyoto Daichi Kagaku, Kyoto, Japan) which requires only 20 µl of blood. Blood ammonia levels were expressed in µmol/l. If necessary, blood samples were diluted with saline, as previously described. In addition, blood was obtained from the inferior caval vein for blood chemistry, and serum was separated by centrifugation and measured by an automated chemistry analyser (AU-5200; Olympus Japan, Tokyo, Japan). Half of the stomach and liver of the gerbils were fixed in 10% phosphate buffered formalin, embedded in paraffin, and sectioned into 4 µm samples. The stomach was stained with haematoxylin-eosin and Giemsa, and the liver with haematoxylin-eosin and Azan for histopathological examination. The remaining half of the stomach was homogenised with 10 ml of phosphate buffered saline. An aliquot (100 µl) of dilution was cultured in TSA-II agar plate (Nippon Becton Dickinson, Tokyo, Japan) for four days at 37°C under micro-aerobic conditions (O₂ 5%, CO₂ 15%, N₂ 80%).

Fisher’s protected least significance difference was used to test the significance of differences between ammonia levels in the different groups. The log rank test was used to compare survival rates of the different groups. The Student’s t test was used to compare the results of blood chemistry and body weight. A p value <0.05 was considered significant.

Results

All Mongolian gerbils fed the choline deficient diet (groups C and D) developed liver cirrhosis with fatty metamorphosis (fig 1A), while all gerbils fed a commercial rodent diet (groups A and B) had a normal liver (fig 1B). All gerbils in groups C and D had slight ascites and the liver parenchyma was subdivided into small pseudolobules. Hepatic cells contained a large amount of fat and were markedly swollen. The sinusoidal lumen was conspicuously compressed. The histological finding of the liver was considered to be stage IIIIB in all gerbils in groups C and D, according to Hobbauer’s report. There were no significant differences between the histological findings of the liver in groups C and D. There were no cases of hepatocellular carcinoma or other cancers in any group.

The gerbils in groups A and C did not have gastric ulcers or gastritis. In all gerbils in groups B and D, which had been inoculated with H pylori, the oedematous thickening of the mucosa was generated in the antrum and adjacent part of the corpus, and the lamina propria of the antrum showed gastritis with massive mononuclear cells and neutrophil infiltration with superficial erosions. In addition, two animals each in groups B and D had gastric ulcers. H pylori was detectable either by culture or by histological findings in the gastric mucosa of all gerbils in groups B and D; however, it was not detected in gerbils in groups A and C. The number of H pylori colonies in each infected gerbil was approximately 6.0 log CFU/stomach.

All Mongolian gerbils in groups A, B, and C survived for 30 weeks. However, in group D, only five of 12 gerbils survived for 30 weeks. The survival rate was 41.7% in group D (fig 2), which was significantly lower than that of the other groups (**p<0.01).

Ammonia levels in blood from the right femoral vein of groups A, B, C, and D were 105 (29), 128 (17), 136 (16), and 180 (30) µmol/l, respectively (fig 3). Ammonia levels in blood from the right femoral vein of group D were significantly higher than those of the other groups (p<0.01). Blood ammonia levels in group C were significantly higher than those of
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Discussion

In the present study, systemic blood ammonia levels in group C were significantly higher than those in group A and systemic blood ammonia levels in group D were significantly higher than those in the other groups. Portal ammonia levels in group D were significantly higher than those of the other groups. All Mongolian gerbils fed the choline deficient diet (groups C and D) developed liver cirrhosis and there were no significant differences between groups C and D in liver function or pathological results. These findings suggest that H pylori infection produces copious amounts of ammonia in the stomach and causes marked elevation of portal ammonia levels in gerbils with liver cirrhosis. In addition, H pylori influences systemic ammonia levels in gerbils with liver cirrhosis.

A choline deficient diet induced liver cirrhosis with fatty metamorphosis in rats in previous studies. Hartroft initially reported that rats developed pathological fatty cirrhosis if fed a choline deficient diet. Hoffbauer reported that after the choline deficient diet was begun, the evolution from fatty liver to cirrhotic liver could be recognised in the rat liver. Wada et al reported that the mechanism of portal hypertension in this model was similar to that of carbon tetrachloride induced rat cirrhosis and to that of portal hypertension in humans. It was confirmed that a choline deficient diet could also induce liver cirrhosis in gerbils in the present study.

The prognosis of group D was significantly worse than that of group C. During this study, seven of 12 gerbils in group D died. In the liver, histological findings that could be determined in these gerbils showed similar or earlier stage findings, compared with gerbils that survived. In addition, systemic ammonia levels in the dying gerbils were very high and in our previous study most showed more than 500 µmol/l in orbital sinus blood before they died. Also, there were no significant differences between H pylori infected and uninfected gerbils with cirrhosis in terms of liver histological findings and blood chemistry at 16 or 24 weeks in those studies. Therefore, we speculated that the difference in ammonia levels in these two groups became more marked as the duration of receiving the choline deficient diet became longer and the liver damage became more severe. The gerbils that died in group D were considered to have developed hepatic encephalopathy with hyperammonaemia. Hepatic encephalopathy leads to disturbances of consciousness and occasionally to hepatic coma. Furthermore, it may cause death in small animals as they are not treated with conventional therapy used in humans. H pylori infection may also influence the prognosis in gerbils with liver cirrhosis.

Hepatic encephalopathy is precipitated by compounds such as urea which can contribute to elevated ammonia levels frequently present in symptomatic patients. Blood ammonia levels and symptomatology increase as liver function declines. Most ammonia is from the gut where venous ammonia levels greatly exceed systemic levels.
showed that gastric mucosal urease activity was much higher than colonic mucosal urease activity, and most of the gut derived ammonia was generated by luminal or fecal urease, although the quantity of ammonia that moved from the stomach to the colon was not considered in their study. Rappoport and Kern showed that gastric mucosal urease activity of patients with cirrhosis was significantly greater than that of normal subjects. In addition, increased gastric ammonia has been reported to be an important source of systemic ammonia.22

Previous studies on the effect of *H pylori* in patients with hyperammonaemia showed such a role23 while other studies showed no such role.24 In humans, most patients had already been treated to various degrees with conventional therapy—for example, a low protein diet, antibiotics, lactulose, and branched chain enriched amino acid solution. These conventional therapies may prevent elucidation of the role of *H pylori* infection in hyperammonaemia, although such patients need to be treated adequately. Therefore, animal models may be preferable for studying the effect of *H pylori* infection on hyperammonaemia as animals have not been treated previously.25

Previously, we examined the effect of instillation of *H pylori* into the stomach on systemic ammonia levels in rats with cirrhosis induced by carbon tetrachloride.26 The study revealed that ammonia levels in portal and venous blood significantly increased after instillation of 10⁸ bacteria CFU in the stomach of cirrhotic rats, and suggested that the ammonia produced by *H pylori* induced hyperammonaemia when this organism was widely distributed and was present in large numbers in the stomach, particularly in the presence of cirrhosis. The study described the short term effect of instillation of *H pylori* into the rat stomach because *H pylori* cannot form colonies in the stomach.27 In the present study, the effect of long term *H pylori* infection in an animal model of hyperammonaemia was demonstrated.

Kusuhara *et al* reported on eradication therapy for *H pylori* infected gerbils and we also tried eradication therapy, consisting of omeprazole, clarithromycin, and amoxicillin, in another group of *H pylori* infected gerbils with liver cirrhosis. However, most of these gerbils died soon after dosing because of liver failure due to drug induced liver injury, although the dose was smaller than previously reported. The establishment of an appropriate dose of these drugs was difficult for gerbils with liver cirrhosis, although trials of various dosages were performed. Therefore, we could not investigate the effect of *H pylori* eradication in infected gerbils with liver cirrhosis. However, the present study clearly indicated that the urease of *H pylori* as well as intestinal bacterial urease caused elevation of systemic and portal ammonia levels in gerbils with liver cirrhosis. We conclude that *H pylori* infection induces hyperammonaemia in gerbils with liver cirrhosis.