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

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 (jejenum, 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 jejenum, 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<0.005 and P<0.005 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.

Li L, Wu ZW, Xiao DS, Sheng JF. Changes of gut flora and endotoxin in rats with D-galactosamine-induced acute liver failure. World J Gastroenterol 2004; 10(14): 2087-2090 http://www.wjgnet.com/1007-9327/10/2087.asp
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 °C 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 v/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). The frozen specimens were cultured within 30 min after collection by modified Mitsuoka’s method[16]. A total volume of 50 µL of the serial dilution (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶) was spread on 3 non-selective agar media: glucose blood liver (BL) (Nissui Pharmacy Co., Tokyo, Japan) agar with 60 mL/L defibrinated sheep blood for all aerobes and facultative anaerobes; Eggerth Gagnon (EG) (Nissui Pharmacy 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 soy (TS) agar (BioMerieux, Paris, France) with 60 mL/L defibrinated sheep blood for all lactic acid-producing bacteria; Eggerth Gagnon (EG) (Nissui Pharmacy 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-taurolactocholate-blood (NBGT) agar prepared with EG agar at our laboratory for members of the family Bacteriodaeae; 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 Enterococcus sp. The plates for the recovery of obligate anaerobes were incubated in an anaerobic chamber (N₂:CO₂:H₂=8:1:1) at 37 °C for 48-72 h. The media used for the isolation of aerobes and facultative species were incubated in air for 48 h at 37 °C. 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 2x10⁸ 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[3]. 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[21]. 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) (Table 1). 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**

| Flora       | Group A (n=10) | Group B (n=11) | Group C (n=10) |
|-------------|----------------|----------------|----------------|
| TP (g/L)    | 62.6±1.3       | 48.9±1.2       | 51.0±3.1       |
| Albumin (g/L) | 34.3±0.7     | 27.8±0.7       | 30.3±1.1       |
| Globulin (g/L) | 28.6±0.8     | 21.1±1.1       | 21.5±2.6       |
| ALT (U/L)   | 60.6±3.3       | 479±114        | 3.183±257      |
| AST (U/L)   | 138.0±7.5      | 5.032±067      | 2.928±843      |
| AKP (U/L)   | 272.0±18.6     | 639±60         | 747±133        |
| TBA (µmol/L)| 11.8±2.6       | 425±32         | 262±58         |
| TBiL (µmol/L)| 7.0±0.4      | 40.6±8.2       | 63.2±18.4     |

Mortality NO 8.33% (1/12) 44.44% (8/18)

Values are expressed as mean±SE or frequency of occurrence (%). *P <0.05, **P <0.01 vs group B, ***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) |
|-------------|----------------|----------------|----------------|
| Bacteriodaeae |                 |                |                |
| 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        | 4.8±0.4        |
| Ileum       | 8.0±0.3        | 6.1±0.4        | 6.2±0.3        |
| Colon       | 9.3±0.2        | 8.1±0.2        | 8.5±0.2        |
| Enterobacteriaceae |         |                |                |
| Jejunum     | 3.6±0.2        | 5.1±0.3        | 4.3±0.3        |
| Ileum       | 5.3±0.3        | 6.9±0.5        | 6.3±0.5        |
| Colon       | 7.6±0.1        | 8.7±0.2        | 7.9±0.3        |
| 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        | 6.7±0.3        |

Data are expressed as mean±SE. ND: Not detected; *P <0.05, **P <0.01 vs group B, ***P <0.01 vs group A.
(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     | 146.7±27.0    |
| Colon (ng/g)   | 1.02±179      | 1.84±163       | 2.44±349      |
| PVB (ng/L)     | 40.6±3.7      | 76.8±9.1       | 45.0±5.3      |
| RVB (ng/L)     | 34.9±6.0      | 37.5±12.3      | 37.1±6.6      |

Data are expressed as mean±SE. *P*<0.05, *P*<0.01 vs group A; *P*<0.05 vs group B, PVB: Portal venous blood; RVB: Right ven- tricle 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[20]. But the exact mechanisms need further study.

The level of endotoxin in the portal vein reflects the extent of translocation of endotoxin from gut[20]. 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[24]. 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.[21] 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 hepatocytosis. Plasma endotoxin is removed primarily by the Kupffer cells. Then, GalN exerted a minor effect on the function of kuffer 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.

**ACKNOWLEDGEMENTS**

We thank Dr. MH Wang (University of Colorado School of Medicine, USA) for revision of the manuscript.

**REFERENCES**

1 Rolando N, Philpott-woward J, Williams R. Bacterial and fun-
Hartemink R, Rombouts FM. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J Microbiol Methods 1999; 36: 181-192

Benno Y, Endo K, Mizutani T, Namba Y, Komori T, Mitsuka T. Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. Appl Environ Microbiol 1989; 55: 1100-1105

Li LJ, Shen ZJ, Lu YL, Fu SZ. The value of endotoxin concentrations in expressed prostatic secretions for the diagnosis and classification of chronic prostatitis. BJU Int 2001; 88: 536-539

Wang XD, Soltesz V, Andersson R. Cisapride prevents enteric bacterial overgrowth and translocation by improvement of intestinal motility in rats with acute liver failure. Eur Surg Res 1996; 28: 402-412

Deitch EA. Bacterial translocation or lymphatic drainage of toxic products from the gut: what is important in human beings? Surgery 2002; 131: 241-244

Barber AE, Jones WG 2nd, Minei JP, Fahey TJ 3rd, Lowry SF, Shires GT. Bacterial overgrowth and intestinal atrophy in the etiology of gut barrier failure in the rat. Am J Surg 1991; 161: 300-304

Stachiewitz RF, Seabra V, Bradford B, Bradham CA, Rusyn I, Germolec D, Thurman RG. Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. Hepatology 1999; 29: 737-745

Riepe SP, Goldstein J, Alpers DH. Effect of secreted Bacteroides proteases on human intestinal brush border hydrolases. J Clin Invest 1980; 66: 314-322

Hashimoto N, Ohyanagi H. Effect of acute portal hypertension on gut mucosa. Hepatogastroenterology 2002; 49: 1567-1570

Nakao A, Taki S, Yasui M, Kimura Y, Nonami T, Harada A, Takagi H. The fate of intravenously injected endotoxin in normal rats and in rats with liver failure. Hepatology 1994; 19: 1251-1256

Hofmann F, Wagle SR, Decker K. Effect of d-galactosamine administration on nucleotide and protein metabolism in isolated rat Kupffer cells. Heppey Selys Z Physiol Chem 1976; 357: 1395-1400

Garcia-Lafuente A, Antolin M, Guarner F, Crespo E, Malagelada JR. Modulation of colonic barrier function by the composition of the commensal flora in the rat. Gut 2001; 48: 503-507

Adawi D, Ahrens S, Molin G. Effects of different porbiotic strains of Lactobacillus and Bifidobacterium on bacterial translocation and liver injury in an acute liver injury model. Int J Food Microbiol 2001; 70: 213-220

Li LJ, Sheng JF. Effect of probiotics bifidobacterium (DM8504) on the plasma endotoxin in patients with chronic hepatitis B. Chin J Microbiol 1996; 8: 20-22

Edited by Kumar M and Chen WW Proofread by Xu FM