

Original Article

Effects of celecoxib on expression of TLR4, Hecpudin, and NF- κ B in rats bearing uremia with peritoneal dialysis treatment

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Received December 30, 2016; Accepted September 24, 2017; Epub November 15, 2017; Published November 30, 2017

Abstract: Objectives: The aim of this study was to probe the effects of celecoxib on expression of toll-like receptor 4 (TLR4), Hecpudin, and NF- κ B in rats bearing uremia with peritoneal dialysis (PD) treatment. Methods: Sixty rats were randomly divided into three groups: control, sham operation, and uremia model. Renal excision was performed to obtain rat model bearing uremia. Levels of urea nitrogen and serum creatinine were determined. Then homemade dialysis catheters were prepared to perform PD. During PD process, celecoxib at various doses (0, 10, 20, and 50 mg/kg) was administrated. After PD treatment, peritoneal tissues were collected and characterized by hematoxylin eosin (HE) staining. The mRNA and protein levels of TLR4, Hecpudin, and NF- κ B in peritoneal tissues were evaluated by RT-PCR and western blot, respectively. Results: In the uremia model group, levels of urea nitrogen and serum creatinine were more than 3 and 2 times of those in the sham operation groups, respectively ($P < 0.05$ and $P < 0.01$), suggesting the successful building of rats bearing uremia. Significant body weights gain of rats bearing uremia after PD treatment on 15 day were found ($P < 0.05$). In rats bearing uremia, we found the severe damage of renal tissues. However, PD treatment obviously alleviated the renal damage in rats bearing uremia. Celecoxib significantly downregulated the expression of TLR4, Hecpudin, and NF- κ B at dose-dependent manners. Conclusion: Celecoxib worked in rats bearing uremia with PD treatment through downregulating the expression of TLR4, Hecpudin, and NF- κ B in TLR4-Hecpudin pathway.

Keywords: Celecoxib, peritoneal dialysis, uremia, TLR4, Hecpudin, NF- κ B

Introduction

In recent years, the morbidity of chronic renal disease (CRD) is raised year by year. It has become another malignant disease harming human health besides cardiovascular and cerebrovascular diseases, malignant cancer, and diabetes. As the end stage of CRD development, end-stage renal disease (ESRD) severely impairs the life safety and living quality of patients with renal disease. At present, how to delay or block the progress of CRD and improve the living quality of ESRD patients are important aspects in the renal disease research [1, 2]. As an important method to treat CRD, peritoneal dialysis (PD) has been widely applied in clinical treatment for its many advantages, such as minor effect on glomerular filtration, good stability of cardiovascular function, and user-friendly control [3]. Although much prog-

ress of PD technology has been achieved now, some troubles such as nutritional imbalance, peritonitis generation, and biocompatibility risk of dialysis fluid induced by PD also impede its further clinical application. Long-term PD is still exposed to multiple challenges.

Toll-like receptor 4 (TLR4) is a kind of pattern recognition receptor in innate immune system mediating diverse inflammatory reactions [4]. TLR4 could activate signal pathways by binding to its ligand lipopolysaccharide and played a crucial role in the oncome of acute renal failure of mice [5]. Abnormal activation of TLR4 signal transduction pathway was able to promote the excessive apoptosis of renal tubular epithelial cells (RTECs) in nephridial tissue, decrease the amount of RTECs, induce long-term and chronic inflammatory reaction, subsequently cause the inflammatory injury, and finally give

rise to the generation of CRD and even renal failure [6, 7]. Hepcidin is a micro-molecule polypeptide with important functions of regulating iron homeostasis and promoting lymphangion genesis. After long-term exposure to non-bio-compatible dialysis stimulation, the neovascularization in peritoneum is greatly enhanced, which may be related to the expression of TLR4 and Hepcidin. Therefore, the investigation of the role of TLR4 and Hepcidin signal pathways in the PD process might be clinically significant for reducing the side effect of PD and enhancing the living quality of CRD patients.

Cyclooxygenase-2 (COX-2) is one of the important factors involved in angiogenesis. COX-2 could induce angiogenesis of various tumors [8]. Inflammatory stimulation promotes the production of COX-2. Meanwhile, COX-2 takes part in the amplification of inflammatory response through many signal pathways such as NF- κ B, where TLR4 and Hepcidin were involved as well. As a COX-2 inhibitor, celecoxib makes anti-inflammatory, analgesic, and antipyretic effects via inhibiting COX-2 and preventing the production of inflammatory prostaglandins. Clinically, celecoxib is also employed as a drug to suppress the peritoneal angiogenesis and improve the morphology and function of the peritoneum [9]. However, the protective effect of celecoxib in PD treatment and the related molecular mechanisms has not yet been clarified.

Therefore, in this study, rat models bearing uremia were established and then received PD treatment. These rats were administrated with various doses of celecoxib by gavage. Peritoneal tissues were separated from parietal layer and omentum and characterized by hematoxylin eosin (HE) staining, where the expressions of TLR4, Hepcidin, NF- κ B were determined by RT-PCR and western blot.

Materials and methods

Materials and animals

BCA kit was from Vazyme Biotech (Nanjing, Jiangsu, China). Celecoxib was bought from Pfizer (Dalian, Liaoning, China). Duplex PD solution (4.25%) was purchased from Baxter (Guangzhou, Guangdong, China). RNA extraction kit was from CoWin Biosciences (Beijing, China). Reverse transcription kit and SYBR[®]

Green RT-PCR Master Mix were bought from Applied Biosystems (Foster city, CA, USA). Ready Prep Protein Extraction Kit was purchased from Bio-Rad (Hercules, CA, USA). Rabbit anti-TLR4, anti-Hepcidin, anti-NF- κ B, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibodies were from Santa Cruz (Dallas, Texas, USA). Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (H+L) was bought from ZSGB-Bio (Beijing, China). Medical silica gel drainage tube was from Xinda Medical Equipments (Suzhou, Jiangsu, China).

Specific pathogen free (SPF) Sprague Dawley (SD) rats (Male, 250 \pm 50 g) were provided by Beijing Vital River Laboratory Animal Technology (Beijing, China). Every 5 rats were homed in a standard cage with free access to food and purified water for drinking. The environment temperature in the feeding room with natural lighting and good ventilation was 18-25°C. The new coming rats were first raised for 7 days to be fitted to the new circumstances before animal experiments. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Linyi People's Hospital, Shandong, China.

Establishment of rat models bearing uremia

Sixty SD rats were randomly divided into three groups (n=20): control group, sham operation group, and uremia model group. Sixty rats were numbered according to their weights from small to large. The random grouping was performed based on the random number table and every group was made up of 20 rats. Rats without any treatment served as the control group. Rats in both sham operation group and uremia model group were anaesthetized by intraperitoneal injection of 10% chloral hydrate. Then rats in sham operation group were received only elimination operation of bilateral renal capsule and 4 \times 10⁵ U penicillin was administrated for infection prevention after the operation. However, rats in the uremia model group suffered nephrectomy. After anesthesia, rats were fixed and an incision of about 1.2 cm which was parallel to the spine was made in the left back near the kidney. Then the skin, muscle, and fascia were successively incised to expose the left kidney. The capsule of left kidney was eliminated and 1/3 tissue in both the top and bottom of left kidney was excised.

Molecular mechanism of celecoxib in peritoneal dialysis treatment

Table 1. Sequences of PCR primers.

Gene	Primer (5'-3')
TLR4 (NM_025817)	For: AGCTCCTGACCTTGGTCTTG Rev: CGCAGGGGAAGCAATGAGG
Hepcidin (NM_032541.2)	For: TGCCTGTCTCCTGCTTCT Rev: AGTTGGTGTCTCGCTTCC
NF-κB	For: CGACGTATTGCTGTGCCTTC Rev: TGAGATCTGCCAGGTGGTAA

Hemostasis by compression was performed in the incision site by cotton ball. After surgery, 4×10^5 U penicillin was administrated for infection prevention. One week later, right kidney was wholly excised and penicillin was also administrated for infection prevention. After 4 weeks, the blood was collected from the caudal vein and angular vein respectively. The urea nitrogen and serum creatinine were determined with an automatic biochemical analyzer (Hitachi 7100, Japan) to judge the successful establishment of rats bearing uremia.

PD treatment

Rats bearing uremia with successful modeling were used to further establish PD models. Rats were anaesthetized in the same way as described above and then fixed in supine position. A section of 2 cm from the needle end of medical silica gel drainage tube was cut and then the tube was sealed with heparin cap. 5-8 side holes (0.2-0.3 cm) were pricked with fine needle from the tail. Every two Krafts were made in the exits of both the head and the enterocoelia. The tubes were sterilized before the animal experiments. Subcutaneous tunnel was done in the original right incision. The site about 1 cm below the midpoint of the ligature between two ears of rats was employed as the exit of the tunnel. Heparin saline was infused through dialysis tube and the solution outflow was observed. 4×10^5 U penicillin was administrated for infection prevention. From 7 days after the surgery, rats were regularly and continuously treated with PD for 20 days by infusing 4.25% duplex PD solution at a volume of 3 ml/100 g body weight of rats. After PD treatment, the body weight was weighed and the serum creatinine was determined.

Pathological examination

Renal tissues of rats in various groups were collected and fixed with 4% paraformaldehyde.

After embedding, the tissues were sliced, dewaxed, and hydrated. Then the slices were stained with hematoxylin for 5 min and washed with running water. After stained with eosin for 3 min, they were washed with running water again and dehydrated. The slices were mounted with neutral resin and subjected to be examined under a microscopy (CX23, Olympus, Japan).

Celecoxib administration and peritoneal tissue separation

Rats with PD treatment were randomly divided into four groups: PD control and celecoxib treatments (10, 20, and 50 mg/kg body weight). Every day, celecoxib at different doses was administrated into rats with PD treatment by gavage, while in the control group, saline was dosed in the same way. 24 h after PD stopped; peritoneal tissues were separated from the parietal layer and omentum respectively.

Real-time (RT) PCR

The expressions of NF-κB, TLR4 and Hepcidin were examined with RT-PCR. Samples of peritoneal tissues were grinded in liquid nitrogen. Total RNA was collected by RNA extraction kit according to its instruction for use. RNA purity was determined with a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). RNA was reversely transcribed into cDNA by using reverse transcription kit according to its instruction for use. Primers were designed according to the mRNA sequences of target genes which were found in NCBI database. They were prepared by Sangon Biotech (Shanghai, China). Their sequences were listed in **Table 1**. PCR system (20 μl) was shown as follows: TaqMan® Gene Expression Assay (20×) 1 μl, cDNA products 5 μl, TaqMan® Gene Expression Master Mix (2×) 10 μl, nuclease-free water 4 μl. Reaction parameters were demonstrated as follows: holding stage 50°C for 2 min, 94.2-97.4°C for 10 min; cycling stage 93-95°C for 15 s, 60°C for 1 min; 37-40 cycles. After the cycling finished, it was assessed on a fluorescent quantitation PCR (Applied Biosystems 7500, Foster city, CA, USA). Cycle threshold (CT) values of genes in RT-PCR were recorded. CT value of internal control gene from that of target gene equaled to ΔCT. Average ΔCT of each specimen from that of the control equaled ΔΔCT. The relative expression level of target

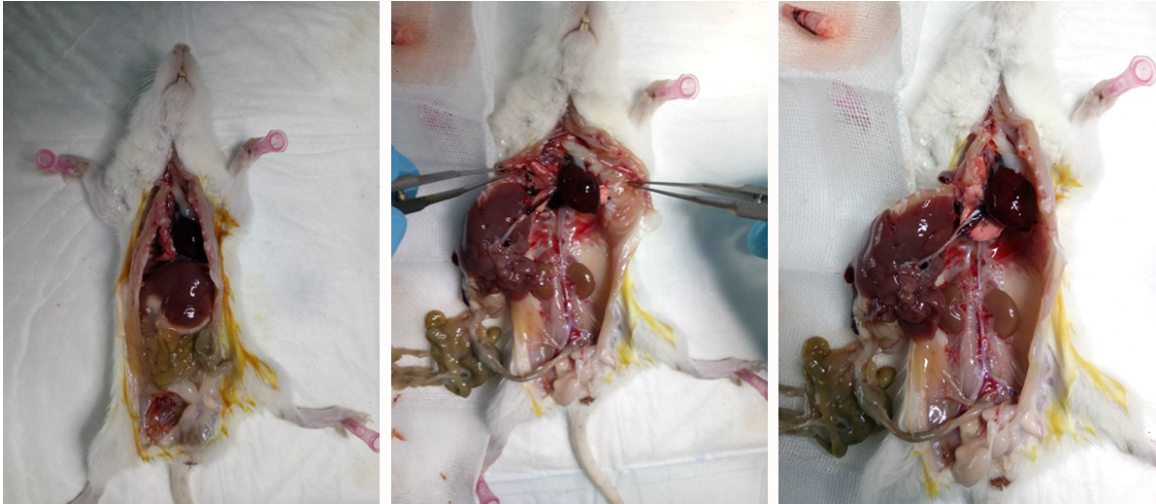


Figure 1. Pictures of rats during the establishment of rat models bearing uremia.

Table 2. Renal function parameters at 4 weeks after the surgery in rats in various groups

Groups	Rats amounts	Urea nitrogen (mmol/l)	Serum creatinine (μ mol/l)
Control	20	2.39 \pm 0.16	27.1 \pm 2.2
Sham operation	20	2.67 \pm 0.33	28.3 \pm 3.7
Uremia model	16	8.81 \pm 0.51*	63.9 \pm 5.9**

*P = 0.029, **P = 0.0049 vs. the sham operation group.

gene was calculated as $2^{-\Delta\Delta CT}$, so the relative expression level of the control gene was $2^0=1$.

Western blot

Samples of peritoneal tissues were grinded in liquid nitrogen. Proteins were collected by using ReadyPrep Protein Extraction Kit according to its instruction for use and shocked briefly at a suitable frequency in ice. The lysate was centrifuged at 13000 rpm and 4°C for 20 min. The supernatant was drawn into a new centrifuge tube. The protein concentration was measured with BCA kit. Protein was loaded quantitatively to perform SDS-PAGE. Subsequently, the gel was immersed in transfer buffer for 10 min to fabricate transfer sandwich. The voltage was 100 V and the membrane was transferred for 60 min. Then the membrane was incubated with properly diluted anti-TLR4, anti-Hepcidin, anti-NF- κ B, and anti-GAPDH antibodies at room temperature for 2 h. It was rinsed and incubated with HRP labeled IgG (H+L) (1:10000) in Tris-buffered saline and tween (TBST) buffer containing 0.0% (w/v) non-fat milk. Then the

membrane was rinsed with TBST for 3 times for 5-10 min every time. Finally, the membrane was exposed on a gel imaging system (ChemiDocTM XRS, Bio-Rad, USA). A software "Quantity one" (v4.62, Bio-Rad, USA) was employed to calculate the gray values of proteins strips. GAPDH served as the internal control.

Statistical analysis

All data were represented as mean \pm standard deviation (SD). Statistical analysis was performed with ANOVA followed by Bonferroni *post hoc* test by using SPSS 17.0 software. Differences were considered to be significant at $P < 0.05$.

Results

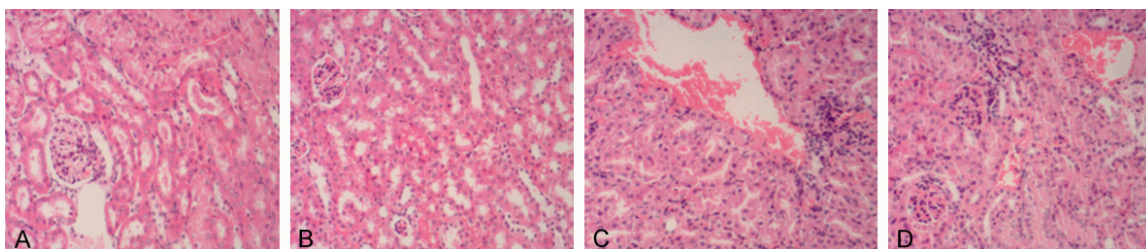
Establishment of rat models bearing uremia

During the model establishing process, four rats in the uremia model group died accidentally, which might be attributed to the postoperative infection or organ injury. Therefore, the successful rate of this PD animal model was 80%. In the control and sham operation groups, no death of rats was observed. Pictures of rats during the surgery were shown in **Figure 1**. The parameters showing renal function (urea nitrogen and serum creatinine) in three groups were measured. If the values of urea nitrogen and serum creatinine in rats suffered any treatment were more than two times of those in the control rats, the rats bearing uremia were

Table 3. Serum creatinines and body weight of rats in various groups on 1 day and 15 day after PD treatment

Groups	Rats amounts	Serum creatinine ($\mu\text{mol/l}$)		Body weight (g)	
		1 d	15 d	1 d	15 d
Control	20	27.1 \pm 2.2	26.2 \pm 1.5	305.0 \pm 1.7	412.6 \pm 10.2
Sham operation	20	28.3 \pm 3.7	26.7 \pm 2.9	296.4 \pm 3.9	436.1 \pm 6.8
Uremia model	3	64.9 \pm 4.3*(P=0.015)	63.8 \pm 3.3*(P=0.027)	317.6 \pm 5.2*(P=0.013)	344.7 \pm 4.8***(P=0.001)
Uremia model with PD treatment	13	65.2 \pm 1.6*(P=0.032)	59.6 \pm 1.8*(P=0.020)	309.8 \pm 2.2*(P=0.032)	389.4 \pm 12.4***(P=0.001) #(P=0.021)

*P < 0.05, **P < 0.01 vs. the sham operation group; #P < 0.05 vs. the uremia model group.


Figure 2. The pathological examination of renal tissues from rats in the control (A), sham operation (B), uremia model (C), uremia model with PD treatment (D) groups (Magnification \times 200).

defined to be obtained successfully. As shown in **Table 2**, the values of both urea nitrogen and serum creatinine were similar between the control and sham operation groups ($P = 0.124$). However, the urea nitrogen and serum creatinine values in the uremia model group were more than 3 and 2 times of those in the sham operation groups, respectively. It was suggested that the model of rats bearing uremia was successfully constructed.

Establishment of rats bearing uremia with PD treatment

The PD catheter was prepared and the successful model of rats bearing uremia was treated with regular PD. Results of the body weight of rats and the serum creatinine after PD treatment were demonstrated in **Table 3**. There was no significant difference in the body weight and serum creatinine between the control and sham operation groups ($P = 0.130$). In comparison with the sham operation group, the serum creatinines in rats bearing uremia only and with PD treatment on both 1 day and 15 day were remarkably elevated. Moreover, although the body weights of rats bearing uremia only and with PD treatment were greater than those in the sham operation group on 1 day, the body weights of rats bearing uremia only and with PD treatment on 15 day were sharply lowered. Fortunately, significant body weights gain of

rats bearing uremia after PD treatment on 15 day were found. It was indicated that the PD treatment raised the body weights of rats bearing uremia from 1 day to 15 day.

Pathological examination

The pathological examination of renal tissues from rats with diverse treatments was shown in **Figure 2**. Rats in the control and sham operation groups demonstrated similar and normal renal pathological results (**Figure 2A** and **2B**). In rats bearing uremia, we found the increase of extracellular matrix, hyaline-like change of minority afferent glomerular arterioles, and foam-like change of endothelial cells (**Figure 2C**), suggesting the severe damage of renal tissues. However, the renal damage in rats bearing uremia after PD treatment was obviously alleviated (**Figure 2D**).

Effects of celecoxib on TLR4 and Hepcidin

Sixteen rats bearing uremia with PD treatment were randomly divided into four groups ($n = 4$): PD control and celecoxib treatments (10, 20, and 50 mg/kg body weight). The effects of celecoxib on the expressions of TLR4 and Hepcidin in peritoneal tissues were evaluated by RT-PCR and western blot, and as the node molecule in the TLR4 signal pathway, NF- κ B was investigated together (**Figure 3**). We found

Molecular mechanism of celecoxib in peritoneal dialysis treatment

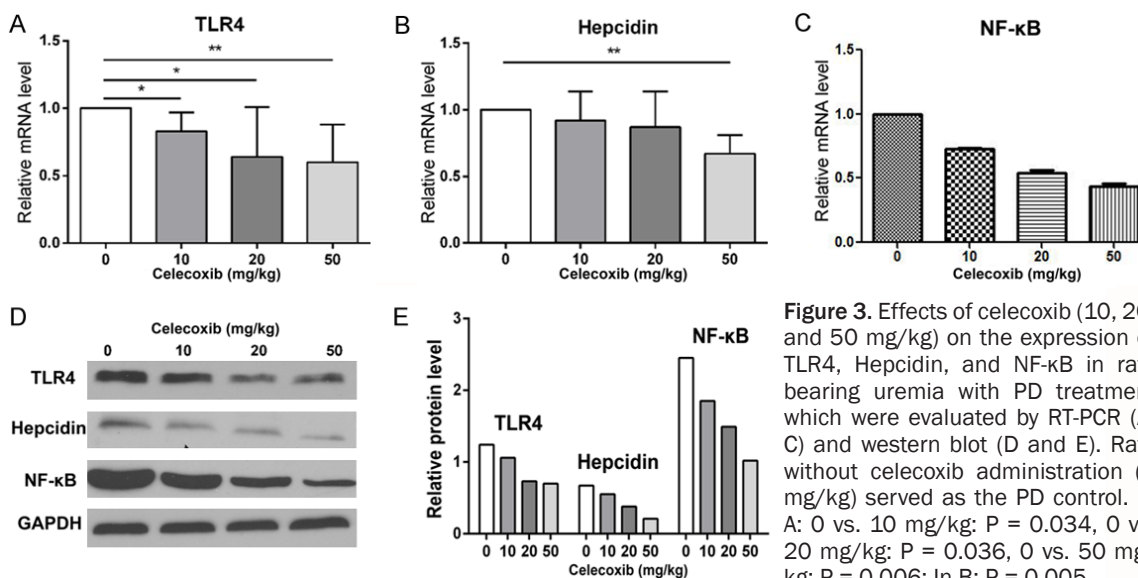


Figure 3. Effects of celecoxib (10, 20, and 50 mg/kg) on the expression of TLR4, Hecpidin, and NF-κB in rats bearing uremia with PD treatment which were evaluated by RT-PCR (A-C) and western blot (D and E). Rats without celecoxib administration (0 mg/kg) served as the PD control. In A: 0 vs. 10 mg/kg; $P = 0.034$, 0 vs. 20 mg/kg; $P = 0.036$, 0 vs. 50 mg/kg; $P = 0.006$; In B: $P = 0.005$.

that celecoxib significantly downregulated the mRNA levels of TLR4, Hecpidin, and NF-κB (Figure 3A-C). Compared to the PD control group, celecoxib at the doses of 10, 20, and 50 mg/kg reduced the level of TLR4 mRNA by 17%, 36%, and 40%, respectively. Celecoxib at the dose of 50 mg/kg rather than 10 and 20 mg/kg decreased the level of Hecpidin mRNA by 33% as compared with the PD control group. Celecoxib gradually reduced the level of NF-κB mRNA along with the progressively increased dosage of celecoxib.

Furthermore, we employed Western blot to probe the effects of celecoxib on the expression of NF-κB, TLR4 and Hecpidin at protein level (Figure 3D and 3E). The results of protein level from Western blot agreed with the above results of mRNA level from RT-PCR. Celecoxib downregulated the expression of TLR4 and Hecpidin and higher dose of celecoxib exerted deeper extent of downregulation. Accordingly, the expression of downstream NF-κB protein was also remarkably inhibited by celecoxib, especially at higher doses.

Discussion

PD is a common method as the renal replacement treatment for CRD patients. However, some troubles such as peritoneal sclerosis and dialysis dysfunction in the PD process would weaken the dialysis efficacy and even induce ultrafiltration failure or peritonitis, which restricted the clinical application of PD. The in-

crease of peritoneal neovascularization and inflammation reaction changed the peritoneal permeability and the osmotic pressure between blood and dialysis solution, which might be an important cause of PD side effects [9]. Therefore, the fundament research about the peritoneal structure and its microenvironment in PD process will facilitate understanding the side effects of PD and guiding the clinical treatment of renal failure.

Animal model is an important tool in basic medical research. The establishment of an easy PD animal model with highly successful rate is extremely significant for the research about the physiology, pathology, and clinic of PD. At present, there are mainly two methods to establish rats bearing uremia including excision of partial or whole kidney by surgery and feed with adenine-rich foods [10, 11]. In this study, we excised the kidney by a modified surgery method to establish the rat model bearing uremia [12]. These rats bearing uremia were treated with PD by homemade dialysis catheters to sufficiently simulate the PD treatment in human. The results of biochemical indexes and pathological characterization confirmed the successful building of rats bearing uremia with a successful rate of 80%. Moreover, the PD treatment significantly alleviated the complications of uremia in rats and improved the normal growth of rats bearing uremia.

TLR is a kind of important proteins participating in nonspecific immunity (innate immunity)

and it is also the bridge connecting nonspecific and specific immunities. TLR4 is a common TLR with wide distribution. At present, TLR is found to be expressed in many immune cells such as macrophage, monocyte, and lymphocyte and various nonimmune cells such as endothelial cells and smooth muscle cells [13, 14]. TLR4 is abnormally overexpressed in chronic renal failure, especially during the disease development of diabetic nephropathy. The abnormal activation of TLR4/NF- κ B signal pathway would trigger a series of immune responses, causing drastic changes of tissue or cellular microenvironment. Meanwhile, these abnormal immune responses usually have the function of cascade amplification, which further damages the related tissues. For example, after stimulated by endogenous and exogenous ligands of TLR4 (such as lipopolysaccharide), renal tubular epithelial cells express and secrete a large number of inflammation factors and the expressions of inflammation factors such as tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and interleukin-8 (IL-8) are remarkably elevated in a short time [15-17]. In clinical research, inflammation factors could be expressed in the dialysis solution used for PD. Hepcidin is a kind of bactericidal peptides. The abnormal activation of TLR4/NF- κ B signal pathway might upregulated the expression of Hepcidin. The glomerular filtration rate in ESRD patients is reported to be correlated with serum Hepcidin [18, 19]. Therefore, the investigation of the activation state of TLR4/NF- κ B signal pathway and the Hepcidin expression in the peritoneal tissues during PD process will contribute significantly to the understanding of the inflammation and microenvironment change during PD process.

As a new generation of nonsteroidal anti-inflammatory drug, celecoxib works by selectively inhibiting the COX-2 and thereby suppressing the production of prostaglandin [20]. As compared with other anti-inflammatory drugs, celecoxib exhibits favorable efficacy and relatively low side effect [20]. Long-term PD causes the inflammation infiltration in peritoneal environment and the increase of neovascularization, consequently resulting in some side effects such as the reduction of filtration effectiveness. Fortunately, as the specific inhibitor of COX-2, celecoxib is deemed to simultaneously have both anti-inflammatory and anti-

angiogenic effects, which plays a crucial role in the protection of peritoneal structure integrity and stable function. In this study, we revealed that celecoxib downregulated the expressions of TLR4 and Hepcidin in both mRNA and protein levels and these downregulation effects were positively dose-dependent. The interference of celecoxib downregulated the TLR4 expression and subsequently suppressed the abnormal activation of TLR4 signal pathway, which contributed to the reduction of inflammation infiltration in peritoneal environment and the protection of peritoneal integrity in rats bearing uremia after PD treatment. Furthermore, as a node molecule in TLR4 signal pathway, NF- κ B is well known as a pro-inflammatory cytokine. Therefore, NF- κ B might also be an important signal molecule involved in the regulation of celecoxib in PD treatment. The alteration of NF- κ B expression after celecoxib treatment at different doses was assessed by western blot. Results also showed that celecoxib downregulated the expression of NF- κ B as a dose-dependent manner, which could be one of the molecular mechanisms involved in the regulation of celecoxib on PD treatment in rats bearing uremia as well.

In conclusion, PD treatment alleviated the complications of uremia in rats. As a drug treating the peritonitis in clinical PD treatment, celecoxib worked through downregulating the expressions of TLR4, Hepcidin, and NF- κ B in TLR4-Hepcidin pathway. These results might serve as guidelines for the rational design of diagnosis reagent and targeting drugs in the combination application with PD treatment for uremia therapy.

Acknowledgements

This work was supported by the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (No. 2011-BAI10B08), Linyi Science & Technology Development Plan Project (No. 201616006).

Disclosure of conflict of interest

None.

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