PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

Changes of gut flora and endotoxin in rats with D-galactosamine-induced acute liver failure

Lan-Juan Li, Zhong-Wen Wu, Dang-Sheng Xiao, Ji-Fang Sheng

Lan-Juan Li, Zhong-Wen Wu, Dang-Sheng Xiao, Ji-Fang Sheng, Department of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
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Correspondence to: Dr. Lan-Juan Li, Department of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. ljli@mail.hz.zj.cn
Telephone: +86-571-87709001 **Fax:** +86-571-87236755
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Abstract

AIM: To investigate the changes of gut microflora and endotoxin levels in rats with acute liver failure (ALF) induced by D-galactosamine (GalN).

METHODS: Flora and endotoxin levels in the jejunum, ileum and colon in normal rats (group A) and rats with GalN - induced ALF were determined at 24 h (group B) or 48 h (group C) after GalN injection, as well as the endotoxin level in portal venous blood (PVB) and right ventricle blood (RVB) were determined by chromogenic limulus amoebocyte assay.

RESULTS: Intestinal (jejunum, ileum, colon) *lactobacillus* count was statistically reduced in group B compared with those in group A (3.4±0.3 *vs* 4.9±0.3, 6.1±0.4 *vs* 8.0±0.3, 8.1±0.2 *vs* 9.3±0.2, $P<0.001$, $P<0.001$ and $P<0.001$ respectively) and recovered partially in the group C compared with those in the group B, whereas the count of *Enterobacteriaceae* in the jejunum, ileum and colon in group B was increased markedly compared with those in the group A (5.1±0.3 *vs* 3.6±0.2, 6.9±0.5 *vs* 5.3±0.3, 8.7±0.2 *vs* 7.6±0.1, $P<0.001$, $P<0.05$ and $P<0.05$ respectively) and restored partially in the group C compared with those in the group B. The endotoxin level in ileum was increased in the group B compared with those in the group A (111.3±22.8 *vs* 51.5±8.9, $P<0.05$). In addition, the endotoxin level in PVB was obviously increased in group B compared with that in the group A (76.8±9.1 *vs* 40.6±7.3, $P<0.01$) and reduced to the baseline at 48 h (group C).

CONCLUSION: Severely disturbed gut flora in rats with GalN-induced acute liver failure plays an important role in the elevation of endotoxin level in PVB.

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INTRODUCTION

Patients with acute liver failure are prone to occurrence of endotoxemia[1], which is usually associated with Gram-negative infection. However, previous studies have showed that some patients with acute liver failure have a high level of endotoxin without clinical evidences of Gram-negative bacterial infection[1,2]. Recent studies have proposed that the elevation of plasma endotoxin is related to the translocation of endotoxin from the gut[3,4].

Gut contains numerous endotoxin, about 90% of which is released by aerobic Gram-negative bacteria, especially the family of enterobacteriaceae[5]. Normally, intestinal anaerobic flora such as *Bifidobacterium* and *Bacteriodes* can prevent the adherence of potential pathogenic enteric bacilli and limit bacterial overgrowth by occupying the space closest to intestinal epithelial cells[6], which is called microbial colonization resistance[7]. Disrupted gut flora observed in severely disease, such as hepatic cirrhosis and hemorrhagic shock, resulted in decrease of microbial colonization resistance and subsequent bacterial overgrowth[8,9]. Wang *et al*.[10] showed the *Escherichia coli* overgrowth in the distal small intestine from 1 h onward after hepatectomy. Researchers suggested that impaired components of the gut barrier, including normal intestinal microflora, could translocate both endotoxin and bacteria from lumen to circulation or other distant organs[6]. Moreover, manipulating gut contents with lactulose or neomycin sulfate with cefazolin could reduce the level of endotoxemia and enhance the survival of rat receiving partial hepatectomy[11]. But to the author's knowledge, the relationship between the changes of gut flora and the fluctuations of the endotoxin levels both in intestine and plasma in rats with GalN-induced acute liver failure has not been reported. In the present study, the changes of gut flora and endotoxin level in the jejunum, ileum and colon, as well as the levels of endotoxin in PVB and RVB of rats with GalN-induced ALF were estimated at various time points.

MATERIALS AND METHODS

Animals and treatment

Male Sprague-Dawley rats weighing about 200-300 g provided by Zhejiang Academy of Medical Sciences, Hangzhou, China, were acclimated to the animal laboratory for 5 d before experiments. They were fed with standard rat chow and water *ad libitum*. All procedures were approved by the Institutional Review Board according to the Animal Protection Act of China.

Acute liver failure in the rats was induced according to the protocol described previously[12]. Briefly, GalN (Chongqing Medical University, Chongqing, China) was dissolved in 0.5 mL of saline and adjusted to pH 6.8 with 1 mol/L NaOH. Then, 20 rats were intraperitoneally given 1.4 g/kg GalN twice at a 12-h interval, and fed with food and water after injection. Since the highest mortality of GalN-induced acute liver failure in rat is between 24 h and 48 h after drug administration[12], and we ensure that the number of survival rat is more than 9[13] at various time points after GalN administration, 40 rats were randomly divided into 3 groups according to the reported mortality[12]: group A with 10 rats without administration of GalN was chosen as normal control, group B with 12 rats that were sacrificed 24 h after GalN injection and group C with 18 rats that were killed at

48 h after injection. Under light ether anesthesia and aseptic conditions, plasma was separated from the portal vein and right ventricle at various time points after laparotomy, then stored immediately at –80 ℃ for analysis of endotoxin. The rats were killed by anaesthetic overdose after blood sampling, the segments of the jejunum, ileum and colon were removed as described by Wang *et al*[10]. Briefly, the segments of the jejunum (5 cm in length, including intestinal wall) and ileum including intestinal wall 5 cm long as well as colon contents were immediately collected from the proximal intestine (5 cm distal of the ligament of Treitz), distal small intestine (5 cm proximal to the ileocecal valve), and descending colon, respectively. After weighting, the samples were placed in pyrogen-free saline (1:9 w/v) in an anaerobic chamber (Forma Scientific Co, USA), mixed by a vortex mixer, and aliquots of 1 mL of mixture were subsequently put into anaerobic solution A (1:9 v/v, decimal dilutions up to 10^{-8}). The remnant was kept at -80 ℃ for detecting endotoxin.

Gut flora analysis

The specimens were cultured within 30 min after collection by modified Mitsuoka's method [14]. A total volume of 50 μL of the serial dilution (10^{-1}, 10^{-3}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) was spread on 3 non-selective agar media: glucose blood liver (BL) (Nissui Pharmcy Co., Tokyo, Japan) agar with 60 mL/L defibrinated sheep blood for all lactic acid-producing bacteria; Eggerth Gagnon (EG) (Nissui Pharmcy Co.) agar with 60 mL/L defibrinated sheep blood for most obligate and facultative anaerobes; and trypticase soy (TS) agar (BioMerieux, Paris, France) with 60 mL/L defibrinated sheep blood for all aerobes and facultative anaerobes, and 4 selective agar media: neomycin sulfate-brilliant green-taurocholate-blood (NBGT) agar prepared with EG agar at our laboratory for members of the family *Bacteriodaceae*; MRS with vancomycin and bromocresol green (LAMVAB) medium [15] for *Lactobacillus sp*.; eosin methylene blue (EMB) agar (Hangzhou microbiological Co., Hangzhou, China) for members of the family of *Enterobacteriaceae*; and *Enterococcus* (Ec) agar prepared at our laboratory for *Enteroococcus sp*. The plates for the recovery of obligate anaerobes were incubated in an anaerobic chamber (N_2:CO_2:H_2=8:1:1) at 37 ℃ for 48-72 h. The media used for the isolation of aerobes and facultative species were incubated in air for 48 h at 37 ℃. After incubation, morphologically distinct colonies were enumerated, isolated and identified. Identification was performed in most cases at family or genus levels using standard bacteriologic techniques [16]. In our study, the lowest detection limit was 2×10^2 organisms per gram of the samples. The results were expressed as the log10 of the number of bacteria per gram weight of the samples.

Chromogenic limulus amoebocyte assay for detecting endotoxin

Intestinal specimens were prepared for endotoxin detection according to the described method[5]. The frozen specimens were thawed at room temperature. Limulus quantitative azo color (LQAC) test[17] with limulus lysate reagent (Eihua Medical Co, Shanghai, China) was used.

Statistical analysis

Data were expressed as the mean±SE. One-way ANOVA was used to compare gut flora data among individual groups. Frequency data were compared using Chi-square test or the Fisher's exact test when necessary. $P<0.05$ was considered statistically significant.

RESULTS

Degree of hepatic injury and mortality

To study the alterations in intestinal microflora and its relationship with plasma endotoxin, ALF model of rat was successfully established by GalN administration as mentioned previously[12]. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bile acid (TBA) were increased significantly at 24 h and gradually decreased at 48 h, whereas those of alkaline phosphatase (AKP) and total bilirubin (TBiL) were increased continuously after GalN injection (Table 1). At the same time, the levels of serum total protein (TP) and albumin at 24 h after GalN administration were lowered markedly compared with those in control group ($P<0.01$, respectively) (Table 1), and increased at 48 h without significance compared with those at 24 h. The mortality rate of group C was higher than group B ($P<0.01$) (Table1). The general conditions, such as activity and appetite, in survivors were restored partially after 48 h.

Table 1 Degree of hepatic injury and mortality in ALF rats at various time points after injection of GalN

	Group A (n=10)	Group B (n=11)	Group C (n=10)
TP (g/L)	62.6±1.3	48.9±1.2[d]	51.0±3.1[d]
Albumin (g/L)	34.3±0.7	27.8±0.7[d]	30.3±1.1[d]
Globulin (g/L)	28.6±0.8	21.1±1.1[d]	21.5±2.6[d]
ALT (U/L)	60.6±3.3	4 798±1 114[d]	3 183±1 257[d]
AST (U/L)	138.0±7.5	5 032±1 067[d]	2 928±843[d]
AKP (U/L)	272.0±18.6	639±60[d]	747±133[d]
TBA (μmol/L)	11.8±2.6	425±33[d]	262±58[da]
TBiL(μmol/L)	7.0±0.4	40.6±8.2[d]	63.2±18.4[d]
Mortality	NO	8.33% (1/12)	44.44% (8/18)[b]

Values are expressed as mean±SE or frequency of occurrence (%). [a]$P<0.05$, [b]$P<0.01$ *vs* group B, [d]$P<0.01$ *vs* group A.

Table 2 Alterations in gut flora in rats with ALF at various time points after GalN administration

Flora	Group A (n=10)	Group B (n=11)	Group C (n=10)
Bacteriodaceae			
Jejunum	ND	ND	ND
Ileum	4.0±0.3	5.0±0.5	4.6±0.7
Colon	9.3±0.3	9.5±0.3	9.7±0.4
Lactobacillus			
Jejunum	4.9±0.3	3.4±0.3[d]	4.8±0.4[a]
Ileum	8.0±0.3	6.1±0.4[f]	6.2±0.3[f]
Colon	9.3±0.2	8.1±0.2[f]	8.5±0.2[d]
Enterobacteriaceae			
Jejunum	3.6±0.2	5.1±0.3[f]	4.3±0.3[b]
Ileum	5.3±0.3	6.9±0.5[c]	6.3±0.5
Colon	7.6±0.1	8.7±0.2[c]	7.9±0.3[a]
Enterococcus			
Jejunum	3.8±0.2	3.9±0.2	3.6±0.2
Ileum	4.9±0.3	5.8±0.3	5.2±0.5
Colon	6.8±0.2	5.8±0.4[c]	6.7±0.3

Data are expressed as mean±SE. ND: Not detected; [a]$P<0.05$, [b]$P<0.01$ *vs* group B, [c]$P<0.05$, [d]$P<0.01$, [f]$P<0.001$ *vs* group A.

Intestinal microflora analysis

To explore the variability in intestinal flora under ALF condition, we analyzed the microbiota from different intestinal segments including jejunum, ileum and colon of the rats at different time points. The count of *Enterobacteriaceae* in the jejunum, ileum and colon was significantly higher in the group A than that in control group ($P<0.001$, $P<0.05$, and $P<0.05$, respectively), whereas the count of *Lactobacillus* in the jejunum, ileum and colon, and that of *Enterococcus* in colon were greatly decreased

(P<0.01, P<0.001, P<0.001, and P<0.05, respectively) (Table 2). The count of *Enterobacteriaceae* in the jejunum and colon was lower in the group C than in the group B (P<0.01 and P<0.05, respectively), but the count of *Lactobacillus* in the jejunum in the group C was significantly elevated (P<0.05) (Table 2). The count of *Lactobacillus* in the ileum and colon in the group C was decreased more evidently than that in the group A (P<0.001 and P<0.01, respectively). These results indicated that the disturbed gut flora existed in rat with ALF, and the extent of changes in flora was correlated with the severity of liver injury.

Changes of endotoxin in intestine, portal venous and right ventricle blood

Gut endotoxin is usually translocated into the portal vein. To determine the relations of the levels of endotoxin in blood and in gut in rats with ALF, we measured the endotoxin levels of the intestine, PVB and RVB at various time points after GalN by LQAC test (Table 3). The levels of endotoxin in the ileum and PVB in group B after administration of GalN were increased more significantly than those in the group A (P<0.05 and P<0.01, respectively). At the same time, the level of endotoxin in the colon was increased, but not significantly. The levels of endotoxin in the ileum and colon in the group C were increased more significantly than those in the group B, and higher than those in the group A (P<0.01, respectively). The level of endotoxin in PVB in the group C was decreased significantly compared with those in the group B (P<0.05). Although there was an increase of endotoxin level in RVB after GalN, no statistically significant difference was observed.

Table 3 Levels of endotoxin in intestine, PVB and RVB in rats with ALF at different time points after injection of GalN

	Group A (n=10)	Group B (n=11)	Group C (n=10)
Jejunum (ng/g)	67.8±13.0	56.7±18.6	88.1±15.2
Ileum (ng/g)	51.5±8.9	111.3±22.8[a]	146.7±27.0[b]
Colon (ng/g)	1 022±179	1 841±363	2 444±349[b]
PVB (ng/L)	40.6±7.3	76.8±9.1[b]	45.0±5.3[c]
RVB (ng/L)	34.9±6.0	37.5±12.3	37.1±6.6

Data are expressed as mean±SE. [a]P<0.05, [b]P<0.01 *vs* group A; [c]P<0.05 *vs* group B. PVB: Portal venous blood; RVB: Right ventricle blood.

DISCUSSION

The present study showed that the count of *Enterobacteriaceae* at 24 h after injection of GalN was significantly increased, whereas that of *Lactobacillus* was markedly lowered. The whole intestinal microflora trended to recover at 48 h after GalN administration. These results indicated that there were intestinal microbial disturbance and overgrowth of *Enterobacteriaceae* in the rats with ALF. The tendency of changes in flora from the jejunum and ileum was consistent with that from the colon, and the extent of imbalance of intestinal microflora was correlated with the severity of liver injury. The alterations of intestinal bacterial flora might be due to the diminished bile secretion and the impaired intestinal motility, which were usually observed in rats with acute liver failure[18]. But the exact mechanisms need further study.

The level of endotoxin in the portal vein reflects the extent of translocation of endotoxin from gut[19]. Our findings showed that the levels of endotoxin in the ileum and colon increased continuously after administration of GalN, and the level of endotoxin in the portal vein was significantly elevated at 24 h, but lowered to the baseline value at 48 h. Interestingly, the elevation of the endotoxin in PVB was accompanied by the imbalance of intestinal flora and the overgrowth of *Enterobacteriaceae*, whereas a decrease of the level of endotoxin in PVB was paralleled with the partial restoration of disturbed flora. The present findings showed that an increase of endotoxin in PVB was closely related to the imbalance of intestinal flora in rats with ALF, and implied that ecological imbalance and bacterial overgrowth in ALF rats might play important roles in dysfunction of gut barrier, which could lead to translocation of endotoxin from gut. Our findings are supported by the previous study[20] that gut bacterial overgrowth is one of the etiological factors of bacteria and endotoxin translocation from gut. Moreover, it has been reported that an increase in gut permeability is related to histamine released by intestinal mast cell in rat with GalN-induced acute liver injury[21]. In addition, effect of bacterial proteases on microvillus membrane proteins contributes to the breakdown of the intestinal barrier[22]. Another reason that should be considered is that edema of intestinal wall resulting from digestive congestion and hypoproteinemia in ALF can injure the function of gut barrier[23]. In the present study, the levels of endotoxin in intestine increased continuously after GalN administration; this might be associated with the *Enterobacteriaceae* overgrowth[5]. Nevertheless, our findings indicated that enlargement of gut endotoxin pool had no impact on endotoxin translocation. In addition, the present data suggested that restoration of the disturbed gut flora might play an important role in inhibiting the endotoxin translocation from gut.

The present data showed there was no significant change of the concentrations of endotoxin in RVB after GalN administration, which was in agreement with Nakao *et al.*[24]. In contrast, the results by van Leeuwen *et al.*[11] showed that arterial plasma endotoxin levels increased in rats with hepatic failure induced by two-third partial hepatectomy. The discrepancy between two types of ALF seemed that the count of Kupffer cell in the latter reduced significantly because of hepatectomy. Plasma endotoxin is removed primarily by the Kupffer cells. Then, GalN exerted a minor effect on the function of kupffer cells, because the nucleotide contents of Kupffer cells were smaller than that those of hepatocytes[25]. Logically, Kupffer cells in rats after GalN administration can phagocytose the gut-derived endotoxin.

At present, we could not measure the pretreatment effect of probiotics on endotoxin translocation for lack of *Lactobacillus* preparation suitable for rats. However, it was demonstrated that administration of the probiotics *Lactobacillus plantarum* could reduce the circulating antibody to endotoxin in patients with ulcerative colitis[26]. Adawi *et al.*[27] showed that administration of *Lactobacillus* could reduce bacterial translocation and hepatocellular damage in rats with acute liver injury. Moreover, our previous work demonstrated that administration of probiotics *Bifidobacterium* (DM8504) could decrease significantly the levels of plasma endotoxin in patients with chronic hepatitis B[28].

In conclusion, severely disturbed intestinal flora disorders are observed in rats with GalN-induced acute liver failure, and related to the extent of injury of liver. Ecological imbalance of intestinal flora is one of the main causes of the translocation of endotoxin from lumen. Additionally, the present study implies that modulation of the intestinal flora using probiotics may be the optional treatment in preventing gut endotoxin translocation in patients with ALF.

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