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Original Paper

Homocysteine Aggravates Intestinal Epithelial Barrier Dysfunction in Rats with Experimental Uremia

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Key Words
Homocysteine • Uremia • Intestinal permeability • Tight junction • Probiotics

Abstract
Background/Aims: Previous studies have shown that homocysteine (Hcy) is an important intestinal-derived uremic toxin. However, whether Hcy is involved in the epithelial barrier dysfunction observed in uremia remains unclear. This study aimed to investigate the effect of Hcy on intestinal permeability and intestinal barrier structure and function in adenine-induced uremic rats. ***Methods:*** Sprague-Dawley rats were divided into five groups: normal control (group NC), Hcy (group H), uremia (group U), uremia + Hcy (group UH), and uremia + Hcy + VSL#3 (group UHV). Experimental uremia was induced by intragastric adenine administration, and Hcy was injected subcutaneously. The animal models were assessed for renal function and pathological tissue staining. The pathological changes of intestinal tissue were observed by hematoxylin and eosin staining and electron microscopy. The serum and intestinal tissue levels of Hcy, interleukin (IL)-6, tumor necrosis factor (TNF)-α, superoxide dismutase (SOD), and malondialdehyde (MDA) as well as serum endotoxin and intestinal permeability were assessed. The levels of the tight junction proteins claudin-1, occludin, and zonula occludens-1 (ZO-1) were assessed by western blotting. ***Results:*** Blood analyses and renal pathology indicated that experimental uremia was induced successfully. Pathological damage to intestinal structure was most obvious in group UH. Serum and tissue Hcy, serum endotoxin, and intestinal permeability were significantly elevated in group UH. The protein levels of claudin-1, occludin, and ZO-1 were decreased to various degrees in group UH compared with groups NC, H, and U. The serum and tissue levels of IL-6, TNF-α, and MDA were significantly increased, while SOD activity was markedly decreased. Supplementation with the probiotic VSL#3 improved these parameters to various degrees and up-regulated the abundance of tight junction proteins, which indicated a role for Hcy in the increase of intestinal permeability and destruction of the epithelial barrier in uremia. ***Conclusion:*** Hcy aggravates the increase of intestinal permeability

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and destruction of the epithelial barrier by stimulating inflammatory and oxidative damage. Probiotic administration can ameliorate this damage by reducing the levels of Hcy-induced inflammation and oxidation.

Introduction

Chronic kidney disease (CKD) is highly associated with damage to any part of the gastrointestinal tract [1], and the destruction of intestinal permeability and barrier structure is a common but serious complication in patients with end-stage renal disease (ESRD) [2, 3]. The intestinal tract is the main source of uremic toxins and gut-derived solutes, such as indoxyl sulfate, p-cresol sulfate, and advanced glycation end products, which not only damage the structure of the intestinal epithelial barrier but also allow bacteria, endotoxin, and other toxic metabolites to enter the blood easily, causing serious damage to blood vessels and accelerating the deterioration of renal function by oxidative stress and inflammatory reactions [4].

Homocysteine (Hcy), a thiol-containing amino acid produced during the metabolism of methionine, has been shown to be associated with many pathological conditions. Hyperhomocysteinemia (HHcy) can aggravate dextran sulfate sodium-induced colitis by stimulating the expression of inflammatory substances [5], impair endothelial cell function by destroying the endothelial barrier, enhance the permeability of endothelial cells, and drive inflammatory reactions [6, 7]. Hcy has also been confirmed to be an important intestinal-derived uremic toxin [8], and elevated plasma Hcy levels are common in patients with uremia. HHcy has been linked to higher cardiovascular risk and mortality in uremic patients on hemodialysis [9, 10]. The level of Hcy is mainly influenced by its nutritional determinants, including folate, vitamin B6, and vitamin B12, and some genetic polymorphisms of enzymes involved in its metabolic cycle [11]. Previous studies showed that folate supplementation decreased the plasma concentration of Hcy and ameliorated Hcy-aggravated colitis [5].

VSL#3 is a probiotic that is manufactured with a carefully designed formulation of billions of bacteria that maintain vitality. Recent studies have shown that supplementation with probiotics can improve intestinal barrier function and local intestinal immunity, enhance antagonism toward pathogens to reduce mucosal dysbiosis, and decrease microinflammation in uremic rats [12-14]. However, it is unclear whether probiotics can decrease Hcy levels and improve HHcy.

The intestinal barrier consists of epithelial cells and the apical junctional complex. The tight junction is the most luminal component of the barrier apparatus and forms an effective barrier against the influx of microbes, microbial toxins, antigens, digestive enzymes, and other noxious substances from the gastrointestinal lumen to the internal milieu. Claudins, occludin, and zonula occludens-1 (ZO-1) are the main transmembrane proteins that connect the junctional complex with cytoskeletal proteins. Intestinal barrier damage can be induced by inflammation, oxidative stress, and toxic metabolites [15]. Hcy, together with indoxyl sulfate and p-cresol sulfate, as the main colon-derived toxins and important pro-inflammatory molecules are risk factors for many chronic inflammatory diseases [16]. Notably, recent studies of Hcy have mainly focused on its effects on myocardial and vascular endothelial cells. However, whether Hcy contributes to the inflammation and destruction of the epithelial barrier in uremia and its underlying mechanisms have rarely received attention.

Therefore, considering the special role of Hcy in uremic toxins and the high homology and similarity in structure and function between the vascular and intestinal epithelium, we developed a chronic experimental model in rats with adenine-induced uremia to study whether Hcy could aggravate intestinal inflammation and destruction of epithelial barrier function in uremia and whether the administration of a probiotic could exert a beneficial effect by inhibiting Hcy-mediated inflammatory and oxidative events.

Materials and Methods

Ethics approval

This study was approved on March 6, 2015 by the "Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University" in Shannxi, China. The study began in January 2016 and will end in December 2019. Animal study was carried out in accordance with the criteria outlined in the Guide for the Care and Use of Laboratory Animals. Ethics approval number is XJTU1AF2015KSL-086.

Animals and main reagents

A total of 65 adult male Sprague Dawley (SD) rats (180~220 g) obtained from the Animal Center of the School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi, China, were used for the experiment. Animals were housed at room temperature with a 12h-12h light-dark cycle with free access to food and water. Adenine and DL-Homocysteine (Hcy) were purchased from Sigma (USA). VSL#3 probiotics capsules (Lot: 05150542; Expiration date: 11 /August /2017) were purchased from Alfa sigma Inc (Sigma-Tau Pharmaceuticals, Inc. USA).

Experiment design and sample collection

The SD rats (180~220 g) were first divided into two groups: a control group (n = 20) and a uremia group (n = 45). Rats in the control group underwent daily intragastric administration of normal saline, while those in the uremia group had experimental uremia induced by intragastric adenine for 28 days, at 250 mg/kg/d continuously in the first 14 days and every other day in the latter 14 days. 28 days after completion of the intragastric adenine, the renal function of 20 rats (randomly selected from the uremic groups) was measured by tail vein blood samples, and the results showed a successful adenine-induced uremic rat model.

Then the normal group rats were randomly re-grouped into normal control group (Group NC, n = 10, underwent daily intragastric administration of normal saline and subcutaneous injection of normal saline) and Hcy group (Group H, n = 10, received daily intragastric normal saline and subcutaneous injection of Hcy). The uremic rats were randomly divided into the following three experimental groups: uremia (Group U, n = 10, received saline instead for injection and intragastric); uremia + Hcy (Group UH, n = 10, rats were injected subcutaneously with Hcy 0.06μmol/g/d from the first day for 30 consecutive days); uremia + Hcy + VSL#3 probiotics (Group UHV, n = 10, 2 capsules contains 225 billion strains per day of each rat for 30 consecutive days and the probiotics powder were dissolved in 1ml saline for intragastric administration). The rats were anesthetized with 10% chloral hydrate by intraperitoneal injection at a dose of 1 ml/kg before collecting specimen. Blood samples were obtained from the abdominal aorta and centrifuged at 3000 rpm for 15 min. The supernatant was collected and stored at -80°C. The intestinal tissues were cut along the longitudinal axis and washed with ice-cold normal saline. Parts of the tissues were used for pathological staining and transmission electron microscopy observation. The remaining was stored at -80°C for other investigations.

Intestinal permeability

Five animals from each of the group were treated by gavage of technetium-99 m-diethylenetriaminepentacetic acid (99mTc-DTPA, Jiangsu Institute of Nuclear Medicine& Jiangyuan Pharmaceutical Factory, Wuxi, China) (5μ Ci in 1mL of water). The rats were placed individually in metabolic cages and allowed to drink water ad libitum for 24h. A total of 500μL of urine and peripheral blood was collected for radioactivity determination by a gamma counter. Intestinal permeability was calculated by the following equations:

$$\text{Radioactivity dose} = \text{Volume (blood or urine)} \times \text{dose per 500 } \mu\text{L} \quad (1)$$

$$\text{Permeability} = \frac{\text{Radio activity dose in blood+urine}}{\text{Total administered dose}} \times 100\% \quad (2).$$

Histological analysis

Intestinal tissues were collected for histological analysis. For staining analysis, each specimen was fixed in 4% paraformaldehyde solution (Dingguo, Beijing, China) for 24h. The tissues were dehydrated, cleared, embedded in paraffin, cut into 4- to 5-μm -thick sections, and stained by Haematoxylin and eosin (H&E, servicebio, Wuhan, China) and Masson's trichrome staining (servicebio, Wuhan, China) separately. For transmission electron microscopy analysis, intestinal tissue pieces were fixed in 2.5% glutaraldehyde solution in 0.15 M phosphate buffer at pH 7.2. Specimens were post-fixed in 1% osmium tetroxide dissolved in saline and impregnated overnight in an aqueous solution of 1% uranyl acetate. Specimens were embedded in polyester resin, and ultrathin sections of the embedded specimens were made. Sections were stained with lead citrate and uranyl acetate and viewed with an H-7650 electron microscope (Hitachi, Tokyo, Japan).

Blood analyses and intestinal homogenate detection

Creatinine and urea nitrogen were tested to assess the kidney function. Detection of homocysteine levels in serum and intestinal tissues homogenate were performed using the Enzyme Cycle-LST Assay Kit (Maker Biotechnology, Sichuan, China) according to the manufacturer's instructions. The serum endotoxin levels were detected by a Limulus Amebocyte assay (Jinshanchuan, Beijing, China). The serum levels of interleukin IL-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were detected by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, USA). Total Superoxide Dismutase (SOD) Assay Kit with WST-8 and malondialdehyde (MDA) test kits were obtained from Beyotime Institute of Biotechnology, Shanghai, China.

Western blotting analysis of tight junction proteins

Total proteins were extracted from intestinal tissues and were solubilized in RIPA buffer (0.1% SDS, 1% NP40, and 0.5% sodium deoxycholate; HEART, Xi'an, China) containing proteinase inhibitors (1% cocktail and 1mmol/L phenylmethylsulfonyl fluoride (PMSF); Sigma-Aldrich, USA). Protein concentrations were determined by BSA assay kit (Pierce, Rockford IL, USA) and 40μg of total protein from each sample were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels at 70V for 30 min and then 110V until the end and transferred to polyvinylidene fluoride (PVDF) membranes (Millpore, USA). Membranes were incubated with the primary antibodies overnight at 4°C (Abcam Technology, UK: claudin-1, 1:1000; occludin, 1:500; Thermo Fisher Scientific, USA: ZO-1, 1:1000). Incubation with the peroxidase-conjugated secondary antibodies (Boster Biological Technology, Wuhan, China) was carried out at a 1:5000 dilution at room temperature for 1 hour. Detection was performed with an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford IL, USA). Bands were scanned and quantitated by densitometry using NIH Image software.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA). Quantitative data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the mean values of more than two groups, with the Student-Newman-Keuls test used for *post hoc* analysis of multiple comparisons. A p-value of less than 0.05 ($p < 0.05$) was considered significant, and a p-value of less than 0.01 ($p < 0.01$) was considered highly significant.

Results

Evaluation of adenine-induced uremia and exogenous Hcy injection-induced mild HHcy

Fifteen rats had died by the end of the experiment, leaving a total of 10 rats in each group. There was a marked decrease of physical and mental activity, food intake, and body weight of the rats after initiating adenine treatment that increased slowly, and this was accompanied with a yellow-haired and dispirited appearance in uremia and uremia + Hcy group rats throughout the experiment. The final body weight (mean ± SD) of the rats in each group was significantly different. Compared with the normal control group (group NC; 370 ± 11.87 g), the final body weight of the Hcy group (group H; 338 ± 12.49 g), uremia group (group U; 285

± 15.66 g), uremia + Hcy group (group UH; 270 ± 10.14 g), and uremia + Hcy + VSL#3 group (group UHV; 293 ± 10.08 g) was significantly reduced ($p < 0.01$). The body weight of group H showed a slower increase compared with group NC ($p < 0.01$), but was much higher than that of groups U, UH, and UHV ($p < 0.01$). At the end of the experiment, the body weight of group UH was also lower than that of group U, but not significantly so ($p > 0.05$). Although the body weight of group UHV was much lower than that of groups NC and H, it was significantly higher than that of group UH ($p < 0.01$) (Fig. 1A). The serum creatinine (Scr) and blood urea nitrogen (BUN) levels of group H were slightly elevated ($p > 0.05$) compared with group NC, while they were significantly increased in groups U, UH and UHV compared with group NC ($p < 0.001$). The Scr and BUN levels of group UH were slightly higher than those of group U ($p > 0.05$); however, the renal function of group UHV was not significantly improved compared with group UH ($p > 0.05$) (Fig. 1B, 1C). Renal pathological changes (Fig. 1F, 1G) detected by hematoxylin and eosin (H&E) and Masson's trichrome staining showed that glomerular integrity and the renal interstitium were basically complete and generally normal in groups NC and H. Renal pathology in groups U, UH, and UHV changed drastically with glomerular mesangial proliferation, extensive expansion of renal tubules and even partial atrophy, interstitial collagen fiber hyperplasia, and a large number of infiltrating inflammatory cells, and these changes were most obvious in group UH.

Hcy concentrations were measured in serum samples (Fig. 1D) and ileum homogenates (Fig. 1E) from the rats of each group. As expected, continuous subcutaneous injection of Hcy significantly increased blood and intestinal tissue Hcy concentrations in group H compared with group NC ($p < 0.05$). HHcy is very common during uremia. Our results showed that there was a moderate and even severe increase of Hcy levels in uremic rats ($p < 0.01$). More importantly, uremia combined with Hcy administration (group UH) had a greater impact on the concentrations of Hcy in both serum and tissue, which were especially high compared with the other groups ($p < 0.001$). Interestingly, after intervention with the probiotic VSL#3, the serum and intestinal tissue levels of Hcy decreased significantly in group UHV ($p < 0.05$).

Histopathological evaluations of the ileum

Fig. 2A shows the H&E staining images used for histological examinations (A1–A4 are representative images from each respective group). Group NC exhibited approximately normal intestinal structure with tall and tightly lined villi. There was no stratification or inflammatory cell infiltration of the submucosa. The intestinal villi in group H showed mild looseness and slight submucosal stratification. The intestinal villi in group U were ruptured and loosely arranged, and the mucosal and submucosal membranes were separated and extended to both sides of the intestinal villi with infiltrating inflammatory cells. In group UH, the damage to the villi and submucosal tissue was more serious, with an arrangement of sparse, ruptured, and bald villi, and the submucosal tissue was more profoundly stratified.

Fig. 2B shows transmission electron microscopy photomicrographs (B1-B4 are representative images from each respective group). Group NC exhibited a tight arrangement of intestinal microvilli and clear intercellular tight junction structures. Group H showed slightly sparse microvilli and basically normal tight junction structures. However, in group U, there were sparse and irregular microvilli, lightly stained tight junctions between epithelial cells, and obvious edema with visible collagen fibers in the intestinal mucosa. More seriously, group UH exhibited profound edema and deposition of many collagen fibers in the mucosa, with decreased or even fractured microvilli and greater disorganization of the epithelial layer.

Hcy aggravates the uremia-induced increase of intestinal permeability and decrease of tight junction protein abundance

Endotoxin level is a commonly used indirect measure of intestinal permeability. Intestinal permeability is an important indicator of the intact structure and function of the intestinal epithelium. We found that endotoxin level (Fig. 3A) and intestinal permeability (Fig. 3B)

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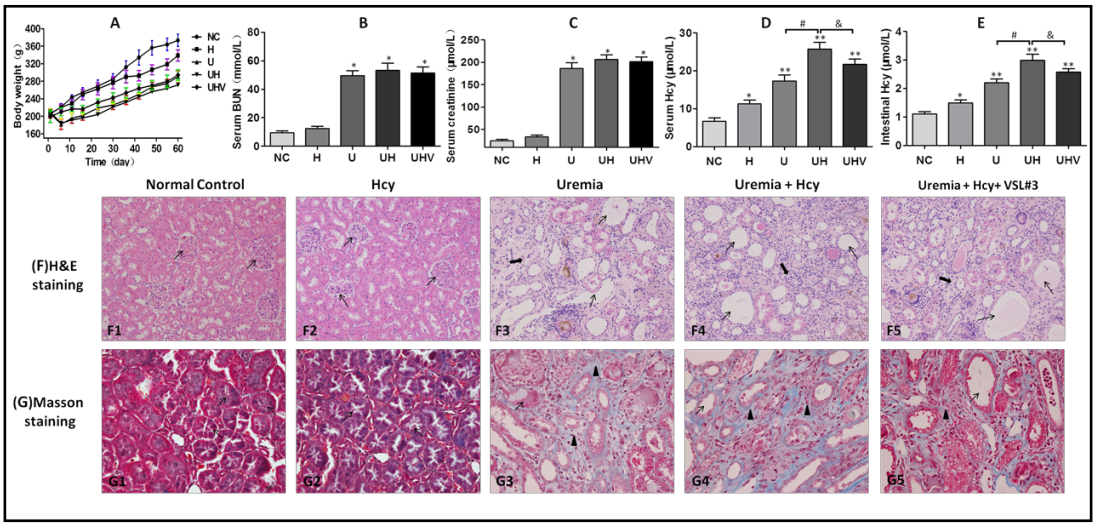

Fig. 1. Body weight changes, renal function, serum and intestinal Hcy, and pathological changes of renal structure of each group. [A] The average daily body weight of rats in each group during the experiment. The body weight of rats began to decrease after initiating treatment with adenine and continued to increase slowly, which was accompanied with a yellow haired and dispirited appearance in groups U and UH throughout the experiment. The final body weight (mean ± SD) of the rats in each group was significantly different: the final body weight of rats in group H (338 ± 12.49 g), group U (285 ± 15.66 g), group UH (270 ± 10.14 g), and group UHV (293 ± 10.08 g) was significantly lower than that of group NC (370 ± 11.87 g) ($p<0.01$); the body weight of group H was lower than that of group NC ($p<0.01$), but was much higher compared with groups U, UH, and UHV ($p<0.01$); the body weight of group UH was smaller than that of group U, but not significantly so ($p > 0.05$); the body weight of group UHV was significantly less than that of groups NC and H, but was significantly higher than that of group UH ($p<0.01$). [B] [C] BUN and Scr. The BUN and Scr levels of group H were slightly elevated ($p > 0.05$) compared with group NC; their levels in groups U, UH and UHV were significantly increased compared with group NC ($p<0.001$); the BUN and Scr levels of group UH were slightly higher than those of group U ($p > 0.05$); there was no significant difference in their levels between groups UHV and UH ($p > 0.05$). [D] [E] Serum and intestinal Hcy. The concentrations of serum and intestinal Hcy were significantly increased in groups H, U, and UH compared with group NC. The levels of Hcy in serum and intestinal tissue were decreased significantly in group UHV compared with group UH. [F] [G] Representative graphs of renal histopathological structural changes. (H&E and Masson's trichrome staining, magnification ×200, scale bar 100 μm.) Glomerular integrity and the renal interstitium were basically complete and generally normal in groups NC and H. Renal pathology changed drastically in groups U, UH, and UHV with glomerular mesangial proliferation, extensive expansion of renal tubules and even partial atrophy, interstitial collagen fiber hyperplasia, and a large number of infiltrating inflammatory cells. Thin arrows indicate the glomeruli; thick arrows indicate the renal interstitium with infiltrating inflammatory cells; small triangles represent the increased collagen fibers in the interstitial tissue of the kidney. Data were analyzed by the one-way ANOVA with a post hoc Tukey's or Dunn's test. *$p<0.05$, vs. group NC; #$p<0.05$, group UH vs. group U; &$p<0.05$, group UHV vs. group UH.

were slightly but significantly increased in group H ($p < 0.05$). Both parameters were clearly increased in groups U and UH compared to group NC ($p < 0.01$); notably, group UH had a more significant increase of endotoxin level and intestinal permeability compared with the other groups ($p < 0.001$). In addition, serum endotoxin level and intestinal permeability were significantly improved after supplementation with the probiotic VSL#3 ($p < 0.05$). Fig. 3C shows the levels of tight junction proteins in intestinal tissues. Consistent with the changes in intestinal permeability, the protein expression of claudin-1 (Fig. 3D; $p < 0.05$), occludin (Fig. 3E; $p < 0.01$), and ZO-1 (Fig. 3F; $p < 0.05$) in intestinal segments was reduced in group H. Moreover, the expression levels of these three tight junction proteins were all significantly decreased in groups U ($P < 0.01$) and UH ($P < 0.001$) compared to group NC.

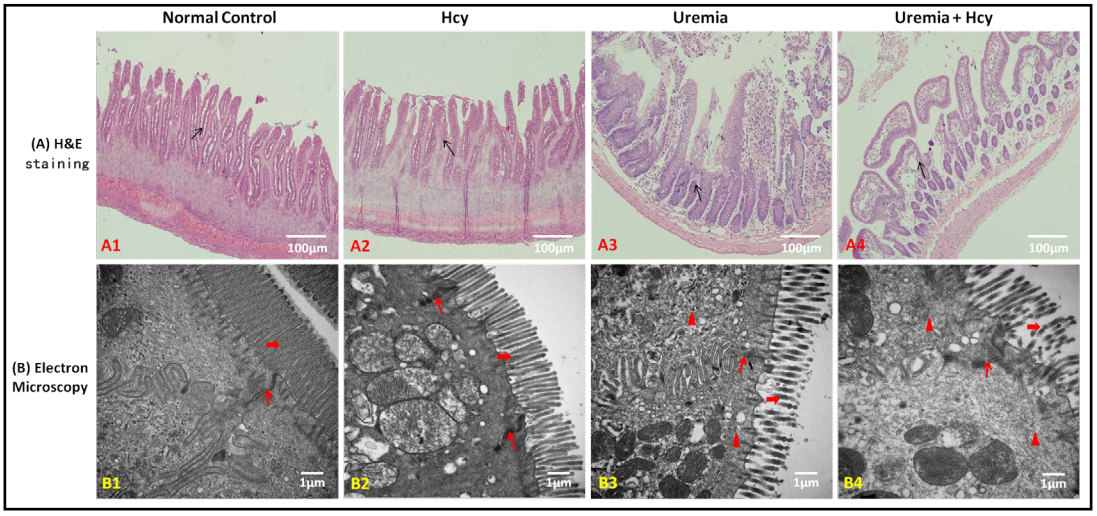

Fig. 2. Representative photomicrographs of H&E staining and transmission electron microscopy detection of the ileum. [A] H&E staining of ileal pathological changes (magnification ×200, scale bar 100 μm). The black thin arrows indicate intestinal villi. Group NC exhibited approximately normal intestinal structure with tall and tightly lined villi. There was no stratification or inflammatory cell infiltration of the submucosa. The intestinal villi in group H showed mild looseness and slight submucosal stratification. The intestinal villi in group U were ruptured and loosely arranged, and the mucosal and submucosal membranes were separated and extended to both sides of the intestinal villi with infiltrating inflammatory cells. In group UH, the damage to the villi and submucosal tissue was more serious, with an arrangement of sparse, ruptured, and bald villi, and the stratification of the submucosal tissue was more severe. [B] Transmission electron microscopy photomicrographs of the ileal mucosa of each group. Thin arrows indicate tight junctions; short thick arrows indicate intestinal villi; triangles indicate inflammatory edema and collagen fibers (magnification ×30,000, scale bar 1 μm). Group NC exhibited a tight arrangement of intestinal microvilli and clear intercellular tight junction structure. Group H showed slightly sparse microvilli and basically normal tight junction structure. Group U exhibited sparse and irregular microvilli, lightly stained tight junctions between epithelial cells, and obvious edema with visible collagen fibers in the intestinal mucosa. Group UH exhibited serious edema and the deposition of many collagen fibers in the mucosa, with decreased or even fractured microvilli and worse organization of the epithelial layer. The tight junction structure was fuzzy and shallow and its continuity was destroyed.

Hcy exacerbates the destruction of intestinal epithelial barrier structure and function by inducing oxidative and inflammatory states

Hcy may aggravate the severity of inflammation and oxidative damage induced by uremia. Treating the rats with a single injection of Hcy (group H) induced mild inflammation and oxidation both in the serum and intestine as compared with group NC ($p < 0.05$). Meanwhile, both the serum and intestinal homogenate levels of IL-6 (Fig. 4A, 4E), tumor necrosis factor (TNF)-α (Fig. 4B, 4F), and malondialdehyde (MDA) (Fig. 4C, 4G) were increased and superoxide dismutase (SOD) activity (Fig. 4D, 4H) was decreased markedly in group UH compared with groups U and NC ($p < 0.001$). The increased levels of inflammation and oxidative cytokines were consistent with the increased levels of Hcy (Fig. 1D, 1E), endotoxin (Fig. 3A), intestinal permeability (Fig. 3B), and tight junction protein damage (Fig. 3D). These results suggested that Hcy may be involved in the deterioration of the barrier function of the intestinal epithelium during uremia by inducing inflammatory and oxidative damage.

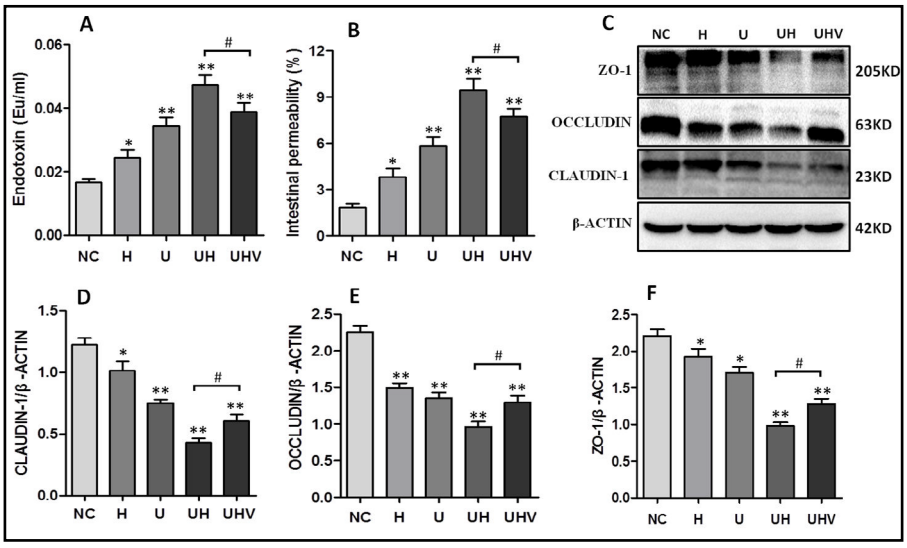

Fig. 3. Effect of Hcy on endotoxin levels, intestinal permeability, and tight junction protein abundance in each group. [A] Endotoxin. [B] Intestinal permeability. [C] Western blotting bands. [D] [E] [F] Quantitative analysis of the protein levels of claudin-1, occludin, and ZO-1, which were normalized to β-actin. Compared with group NC, serum endotoxin levels and intestinal permeability were all increased in groups H, U, and UH, especially in group UH. The protein expression of claudin-1, occludin, and ZO-1 in intestinal segments was significantly reduced in groups U and UH compared with groups NC and H. Endotoxin levels and intestinal permeability were significantly decreased in group UHV with improved tight junction protein levels compared to group UH. Data were analyzed by one-way ANOVA with a post hoc Tukey's or Dunn's test. *p<0.05, **p<0.01 vs. group NC; #p<0.05, group UHV vs. group UH.

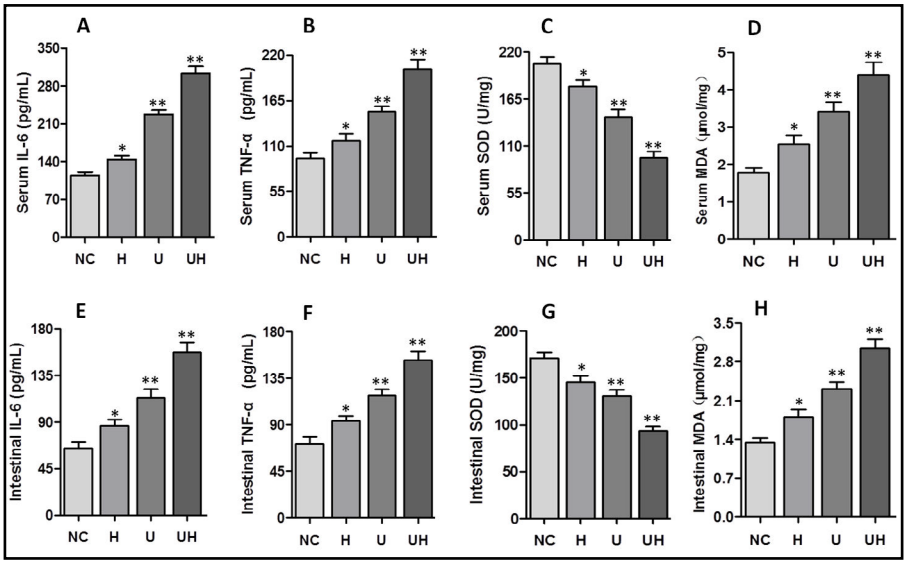

Fig. 4. Levels of serum and intestinal inflammatory and oxidative markers in each group. [A] [B] Serum IL-6 and TNF-α. [C] [D] Serum SOD and MDA. [E] [F] Intestinal IL-6 and TNF-α. [G] [H] Intestinal SOD and MDA. The serum and intestinal homogenate levels of IL-6, TNF-α, and MDA were all increased with decreased SOD activity in groups H, U, and UH compared with group NC. Data were analyzed by one-way ANOVA with a post hoc Tukey's or Dunn's test. *p<0.05, **p<0.01 vs. group NC.

VSL#3 ameliorates Hcy-aggravated intestinal barrier dysfunction in uremic rats

VSL#3 is manufactured with a carefully designed formulation of billions of eight strains of live freeze-dried lactic acid bacteria. In our study, we found that after intervention with probiotic VSL#3, the serum and intestinal tissue levels of Hcy were decreased significantly in group UHV compared with group UH (Fig. 1D, 1E; $p < 0.05$). Interestingly,

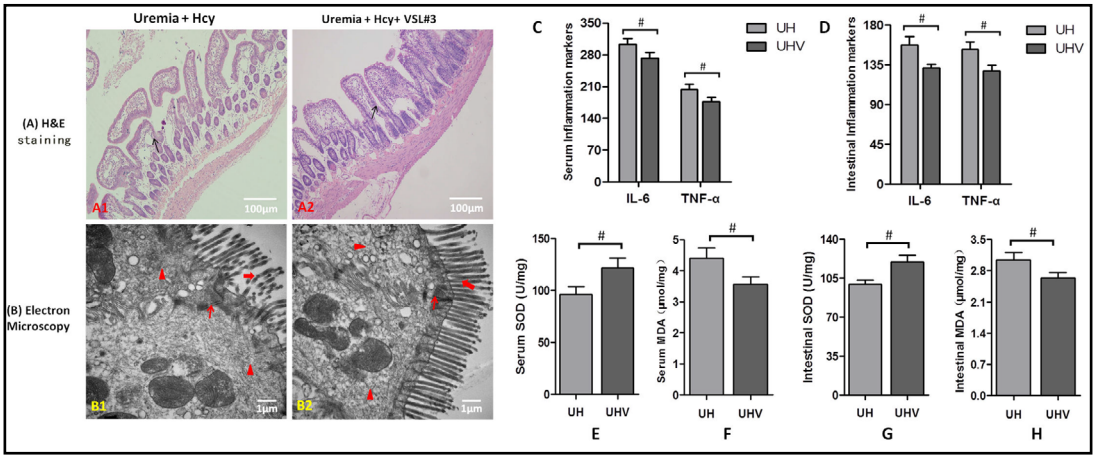

Fig. 5. Representative intestinal photomicrographs of H&E staining and transmission electron microscopy analysis of groups UH and UHV. [A] H&E staining photomicrographs. In group UHV, the damage to the intestinal mucosa showed an obvious improvement. Submucosal stratification was discontinuous and the broken and disrupted villi were reduced compared with group UH. [B] Transmission electron microscopy photomicrographs. In group UHV, the destruction of the intestinal epithelium and mucous membranes was reduced considerably with better organization of the microvilli and decreased collagen deposition compared with group UH. [C] Serum interleukin (IL)-6 and TNF-α. [D] Intestinal IL-6 and TNF-α. [E] Serum SOD. [F] Serum MDA. [G] Intestinal SOD. [H] Intestinal MDA. Compared with group UH, the serum and intestinal tissue levels of IL-6, TNF-α, and MDA were decreased with an improvement of SOD to different degrees in group UHV. Student's t-test was used to examine the differences in inflammatory and oxidative markers between groups UHV and UH. #p<0.05, group UHV vs. group UH.

we also found that damage to the intestinal mucosa was improved slightly in group UHV, and submucosal stratification was discontinuous and the broken and disrupted villi were reduced compared with group UH according to H&E staining (Fig. 5A). Transmission electron microscopy observation demonstrated that tight junction structure was fuzzy and shallow and its continuity was clearly destroyed in group UH. However, in group UHV, the destruction of the intestinal epithelium and mucosal membranes was reduced considerably with an improvement in the organization of microvilli and decreased collagen deposition (Fig. 5B). More importantly, after supplementation with the probiotic VSL#3, the protein levels of the three tight junction markers claudin-1, occludin, and ZO-1 were significantly increased compared with group UH (Fig. 3C-F; $p < 0.05$). However, the expression levels of these proteins were still significantly different between groups NC and UHV ($p < 0.05$). In addition, in group UHV, the serum and tissue levels of the above-mentioned inflammatory and oxidative markers were significantly improved to different degrees compared with group UH (Fig. 5C-H; $p < 0.05$).

Discussion

In this study, we explored the effect of Hcy on intestinal epithelial structure and function in uremic rats. The main results were as follows. (1) Hcy can aggravate the increase of intestinal epithelial permeability and tight junction structural dysfunction in uremic rats. (2) The deterioration of intestinal epithelial structure and function by Hcy occurred through inflammation and oxidative damage. (3) Supplementation with the probiotic VSL#3 can reduce the serum and intestinal levels of Hcy, and thus alleviate Hcy-aggravated inflammation and oxidative injury to ameliorate intestinal epithelial barrier function in uremic rats. These results might have important implications in our understanding of the toxicity of Hcy on the intestinal epithelium and the importance of reducing HHcy in uremia.

The epithelial cells of the gastrointestinal tract are critical in preventing the entry of microbial toxins and harmful metabolic products into sub-epithelial tissues and the internal milieu. Hcy is an important intestinal-derived uremic toxin originating from endogenous metabolism and intestinal bacterial metabolism [8]. Plasma Hcy levels are clearly elevated in patients with ESRD, and the relationship between Hcy and CKD-associated cardio-cerebrovascular complications has been studied extensively [10, 17]. Unfortunately, the involvement of Hcy in intestinal epithelial barrier dysfunction in uremia is understood poorly. Accordingly, in this study, we speculated that Hcy may play a critical role in the intestinal epithelial barrier injury that occurs during uremia, and we found that both serum and intestinal Hcy levels were significantly increased in groups H and U, which is in line with previous studies. Interestingly, we also found that Hcy levels were evidently increased in group UH, indicating that Hcy may accumulate more easily in a state of illness such as uremia. Meanwhile, we also found that the intestinal histopathological changes observed in group UH rats exhibited more serious inflammatory cell infiltration, collagen fiber deposition, and decrease or facture of intestinal microvilli compared to groups H and U. Of note, the decreased expression of tight junction proteins was also accompanied by the marked increase of endotoxin levels and intestinal permeability in group UH, indicating that elevated Hcy levels may be associated with the aggravation of increased intestinal permeability and epithelial barrier dysfunction in adenine-induced uremia.

Previous studies reported that Hcy promotes the inflammatory reaction of intestinal microvascular endothelial cells *in vitro* [18] and aggravates colonic inflammation in colitis rats [5]. Previous studies also demonstrated that uremia is characterized by chronic local and systemic inflammation [19], which may cause intestinal bacterial translocation, microbiome dysbiosis, increased levels of toxic metabolites, and destruction of intestinal epithelial structure [12, 20]. Hcy plays a critical role in the denaturation of key molecules through DNA and protein hypomethylation and can induce the generation of reactive oxygen species and elicit oxidative damage by various means [21]. It can also up-regulate the expression of many pro-inflammatory factors, such as TNF-α, IL-6, IL-1β, and interferon-γ, and these proteins can conversely affect the intestinal mucosa and enhance its permeability by destroying cell-cell tight junctions [22]. Our study firstly revealed that rats in group UH had much higher Hcy concentrations than rats in groups H and U, and the concentration of Hcy was tightly related to the destruction of the intestinal epithelium of uremic rats. In order to explore further the mechanism by which Hcy destroys tight junction structure and function in uremic conditions, we detected the levels of the inflammatory and oxidative markers TNF-α, IL-6, MDA, and SOD in both serum and intestinal homogenates, and the results showed that their serum and intestinal tissue levels were increased evidently in group UH, suggesting that there was a positive correlation between the concentration of Hcy and the levels of oxidation and inflammation as well as intestinal permeability and the degree of intestinal epithelial tight junction dysfunction. Taken together, these results showed that Hcy significantly increased the serum and intestine levels of inflammatory and oxidative markers in uremic rats. In other words, Hcy can aggravate the increased permeability of the intestinal barrier and exacerbate intestinal epithelial damage through the induction of oxidative and inflammatory lesions in uremia.

HHcy can be found in any stage of chronic renal insufficiency and in uremic patients treated with various therapeutic methods [23]. The majority of patients with uremia, especially those who receive maintenance hemodialysis, have metabolic disorders, dietary restriction, and therapeutic factors that not only lead to reduced intake of folic acid and vitamins but also problems with their absorption [24]. Thus, abnormal Hcy metabolism and the weakened effect of vitamins combine to promote the formation of HHcy in uremia. Folate, vitamin B6, and vitamin B12, as important regulators of Hcy metabolism, have a strong influence on Hcy levels and play crucial roles in multiple key metabolic pathways [25]. Ramalingam et al. demonstrated that restricting the intake of methionine could improve intestinal barrier function by changing the composition of tight junction proteins

[26]. Waskiewicz et al. found that a deficiency of folate, vitamin B6, and vitamin B12 can lead to elevated plasma Hcy levels, and their supplementation can reduce plasma Hcy levels [27].

Probiotics have been shown to regulate gut intestinal microbiota [28] and improve intestinal epithelial cell structure and function in pathologic conditions [29]. It has been reported that some commensal bacterial species and probiotic bacteria can produce many kinds of vitamins, such as vitamins in the B and K groups, folate, and nicotinic acid, which are directly involved in essential energy metabolism in the gut [30, 31]. VSL#3 is manufactured with a carefully designed formulation of billions of eight strains of live freeze-dried lactic acid bacteria. Valentini et al. reported that diet plus VSL#3 was associated with a clinically relevant reduction in Hcy levels and increased levels of folate and vitamin B12 [32]. In the present study, after the administration of the probiotic VSL#3 for 30 days, rats in group UHV showed an outstanding systemic improvement. On the one hand, body weight gain, hair color, appetite, and activity all improved after VSL#3 treatment; on the other hand, Hcy levels as well as inflammatory and oxidative cytokines in serum and intestinal mucosa were significantly reduced, and the abundance of tight junction proteins was ameliorated and accompanied with decreased intestinal permeability. These results indicated that probiotics can not only regulate the intestinal microbiota and promote intestinal bacteria to produce vitamin B, folic acid, and other vitamins so as to participate in the metabolism of Hcy and decrease Hcy levels but also improve intestinal tight junction structure and function in uremia by alleviating Hcy-aggravated inflammatory and oxidative damage. Unfortunately, despite the significant improvements in local intestinal changes and decreased toxin levels after probiotic supplementation, only 1 month of probiotic intervention did not significantly improve the damage to renal function. This result is basically consistent with the results of our previous research as well as some other studies [14]. Probiotics can delay the progress of impaired renal function by reducing the permeability of the intestinal barrier at the early stage of CKD, which may be related to the regulation of mucosal immunity and reduction of systemic inflammation, but they cannot reverse the damage to renal function when there is obvious renal failure. The damage to renal pathological structures as well as renal function was severe in our study. Moreover, the duration of probiotic intervention in this study was relatively short; therefore, changes to renal function after probiotic intervention were unlikely to be significant. In order to clarify this problem further, a longer period of probiotic intervention in uremic rats should be considered in future studies so as to explore the influence of probiotics on the degree of serious damage to renal pathological structures and function.

Conclusion

Our study showed that Hcy aggravated the increased intestinal permeability and damage to tight junction structure induced by uremia by stimulating the expression of the inflammatory and oxidative stress markers TNF-α, IL-6, SOD, and MDA. VSL#3 probiotic supplementation clearly reduced Hcy levels and partly alleviated inflammatory and oxidative damage in both the serum and intestine by regulating the intestinal microbiota and vitamin production so as to improve intestinal permeability and tight junction structure and function in uremia. This study might provide a new clue for the variety of systemic complications caused by Hcy and suggests it may be a novel target for future therapy. Further investigations to identify the detailed roles and signaling pathways underlying the action of Hcy on intestinal cells in uremia should be conducted.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare and nothing to disclose.

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