Original Article
The study of Tongfu Xiezhuo recipe on regulating intestinal barrier function in chronic kidney disease

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Abstract: Objective: To investigate the effect and mechanism of Tongfu Xiezhuo (TFXZ) Recipe on regulating intestinal barrier function and delaying the progress of renal disfunction in rats with chronic kidney disease (CKD) induced by adenine. Methods: In total, 38 male rats were involved in this study. Among them, 30 SD male rats were randomly selected for disease modeling by adenine gavage for four weeks, and the other 8 rats were set as the blank control group. After successful modeling, 30 rats were randomly divided into a model group, TFXZ group, and rhubarb group (RG). All the rats in each group were given corresponding doses of drug treatment. After 8 weeks, the changes of general condition, colon pathology, a uremic toxin, DAO, DLA, ET, 24-hour urine volume and 24-hour urinary protein were observed. The expression of ZO-1, Claudin-1 and Occludin protein in the colon tissue was detected by immunohistochemistry and Western blot. Results: Compared with the model group, in the TFXZ group the levels of BUN, Scr, UA, CysC, IS, DAO, DLA, ET, and 24-hour urinary protein decreased significantly (P<0.05) and the expression of ZO-1 protein and Claudin-1 protein in the intestinal mucosa increased significantly (P<0.05). While in the RG, the levels of BUN, Scr, CysC, IS, DAO, DLA, ET, and 24-hour urinary protein decreased significantly (P<0.05) and ZO-1 protein in the intestinal mucosa decreased significantly (P<0.05). When it came to decreasing UA level and increasing expression of Claudin-1 protein, the RG was not as good as that of TFXZ group. Conclusion: TFXZ recipe reduced the level of uremic toxin and increased the expression of intestinal tight junction protein which resulted in delaying the progress of renal disfunction in CKD rats. Its potential mechanism may be related to reducing the damage of the intestinal mucosal barrier and the absorption of endogenous toxin.

Keywords: Tongfu Xiezhuo recipe, intestinal barrier, chronic kidney disease

Introduction

Chronic kidney disease (CKD) is a chronic progressive disease of renal structural abnormalities and dysfunction caused by multiple causes. Its incidence rate is increasing year by year and it has become an important global health problem [1]. Finding effective treatments for chronic kidney disease has important clinical and social significance. More and more studies have shown that the intestine is one of the important organs producing uremic toxins. Toxins derived from the intestines and endogenous uremic toxins can not only aggravate the progression of CKD, but are also closely related to death in CKD [2]. With the decline of renal function, the uremic toxins accumulated in the blood causes damage to the intestinal barrier function through a series of mechanisms, and a large number of intestinal toxins such as endotoxin and sulfate enter the systemic circulation through the damaged intestinal barrier, resulting in toxin aggregation and inflammatory states, which further aggravate kidney damage and eventually form a vicious cycle [3]. Based on this, Meijers et al. [4] proposed a theory of intestinal and renal axis for CKD progression and intervention, and it has become a new strategy and direction for delaying the progress of CKD in recent years.

Tongyu Xiehuo is a way of treating renal failure in the Department of Nephrology, Nanjing Traditional Chinese Medicine Hospital. It
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consists of raw rhubarb, cooked aconite, raw oyster, dandelion, raw glutinous rice, June snow, and rhubarb as the monarch. The specific application of the theory of “opening the ghost door, cleansing the house, going to Chen Chen”, has been clinically applied for many years and has a remarkable curative effect. Our previous experimental studies have shown that this prescription can reduce the levels of uremic toxins such as urea nitrogen, creatinine, and sulfate in rats with chronic kidney disease, reduce renal fibrosis and delay the progression of renal damage \[5, 6\], but the mechanism is not yet clear. Therefore, based on the previous research, we carried out this study further to observe the regulation of Tongfu Xiezhuo Recipe on intestinal barrier function in rats with chronic kidney disease, and to explore its related mechanism of delaying the progression of chronic kidney disease.

Materials and methods

Experimental animals

In total we used 38 healthy SD male rats, SPF grade, 8 weeks old, body weight (200 ± 20) g, purchased from Zhejiang Animal Experimental Center (license number: SCXK (Zhejiang) 2014-0001), that were housed in the Animal Experimental Center of Nanjing University of Traditional Chinese Medicine (license number: SYKX (Su) 2014-0001). The animals were given free access to diet and drinking water and allowed to adapt to feeding for a week. Every procedure was approved by the Animal Care and Use Committee of the Third affiliated Hospital of Nanjing University of Chinese Medicine.

Experimental drugs and reagents

TFXZ (composition: raw rhubarb, raw oysters, dandelion, raw glutinous rice, June snow, cooked aconite, Chinese herbal medicine granules produced by Jiangyin Tianjiang Pharmaceutical Co., Ltd.); adenine (AMRESCO, 0183); sulphate standard (Sigma); rat cystatin C ELISA kit (Abeam, ab201281); diamine oxidase/lactic acid/bacterial endotoxin test kit (Zhongsheng Jinyu), ZO-1 antibody (Abeam, ab214228), Claudin-1 antibody (Abeam, ab15098), Occludin antibody (Abeam, ab216327), ACTIN antibody (Service, GB12001), HRP-labeled goat anti-rabbit and goat anti-mouse antibody (service).

Instruments and equipment

We used a 515 high performance liquid chromatography (Waters), 2475 fluorescence detector (Waters), XBridge BEH C18 Column (Waters), JY-DLT intestinal barrier function biochemical index analysis system (Zhongsheng Jinyu), Rt2100c microplate reader (Rayto), TSY-B decolorization shaker (Service), PL-203 electronic analytical balance (Mettler Toledo), AJC-0501-P pure water meter (Chongqing Aikepu), refuge 13R refrigerated centrifuge (Heal force), XSP-C204 microscope (CIC), DYCZ-24DN double vertical electrophoresis instrument (Beijing Liuyi Instrument Factory).

Model preparation, grouping and intervention methods

Thirty out of 38 SD rats were randomly selected, and an animal model of chronic kidney disease was made according to Yokozawa [7] with the adenine gavage method. Rats were intragastrically administered with a 2.5% adenine suspension (200 mg/kg/d) once a day for four weeks. Another 8 rats were orally administered with an equal amount of distilled water and were set as a blank group (sham). On the fourth week, four model rats were randomly selected for blood sampling to detect Scr and BUN, and the model was evaluated. After successful modeling, 30 rats were randomly divided into a model group (MG), a Tongfu turbidity group (TFXZ) and a rhubarb group (RG), with 10 rats in each group. At the fifth week, the model rats were continued to be administered with 2.5% adenine suspension (200 mg/d/kg) every other day to maintain the progression of the disease. By the 7th week, the adenine dosing was stopped, and the blank group was given the corresponding volume. Distilled water was administered to the stomach. At the same time, each group of rats was given corresponding drug treatment every afternoon. The dose for the rats was converted according to the equivalent dose of human to rat (rat dose = human dose × 0.018 × 5) [8]. After calculation, the Tongfu Xiezhuo Recipe group was administered according to the following dosages: rhubarb 0.6 g/kg, aconite 0.15 g/kg, oyster 0.045 g/kg, dandelion 0.36 g/kg, glutinous rice 0.54 g/kg, June snow 0.09 g/kg; RG was administered with rhubarb granules at a dose of 0.6 g/kg, and the above granules were dissolved in 10 ml of dis-
tilled water to prepare a suspension of traditional Chinese medicine, which was administered at a dose of 10 ml/kg/d, and the blank group and the MG were large. Rats were given an equal volume of distilled water in the afternoon. Rats were free to eat and water during treatment. During the experiment, due to improper operation of the gavage, a trachea was inserted, and a total of 5 deaths occurred. Among them, 2 died in the MG, 1 died in the Tongqiao stagnation group, and 2 died in the RG.

**Specimen collection**

After the end of the eighth week of the experiment, the metabolic cage collected 24-hour urine and detected 24-hour urine protein. The rats were fasted for 12 hours, weighed, and then anesthetized with 3% pentobarbital sodium according to 0.2 ml/100 g intraperitoneal injection. After anesthesia, the abdominal aorta was used for blood taken, which was then preserved in blood collection tubes, and allowed to stand for 30 minutes, and was centrifuged at 3000 r/min for 10 minutes in order to take the supernatant for examination. After the blood collection is completed, the abdominal cavity is fully exposed, and the proximal colon adjacent to the ileocecal area (<5 cm) is quickly taken, about 4 cm. The contents of the intestinal tract are carefully washed in physiological saline, and about 1.5 cm of the colon is placed in 4% paraformaldehyde. After fixation for 24 hours, paraffin sections were prepared and subjected to H&E staining. The rest of the tissue was placed in a sterile cryotube and placed in liquid nitrogen for quick freezing, and then stored at -80°C for later use.

**Detection indicators and methods**

1. Pathological changes of colon tissue. The colon tissue fixed by paraformaldehyde was routinely dehydrated, embedded in paraffin, coronal sectioned, and stained with hematoxylin-eosin (H&E). The histopathological changes of colon tissue were observed under a light microscope. The degree of intestinal mucosal damage was assessed by the integration method reported by Haglund et al. [9]. Zero is scored as normal villi and gland; 1 is scored as partial villus top epithelial mildly damaged; 2 is scored as subepithelial gland slightly damaged; 3 is scored as subepithelial space enlargement, capillary congestion; 4 is scored as epithelium and the lamina propria is moderately separated, the gland is damaged; 5 is scored as partial villus topping; 6 is scored as villi detachment, telangiectasia; 7 is scored as lamina propria, and gland is damaged; 8 is scored as lamina propria Digestion and decomposition; and 9 is scored as bleeding and ulcers.

2. Immunohistochemical detection of colon tissue ZO-1, Claudin-1, Occludin protein expression. Paraffin-embedded sections were made with xylene dewaxing water washing, antigen microwave heat repair for 15 min, 3% hydrogen peroxide incubation at room temperature for 15 min, BSA. Add ZO-1, Claudin-1, Occludin antibody at 4°C overnight, then add secondary antibody, incubate for 50 min at room temperature, using DAB for coloration visualized under a microscope, counterstained with hematoxylin, and finally dehydrated to the slide. Six 200-fold fields of view were randomly selected for each slice in each group. Attempted to make the individual fields fill the entire field of view when taking pictures, and ensure that the background light of each photo is the same. Image-Pro Plus 6.0 software was used to select the same brown color as the unified standard for judging all photos. Each photo was analyzed to obtain the cumulative optical density (IOD) of each photo, and the average value was taken as a group of proteins. The greater the relative content, the greater the IOD value indicates the higher the protein expression level.

3. Western blot was used to detect the expression of ZO-1, Claudin-1, and Occludin in the colon tissue. One hundred mg colon tissue was cut, RIPA lysate containing protease inhibitor was added, the homogenate was made, an ice bath was performed for 30 min, centrifuged at 12000 rpm for 10 min at 4°C, and samples were collected. From the supernatant, the protein concentration was determined by BCA kit, and SDS-PAGE electrophoresis was carried out, then the membrane was transferred, and 5% skim milk was used to block at room temperature for 1 h. We added anti-ZO-1 (1:1000), Claudin-1 (1:800), Occludin (1:1000) dilutions and an internal reference of β-Actin (1:3000), incubated overnight at 4°C, and added 1:3000 secondary antibody which was incubated for 2 h at room temperature. After washing, developing, fixing, and scanning, the results were ana-
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lyzed by a gel image analysis system: protein content = sample protein gray value/same sample β-Actin gray value.

(4) Blood and urine specimens. Determination of indole sulfate (IS) by high performance liquid chromatography-fluorescence analysis, diamine oxidase (DAO), D-lactic acid (DLA), endotoxin (ET) using diamine oxidase/Lactic acid/bacterial endotoxin test kit (enzymatic method) detection. ELISA detection of cystatin C (CysC), serum creatinine (Scr), urea nitrogen (BUN), homocysteine (HCY), 24-hour urine protein quantification was commissioned by the laboratory inspection of the Third Affiliated Hospital of Nanjing University of Traditional Chinese Medicine.

Statistical methods

Statistical analysis was performed using SPSS 22.0 software. The measurement data were expressed. One-way analysis of variance was used for comparison between groups. The LSD method was used for comparison between groups. P<0.05 was considered statistically significant.

Results

General situation of animals

In the blank group, the weight of the rats increased rapidly, their spirit was good, the color of the fur was bright, their reactions were sensitive, their activity was normal, and both eating and drinking were normal. In the MG, the weight gain of the rats was slow, their spirit was poor, the fur was yellow and scattered, their reactions were slow, contraction was common, their activity was reduced, the polydipsia and polyuria were decreased, diet intake was decreased, and the feces were stinky, thin and not formed. In the Tongxie Xiezhuofang group and the RG, the hair color and mental state of the rats were better than those in the MG, and the bodyweight was significantly higher than that in the MG (P<0.05). The changes in body weight of the rats in each group at baseline at 8 weeks are shown in Figure 1.

Figure 1. Changes in body weight of rats in each group at 8 weeks. Note: **P<0.01 compared with the blank group; #P<0.05 compared with the MG.

Comparison of BUN, Scr, UA, HCY, CysC, and IS in each group

The levels of uremic toxins in the rats in each group were significantly higher than those in the blank group (P<0.05), and the BUN, Scr, UA, CysC groups in Tongyu bleed turbidity group was significantly higher than those in the MG. PUN, Scr, UA, HCY, CysC, and IS were significantly higher than those in the MG. The IS was lower than the MG (P<0.05), and the BUN, Scr, CysC, and IS in the RG were lower than those in the MG (P<0.05) (Table 1).

Urine index of rats in each group

Compared with the blank group, the 24-hour urine volume and 24-hour urine protein quantitation in the MG were significantly increased. Compared with the MG, both 24-hour urine volume and 24-hour urine protein quantitation were reduced in Tongfu Xiezhuo Recipe group and RG (P<0.05) (Table 2).

Comparison of DAO, DLA, and ET in each group

Intestinal mucosal permeability index changes in the intestinal mucosa permeability index DAO, DLA, ET were significantly higher than the blank group (P<0.01) with the DAO, DLA, ET comparison in the Tongyu bleed turbidity group was significantly lower (P<0.01). The DAO, DLA, and ET in the RG were lower than those in the MG (P<0.05) (Table 3).

Pathological changes of intestinal mucosa in each group

Under light microscopy, the colonic mucosal cells of the rats in the blank group were neatly arranged, the structure was intact, the villi and
glands were normal, and there was no inflammatory cell infiltration, telangiectasia, tissue edema, and necrosis. In the MG, the surface epithelium of the colonic mucosa was exfoliated, a large number of inflammatory cells infiltrated, telangiectasia, hyperemia, glandular gland destruction, and unclear structure. The pathological damage of colonic mucosa in Tongfu Xiezhuofang group and RG was significantly reduced compared with the MG (Figure 2).

Expression levels of ZO-1, Claudin-1, and Occludin in the colon of each group

(1) Immunohistochemical analysis results. ZO-1, Claudin-1 and Occludin proteins in the colonic mucosa of the blank group were uniformly distributed continuously at the edge of the intestinal epithelial cells, and distributed uniformly along the villus at the top of the cell membrane, which was honeycomb. Point-like aggregation; visible in the whole section; ZO-1, Claudin-1 and Occludin proteins in the colonic mucosa of the MG were scattered at the top of intestinal epithelial cells, and their staining distribution was uneven, faded. The IOD values of ZO-1, Claudin-1 and Occludin protein in the MG were lower than those in the normal control group (P<0.01). The IOD values of ZO-1, Claudin-1 protein and RG ZO-1 protein in the turbidity group were higher than those in the MG (P<0.01 or P<0.05). The Occludin protein in the turbidity group and the Ilorin-1 and Occludin protein IOD values in the RG were also higher than that in the MG (Figure 3).

(2) Western-blot analysis results. With β-Actin as the internal reference, the ZO-1 protein band appeared at around 230,000 kDa. The Occludin protein band appeared at around 59,000 kDa and the Claudin-1 protein band appeared at around 23,000 kDa. Gray-scale analysis of each strip showed that the results of Western-blot were consistent with the results of immunohistochemistry. The expression of ZO-1, Claudin-1, and Occludin in the MG was lower than that in the blank group (P<0.05). The expression of ZO-1 protein in the turbidity group and rhubarb was higher than that in the MG (P<0.05). The expression of Claudin-1 and Occludin in the turbidity group and the RG was also higher than that in the MG (Figure 4).
Discussion

The intestinal mucosal barrier is a functional structural system with a highly selective barrier effect. The mechanical barrier is the most important component of the system. It consists of a connective complex between intestinal epithelial cells and other epithelial cells. The tight junction protein is the junctional complex. The expression level of tight junction proteins in epithelial cells can not only reflect the degree of damage of the intestinal barrier but also reflect the degree of recovery of the intestinal barrier [10]. The structural integrity of the mechanical barrier ensures a stable play of intestinal epithelial permeability.

In recent years, studies have shown that the tight junction proteins between intestinal mucosal epithelial cells of CKD rats are significantly reduced or absent, intestinal mucosal permeability is increased, and a large number of endotoxin-induced uremic toxins such as endotoxin and sulfate are absorbed into the blood [11]. After entering the systemic circulation, these enterotoxins can cause a systemic inflammatory response and oxidative stress, aggravate renal interstitial fibrosis and tubular damage, accelerate the progression of CKD, induce other concomitant diseases, and forms a vicious cycle [12]. Therefore, the intestinal mucosal barrier is closely related to the progression of CKD. Repairing intestinal mucosal barrier damage, reducing the production and absorption of intestinal uremic toxins may improve the clinical symptoms of CKD patients and delay the progression of CKD.

There is no record of the names of “chronic kidney failure” and “chronic kidney disease” in traditional Chinese medicine. According to its clinical manifestations, it belongs to the category of disease such as edema, phlegm, stagnation, labor, and scorpion in Chinese medicine. The disease is in the kidney, often affecting the spleen and stomach. Although the pathogenesis of chronic renal failure is complicated, “cloudy poisoning” is one of its basic pathogenesis. The treatment of “turbidity” should be based on the platoon, but in the later stage of CKD, the function of viscera is declining, and the poison of wet turbidity is not be small. It is discharged, so another way to ventilate the turbidity is to replace it with turbidity, that is, the “Yellow Emperor’s Internal Classic” “to swear by Chen Yu”, so that “wet turbidity
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and evil spirits” are excreted from the stool. This coincides with the modern medical “intestinal and kidney axis” theory.

Rhubarb is a representative drug of Tongqiao turbidity method, and its curative effect on chronic renal failure is definite and has been widely used in clinical treatment. Modern research has shown that rhubarb can stimulate bowel movements, enhance gastrointestinal motility, prevent ammonia from being absorbed from the intestine, promote nitrogen excretion from the intestine, and have a good protective function against the intestinal mucosal barrier. Pathogenic bacteria and enterococci significantly reduce the level of endotoxins in the body. It can reduce the apoptosis of intestinal epithelial cells and increase the expression of tight junction proteins through anti-inflammatory and anti-oxidative damage, and prevent the

Figure 3. Expression of ZO-1, Claudin-1 and Occludin protein in the colon of each group (immunohistochemical staining, ×200). (A) The IF staining results of Expression of ZO-1, Claudin-1 and Occludin protein in the colon of each group, (immunohistochemical staining, ×200). (B) The date analysis of Claudin-1 expression level of the (A) results. (C) The date analysis of Occludin expression level of the (A) results. (D) The date analysis of ZO-1 expression level of the (A) results. Note: **P<0.01 compared with the blank group; #P<0.05, ##P<0.01 compared with the MG.
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Intestinal mucosal barrier from being indirectly prevented.

The role of intestinal flora shifts [13]. Tongyu bleeds the phlegm using rhubarb as a medicinal herb, which means venting turbidity through the sputum, so that the evil of wet turbidity emerges from the stool. The oysters of the compatibility oysters converge, if the diarrhea is too strong, and the diarrhea is too much, then positive gas occurs, and in order to prevent the cold and diarrhea from the drug, then dandelion heat detoxification is used, loosening the swelling, and raw glutinous rice cools blood to stop bleeding, and June snow heats the dampness, with Shujin active. The groups are reasonable and well-matched, for the basic pathogenesis of chronic renal failure turbidity, which is in line with the modern medical “intestinal and kidney axis” theory.

In this study, it was confirmed by adenine-induced CKD rat model that urinary toxin accumulation, serum diamine oxidase, D-lactic acid, endotoxin levels were significantly increased, and intestinal mucosal permeability was in-

Figure 4. Expression of ZO-1, Claudin-1, and Occludin in the colon of rats in each group (Western-blot method). (A) Western blotting results of Claudin-1 expression levels in the four groups. (B) Western blotting results of Occludin expression levels in the four groups. (C) Western blotting results of ZO-1 expression levels in the four groups. Note: Compared with the blank group, *P<0.05, **P<0.01; compared with the MG, #P<0.05.
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creased in CKD. The expression level of tight junction proteins such as ZO-1 protein, Occludin protein and Claudin-1 protein in the intestinal mucosa were significantly decreased, suggesting that the tight junction was destroyed and the intestinal mucosal barrier was damaged. After the administration of Tongqiao Xiezhuo Recipe, the bodyweight growth was slow, and the blood BUN, Scr, UA, CysC, IS, diamine oxidase, D-lactic acid, endotoxin levels, and 24-hour urine protein were significantly decreased. The expression of ZO-1 protein and Claudin-1 protein in intestinal mucosa was significantly increased, indicating that the intestinal mucosal barrier damage and intestinal mucosal permeability were improved, and renal function was restored. However, after administration of rhubarb alone, it was also improved the slow growth of weight in rats, reduced the level of uremic toxin, and increase the expression of ZO-1 protein in colonic mucosa. However, it is not as obvious as Tongqiao phlegm, and the RG reduced UA level and increase the expression of Claudin-1 protein. The slight insufficiency may be related to the small sample size, but it also suggests that although single rhubarb is effective in treating CKD, it can repair intestinal mucosal barrier damage, but its has effectiveness compared to Tongxie Xiezhuo Recipe.

The improvement of each index is relatively clear, and the possible mechanism is related to the multi-channel multi-target treatment effect after the treatment, and it also suggests that the clinical single-drug treatment still has some limitations. These limitations need to be differentiated according to the actual situation.

In summary, the results of this experiment show that CKD is closely related to intestinal mucosal barrier structure damage. Tongxie Xiezhuo Recipe can remove uremic toxin levels, especially intestinal toxins, reduce 24-hour urine protein levels, increase the expression of intestinal mucosal tight junction proteins, especially ZO-1 protein and Claudin-1 protein, and restore intestinal epithelial tightness. Therefore, delaying the progression of renal function in CKD rats, may be related to the reduction of intestinal mucosal barrier damage and the reduction of intestinal uremic toxin absorption.

Disclosure of conflict of interest

None.

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References


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