Original Article Intestinal mucosal barrier dysfunction participates in the progress of nonalcoholic fatty liver disease

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Abstract: Intestinal mucosal barrier dysfunction is closely related to liver diseases, which implies impaired gutliver axis may play a role in the pathogenesis of NAFLD. In our study, rats were divided into three groups: normal chow diet (NCD) group, high-fat diet (HFD) group and TNBS-induced colitis with high-fat diet (C-HFD) group. Liver tissues were obtained for histological observation and TNF- α , IL-6 mRNA determination and blood samples were collected for liver enzymes and LPS analysis. Ultrastructural changes of jejuna epithelium, SIBO and amounts of CD103⁺MHCII⁺DCs and CD4⁺CD25⁺FoxP3⁺T-regs in terms of percentage in mesenteric lymph nodes (MLN) were observed by electron microscope, bacterial cultivation and flow cytometry, respectively. The results demonstrated the pathological characteristics accorded with nonalcoholic simple fatty liver (NAFL) and NASH in HFD group by week 8 and 12, respectively. Besides, the degree of hepatic steatosis and steatohepatitis was more severe in C-HFD group compared with HFD-group at the same time point. NAFLD activity score (NAS), liver enzymes, concentration of LPS and mRNA expressions of TNF- α , IL-6 were higher significantly in C-HFD group compared with HFD and NCD group at week 4, 8 and 12, respectively. In HFD group, epithelium microvilli atrophy, disruptive tight junctions and SIBO were present, and these changes were more severe in NASH compared with NAFL. The percentage of CD103⁺MHCII⁺DCs and CD4⁺CD25⁺FoxP3⁺T-regs decreased significantly in NAFL and NASH compared with NCD group. Our conclusion was that gut-liver axis was impaired in NAFLD, which played crucial role in the pathogenesis of NAFLD.

Keywords: Nonalcoholic fatty liver disease, intestinal mucosal barrier, TNF-α, IL-6, dendritic cell, regulatory T cell

Introduction

NAFLD is a chronic liver disorder and hepatic manifestation of metabolic syndrome that is increasing in prevalence with the worldwide epidemic of obesity. It refers to a wide spectrum of fatty degenerative disorders of the liver in the absence of alcohol intake, which describes a spectrum of liver pathology ranging from nonalcoholic simple fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) and even cirrhosis or hepatocellular carcinoma (HCC) [1].

NAFLD is a multifactorial disease that is related with insulin resistance (IR) and genetic predisposition, and a double-hit hypothesis has been proposed, where the first hit includes lipid accumulation in the liver, followed by a second hit in which pro-inflammatory mediators induce inflammation, hepatocellular injury and fibrosis [2]. But the underlying molecular mechanisms are incompletely understood. Nowadays, a multiple parallel hits hypothesis including genes, lipotoxic agents, endoplasmic reticulum stress, role of oxidative stress, inflammation and adipokines as well as gut-liver axis has also been suggested [3, 4]. Among these factors, interactions between intestinal mucosal barrier and liver, also named as gut-liver axis, has been widely investigated in the pathogenesis of NAFLD [5].

Given that the liver and gut are connected by the portal vein, it makes the liver more vulnerable to translocation of bacteria, bacterial products, LPS and inflammatory mediators. In normal physiological conditions, intestinal mucosal barrier builds up the first defensive

line between organism and exogenous substances, and the liver contributes the second line of defense to those antigens and inflammatory factors which escaped from gut mucosal immune surveillance [6]. However, in some pathological conditions, disruption of these defense mechanisms can induced bacterial translocation to extra-intestinal space and aberrant activation of immune system, which can trigger inflammations and damages in the liver [7]. In addition, interactions between the gut and liver are bidirectional, which means liver-derived inflammatory cytokines can further injure the intestinal mucosal barrier function by disruption of gut epithelial tight junctions and so on [8].

The theory of gut-liver axis makes the gut and liver which were previously considered disparate associate with each other. In our study, NAFLD models with intestinal mucosal barrier dysfunction were established to investigate whether or not the gut barrier dysfunction promoted the development of NAFLD, and then, experiments were carried on to demonstrate whether or not gut barrier dysfunction was involved in the process of NAFLD. We preliminarily explored the relationships between intestinal mucosal barrier and NAFLD, and illustrated impaired gut-liver axis played an important role in the pathogenesis of the disease.

Therefore, understanding the gut-liver axis and the signaling pathway may provide new targets and strategies for the prevention and treatment of NAFLD.

Materials and methods

Animals

Healthy male Wistar rats weighing about 180 g to 220 g were supplied by the Specific Pathogen Free (SPF) laboratory animal center of Dalian Medical University. Rats were randomly divided into three groups: normal chow diet (NCD) group, high-fat diet (HFD) group and TNBS (Sigma)-induced colitis with high-fat diet (C-HFD) group. Rats in NCD and HFD group were fed with ordinary diet and a HFD consisting of 88 g normal diet, 10 g lard oil, and 2 g cholesterol high-fat respectively. In C-HFD group, models were established by administering the mixture solution of TNBS (100 mg/kg) and 50% ethanol (0.25 ml) through enema, and

then fed with high-fat diet. Rats from all groups were sacrificed at week 4, 8, 12 after the highfat diet. Liver tissues, blood samples in inferior vena cava and portal vein, jejunal tissues and contents as well as MLN were collected for further examinations. Rats were kept in a normally controlled breeding room with standard laboratory food and water for one week before the experiments. All experimental procedures were conducted according to the institutional guidelines for the care and use of laboratory animals of Dalian Medical University, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Determination of histopathology and NAFLD activity score (NAS)

After rats were sacrificed, the right part of liver samples was obtained and formalin-fixed, paraffin-embedded and cut into 4- μ m thick sections for HE staining and then analyzed by an expert pathologist. Slides were examined and scored in a blinded fashion using published grading system. The Kleiner classification was used to establish NAS (from 0 to 8), including steatosis (0 to 3), lobular inflammation (0 to 3) and hepatocelluar ballooning (0 to 2). NASH was considered if the NAS was >4 [9].

Determination of liver enzymes and LPS

Blood sampling was performed at 4, 8, and 12 weeks in rats fasted overnight. Under pentobarbital anesthesia by intraperitoneal injection, blood samples were collected from the inferior vena cava and portal vein for determination of liver enzymes and LPS concentration respectively. Blood samples from inferior vena cava were centrifuged at 10000 rpm for 10 min at 4°C after 30 min standing, and then the serum was collected for the levels of ALT and AST assay by automatic biochemistry analyzer. Anticoagulant blood samples from portal vein were centrifuged at 3000 rpm for 10 min and the plasma was collected and stored at -80°C until LPS concentration determination using a rat LPS ELISA kit (Sigma).

Liver TNF- α and IL-6 mRNA expression by real-time PCR

Total RNA was extracted from hepatic tissue samples using Trizol (Invitrogen) according to the manufacturer's protocols. All RNA samples

were checked for RNA quality by gel electrophoultraviolet spectrophotometer. resis and Reverse Transcription (RT) reactions were performed using 0.5 µl total RNA, 2 µl PrimeScript® RT Master Mix (Dalian, Takara Co. Ltd.) and 7.5 µl RNase Free dH₂O. An equal amount of cDNA from each sample was amplified using primers specific to each gene. Amplification was performed in a total volume of 20 µl containing 0.4 ul of PCR forward primer and reverse primer, 0.4 µl of ROX Reference Dyell (Dalian, Takara Co. Ltd.), 10 µl of SYBR[®] Premix Ex Taq[™] (Dalian, Takara Co. Ltd.), 6.8 µl of d H₂O and 2.0 µl of DNA. The Real-time PCR primer sequences (Dalian, Takara Co. Ltd.) for TNF-α were TNFα-F: 5'-TCA GTT CCA TGG CCC AGA C-3' and TNF-α-R: 5'-GTT GTC TTT GAG ATC CAT GCC ATT-3', for IL-6 were IL-6-F: 5'-ATT GTA TGA ACA GCG ATG ATG CAC-3' and IL-6-R: 5'-CCA GGT AGA AAC GGA ACT CCA GA-3', for B-action were β-action -F: 5'- AAG CCT AAG GCC AAC CGT GAA AAG -3' and β-action -R: 5'- TCA ATG AGG TAG TCT GTC AGG T-3'. The PCR reactions were done under the following conditions: 35 cycles of initial denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 34 s and extension at 72°C for 60 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction. All Real time-PCR assays were performed in triplicate.

Ultrastructural changes of jejunal epithelium and tight junctions

Jejunal tissues were collected and made the shapes about 1 mm×1 mm×2 mm, fixed with glutaraldehyde and osmic acid, dehydrated with ethanol and embedded with ethoxyline resin for electron microscope examination.

Bacterial cultivation of intestinal contents

Jejunal contents about 0.1 ml were obtained in sterile conditions, diluted 100 times with normal saline, and then the diluent about 50 μ l were vaccinated on blood AGAR medium plate for 48 h incubation at degree of 35°C. Colonyforming units (CFU) per plate were counted and the results were demonstrated as 10⁶ CFU/ml.

Measurement of CD103⁺MHCII⁺DC, CD4⁺CD25⁺Foxp3⁺Treg by flow cytometry

MLN were obtained and mechanically disaggregated into a single cell suspension by using 74

µm cell strainers. MACS buffer consisting of phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA) and 2 mM EDTA was added to the cells for a concentration of 1×106/ ml. Cells were incubated with PE-conjugated anti-rat I-A/I-E (BioLegend Company) and FITCconjugated anti-rat CD103 (BioLegend Company), and then washed, centrifuged and resuspended with PBS before subjecting them to FCM for assaying. For detecting Tregs, cells were stained with PE-conjugated anti-rate CD4 (BioLegend Company) and PerCP-conjugated anti-rat CD25 (BioLegend Company) and cells were subsequently washed in staining buffer twice prior to fixation and permeabilization using eBioscience's buffers for intracellular staining of FoxP3 according to the manufacturer's instructions. The cells were then washed, centrifuged and re-suspended in PBS before subjecting them to flow cytometry. Data were acquired on a FACS Calibur flow cytometer with CELLQuest software.

Statistical analysis

Data showed a normal distribution and are expressed as means \pm standard deviation. The results in different experimental groups were analyzed using one-way ANOVA. A value of *P*<0.05 was considered to be statistically significant between different groups. Data analysis was performed using SPSS version 18.0 statistical software.

Results

Changes of histopathology

No abnormalities were observed in the liver tissues of the NCD rats at different times microscopically. In HFD group, the pathology showed hepatic microvesicular steatosis at week 4, and a more severe degree and macrovesicular steatosis present in hepatic cells; meanwhile, neutrophil infiltration may be seen occasionally at week 8, which accorded with pathological changes of NAFL. In HFD group, at week 12, all rats developed hepatic changes as obvious hepatocyte ballooning, moderate to severe steatosis and lobular inflammation as well as focal necrosis including both acute and chronic hepatic inflammation as demonstrated by infiltration with polymorphs and mononuclear cells, respectively, which accorded with NASH (Figure **1**). In C-HFD group, it showed mixed diffusely



Figure 1. No abnormalities were observed in the liver of the NCD rats. In HFD group, hepatic microvesicular steatosis was showed at week 4 and macrovesicular steatosis were present in hepatic cells at week 8, which accorded with pathological changes of NAFL. Hepatocyte ballooning, moderate to severe steatosis and hepatic inflammation

Impaired gut-liver axis in NAFLD

as well as focal necrosis were showed at week 12 accorded with NASH changes. In C-HFD group, the pathological result showed mixed diffusely hepatic steatosis at week 4. Hepatocyte ballooning, moderate to severe macrovesicular steatosis, lobular inflammation and focal necrosis as well as mild portal zone inflammation were observed at week 8, and an even greater degree of hepatic steatosis, steatohepatitis and lobular inflammation were present at week 12. Pathological changes in the NCD, HFD and C-HFD groups at week 4, 8 and 12 under light microscope (A: HE×100, B: HE×200).



Figure 2. Both in HFD and C-HFD group, the NAS at week 12 was significantly higher than that at week 8 and 4, and the score was significantly higher at week 8 compared with week 4 (*P<0.05 versus 4 wk, *P<0.05 versus 8 wk). The score was significantly higher in C-HFD group compared with HFD group at week 4, 8, 12, respectively (*P<0.05 versus HFD group). NAFLD activity score (NAS) in HFD and C-HFD group at week 4, 8, and 12.

| | NCD group | | HFD group | | C-HFD group | |
|-------|------------|--------------|------------------------------|-------------------|---------------------------------|----------------------------------|
| | ALT | AST | ALT | AST | ALT | AST |
| 4 wk | 63.33±1.21 | 167.67±7.12 | 86.33±5.32* | 186.17±9.58* | 103.00±12.05*,# | 239.17±14.52 ^{*,#} |
| 8 wk | 69.50±4.20 | 169.33±10.99 | 125.50±8.23*,△ | 228.83±6.55*,△ | 146.75±7.00 ^{*,#,△} | 283.40±12.07*,#,△ |
| 12 wk | 68.75±4.57 | 167.17±10.57 | 138.00±2.00 ^{*,△,▲} | 286.50±14.94*,△,▲ | 178.25±7.59 ^{*,#,∆,} ▲ | 312.50±16.05 ^{*,#,△,} ▲ |

Note: *P<0.05 versus NCD group; #P<0.05 versus HFD group; \triangle P<0.05 versus 4 wk; \triangle P<0.05 versus 8 wk.

hepatic steatosis at week 4, which was more severe compared with HFD group. Hepatocyte ballooning, moderate to severe macrovesicular steatosis, lobular inflammation and focal necrosis as well as mild portal zone inflammation were observed at week 8, and an even greater degree of hepatic steatosis, steatohepatitis and lobular inflammation were present at week 12 (Figure 1).

NAS

Both in HFD and C-HFD groups, the NAS at week 12 was significantly higher than that at week 8 and 4 (NAS in HFD group: 6.17 ± 0.75 versus 3.67 ± 0.82 , 6.17 ± 0.75 versus $2.00\pm$ 0.89, respectively, *P*<0.05; NAS in C-HFD group: 7.67 ± 0.82 versus 5.83 ± 0.75 , 7.67 ± 0.82 versus 3.50 ± 055 , respectively, *P*<0.05), and at week 8 NAS was significantly higher than that at week 4 (NAS in HFD group: 3.67 ± 0.82 versus

2.00 \pm 0.89, *P*<0.05; NAS in C-HFD group: 5.83 \pm 0.75 versus 3.50 \pm 0.55, *P*<0.05) (**Figure 2**). In addition, the score was significantly higher in C-HFD group compared with HFD group at the same time points (NAS at week 4, 8 and 12=3.50 \pm 0.55, 5.83 \pm 0.75, 7.67 \pm 0.82 versus 2.00 \pm 0.89, 3.67 \pm 0.82, 6.17 \pm 0.75, respectively, *P*<0.05) (**Figure 2**).

Serum levels of ALT and AST in inferior vena cava

The serum levels of ALT and AST had no significant difference in NCD group at different times. Both in HFD and C-HFD groups, levels of ALT and AST at week 12 were significantly higher than that at week 8 and 4, and at week 8 levels were significantly higher than week 4 (P<0.05). Furthermore, the ALT and AST levels were significantly higher in C-HFD group compared with HFD group at the same time points (P<0.05) (**Table 1**).



Figure 3. The LPS concentration had no significant difference in NCD group at different times. In HFD and C-HFD group, the LPS level was significantly higher at week 12 compared with week 8 and 4, and the concentration was significantly higher at week 8 compared with week 4 (**P*<0.05 versus 4 wk, **P*<0.05 versus 8 wk). The LPS concentration was significantly higher in C-HFD group compared with HFD and NCD group at week 4, 8, 12, respectively (**P*<0.05 versus NCD group, **P*<0.05 versus HFD group). Plasma concentration of LPS in NCD, HFD and C-HFD group at week 4, 8 and 12.

Plasma concentrations of LPS in portal vein

The LPS concentrations had no significant difference in NCD group at different times. There were statistical differences both in HFD and C-HFD groups at week 4, 8 and 12, respectively. The LPS levels were significantly higher at week 12 compared with levels at week 8 and 4 (LPS in HFD group: 37.25 ± 1.50 versus 29.60 ±0.86 , 37.25 ± 1.50 versus 24.49 ±1.12 , respectively, *P*<0.05; LPS in C-HFD group: 42.64 ±1.89 versus 38.01 ±0.87 , 42.64 ±1.89 versus 31.12 ±1.08 , respectively, *P*<0.05), and the concentration was significantly higher at

lular tight junctions

Rats in HFD group at week 8 and 12 represent NAFL and NASH, respectively. Regular and tight jejunum epithelium cells alignment, rich and orderly microvilli of epithelial cells as well as normal intercellular tight junctions were observed by electron microscope in NCD rats. Some lodging and irregular epithelial microvilli and slight increased gap of tight junctions were discovered in NAFL. The jejunal epithelium cells and their organelle became swollen and disruptive; microvilli were atrophic and even deficient as well as gap of intercellular tight junctions became significantly larger in NASH (**Figure 4**).

week 8 than that at week 4 (LPS in HFD group: 29.60± 0.86 versus 24.49±1.12, p<0.05; LPS in C-HFD group: 38.01±0.87 versus 31.12±1.08, P<0.05) (Figure 3). Furthermore, the LPS level was significantly higher in C-HFD group compared with HFD group at the same time points (LPS at week 4, 8 and 12= 31.12±1.08, 38.01±0.87 and 42.64±1.89 versus 24.49±1.12, 29.60±0.86 and 37.25±1.50, respectively, *P*<0.05) (**Figure 3**).

Liver TNF-α and IL-6 mRNA expressions in different groups

Expressions of TNF- α and IL-6 had no significant difference in NCD group at different times. In HFD group, TNF- α and IL-6 expressions were significantly higher at week 12 than that at week 8 and 4; meanwhile, at week 8, the expressions were significantly higher than that at week 4. In C-HFD group, TNF- α and IL-6 mRNA expressions were higher compared with HFD group (**Table 2**).

Disruptions of jejunal epithelium cell and intercel-

| | NCD group | | HFD group | | C-HFD group | |
|-------|-----------|-----------|----------------------------|-----------------------------|-------------------------------|-------------------------------|
| | TNF-α | IL-6 | TNF-α | IL-6 | TNF-α | IL-6 |
| 4 wk | 0.35±0.11 | 0.17±0.03 | 3.30±0.10* | 2.45±0.06* | 4.90±0.08 ^{*,#} | 4.49±0.12 ^{*,#} |
| 8 wk | 0.36±0.05 | 0.18±0.02 | 5.11±0.20 ^{*,∆} | 4.67±0.16 ^{*,∆} | 6.13±0.31 ^{*,#,∆} | 5.75±0.75 ^{*,#,∆} |
| 12 wk | 0.39±0.03 | 0.19±0.06 | 6.09±0.17 ^{*,∆,▲} | 5.70±0.28 ^{∗,∆,} ▲ | 6.96±0.08 ^{*,#,∆,} ▲ | 6.36±0.07 ^{*,#,∆,} ▲ |

Table 2. Liver TNF- α and IL-6 mRNA expressions in different groups at week 4, 8, and 12 ($\overline{x} \pm s$)

Note: *P<0.05 versus NCD group; #P<0.05 versus HFD group; AP<0.05 versus 4 wk; AP<0.05 versus 8 wk.



Figure 4. Regular and tight jejunum epithelium cells alignment, rich and normal microvilli and intercellular tight junctions were observed in NCD group. Lodging and irregular epithelial microvilli and slight increased gap of tight junctions were observed in NAFL. The jejunum epithelium cells and their organelle became swollen and disruptive, and microvilli were atrophic and even deficient as well as gap of intercellular tight junctions became significantly larger in NASH. Changes of jejunum epithelium cell and intercellular tight junctions by electron microscope.

Table 3. Colony-forming units(CFU) in different group at week 4, 8 and 12 (10^5 CFU/ml) ($\overline{X} \pm s$)

| | NCD group | HFD group | C-HFD group |
|-------|-----------|----------------------------|------------------------------|
| 4 wk | 1.62±0.09 | 2.72±0.43* | 3.92±0.21 ^{*,#} |
| 8 wk | 1.65±0.07 | 3.43±0.34 ^{∗,∆} | 5.18±0.46*,#,△ |
| 12 wk | 1.65±0.01 | 4.08±0.40 ^{*,△,▲} | 5.85±0.12 ^{*,#,△,▲} |

Note: *P<0.05 versus NCD group; #P<0.05 versus HFD group; $^{\Delta}$ P<0.05 versus 4 wk; **^**P<0.05 versus 8 wk.

Numbers of jejunal CFU in different groups

There had no statistic difference of CFU in NCD group at different time points. In HFD group, CFU increased significantly at week 12 compared with that at week 8 and 4. In C-HFD group, CFU increased significantly compared with HFD group at week 4, 8 and 12, respectively (**Table 3**).

Amounts of CD103+MHCII+DCs and

CD4+CD25+FoxP3+T-regs in terms of percentage

In the NAFL group and NASH group, the percentage of CD103+MHCII+DCs of total cells

were $(1.12\pm0.15)\%$ and $(0.52\pm0.09)\%$, respectively, and the percentage of CD4+CD25+ FoxP3+T-regs in CD4+T cells were $(1.07\pm0.08)\%$ and $(0.71\pm0.21)\%$, respectively. Statistical analysis showed the percentage of DCs and T-regs in NASH and NAFL group decreased significantly compared to NCD group (P<0.05). The percentage of DCs in NASH group decreased significantly compared to NAFL group; but the percentage of T-regs had no significant difference between NAFL and NASH group (Figure 5).

Discussion

NAFLD is a rising worldwide public health problem characterized by a typical sequence of disease stages ranging from steatosis to inflammation, fibrosis, cirrhosis and even liver cancer, which worldwide prevalence is estimated to be 20%-30% [10]. In the pathogenesis of NAFLD, double-hit hypothesis has been proposed, but the underlying molecular mechanisms are incompletely understood. Nowadays, a multiple parallel hits hypothesis which including genes, lipotoxic agents, endoplasmic reticulum stress,



Figure 5. The percentage of DCs and T-regs in NASH and NAFL group decreased significantly compared to NCD group (P<0.05). The percentage of DCs in NASH group decreased significantly compared to NAFL group; but the percentage of T-regs had no significant difference between NAFL and NASH group. The amount of CD103+MHCII+DCs and CD4+CD25+FoxP3+T-regs in MLN in terms of percentage by flow cytometry.

role of oxidative stress, inflammation and adipokines as well as gut-liver axis has also been suggested [3, 4]. Among these factors, interactions between intestinal mucosal barrier and liver had been widely investigated in the pathogenesis of NAFLD [5].

Normally, translocation of gut bacteria or its products to extraintestinal space is effectively prevented by the defense mechanisms as the complete intestinal barrier function and cleansing function of the liver [6]. However, disruption of these defense mechanisms can lead bacterial translocation and aberrant activation of immune system, which can trigger harmful or inflammations in the liver [7].

To study whether or not the intestinal mucosal barrier dysfunction was involved in the pathogenesis of NAFLD, we established the rat models of NAFLD with intestinal mucosal barrier dysfunction by administering the mixture solution of TNBS and ethanol through enema and then fed with high-fat diet. Our previous works have indicated that the above modeling method could induce obviously impaired intestinal barrier which histological appearance present as severe colonic mucosal inflammation, erosion and ulcer from 1 to 2 weeks and the changes last 2-3 months [11-13].

The HE staining results indicated a more severe degree macrovesicular steatosis in hepatic cells at week 8 and obvious hepatocyte ballooning, moderate to severe steatosis, lobular inflammation as well as focal necrosis including both acute and chronic hepatic inflammation at week 12 in HFD group, which accorded with the typical characteristics of NAFL and NASH, respectively. While in C-HFD group, the degree of hepatocyte steatosis and steatohepatitis were more severe than which in HFD group at the same time point. Furthermore, NAS and serum levels of ALT and AST in inferior vena cava were significantly higher in C-HFD group compared with HFD group at the same time, which illustrated the degree of liver function damage and liver inflammation were completely conformed. These data first declared intestinal mucosal barrier dysfunction aggravated the degree of steatosis and steatohepatitis based on histology and biochemistry index.

As everyone knows, the homeostasis of acting cytokines is fundamental in the control of sys-

temic and hepatic insulin action, and as a consequence, in the development of NAFLD [14]. TNF- α and IL-6, important cytokines related with live inflammation, were considered as the factors of contributing to steatohepatitis.

Recent studies have indicated that the serum or plasma TNF-α levels was higher in NASH compared with healthy subjects and it was shown to correlate with liver fibrosis in advanced NAFLD [15, 16]. Besides, certain TNF-α polygenetic polymorphisms have been found to have higher IR indices, a higher susceptibility to the development of NAFLD [17]. In our study, both in HFD and C-HFD group, TNF- α and IL-6 expressions were significantly higher at week 12 compared with week 4 and 8. And more important, mRNA expressions of TNF- α and IL-6 increased significantly in C-HFD group compared with HFD group at week 4, 8 and 12, respectively. These results verified the important role of TNF- α and IL-6 in the pathogenesis of NAFLD, and also indicated that the intestinal mucosal barrier dysfunction promoted the development of NAFLD.

To investigate the mechanism about gut barrier dysfunction aggravating the state of NAFLD, jejunal CFU and concentration of LPS in portal vein were observed. The results showed that higher numbers of CFU and concentration of LPS contributed to severe steatosis and steatohepatitis. SIBO, an increase in the number and/ or alteration in the composition of bacteria in the proximal gastrointestinal tract, was related with the liver steatosis in fatty liver [18]. Recent study suggested that modulation of the gut microbiota was a critical determinant of NAFLD as well as multiple other aspects of metabolic syndrome [19]. In addition, SIBO and changes in the composition of bacterial flora in the gut could promote bacterial translocation [20]. which was defined as the migration of bacteria or bacterial products as LPS from the gut to the extra-intestinal space.

In normal condition, only very small amount of bacteria and LPS can enter the liver, where they were cleared by hepatic immune cells, particularly by Kupffer cells. However, in some pathologic conditions with impaired intestinal mucosal barrier function, the entry of bacteria and LPS to the liver were increased significantly, which eventually induced activation of liver immune cells to produce massive pro-inflammatory mediators as TNF- α and IL-6, and then led to liver damage [21]. Then some aspects of mechanical, biological and immunologic barrier were observed to identify whether or not intestinal mucosal barrier dysfunction was involved in the process of NAFLD.

Ultrastructural changes of jejunal epithelium as well as bacterium numbers were observed by electron microscope and germiculture to demonstrate the gut mechanical and biological barrier dysfunction. Lodging epithelial microvilli and slight increased gap of tight junctions were discovered in NAFL. The epithelium cells and their organelle became swollen and disruptive; microvilli were atrophic and even deficient as well as gap of tight junctions become larger in NASH. Numbers of CFU increased significantly in NASH compared with NAFL. Miele et al [18] had reported that patients with fatty liver disease had increased intestinal permeability related to SIBO and disrupted tight junctions compared to the normal controls. Besides, they also demonstrated that intestinal permeability and SIBO were correlated with the severity of hepatic steatosis. Intestinal bacterial overgrowth and the composition changes of bacterial flora in the gut were present due to the decrease in gastric acidity, intestinal motility, and biliary secretions [22]. In addition, liverderived inflammatory cytokines can further increase intestinal permeability by disruption of epithelial tight junctions [23]. Increased intestinal permeability and SIBO contributed to the development of bacterial translocation [20].

Besides mechanical and biological barrier, immune barrier participated in the process of NAFLD. Recent study had showed intra-abdominal lymph node morphology was altered and that CD4+ and CD8+ T cell counts were reduced in HFD fed mice [24]. Su Lin et al [25] also reported that progression of NAFLD in obese rats was associated with initial immune responses that rapidly declined. In our study, the amounts of CD103+MHCII+DCs and CD4+CD25+FoxP3+T-regs in terms of percentage in MLN were measured by flow cytometry to investigate the gut immune barrier in NAFLD. The result showed the percentage of DCs and T-regs in NASH and NAFL group decreased significantly compared to NCD group. DCs are pivotal in tolerance induction and direct the differentiation of T cells. Microbial antigen handled by DCs is believed to be of critical importance for gut immunity. T-regs have an essential role

in maintaining immune tolerance in the gut. This result indicated that CD103+MHCII+DCs and CD4+CD25+FoxP3+T-regs were involved in the imbalance of colonic mucosal immunology in the process of NAFLD. In accordance with the decrease of DCs and T-regs in MLN in HFD rats, concentration of LPS was increased, confirming the importance of intestinal immune barrier function. With the progress of NAFLD, the numbers of DCs decreased significantly, which implied the impaired immune barrier still existed, but the numbers of T-regs did not reduced significantly, which indicated decreased ratio of DCs and T-regs may led to the imbalance between pro-inflammatory and antiinflammatory factors. However, the specific function of intestinal cellular immune and the definite regulatory mechanism between DCs and T-regs in NAFLD are still unknown and need to be clarified in future work.

Our study indicated that due to intestinal mucosal barrier dysfunction in NAFLD, the entry of bacteria and LPS to the liver were increased significantly which eventually induced activation of liver immune cells to produce massive pro-inflammatory mediators as TNF- α and IL-6, and then led to liver more serious damage. In conclusion, gut-liver axis was impaired in NAFLD, which played crucial role in the pathogenesis of NAFLD.

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Disclosure of conflict of interest

None.

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