The possible association between AQP9 in the intestinal epithelium and acute liver injury-induced intestinal epithelium damage

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Abstract. The present study aimed to investigate the expression and function of aquaporin (AQP)9 in the intestinal tract of acute liver injury rat models. A total of 20 Sprague Dawley rats were randomly divided into four groups: Normal control (NC) group and acute liver injury groups (24, 48 and 72 h). Acute liver injury rat models were established using D-amino galactose, and the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (Tbil) and albumin were determined using an automatic biochemical analyzer. Proteins levels of myosin light chain kinase (MLCK) in rat intestinal mucosa were investigated via immunohistochemistry. Pathological features were observed using hematoxylin and eosin (H&E) staining. MLCK, AQP9 and claudin-1 protein expression levels were detected via western blotting. Levels of ALT and AST in acute liver injury rats were revealed to steadily increase between 24 and 48 h time intervals, reaching a peak level at 48 h. Furthermore, TBil levels increased significantly until 72 h. Levels of ALT were revealed to significantly increase until the 48 h time interval, and then steadily decreased until the 72 h time interval. The acute liver injury 72 h group exhibited the greatest levels of MLCK expression among the three acute liver injury groups; however, all three acute liver injury groups exhibited enhanced levels of MLCK expression compared with the NC group. Protein levels of AQP9 and claudin-1 were enhanced in the NC group compared with the three acute liver injury groups. H&E staining demonstrated that terminal ileum mucosal layer tissues obtained from the acute liver injury rats exhibited visible neutrophil infiltration. Furthermore, the results revealed that levels of tumor necrosis factor-α, interleukin (IL)-6 and IL-10 serum cytokines were significantly increased in the acute liver injury groups. In addition, AQP9 protein expression was suppressed in acute liver injury rats, which induced pathological alterations in terminal ileum tissues may be associated with changes of claudin-1 and MLCK protein levels.

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Introduction

The major function of the intestinal epithelial barrier is to regulate the transport of molecules through transcellular and paracellular pathways between the hostile environment of the intestinal epithelium and the sub-epithelial tissue (1). The intestinal epithelial barrier has a selective permeable barrier that limits the permeation of luminal noxious molecules, including toxins, pathogens and antigens, while regulating the absorption of nutrients and water. This barrier is established by intercellular tight junction (TJ) structures (2). TJ assembly involves at least four types of transmembrane proteins: Tricellulin, claudins, occludin and junctional adhesion molecules. Claudins are structural proteins present in TJs and are involved in the regulation of paracellular movement by forming a barrier across the epithelial monolayer (3). Altered functions of claudins are associated with different forms of cancer, inflammatory bowel diseases and diarrhea (4). Claudin-1, a major structural and functional TJ protein is responsible for modulating the permeability of the epidermis (5).

Myosin light chain kinase (MLCK) is a calcium-dependent enzyme present in the cytoskeleton and the cytoplasm (6). MLCK may promote phosphorylation of the myosin light chain, which regulates the cytoskeleton (6). The MLCK pathway mediates intestinal epithelial barrier permeability by regulating TJs (7). A previous study revealed that downregulation of MLCK may attenuate damage to TJs (8). The results of the aforementioned studies suggest that MLCK has an important role in TJ damage under endotoxemic conditions.

Aquaporins (AQPs), including AQP0-AQP12, are a family of small integral membrane proteins that transport water across the cytoplasmic membrane. AQP9 is a subtype of the AQP protein family that is present in mammalian cells (9), and murine AQP9 protein expression has been demonstrated in liver, skin, epididymal, epidermal and neuronal cells (10,11). It has been well established that AQP9 functions as an

aquaglyceroporin protein in the liver, facilitates hepatic uptake of glycerol (12-14), and attenuates glaucomatous damage (15). AQP9 was first reported to be present in the duodenum, jejunum, ileum and colon (16) and a more recent study demonstrated that AQP9 has important roles in the digestive system (17). Therefore, AQP9 may have an important role in energy metabolism by functioning as channels in metabolic pathways.

The present study aimed to determine the association between AQP9 expression in the intestinal mucosa and conditions in which TJs are disrupted. This was investigated by comparing the expression levels of claudin-1 and MLCK; revealing the pathological features of ileum tissue in the normal control (NC) group, and acute liver injury groups at 2, 48, 72 h time intervals; and determining the association between the expression levels of AQP, Claudin-1 and MLCK.

Materials and methods

Materials. A total of 20 male Sprague Dawley rats (age, 3 months; body weight, 180-240 g), were purchased from the Department of Animal Science and Technology, Nanchang University (Nanchang, China). D-galactosamine was purchased from Shanghai Oripharm Co. Ltd. (Shanghai, China). Antibodies against claudin 1 (cat. no. 4933s; Cell Signaling Technology, Inc., Danvers, MA, USA), MLCK (cat. no. ab76092), AQP9 (cat. no. ab15129) and β-actin (cat. no. ab8228; all Abcam, Cambridge, UK) were used. An immunohistochemical staining kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China).

Establishment of an acute liver injury animal model and preparation of tissue specimens. All animal experiments were approved by the Animal Ethics Association of the First Affiliated Hospital of Nanchang University. Rats were housed in a specific pathogen-free environment at 23±2˚C with 45-65% humidity and under a 12 h light/dark cycle. The animals had free access to food and water. The rats were randomly divided into two groups following one week of adaptive feeding: An acute liver injury group (15 rats) and a NC group (5 rats). Animal models of acute liver injury were established by one intraperitoneal injection of D-galactosamine (D-GalN; 1,200 mg/kg). A total of five animals were sacrificed at 24, 48 and 72 h time intervals post-injection. Rats in the NC group were subjected to intraperitoneal injection of normal saline (12 ml/kg) and then sacrificed at the 72 h time interval. Following this, serum, liver and ileum tissue specimens from all rats were collected for subsequent investigation.

Serum biochemical examination. Whole blood samples were centrifuged at a speed of 1,500 x g for 15 min at 4˚C to separate the serum. Following this, levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (Tbil) and albumin (ALB) were detected in serum samples using an automatic biochemical analyzer (Beckman Coulter Chemistry Analyzer AU5800 Series; Beckman Coulter, Inc., Brea, CA, USA).

Pathological examination of liver and ileum tissues. Liver and ileal tissues (~0.5 g) were collected, washed with PBS, fixed in 10% neutral formaldehyde solution for 24 h at 4˚C and then embedded in paraffin sections. The pathological alterations in liver and ileum tissue were observed under a light microscope (BX41; Olympus, Corporation, Tokyo, Japan), following hematoxylin and eosin staining for 5 min at room temperature. At least five fields of view were captured and analyzed.

Western blot analysis. The expression levels of MLCK, claudin-1 and AQP9 in terminal ileum tissues were detected via western blotting. The same weight (~30 mg) of terminal ileum tissue samples was obtained from rats belonging to each group and total protein was isolated using the illustra triplePrep kit (cat. no. 28-9425-44; GE Healthcare Life Sciences, Little Chalfont, UK). The bicinchoninic acid assay method (BCA Protein Assay kit; cat. no. P0011; Beyotime Institute of Biotechnology, Haimen, China) was used for the determination of protein concentration in the supernatant. Equal amounts of protein (20 μg) were loaded into lanes of 6 and 12% SDS-PAGE. Once the proteins had migrated through the gel, the current was terminated and gels were transferred to polyvinylidene difluoride (PVDF) membranes in transfer buffer at 18 V for 60 min. PVDF membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 1 h. Membranes were incubated overnight at 4˚C with primary antibodies against the following proteins: MLCK (1:250; cat. no. ab76092), AQP9 (1:250; cat. no. ab15129; both Abcam), claudin-1 (1:250; cat. no. 4933s; Cell Signaling Technology, Inc.) and β-actin (1:5,000; cat. no. ab8228; Abcam). Following this, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5,000; cat. no. ab6721; Abcam) for 2 h at room temperature. Membranes were subsequently washed three times with Tris-buffered saline containing 0.1% Tween-20 and proteins were then visualized using Pierce™ ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Each sample was normalized against β-actin expression. The protein bands were analyzed for densitometry using Quantity One software version 1.4.6 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemistry (IHC). IHC assays were performed to determine protein expression levels of claudin-1 and MLCK in terminal ileum tissues obtained from each group. Tissue sections (4 μm) were fixed with 10% neutral-buffered formalin for 24 h at 4˚C and then embedded in paraffin. Paraffin sections were incubated for 2 h at 67˚C and then subjected to dewaxing, boiling and washing with PBS. Following this, sections were incubated with rabbit anti-mouse monoclonal primary antibodies against MLCK (1:250; cat. no. ab76092; Abcam) for 60 min at room temperature, followed by incubation with anti-rabbit IgG horseradish peroxidase-linked antibody (1:200; cat. no. 7074; Cell Signaling Technology, Inc.) at 37˚C for 15 min. Immunohistochemical staining was visualized by incubating sections with 3,3'-diaminobenzidine chromogen for 3 min at room temperature. A 50i Nikon Fluorescence Microscope (Nikon Corporation, Tokyo, Japan) and Adobe Photoshop CS4 software version 11.0 (Adobe Systems, Inc., San Jose, CA, USA) were used to capture and

Table I. Comparative analysis of serum biochemical index levels in acute liver injury model rats at three time intervals and NC rats.

Groups	Rats (n)	ALT (U/l)	AST (U/l)	Tbil (μmol/l)	Alb (g/l)
NC	5	26.67±5.03	113.33±7.02	0.53±0.21	23.33±1.79
M-24 h	5	1,633.53±449.11[a]	3,472.67±314.42[a]	22.15±6.02[a]	23.83±2.78
M-48 h	5	3,886.64±255.62[a]	5,013.50±749.38[a]	60.47±11.74[a]	21.50±1.89
M-72 h	5	935.57±173.05[a]	2,230.75±297.13[a]	114.45±24.46[a]	20.88±2.38[a]

[a]P<0.05 vs. the NC group. M, model group; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Tbil, total bilirubin; ALB, albumin; NC, normal control.

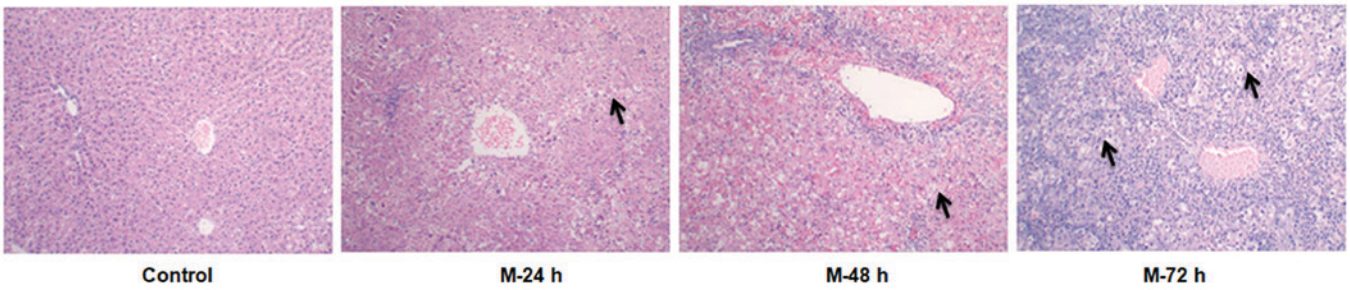

Figure 1. Liver tissues were pathologically examined using hematoxylin and eosin staining (magnification, x200). Acute liver injury models were established by intraperitoneal injection of D-galactosamine (200 mg/kg), and acute liver injury model rats were then separated into three groups and sacrificed at 24, 48 and 72 h time intervals. Arrows indicate areas of necrosis. M, model.

analyze the images. Finally, samples were counterstained with 1 mg/ml hematoxylin for 3 min at room temperature, dehydrated in alcohol and then mounted. Two investigators blinded to the clinical data, semi-quantitatively scored the slides by evaluating the staining intensity and percentage of stained cells in selected areas. At least five fields of view were captured at magnification x200. The staining intensity was scored as 0 (no signal), 1 (weak), 2 (moderate), or 3 (high). The percentage of cells stained was scored as 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). A final score of 0-12 was obtained by multiplying the intensity and percentage scores (18).

ELISA. Expression levels of secreted tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 in the supernatant were investigated using rat TNF-α (cat. no. ab100785), IL-6 (cat. no. ab100772) and IL-10 (cat. no. ab100765; all Abcam) ELISA kits according to the manufacturer's protocol. Absorbance values were determined using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm, and IL-10 concentrations were calculated according to a standard curve and dilution ratio. All assays were performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS software (version 19.0; IBM Corps., Armonk, NY, USA). Experiments were repeated six times and all data are expressed as the mean ± standard error of the mean. For statistical comparison of normal distributed data, a one-way analysis of variance followed by Tukey's post hoc test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Levels of serum biochemical markers in the NC and acute liver injury model groups. The results demonstrated that ALT and AST levels detected in rats in the acute liver injury groups increased in a time-dependent manner until the 48 h time interval; then were levels of ALT and AST were decreased at the 72 h time interval. The levels of Tbil were revealed to increase in a time-dependent manner, and the results also demonstrated that Alb levels were significantly reduced at the 72 h time interval (Table I). These results suggested that rats were suffering from acute liver damage (19). Compared with the NC group, differences regarding levels of ALT, AST and Tbil in acute liver injury groups were statistically significant compared with the (P<0.05; Table I).

Pathological features of liver tissues obtained from rats in each group. The liver tissues obtained from rats in the NC group exhibited intact hepatic lobules, with the lobular central vein presented at the center and the hepatic cord radially arranged, in the absence of marked levels of inflammation, necrosis and degeneration (Fig. 1). The normal structure of liver tissues obtained from rats with acute liver injury at the 72 h time interval disappeared, with inflammatory cell infiltration, liver sinus congestion and hemorrhaging, and the liver cells displayed signs of necrosis (Fig. 1).

Pathological features of terminal ileum tissues in NC rats and acute liver injury rats. Complete mucosal structure was demonstrated in terminal ileum tissues obtained from

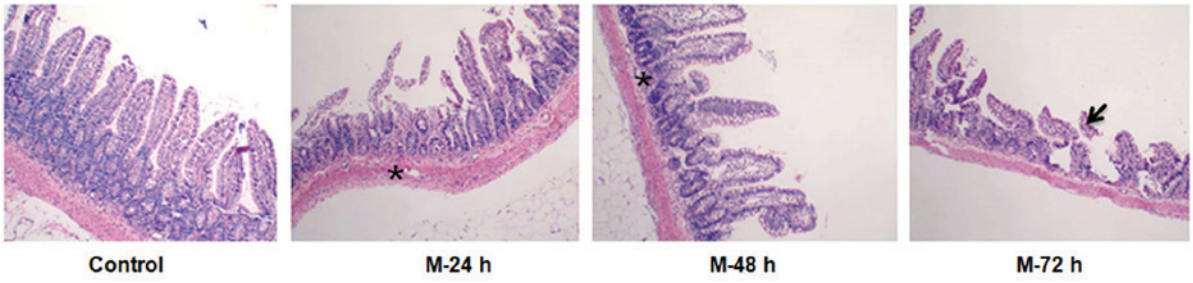

Figure 2. Terminal ileum tissues were investigated via histopathology using H&E staining (magnification, x200). The complete mucosal structure was visualized in terminal ileum tissue obtained from the normal control group, in the absence of inflammation, necrosis and shedding. However, terminal ileum tissue obtained from rats in acute liver injury model groups exhibited increasing levels of neutrophil infiltration (star) in a time-dependent manner, interstitial edema in the villus mucosa and submucosa. Damage to villi (arrow) was observed in terminal ileum tissue obtained from rats in the 72 h acute liver injury model group. M, model; H&E, hematoxylin and eosin.

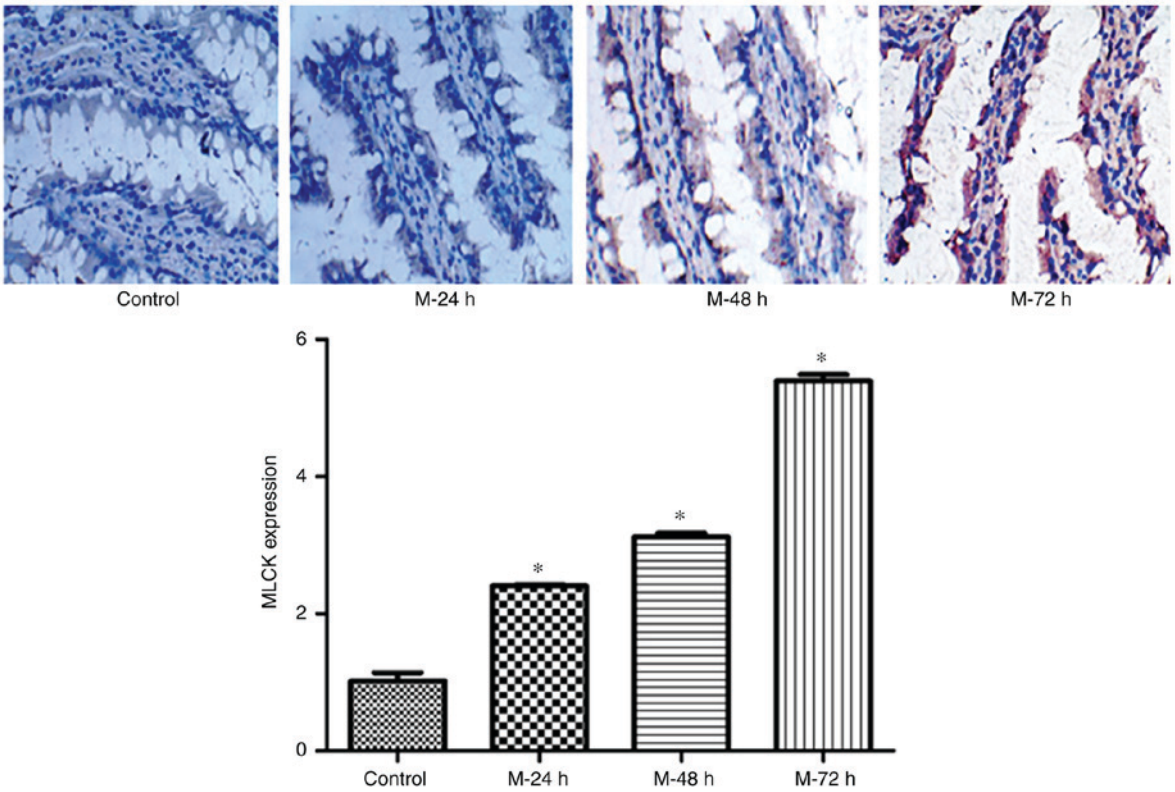

Figure 3. MLCK protein expression was detected by immunohistochemistry (magnification, x400). Terminal ileum tissues obtained from the four experimental groups were used for immunohistochemistry analysis. Quantification data of MLCK expression is presented. *P<0.05 vs. the normal control group. M, model; MLCK, myosin light chain kinase.

NC rats, in the absence of inflammation, necrosis and shedding (Fig. 2). However, terminal ileum tissues obtained from rats belonging to the acute liver injury groups exhibited visibly increased levels of neutrophil infiltration in a time-dependent manner, enhanced levels of interstitial edema in mucosa and submucosa villi, and, at the 72 h time interval, part of the tip of the villi was eroded in the terminal ileum mucosa model 24, 48 and 72 h (Fig. 2).

MLCK, AQP9 and claudin-1 expression levels in terminal ileum tissues obtained from each group as determined by IHC and western blotting. IHC staining was used to determine MLCK protein expression levels, and the results demonstrated that in the sections of ileum obtained from NC rats, slight positive brown staining revealing MLCK protein expression in the cytoplasm was observed; however, positive staining in the mucosa of terminal ileum tissues obtained from acute liver injury rats at 24, 48 and 72 h time intervals was revealed to be significantly increased in a time-dependent manner (P<0.05; Fig. 3). The greatest protein expression levels of MLCK were demonstrated in the model 72 h group (Fig. 3). The results of western blotting revealed that MLCK protein expression levels were significantly increased compared with the NC group in a time-dependent manner (Fig. 4).

Western blotting was also used to investigate AQP9 and claudin-1 protein expression levels in terminal ileum tissues obtained from all rats. The results revealed that both AQP9

Table II. Concentrations of TNF-α, IL-6 and IL-10 in acute liver injury model rats at three time intervals and normal control rats.

	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
NC	8.20±3.12	5.12±1.12	7.12±2.15
M-24 h	29.20±3.54[a]	21.60±3.59[a]	18.69±4.56[a]
M-48 h	53.40±5.21[a]	46.52±3.26[a]	25.78±5.89[a]
M-72 h	30.60±3.58[a]	36.89±4.69[a]	34.65±5.68[a]

[a]P<0.05 vs. the NC group. (x±s; n=5). NC, normal control; M, model group; IL, interleukin; TNF-α, tumor necrosis factor-α.

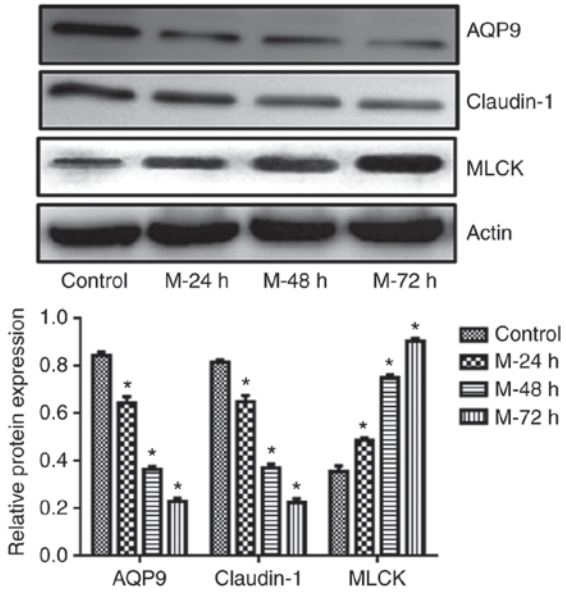

Figure 4. MLCK, AQP9 and claudin-1 protein expression levels were detected via western blotting. Representative blots are presented in the upper panel; Bottom panel: Quantification data are presented. *P<0.05 vs. the normal control group. AQP9, aquaporin 9; MLCK, myosin light chain kinase.

and claudin-1 expression levels were significantly decreased in the terminal ileum of model groups compared with the NC group in a time-dependent manner (P<0.05; Fig. 4).

Concentrations of serum cytokines in NC rats and acute liver injury model rats. Levels of serum TNF-α, IL-6 and IL-10 in each group were determined using ELISA. The results demonstrated that levels of serum TNF-α, IL-6 and IL-10 were significantly increased in the 24 h model group compared with the NC group (P<0.05; Table II). The serum levels of TNF-α, IL-6 and IL-10 were further increased in the 48 h model group. However, in the 72 h model group, levels of TNF-a and IL-6 were suppressed compared with the 48 h group; whereas levels of IL-10 continued to increase in a time-dependent manner (Table II).

Discussion

Liver diseases have previously been reported to be associated with gastrointestinal function (20). When liver function is severely impaired, it is frequently accompanied by endotoxemia; meanwhile in intestinal barrier function disorders, a large number of intestinal bacteria translocate from the intestinal lumen causing infection, and increasing the production of endotoxins (21). In addition, the intestinal canal represents a bacterial repository, and the largest gram-negative bacterial endotoxin pool (22,23). Therefore, it is very important to maintain intestinal barrier function, limit bacterial translocation and reduce endotoxemia in the treatment of liver disease (23,24).

Intestinal mucosal barrier function is essential for the working of the intestine (25). The normal intestinal mucosal barrier is composed of mechanical, chemical, immune and biological barriers, of which the intestinal mechanical barrier is the most important, and is predominantly composed of intestinal epithelial cells and TJs between the epithelial cells. The mechanical barrier, which physically separates the lumen and the internal milieu, facilitates vectorial transport of nutrients ions, and other substances (26). In addition, there is communication between epithelial cells and the immune system (27). Therefore, the mucosal barrier exhibits physical, biochemical and immune features (25). However, numerous inflammatory diseases may induce intestinal mucosal barrier dysfunction, including fulminant hepatitis (28), chronic obstructive pulmonary disease, uremia, severe acute pancreatitis and peritoneal air exposure (29-31). A number of traditional Chinese medicines have been demonstrated to improve intestinal barrier function via inhibition of the nuclear factor-κB-mediated signaling pathway or antioxidative action, inhibiting the production of inflammatory cytokines and upregulation of zona occludens protein 1 mRNA and protein expression levels (29,32-34). The involvement of inflammatory cells and inflammatory factors, as well as the immune response, results in damage to the intestinal epithelial barrier caused by liver injury, and our model displays these pathophysiological changes.

The most important element of the intestinal barrier is TJs, which are composed of both cytosolic and integral membrane proteins. TJs represent the main component of the intestinal mucosal mechanical barrier structure and have an important role in maintaining the integrity and permeability of the intestinal mucosa (1,2). Claudin-1 is an important structural protein within TJs (5,35). In liver injury, the permeability of TJs between intestinal epithelia is increased and the distribution of the TJ proteins are markedly altered on the intestinal mucosal surface, as well as in membrane microdomains (36).

TNF-α is an important regulator of intestinal inflammation that induces an increase in intestinal permeability via extracellular signal-regulated kinase 1/2-dependent activation of ETS transcription factor (Elk-1), which subsequently activates the MLCK gene in the nucleus by binding to the promoter region of MLCK (37,38). However, restoration of impaired mucosal barrier function may be achieved via downregulation of MLCK expression (7,8). The results of the present study demonstrated that levels of serum TNF-α in acute liver injury rats increased in a time-dependent manner. All the aforementioned results suggested that the model of acute liver injury-induced impaired mucosal barrier was successfully established.

Water homeostasis is important for neural signal transduction in the nervous system and the function of the intestinal epithelial barrier (4). AQPs were first observed in the nervous

system (39). Following this, AQPs were revealed to be widely distributed in the liver, duodenum, jejunum, ileum and colon. AQP9 is a transmembrane protein composed of a single polypeptide transmembrane peptide 6 (40). Both rat and human AQP9 are abundantly expressed in the liver, whereas in contrast to rat AQP9 (41), human AQP9 is also expressed in peripheral leukocytes and in tissues that accumulate leukocytes, including the lung, spleen and bone marrow (10,11-14). The promoter region of AQP9 contains putative tonicity and glucocorticoid-responsive elements, suggesting that AQP9 may be regulated by osmolality and catabolism (42), involving osmoregulation of small molecules (43). This mechanism is important to the function of intestinal epithelial barrier (44).

To the best of our knowledge, the present study is the first to investigate the expression and function of AQP9 in the ileum mucosa, and the results demonstrated that expression levels of AQP9 in damaged ileal mucosa were decreased compared with the NC group. Furthermore, the results revealed that claudin-1 expression levels in damaged ileum mucosa were also decreased compared with the NC group; however, MLCK expression levels were increased. These results suggested that AQP9 may have various associations with ileal mucosal damage.

However, the results of the present study have predominantly focused on the association between AQP9 expression levels and the expression of two important makers of the intestinal epithelium; however, the specific molecular mechanism has not been determined. In addition, the present study only investigated the ileum mucosa; the situation of the rest of the small intestine remains unclear.

The results of the present study revealed that AQP9 in the intestinal epithelium has an important role in the development of acute liver injury-induced intestinal epithelium damage.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

TX and NC conceived and designed the study. TX, SG, JW, JX, LY and XW performed the experiments. TX and NC wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Ethics Association of the First Affiliated Hospital of Nanchang University.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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