LABORATORY STUDY

The influence of mutant lactobacilli on serum creatinine and urea nitrogen concentrations and renal pathology in 5/6 nephrectomized rats

Fang Wanga, Yun-Sheng Jiangb and Fang Liuc

aDivision of Nephrology, Ningbo Medical Center Lihuili Eastern Hospital, Taipei Medical University Ningbo Medical Center, Ningbo, PR China; bDivision of Nephrology, The Second Xiangya Hospital, Research Institute of Nephrology, Central South University, Changsha, PR China; cDivision of Health Management Center, Xiangya Hospital, Central South University, Changsha, PR China

ABSTRACT

Objectives: To explore the capacity of mutant lactobacilli to remove creatinine (Cr) and urea nitrogen (UN) via the gastrointestinal tract and its effects on renal pathology in the 5/6 nephrectomized rat model of chronic renal failure.

Methods: Sixty Sprague–Dawley rats were randomly divided into a Sham group, a Model group, a wide-type Lactobacilli group (L.B group), and a Mutant Lactobacilli group (Mut-L.B group). The rats in the Model, L.B and Mut-L.B groups underwent 5/6 nephrectomy. Eight weeks after administration, 24-h urine, orbital blood and digestive secretions were collected to analyze Cr and UN levels. Pathological changes in nephridial tissues were observed by hematoxylin and eosin and Masson trichrome staining, and the expression of TGF-β1 and FN was detected by immunohistochemistry.

Results: There were no significant differences in urinary Cr and UN levels among the Sham, L.B and Mut-L.B groups (p > .05), while serum and digestive Cr and UN levels were significantly decreased in the Mut-L.B group (p < .01). Furthermore, renal tubular injury and interstitial fibrosis were significantly reduced and TGF-β1 and FN expression was decreased (p < .05) in the Mut-L.B group.

Conclusion: Mutant lactobacilli decreased serum Cr and UN levels, reduced the expression of TGF-β1 and FN in renal tissues and alleviated renal interstitial injury and fibrosis in a rat model of chronic renal failure in a mechanism that may involve decomposition and not just excretion of small molecule toxins in the gastrointestinal tract.

Introduction

Chronic kidney disease (CKD), marked by a progressive loss of renal function, is a leading cause of hemodialysis initiation. There are currently 13.3 million patients with CKD and 300,000 patients are currently undergoing hemodialysis in Japan. Therefore, the economic implications of delaying the initiation of dialysis is a high-priority issue from the viewpoint of public health. The key for controlling the number of dialysis patients is to delay the progress of CKD and protect residual renal function. Emerging evidence suggests that uremic toxins may be one of the main factors responsible for the progression of CKD and loss of residual renal function. Currently, hemodialysis, which is the method by which the blood is purged of uremic toxins, remains the main strategy for alleviating uremia symptoms. However, hemodialysis may accelerate the loss of residual kidney function and increase the risk of cardiovascular events. Moreover, because of the high cost of this approach, hemodialysis has not been widely adopted in developing areas, and alternative strategies for the removal of uremic toxins via the gastrointestinal tract are increasingly being sought. In addition to the traditional methods, the use of oral adsorbents, and intestinal bacteria therapy, especially probiotics, can lower plasma toxin levels, prevent replication of intestinal pathogenic bacteria, reduce production of entero- genic uremic toxins, improve blood lipids and strengthen immunity, thereby compensating for the defect in CKD. Thus, probiotic treatment of CKD has become a focus of research in this field.

We have previously shown that oral supplementation with wild-type Lactobacillus leads to reductions in small molecule uremic toxin levels in both the serum and digestive secretions in 5/6 nephrectomized rats as a result of colonization on the gastrointestinal mucosa. To enhance the efficacy of this approach, we have successfully generated a Lactobacillus bulgaricus mutant...
with greater capacity to decompose uremic toxins after
directional induction and compound mutation in vitro. In
the present study, we investigated the capacity of
the mutant lactobacilli to colonize the intestinal mucosa
and decompose toxins in the 5/6 nephrectomized rat
model of chronic renal failure by analyzing serum
uremic toxin levels. Furthermore, we investigated the
potential of this approach to relieve the damage to
residual nephrons caused by toxins through observa-
tion of the expression of renal fibrosis factors and renal
pathological changes.

Materials and methods

Materials

*Lactobacillus bulgaricus* was provided by the
Agricultural Food and Microorganisms Laboratory of
Hunan (China). Mutated Lactobacilli were generated
and stored in our laboratory. Rabbit anti-TGF-
1 and anti-FN were purchased from Santa Cruz Biotechnology,
Inc. (Santa Cruz, CA).

Animals and groups

Sixty healthy male Sprague–Dawley rats (aged 6 weeks,
180–200 g) were purchased from the Animal
Department of Central South University Xiangya School
of Medicine. Two operations were performed in which
rats were anesthetized by intraperitoneal injection of
chloral hydrate 10% (0.35 mL/100 g). In the first oper-
ation, the upper and lower poles (each corresponding
to 1/3 of the kidney) of the left kidney were removed.
After 7 days, the entire right kidney was removed in a
second operation. Fifteen control rats underwent sham
operations simultaneously. Two weeks after the second
operation, the remaining 5/6 nephrectomized rats
were randomly divided into four groups and treated via
intragastric administration as follows: (1) the Sham
group received 2 mL/d saline (*n* = 15); (2) the Model
group received 2 mL/d saline (*n* = 13); (3) the L.B group
received 2 mL/d wide-type *L. bulgaricus* suspension
(1.5 × 10^8 cfu/mL) (*n* = 14); and (4) the Mut-L.B group
received 2 mL/d mutated lactobacilli suspension
(1.5 × 10^8 cfu/mL) (*n* = 14). Food and feeding methods
were the same among the four groups. All rats were
sacrificed after 8 weeks of therapy. All protocols involv-
ing animals were approved by the appropriate institu-
tional animal care and use committee.

Specimens collections and measurements

Rats were placed in metabolic cages with free access to
food and water. Twenty-four hour urine samples were
collected before sacrifice, and orbital blood samples
were obtained at the time of sacrifice. The remnants
of the left kidney were resected and fixed in 4% parafor-
maldehyde for subsequent experiments. Bowel loops
(including contents) under the stomach were removed,
cut into small pieces, and then homogenized with
saline. After centrifugation, supernatants containing the
digestive secretions were collected for subsequent ana-
lysis. UN and Cr levels in serum, urine and digestive
secretions were determined using an automatic bio-
chemistry analyzer (Hitachi 7170A, Tokyo, Japan).
Pathological changes of the nephridial tissue were
observed by hematoxylin and eosin (H&E) and Masson
trichrome staining, and the expression of TGF-β1 and
FN in nephridial tissue was detected by immunohistochemistry.

Histopathology

The kidney tissues were fixed in 4% paraformaldehyde,
embedded in paraffin and sectioned (thickness,
2–3 μm). The sections were stained with H&E for histo-
pathological observations and evaluation of tubulointer-
stitial damage and with Masson trichrome stain for
evaluation of collagens.

To evaluate tubulointerstitial damage, a total of five
unique and randomly selected renal tubulointerstitial
regions were observed for each specimen under light
microscopy (magnification, 200 ×). Injury grades were
then assigned according to the following eight indica-
tors: tubular expansion, tubular atrophy, vacuolar
degeneration of renal tubular epithelial cells, red cell
casts, protein casts, interstitial fibrosis, interstitial
edema, and interstitial infiltration of inflammatory cells.
The mean values were calculated as the tubulointersti-
tial damage index. To evaluate renal interstitial fibrosis,
the fibrotic area stained blue by Masson trichrome stain
was observed and photographed under a microscope
(OLYMPUS CX41, Tokyo, Japan). In each section, 20 non-
overlapping interstitial fields of visions were collected.
The HMIAS-2000 high-definition color medical analysis
system was used for automatic measurement acquisi-
tion and analysis, and the percentage of tubulointersti-
tial areas in the total fields of vision analyzed was
calculated for each section.

Immunohistochemical staining of TGF-β1 and FN
in the kidney

Paraffin-embedded tissue sections were dewaxed,
hydrated and treated with 5 mmol/L levamisole to block
endogenous alkaline phosphatase. Sections were then
incubated with blocking serum for 30 min at room
temperature to reduce nonspecific background staining. Sections were rehydrated in PBS including 0.1% BSA for 15 min before the addition of the appropriate blocking serum for an additional 15 min. Sections were then incubated with rabbit anti-rat FN or rabbit anti-TGF-β1 polyclonal antibodies (diluted 1:50) overnight at 4 °C. After rinsing, the sections were incubated with biotinylated goat anti-rabbit IgG (Abcam Inc., London, UK) and processed using an alkaline phosphatase–streptavidin–biotin immunoperoxidase method (Maixin Biotechnological Company, Shenzhen, China). The tissue sections were then counterstained with hematoxylin. Negative controls for specific labeling were prepared in parallel by replacing the primary antibody with normal rabbit serum. All the images were analyzed semiquanti-tatively by observation under a light microscope (magnification, ×200). Normal cell brown in color was observed. In each group, 10 fields of vision without glomerular and tubulointerstitial arteries were evaluated. The Image-Pro P1uS Version 6.0 image analysis system was used to measure the percentage of the optical density (OD) of the view of positive OD and positive area. The average OD(AOD)/area was used to represent the degree of expression of the respective index.

Statistical analysis

All data were analyzed by the SPSS17.0 statistical software (Chicago, IL). Data are expressed as the mean± standard deviation (SD). Single factor analysis of variance (ANOVA) was used to analyze the differences between groups. The LSD test was also used in cases of homogeneity of variance between two groups. Differences in means with \( p < .05 \) were considered statistically significant.

Results

The effect of Mut-L.B on UN and Cr levels in blood, urine and digestive secretions of 5/6 nephrectomized rats

Compared with the Sham group, the urinary creatinine clearance (U-Ccr) and urinary nitrogen clearance (U-CUN) rates were significantly decreased after 8 weeks of treatment, while Cr and UN levels in serum and digestive secretions were significantly increased in the Model group (\( p < .01 \)). Compared with the Model and LB groups, Cr and UN levels in serum and digestive secretions in the Mut-L.B group were significantly decreased (\( p < .01 \)), while there were no significant differences in U-Cr and U-CUN among the groups (\( p > .05 \)). These results indicated that the mutated lactobacilli decrease serum Cr and UN levels in 5/6 nephrectomized rats by decomposing the toxins present in the gastrointestinal tract with significantly greater efficiency than that of the wild-type Lactobacillus (Table 1).

H&E and Masson trichrome staining of the kidney

No glomerular sclerosis, tubular expansion, interstitial infiltration of inflammatory cells, or fibrous proliferation was observed in the Sham group. After 8 weeks of treatment, some of the kidney samples in the Model group showed enlargement of the glomerular volume and increased mesangial matrix. There was some capillary expansion or occlusion and glomerular wall thickening as well as some segmental sclerosis of the glomeruli. The brush border of the proximal tubular epithelial cells was diminished or lost, and in some renal tubular epithelial cells showed vacuolar degeneration or, in some cases, complete destruction. A large amount of interstitial fibrosis was also observed (Figure 1(A)). Areas stained blue by Masson trichrome staining mainly represent collagen, and the degree of interstitial fibrosis can be inferred by determining blue-stained interstitial areas. After 8 weeks of treatment, renal tubular injury and interstitial fibrosis was significantly ameliorated in the two treatment groups compared with the Model groups (\( p < .05 \)), with a significantly greater effect observed in the Mut-L.B group (\( p < .05 \)) compared with that in the LB group (Figure 1(B,C)).

Expression of TGF-β1 and FN protein

Low levels of TGF-β1 were detected in the normal collecting duct, distal tubule, and proximal tubule cells, while significantly higher levels were observed in these regions in the Model group (\( p < .05 \)). However, TGF-β1 levels were significantly reduced in the corresponding regions in the LB and Mut-L.B groups (\( p < .05 \)), with a significantly greater effect observed in the Mut-L.B group (\( p < .05 \) vs. LB group) (Figure 2).

FN was deposited mainly at the glomerular and tubular basement membranes in the Sham group, with significantly higher levels of FN protein observed in the tubules in the Model group (\( p < .05 \)). However, tubular FN protein levels were significantly lower in both the LB and Mut-L.B groups (\( p < .05 \)), with a significantly greater effect observed in the Mut-L.B group (\( p < .05 \) vs. LB group) (Figure 3).
There is abundant evidence that uremia profoundly alters the composition of the gut microbiome in both humans and animals. Patients with CKD show increased levels of aerobic bacteria in the bowel that produce uremic toxins and decreased levels of anaerobic bacteria, such as Bifidobacteria and Lactobacillus, indicating the absence of the normal symbiotic relationship among the intestinal microbial flora. Recent studies conducted in laboratories have demonstrated marked disintegration of the colonic epithelial barrier structure because of nutritional metabolism and toxins in humans and animals with advanced CKD. This phenomenon may play an important role in the development of systemic inflammation by enabling an influx of endotoxins and other noxious luminal contents into the systemic circulation, thus accelerating the progression of CKD. The excessive proliferation of aerobic bacteria is the main source of gut-derived uremic toxins.12

Figure 1. Pathological changes in the kidney. A: (a–d) H&E staining (×200); (e–h) Masson trichrome staining (×200). (a and e) Sham group; (b and f) Model group; (c and g) L.B group; (d and h) Mut-L.B group. *p < .01 compared with Sham group, #p < .05 vs Model group, ▲p < .01 compared with L.B group.

Figure 2. Expression of TGF-β1 protein (×400). *p < .05 compared with the Sham group, ▲p < .01 compared with Model group, ▲▲p < .05 compared with L.B group: (a) Sham group; (b) Model group; (c) L.B group; (d) Mut-L.B group.

### Table 1. UN and Cr levels in urine, serum and digestive secretions of 5/6 nephrectomized rats.

| Group  | N  | U-Cr (ml/min) | Scr (μmol/l) | Digestive juice Cr (μmol/l) | U-CrUN (ml/min) | BUN (mmol/l) | Digestive juice UN (mmol/l) |
|--------|----|---------------|--------------|----------------------------|----------------|--------------|-----------------------------|
| Sham   | 15 | 5.04 ± 1.39   | 38.60 ± 5.05 | 32.91 ± 6.38               | 1.41 ± 0.78    | 6.97 ± 5.05  | 5.97 ± 1.99                |
| Model  | 12 | 1.9 ± 0.52#   | 104.04 ± 10.66# | 68.88 ± 13.33#            | 0.65 ± 0.26#   | 18.08 ± 1.41# | 13.67 ± 2.91#             |
| L.B    | 13 | 2.22 ± 0.44   | 91.76 ± 8.52△ | 61.00 ± 8.87△             | 0.75 ± 0.27△   | 16.50 ± 0.98△ | 10.17 ± 2.24△             |
| Mut-L.B| 14 | 2.65 ± 0.51   | 69.83 ± 5.24△ ▲ | 40.38 ± 8.67△ ▲         | 0.87 ± 0.26     | 12.66 ± 0.98△ ▲ | 7.55 ± 1.53△ ▲            |

U-Cr: Urinary creatinine clearance rate = urinary creatinine/Scr × urine volume/24/60, U-CrUN: Urinary nitrogen clearance rate = urinary nitrogen/BUN × urine volume/24/60.

* p < .01 vs Sham group.
#p < .05 vs Model group.
△p < .01 vs L.B group.

Discussion

There is abundant evidence that uremia profoundly alters the composition of the gut microbiome in both humans and animals. Patients with CKD show increased levels of aerobic bacteria in the bowel that produce uremic toxins and decreased levels of anaerobic bacteria, such as Bifidobacteria and Lactobacillus, indicating the absence of the normal symbiotic relationship among the intestinal microbial flora. Recent studies conducted in laboratories have demonstrated marked disintegration of the colonic epithelial barrier structure because of nutritional metabolism and toxins in humans and animals with advanced CKD. This phenomenon may play an important role in the development of systemic inflammation by enabling an influx of endotoxins and other noxious luminal contents into the systemic circulation, thus accelerating the progression of CKD. The excessive proliferation of aerobic bacteria is the main source of gut-derived uremic toxins.
-cresidyl sulfate. Creatinine and urea nitrogen have traditionally been regarded as the index of renal function damage. However, in recent years, many studies have shown that their metabolites are involved in the progression of kidney disease. Creatinine is converted to creatinine hydroxide in the presence of free radicals, which increases the expression of TGF-β1, CTGF and FN in renal tubular epithelial cells and exacerbates renal tubular interstitial fibrosis. In this way, creatinine indirectly promotes fibrosis. A high concentration of urea greatly increases hydroxylation of proteins by increasing reactive oxygen species (ROS) in mouse renal inner medullary (mIMCD3) cells in culture. Hydroxylation of proteins plays a very important role in the synthesis and stability of collagen. Pyridinoline cross-links are derived from hydroxylated lysine residues located within the collagen telopeptides, which can induce an excessive accumulation of collagen and irreversibility of fibrosis.

In the present study, we also found high levels of blood urea secreted into the gastrointestinal tract due to impaired excretion in 5/6 nephrectomized rats. The influx of urea and its conversion to ammonia by microbial urease in CKD lead to degradation of the intestinal epithelial tight junction (TJ) and intestinal barrier dysfunction. This, in turn, leads to system inflammation and progressive damage to the renal function by allowing absorption of plasma endotoxin, IL-6, TNF-α, MCP-1, CINC-3, L-selectin, ICAM-1, and malondialdehyde into the circulatory system. Experiments in a rat model of chronic kidney failure showed that urea directly enhances oxidative stress and insulin resistance, which play a central role in the pathogenesis of accelerated cardiovascular disease and numerous other CKD-associated complications. It was also observed that proteinuria was reduced and the decline in renal function was delayed by lowering the levels of blood urea in patients with CKD.

In addition to increased enterogenous inflammatory factor levels, the influence of creatinine and urea on the fibrosis-related factors in CKD in vivo is occasionally reported. In our study, we observed increased expression of TGF-β1 and FN in damaged renal tissue. TGF-β1 is a key factor in fibrosis, which can promote proliferation and activation of renal tubular epithelial cells and fibroblasts leading to autocrine and paracrine induction of TGF-β1, collagen type IV and FN deposition, LN, and multiplying the extracellular matrix (ECM). On the other hand, TGF-β1 can accelerate fibrosis by promoting secretion of collagen degradation inhibitor. FN protein, as a main component of ECM, plays an important role in mediating cell proliferation and fibrosis by binding with integrins. In accordance with these reports, our study showed that serum creatinine and BUN levels were increased, renal tubular interstitial damage was exacerbated and the expression of TGF-β1, FN in renal tissues was enhanced in the Model group.

Probiotics that colonize the gastrointestinal tract perform a number of functions that include regulating the normal development and function of the mucosal barriers and assisting with maturation of immunological tissues, which in turn promotes immunological tolerance to antigens from foods, the environment, or potentially pathogenic organisms. Probiotics are also important in controlling nutrient uptake and metabolism and preventing propagation of pathogenic microorganisms. A decrease in the blood urea concentration of more than 10% was found in patients with KDOQI stage 3 and stage 4 CKD following oral administration of the Lactobacillus Casei shirota. Similar studies have provided further evidence that probiotics represent a potential intervention to minimize the adverse effects on residual renal function caused by gut-derived uremic toxins in CKD. Our results show that serum creatinine and urea levels were significantly reduced, while no changes were observed in the digestive secretions in rats treated with mutant lactobacilli. This observation suggests that the mutant lactobacilli produce enzymes...
that degrade creatinine and urea and do not increase intestinal excretion. Furthermore, the ability of the mutant lactobacilli to mediate this effect is significantly higher than that of the wild-type lactobacilli. This speculation is consistent with the results reported by Wong, which showed that ESRD patients exhibited significant expansion of bacterial families possessing urease, uricase, and indole and p-cresol forming enzymes in digestive secretions after oral administration of probiotics.

Reports of the potential efficacy of probiotics in attenuating renal pathological changes in chronic renal failure are rare. In this preliminary study, we show that oral administration of the mutant or wild-type lactobacilli decreased renal tubular interstitial damage and collagen deposition, relieved interstitial fibrosis, and significantly decreased TGF-β1 expression and FN deposition in 5/6 nephrectomized rats compared with those that were untreated, with a significantly greater effect observed following administration of the mutant lactobacilli. It can be speculated that this effect is related to a reduction in gut-derived uremic toxins and some inflammatory factors mediated by damage; however, the exact mechanism requires further investigation.

In summary, it can be suggested that the Lactobacillus bulgaricus mutant with greater capacity to decompose uremic toxins might be a prospective and useful drug to reduce serum uremic toxins and prevent the tubulointerstitial fibrosis in CKD. In future studies, it is needed to focus on the possible toxicity and side effects of this mutant and the molecular mechanisms of action.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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