Chronic bile duct hyperplasia is a chronic graft dysfunction following liver transplantation

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AIM: To investigate pathological types and influential factors of chronic graft dysfunction (CGD) following liver transplantation (LT) in rats.

METHODS: The whole experiment was divided into three groups: (1) normal group (n = 12): normal BN rats without any drug or operation; (2) syngeneic transplant group (SGT of BN-BN, n = 12): both donors and recipients were BN rats; and (3) allogeneic transplant group (AGT of LEW-BN, n = 12): Donors were Lewis and recipients were BN rats. In the AGT group, all recipients were subcutaneously injected by Cyclosporin A after LT. Survival time was observed for 1 year. All the dying rats were sampled, biliary tract tissues were performed bacterial culture and liver tissues for histological study. Twenty-one day after LT, 8 rats were selected randomly in each group for sampling. Blood samples from caudal veins were collected for measurements of plasma endotoxin, cytokines and metabonomic analysis, and faeces were analyzed for intestinal microflora.

RESULTS: During the surgery of LT, no complications of blood vessels or bile duct happened, and all rats in each group were still alive in the next 2 wk. The long term observation revealed that a total of 8 rats in the SGT and AGT groups died of hepatic graft diseases, 5 rats in which died of chronic bile duct hyperplasia. Compared to the SGT and normal groups, survival ratio of rats significantly decreased in the AGT group (P < 0.01). Moreover, liver necrosis, liver infection, and severe chronic bile duct hyperplasia were observed in the AGT group by H and E stain. On 21 d after LT, compared with the normal group (25.38 ± 7.09 ng/L) and SGT group (33.12 ± 10.26 ng/L), plasma endotoxin in the AGT group was remarkably increased (142.86 ± 30.85 ng/L) (both P < 0.01). Plasma tumor necrosis factor-α and interleukin-6 were also significantly elevated in the AGT group (593.6 ± 171.67 pg/mL, 323.8 ± 68.30 pg/mL) vs the normal (225.5 ± 72.07 pg/mL, 114.6 ± 36.67 pg/mL) (both P < 0.01). Furthermore, Bacterial cultures of bile duct tissues revealed that the rats close to death from the SGT and AGT groups were strongly positive, while those from the normal group were negative. The analysis of intestinal microflora was performed. Compared to the normal group (9.62 ± 1.60, 9.93 ± 1.10) and SGT group (8.95 ± 0.04, 9.02 ± 1.14), the numbers of Bifidobacterium and Lac-
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**INTRODUCTION**

Liver transplantation (LT) has become an established therapy for various end-stage liver diseases for more than three decades[1-3]. Numerous advances in surgical technique, organ preservation, perioperative anesthesia, postoperative care, and clinical immunosuppression, as well as improved recipient selection and donor management have together significantly increased the survival rates of allograft and improved life quality of patients following LT[4]. Currently, approximately 90% of liver transplant patients are alive after 1 year and 75% after 5 years with majority living a full and near-normal life[5]. However, although early mortality rates after transplantation have fallen dramatically, long-term graft survival has barely improved over the last two decades[6,7]. That is to say, the incidence of chronic graft dysfunction (CGD) and the mortality of patients after LT have remained constant, and CGD has become the biggest obstacle for long-term function of allograft and better life quality of patients. Thus, it is essential to investigate the causes and relevant mechanisms of CGD to improve long-term outcomes of patients following LT.

In the early day after LT, common causes of hepatic allograft dysfunction include ischemia and reperfusion injury, infection, technical complications such as hepatic artery thrombosis and recipient diseases[8,9]. Thereafter the causes of allograft dysfunction are variable with disease recurrence and chronic rejection as major causes of graft loss[8]. Moreover, the biliary tract is still the most common site for postoperative complications. The importance of this condition lies in the fact that the biliary tract complications can be a serious source of morbidity and sometimes mortality[10]. These complications not only affect allograft survival but also have a major impact on the life quality for a hepatic allograft recipient.

However, so far little is known on biliary tract variation of CGD and its influential factors in recipients following LT. In this study, we want to explore hepatic graft pathology and the relevant mechanisms of CGD from aspects of inflammation, intestinal barrier function, intestinal microflora and metabolomics following LT.

**MATERIALS AND METHODS**

**Animals**

Specific pathogen-free (SPF) male inbred Lewis and BN rats (weight 220-250 g, 12-15 wk) were purchased from Beijing Vital River Laboratories (Beijing, China). All rats were housed in a SPF lab (Zhejiang Academy of Medical Sciences, China). The rats were caged in 21 ℃, 12 h light/dark cycle, and fed with sterilized standard rat chow and water. All animals received humane care and the study was conducted according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

**Experimental design**

Sixty rats were raised in SPF animal facility. Twelve inbred BN rats were served as normal controls; 12 inbred Lewis and 12 inbred BN rats as donors; 24 inbred BN rats as recipients. All donors and recipients were randomly performed orthotopic LT under strict sterile conditions. The remaining 36 BN rats were divided into three groups: (1) normal group (n = 12): normal BN rats without any drug or operation; (2) syngeneic transplant group (SGT of BN-BN, n = 12): both donors and recipients were BN rats; and (3) allogeneic transplant group (AGT of LEW-BN, n = 12): donors were Lewis and recipients were BN rats. In the AGT group, all recipients were subcutaneously injected by Cyclosporin A at 1 mg/kg daily in the first 30 d, and then at 2 mg/kg daily for the next 100 d after orthotopic LT. All the recipients in the SGT and AGT groups were received alanine (ALA) daily by gastric perfusion for preoperative 3 d and postoperative 130 d. Survival time was observed for 1 year.

**Surgery procedures**

All rats were fasted for 12 h before the operation. The initial anesthesia of rats was performed by intraperitoneal injection of Ketamine Hydrochloride (100 mg/kg) and Atropine (1 mg/kg) (Shanghai No. 1 Biochemical and Pharmaceutical, China), and then ether was inhaled to maintain anesthesia. The profiles of rats with orthotopic LT were established according to the previous techniques[11,12], with slight modifications. Briefly, after the
liver of the donor was dissociated, the graft was perfused with chilled saline containing 25 μg/mL heparin via the portal vein, and then preserved in cold normal saline for no more than 1 h before being placed in the abdomen of recipient. After the anastomosis of suprarehepatic vena cava and portal vein was finished, the graft was reperfused. The common bile duct was reconstructed by tying the duct over a stent. All recipients recovered in a short time, and no further treatment was performed.

Sample collections

The survival conditions of rats were monitored continuously. When any rat was dying, the following samples were collected under strict sterile condition; the liver was fixed in 40 g/L neutral formaldehyde for later histological study and the biliary tract tissue for bacterial culture. Moreover, on 21 d after the surgery, biliary tract tissues of 4 rats selected randomly in the normal group were collected for bacterial culture. Eight rats were selected randomly in each group for sampling. Blood samples from the caudal veins were gained for measurements of plasma endotoxin, cytokines and analysis of ultra performance liquid chromatography-mass spectrometry (UPLC-MS), and facaces were collected for the determination of intestinal microflora.

Graft histopathology

The sample from the left lobe of hepatic graft was fixed in 40 g/L neutral formaldehyde and embedded in paraffin, cut into 3 μm slices, stained with hematoxylin and eosin (HE), and then observed under light microscopy by a pathologist.

Plasma endotoxin and cytokines

The blood sample (100 μL) was placed in the pyrogen-free heparin-containing tube, and then centrifuged at 3000 g for 15 min at 4 ℃. Plasma endotoxin of the caudal vein was determined using a quantitative, chromogenic Limulus Amebocyte Lysate assay according to the manufacturer’s instruction (Eihua Medical, Shanghai, China). The value was expressed as nanogram per liter of plasma (ng/L).

The levels of plasma tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were tested with enzyme-linked immunosorbent assay (ELISA) (Groundwork Biotechnologies Diagnosticate Ltd, United States) according to the protocol of manufacturer. The result was expressed as ng/L.

Bacterial culture and identification

Biliary tract tissues from the grafts were weighed and placed in a germ-free glass homogenizer containing a nine-fold amount of anaerobic buffer (phosphate buffered saline with 0.5 g cysteine HCl, 0.5 mL tween 80, and 0.5 g agar). They were homogenized and 50 μL of 10% homogenate were placed on the agar base of Colombian culture medium within 30 min, incubated for 48 h at 37 ℃. Bacterial colonies were evaluated qualitatively according to their growth conditions respectively at the end of the culture. In addition, bacterial colonies from biliary tract tissues were identified by the Automatic analyzer of bacteria (Model Viger 60, France) to identify bacterial species according to the report[15].

Determination of intestinal microflora

Intestinal microflora was studied with 4 selected agar media according to the reports[14]. Samples from colorectal contents were placed in sterile tubes, weighed and transferred into other sterile tubes containing appropriate anaerobic buffer (as described above) to approach a 10-fold dilution of samples, and then serial decimal dilutions were taken in the same way from 10^-2 to 10^-8. Within 30 min of sample collection, bacterial cultures were finished with placing 50 μL dilutions on 4 agar media. According to the instructions, TPY agar medium, LBS agar medium, EC medium, and Eosin-Methylene Blue Agar (EMB) were used for Bifidobacterium, Lactobacillus, Enterococcus and Enterobacter, respectively. Anaerobic bacteria were incubated in Anaerobic Box System including AnaeroPack (MGC, Japan) and GENbox anaer (BioMérieux, France), and aerobic bacteria were incubated aerobically for 48 h at 37 ℃, respectively. Bacterial colonies on every plate were counted and calculated at the primal weight of samples. The results were expressed as bacterial colony forming units per gram content (log_10 CFU/g).

UPLC-MS analysis

Blood samples from caudal veins of rats were collected for metabonomics analysis according to the method of UPLC-MS described by Wang et al[8] and Yang et al[10]. Prior to analysis, blood samples were defrosted at room temperature and mixed with acetonitrile (ACN) at the ratio of 1:3 (v/v), the mixture was vortered and centrifuged at 10 000 g for 10 min. The supernatant was transferred to sample bottles for UPLC separation. Chromatographic separations were performed at a 100 mm × 2.1 mm ACQUITY-1.7 lm C18 column (Waters Co., Milford, United States) using an ACQUITY-Ultra Performance Liquid Chromatography system (Waters). Mass spectrometry was performed on a Premier-Q-Tof (Waters MS Technologies, Milford, United States).

Partial least squares-discriminate analysis

The UPLC-MS data were analyzed with the SIMCA-P+ 12 Software (Umetrics, Sweden). An ApexTrack-peak detection algorithm was adopted in MarkerLynx V4.1 software (Waters, United States) to measure peaks and align retention times of the peaks for all chromatograms. The results were transferred into a single data matrix by aligning peaks with the same mass/retention time pair together from each data file in the dataset, along with their relevant intensities. The resulting dataset containing peak numbers (RT-m/z pair), sample names, and ion intensities was analyzed by partial least squares-discriminate analysis (PLS-DA) with the SIMCA-P+ 12 Software.
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***Inflammatory cells*** (Figure 2A). The rat died of chronic bile duct hyperplasia on the 25th, 34th, and 35th day post-operation, respectively; the remaining 4 rats died of abdominal infection on the 15th and 23th day post-operation. It was worth noting that a total of 8 rats in the AGT group were dead: two died of hepatic necrosis on the 25th and 26th day post-operation; the others were determined using Students T-test. These analysis were performed with the statistical software SAS 9.1.3 (SAS Institute Inc., North Carolina State University, United States), and UPLC-MS data were analyzed by PLS-DA with the SIMCA-P+ 12 Software (Umetrics, Sweden). A P-value of less than 0.05 was considered statistically significant.

**RESULTS**

During the surgery of LT, no complication of blood vessels or bile duct happened, and all rats in each group were still alive in the next 2 wk.

**Survival distribution function**

The survival distribution function in each group was shown in Figure 1. A total 8 rats in the normal group were alive for more than one year. Two rats in the SGT group were dead of hepatic injury and CGD of recipients after LT. As showed in Figure 3, there was no significant difference in plasma endotoxin between the normal group (25.38 ± 7.09 ng/L) and SGT group (33.12 ± 10.26 ng/L). By contrast, plasma level of endotoxin in the AGT group was remarkably increased (142.86 ± 30.85 ng/L) vs the other two groups (P < 0.01).

Both TNF-α and IL-6 are important pro-inflammatory cytokines and can directly or indirectly cause graft injury and CGD of recipients after LT. As shown in Figure 3 (pg/mL), compared to the normal group (225.5 ± 72.07 ng/L, 114.6 ± 36.67 ng/L) and SGT group (321.3 ± 88.47 ng/L, 205.2 ± 53.06 ng/L), the levels of plasma TNF-α and IL-6 were significantly elevated in the AGT group (593.6 ± 171.67 ng/L, 323.8 ± 68.30 ng/L) (P < 0.01). In addition, plasma levels of both TNF-α and IL-6 in the SGT group were also increased vs those in the normal group (P < 0.05, P < 0.01, respectively).

**Bacterial culture and identification**

Bacterial translocation may participate in some physiological and pathological procedures when recipient suffers from CGD. Samples from biliary duct tissues of hepatic graft were cultured and results were summarized in Table 1. There was rare bacterial colony in the 4 rats from the normal group, while bacterial culture of samples from the SGT and AGT groups were strongly positive. Bacterial identification revealed significantly increased aerobic bacteria, such as *Escherichia coli* and *Enterococcus* in the groups of LT. Moreover, *Proteus vulgaris*, *Streptococcus agalactiae* and *Proteus mirabilis* presented remarkably positive in the AGT group, which suggested that more kinds of bacteria translocated to biliary duct tissues when recipients suffered from CGD following allogeneic LT.

**Intestinal microflora**

To determine the alterations of intestinal microflora when recipients suffered from CGD after LT, bacterial

![Kaplan-Meier survival curve in the different groups.](image)

**Statistical analysis**

All the data were presented as mean ± SD. The survival distribution function was evaluated by the Kaplan-Meier survival curve and the others were determined using Students T-test. These analysis were performed with the statistical software SAS 9.1.3 (SAS Institute Inc., North Carolina State University, United States), and UPLC-MS data were analyzed by PLS-DA with the SIMCA-P+ 12 Software (Umetrics, Sweden). A P-value of less than 0.05 was considered statistically significant.
species and numbers in colorectal contents were analyzed. As shown in Table 2, compared to the normal group (7.98 ± 0.92, 8.90 ± 1.44) and SGT group (8.51 ± 0.46, 9.43 ± 1.10), the numbers of *Enterococcus* and *Enterobacteria* in the AGT group (8.76 ± 1.93, 10.18 ± 1.64) were significantly increased (both $^aP < 0.01$, $^bP < 0.05$, respectively). Meanwhile, compared to the normal group (9.62 ± 1.60, 9.93 ± 1.10) and SGT group (8.95 ± 0.04, 9.02 ± 1.14), the numbers of *Bifidobacterium* and *Lactobacillus* in the AGT group (7.83 ± 0.72, 8.87 ± 0.13) were remarkably reduced (both $^aP < 0.01$, $^bP < 0.05$, respectively). There were no statistical differences in bacterial species and counts between the normal group and the SGT group.

### UPLC-MS analysis and PLS-DA

In order to analyze the interactions between metabolic profile and CGD, caudal vein plasma was collected to perform metabonomics analysis by the method of UPLC-MS and PLS-DA. As shown in Figures 4-6, the metabolic profiles of plasma in rats from the SGT and AGT groups were deviated from those of the normal group gradually. We also found that metabolic alterations were more severely deviated in the AGT group vs the normal group, which suggested that the degree of metabolic change was positively associated with the severity of CGD in recipients following LT.

### DISCUSSION

Organ transplantation has become the radical method for treatment of many end-stage organic diseases. Since the early experiences of the 1960s, liver transplant surgery has evolved over the decades and is now the standard of care in patients with end-stage liver disease. Although there has been consistent improvement in the overall survival rates for transplant recipients, organ shortages and CGD are still two major problems which hinder the development of organ transplantation.
In general, chronic hepatic allograft dysfunction is defined as the declining of hepatic graft function irreversibly and gradually, expressed by increasing or persistent elevations in serum levels of alanine aminotransferase, alkaline phosphatase, or bilirubin (greater than two times the upper limit of normal). Chronic hepatic allograft dysfunction may result from a variety of causes including rejection, vascular stenosis/thrombosis, de novo or recurrent infection, biliary complications including stricture or stenosis, recurrent disease related to autoimmune mechanisms such as that seen in primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune chronic active hepatitis. In addition, drug hepatotoxicity and the development of neoplasms such as a posttransplant lymphoproliferative disorder or the recurrences of hepatocellular carcinoma are important considerations.

With the efforts in recent decades, survival after LT is 90% at 1 year and approximately 75% at 5 years. However, biliary tract complications are still the “Achilles heel” of LT. Despite great improvements in the surgical techniques and standardization of the method of biliary reconstruction, the biliary tract is still the most common site for postoperative complications. So far little is known about alterations of biliary tract when recipients suffer from CGD following LT. Thus, it has been indispensable to explore changes of biliary tract and relevant mechanisms of CGD to improve survival conditions of recipients following LT for a long time.

In our study, no rats died within 2 wk after LT since applications of immunosuppressants and sterile surgery in SPF-class laboratory. Life span of human is usually 60–90 years, while life expectancy of rats typically is 2–3 years, so we can define CGD of rats as loss of graft function after about one month post-operation. During the whole process of observation, a total of 8 rats in the LT groups died of hepatic graft diseases, such as partial hepatic necrosis and chronic bile duct hyperplasia, and the other 2 rats died of abdominal infection. Notably, 5 rats in these 8 rats that died of hepatic graft diseases died of chronic bile duct hyperplasia, which accounted for 5/8 (62.5%). We concluded that chronic bile duct hyperplasia was a pathological type of CGD and its occurrence frequency was closely associated with the causes of CGD following LT. This finding is consistent with the reports that bile duct hyperplasia extending progressively is a pathology finding in a profile of auxiliary LT with portal vein arterIALIZATION in pigs.

In order to explore influential factors and possible mechanisms of chronic bile duct hyperplasia, as a special type of CGD following LT, we further investigated alterations of serum endotoxin and cytokines, bacterial translocation, intestinal microflora, and metabolic profile in the different groups.

Endotoxin, which mainly originated from non-viable intestinal gram-negative bacteria, is a crucial medium to aggravate hepatic graft injury. Plasma level of endotoxin not only can reflect intestinal barrier function, but also can predict the defensive ability of body and the injury degree of hepatic graft. Our research found that plasma level of endotoxin in the AGT group remarkably increased compared to the other two groups, which suggested that intestinal barrier function was destroyed when recipients suffered from CGD following LT. Meanwhile, we also speculated that the elevation of plasma endotoxin was closely associated with the occurrence of CGD after LT in rats.

Inflammation plays a vital role in the progression of liver injury. TNF-α and IL-6 are important proinflammatory mediators and can directly, or by inducing inflammatory cascades and enhancing the microvascular dysfunction of liver and intestine, aggravate the injury of hepatic graft. Inflammatory mediators such as TNF-α, interferon-γ (INF-γ) and interleukin (IL) have cytotoxicity, thus being considered as effectors of liver injury. Our results revealed that plasma levels of cytokines gradually increase as the severity of hepatic graft dysfunction increasing.
increased from the rats in the normal group to those in the syngeneic transplant group, and even to those in the allogeneic transplant group, suggesting that inflammatory reaction was positively associated with the severity of CGD in recipients. Thus, our findings support the concept that inflammation may be a component of the pathogenesis of CGD, which is in line with the report on inflammation and graft deterioration by Dahl et al.\textsuperscript{29} However, the exact pathogenetic role of inflammatory cytokines in graft failure is still elusive.

Under certain conditions, the original bacteria in the intestine would cross a relatively complete intestinal epithelium to reach the sites of MLN, abdominal internal and external organs (such as liver, spleen and lung) as well as blood, and may cause the occurrence of infection, which is called "bacterial translocation"\textsuperscript{30}. Normally, a small amount of bacteria and endotoxin can go through the intestinal wall, which may be associated with the maintenance of normal intestinal immune response and the activity of reticuloendothelial system\textsuperscript{31,32}. In gen-

**Figure 4** Loading plot of all the different plasma samples. The marked numbers beside the points were the corresponding retention time and the mass-to-charge ratio (m/z) of each possible biomarkers.

**Figure 5** The partial least squares-discriminate analysis scores plot of the different groups. According to this plot, the metabolic profiles of the SGT and AGT groups deviated from those of the normal group gradually.
In conclusion, according to the observation of one year for recipients following LT in rats, we have found that chronic bile duct hyperplasia is a pathological type of CGD following LT. The mechanism of this kind of CGD is associated with the alterations of inflammation, intestinal barrier function, intestinal microflora, and plasma metabolic profile, which will be the possible therapeutic targets for LT.

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COMMENTS

Background

Although early mortality rates after liver transplantation (LT) have fallen dramatically, the paradox is that long-term graft survival has barely improved over the last two decades. Chronic graft dysfunction (CGD) has become the biggest obstacle for long-term function of allograft and better life quality of patients. The biliary tract is still the most common site for postoperative complications. These complications not only affect allograft survival, but also have a major impact on the life quality for a hepatic allograft recipient.

Research frontiers

So far little is known on biliary tract variation of CGD and its influential factors in recipients following LT. In our experimental study, according to the observation of one year for recipients following LT, we have found that chronic bile duct hyperplasia is a pathological type of CGD following LT. The mechanism of this kind of CGD is associated with the alterations of inflammation, intestinal barrier function, intestinal microflora, and plasma metabolic profile. The three-dimensional partial least squares-discriminate analysis scores plot of the different groups. According to this three-dimensional plot, the metabolic profiles of the AGT groups deviated more than those of the SGT group from those of the normal group. PLS: Partial least squares.

Figure 6

R2 × [1] = 0.311089  R2 × [2] = 0.0808523

In conclusion, according to the observation of one year for recipients following LT in rats, we have found that chronic bile duct hyperplasia is a pathological type of CGD following LT. The mechanism of this kind of CGD is associated with the alterations of inflammatory response, intestinal microflora, and plasma in recipients following LT. As a system analysis approach, metabolomics can provide comprehensive information on the dynamic process of postoperative physiopathological development\[15\]. The systemic detection of chronic diseases can be obtained with metabolomics at an earlier stage compared to the clinical chemistry and histopathological assessment\[13\]. Our results on metabolomics analysis revealed that the metabolic profile of plasma gradually deviated from the normal parameters, from the rats in the syngeneic transplant group to those in the allogeneic transplant group. To some extent, this finding suggested that the mechanism of CGD might be explained by the alterations of plasma metabolic profile in recipients following LT. However, further exploration need to be taken for the accurate relationship between metabolic changes and CGD of recipients following LT.

In conclusion, according to the observation of one year for recipients following LT in rats, we have found that chronic bile duct hyperplasia is a pathological type of CGD following LT. The mechanism of this kind of CGD is associated with the alterations of inflammation, intestinal barrier function, intestinal microflora, and plasma metabolic profile, which will be the possible therapeutic targets for LT.
barrier function, intestinal microflora, and plasma metabolic profile. Furthermore, through the technique of ultra performance liquid chromatography-mass spectrometry analysis and partial least squares-discriminate analysis (PLS-DA), we explored the plasma metabolomics alterations during the period of CGD after LT, and indicated the relevance between plasma metabolomics and CGD after LT in rats.

**Applications**

This study provides the experimental data for the research of CGD after organ transplantation in rats, and indicated that chronic bile duct hyperplasia is a kind of CGD following LT in rats. The relative influence factors will be the possible therapeutic targets to prevent or alleviate CGD after LT.

**Terminology**

Chronic hepatic allograft dysfunction is defined as the declining of hepatic graft function irreversibly and gradually, expressed by increasing or persistent increase in serum levels of alanine aminotransferase, alkaline phosphatase, or bilirubin (greater than two times the upper limit of normal). PLS-DA is a statistical method that can grasp the principal contradiction from the complexity, and thereby simplify something complexity, which can generate models that are tightly focused on the effects of interest.

**Peer review**

This is a well designed experimental report on chronic allograft dysfunction after LT. The topic is of some interest due to the prolonged survival now being constantly achieved after LT. The authors described chronic bile duct hyperplasia as a type of chronic allograft dysfunction associated to inflammation of the intestinal mucosa.

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