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Original Article

Effects and functional mechanism of Tripterygium Wilfordii polyglycoside with catalpol on diabetic nephropathy

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Abstract: Diabetic nephropathy (DN) is acommon and severe complication of diabetes. Tripterygium Wilfordii has dual roles in immune suppression and anti-inflammation, whilst catalpol can resist inflammation and oxidation. The effect of Tripterygium Wilfordii combined with catalpol in treating DN, has not been reported. Wistar rats were treated with Tripterygium Wilfordii polyglycoside and or catalpol after DN model was established. Serum levels of creatine (Scr), urea nitrogen (BUN), urea albumin (UAlb), lactate hydrogenase (LDH) and superoxide peroxidase (SOD) were measured at 4 weeks after treatment. Real-time PCR was employed for detecting the mRNA level of transformation growth factor (TGF)-β1, whilst caspase 3 activity, secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-1β were measured by ELISA. DN rats had elevated levels of Scr, BUN and UAlb, plus enhanced expression of TGF-β1, TNF-α and IL-1β. Caspase 3 and LDH activity were also increased, with lower SOD level ($P<0.05$ compared with control group). Either Tripterygium Wilfordii polyglycoside or catalpol treatment significantly decreased the levels of Scr, BUN or UAlb, suppressed Caspase 3 activity, decreased expression of TGF-β1, TNF-α and IL-1β expression, suppressed LDH activity whilst increased SOD level ($P<0.05$ compared with DN model rats). Combined treatment group had better improvements than single usage ($P<0.01$). In conclusion, both Tripterygium Wilfordii polyglycoside and catalpol can regulate the balance between oxidation and anti-oxidation, suppress apoptosis and inflammation, with combined therapy having better efficiency.

Keywords: Tripterygium Wilfordii polyglycoside, catalpol, diabetic nephropathy, anti-oxidation, inflammation

Introduction

Diabetes is the most common metabolic syndrome worldwide, with rapidly increased incidence due to the influence of life style and food habit transition [1, 2]. China is an epidemic area of diabetes, whose onset age is becoming younger [3]. Common complications of diabetes include cardiovascular or peripheral vascular incidence as a result of major vessel malformation, plus micro-vessel related complications such as diabetic retinopathy, diabetic nephropathy (DN) and neural disorders, leading to higher morbidity or mortality [4, 5]. DN is a common and severe chronic complication of diabetes. Due to its high incidence and slow progression, it may eventually develop into end-stage renal disease (ESRD) and subsequent death. Pathological features of DN mainly manifest as renal vessel damage as a result of diabetes or other factors, further leading to hyperplasia of extracellular matrix (ECM), thickening of glomerular basal membrane, sclerosis of glomerulus, eventually developing to DN [6, 7]. Although various treatment have been developed to treat diabetes and complications, including blood glucose management or symptomatic treatment, the retard of DN progression cannot prevent the development of DN into renal failure, which requires dialysis or kidney transplant [8, 9].

DN has a very complicated pathogenesis mechanism, involving genetics, physics, chemistry and environmental factors. Previous study indicated the involvement of inflammatory cytokines, oxidative stress and growth factors in the DN occurrence and progression [10]. Therefore,

preventive treatment approaches for DN have become a major challenge. Tripterygium Wilfordii polyglycoside is a major component extracted from Tripterygium Wilfordii [11, 12], and belongs to non-steroid immune suppressant with pluripotent pharmaceutical activities especially having prominent anti-inflammatory functions [13]. Catalpol is the major effective component of radix rehmanniae, and a small molecule iridoid glucosides compound [14]. Previous studies revealed pluripotent bio-activities of catalpol, including anti-tumor, toxicity against fungus, virus, treating Alzheimer's Disease, suppressing microvascular permeability, as well as anti-inflammation [15, 16]. The effect of Tripterygium Wilfordii polyglycoside and catalpol on DN, however, has not been reported yet.

Materials and methods

Experimental animals

A total of 40 healthy male Wistar rats (aged 3 months, SPF grade, weighted 250±30 g) were purchased from Laboratory Animal Center of Beijing University of Chinese Medicine and were kept in an SPF grade facility. The facility was kept at a fixed temperature (21±1°C) and relative humidity (50%~70%) with 12 h light/dark cycle.

Rats were used for all experiments and all procedures were approved by the Animal Ethics Committee of Dongfang Hospital of Beijing University of Chinese Medicine.

Major materials and equipment

Tripterygium Wilfordii polyglycoside was purchased from Huitain Bio (China). Catalpol was purchased from Biological Product Institute of China. Surgical instrument was purchased from Suzhou Med Instrument (China). Trizol reagent was purchased from Invitrogen (US). Streptozotocin (STZ) was purchased from Sigma (US). Serum creatine assay kit was purchased from Roche (US). RSV nucleic acid test kit was purchased from Zhijiang Biotech (China). PVDF membrane was purchased from Pall Life Sciences (US). Chemical reagents for Western blotting were purchased from Beyotime (China). ECL kit was purchased from Amersham Biosciences (US). Rat anti-mouse TGF-β1 monoclonal antibody, and goat anti-mouse horseradish peroxidase (HRP) conjugated IgG antibody were obtained from Cell Signaling (US). RNA extraction kit and reverse transcription kit were purchased from Axygen (US). Test kits for TNF-α, IL-1β were purchased from R&D (US). Caspase 3 activity kit was purchased from Pall Life Science. Rat urea protein assay for albumin was purchased from Furui (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). DNA amplification cycler was purchased from PE Gene Amp System 2400. Electric blood glucose meter was purchased from Advantage (US). Automatic biochemical analyzer was purchased from Beckman (Germany). Other common reagents were purchased from Sangon (China).

Animal grouping

40 rats were randomly assigned into five groups (N=8 in each group). DN group was prepared for establishing disease model using 45 mg/kg STZ. Model rats then received Tripterygium Wilfordii polyglycoside (10 mg/kg daily by gavage as previously described [13]), catalpol (20 mg/kg daily by gavage for 4 weeks [14]) or combined treatment (daily for four consecutive weeks [15]).

DN model preparation

After one week acclimation, rats were fasted for 12 h. 0.5% STZ prepared in sterile citric acid-citric acid sodium buffer was injected via the tail vein at a dose of 40 mg/kg. Control group received equal volume of citrate buffer. Two weeks later, blood glucose and urea sugar levels were measured. Those rats with higher than 16.7 mmol/L blood glucose, urea sugar below "++", plus at least one-fold increase of urea volume were determined as successfully established DN model [17].

Sample collection

Rat abdominal aorta blood samples were collected in a negative pressure tube and left at room temperature for 30 min incubation. After blood clotting, samples were centrifuged at 3600 rpm at 4°C for 10 min. The supernatant was saved and frozen at -20°C for further use. Rats in all groups were sacrificed and left renal tissues were collected, which were kept at -80°C for further assays.

Table 1. Primer sequences

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	ACCAGGTATCTTGGTTG	TAACCATGTCAGCGTGGT
TGF-β1	CAGTAGTGGTCTCTACCGCC	TCATTAACCCTCTCACAGAACC

Kidney function index assay

A fully automatic biochemical analyzer was used to analyze serum levels of Scr and BUN. Radioimmunology assay was used to quantify UAlb. Kidney/body weight ratio was then calculated.

LDH and SOD activity in renal tissues

Activity of SOD and LDH in rat kidney tissues was measured following the manual instruction of the test kit. In brief, tissue proteins were extracted by 95°C for 40 min. Proteins were rinsed in cold water and centrifuged at 4000 rpm for 10 min. Ethanol-chloroform mixture (5:3 v/v) was used to extract the ethanol phase for testing LDH and total SOD activity.

Caspase 3 activity assay

Caspase 3 activity in rat renal tissues was evaluated following manual instruction of test kit. In brief, cells were digested by trypsin, and centrifuged at 600 g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20 000 g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. Optical density (OD) values at 450 nm wavelength were measured to evaluate the caspase 3 activity.

ELISA for serum inflammatory factors TNF-α and IL-1β

Serum samples from all groups of rats were collected and tested for expression levels of TNF-α and IL-1β following the manual instruction of ELISA kits. In brief, 96-well plate was added with 50 μl serially diluted samples, which were used to plot standard curves. 50 μl test samples were then added into test wells in triplicates. After washing for 5 times, liquids were discarded to fill with washing buffer for 30 sec vortex. The rinsing procedure was repeated for 5 times. 50 μl enzyme-labeled reagent was then added into each well except blank control. After gentle mixture, the well was incubated for 30 min at 37°C. Chromogenic substrates A and B were sequentially added (50 μl each), followed by 37°C dark incubation for 10 min. The test plate was then mixed with 50 μl quenching buffer as the blue color turned into yellow. Using blank control well as the reference, absorbance (A) values at 450 nm wavelength were measured by a microplate reader within 15 min after adding quenching buffer. Linear regression model was then plotted based on the concentrations of standard samples and respective A values. Sample concentration was further deduced based on A values and regression function.

Real-time PCR for renal expression of TGF-β1 mRNA expression

Trizol reagent was used to extract RNA from rat renal tissues from all groups. DNA reverse transcription was performed following the manual instruction, using primers designed by Primer-Primer6.0 and synthesized by Invitrogen (China) as shown in **Table 1**. Real-time PCR was performed under the following conditions: 5 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the internal control. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis using $2^{-\Delta Ct}$ method.

Statistical analysis

SPSS 19.0 statistical software was used for analysis. Measurement data were presented as mean ± standard deviation (SD). Comparison of means across multiple groups was performed using one-way analysis of variance (ANOVA). A statistical significance was defined when $P<0.05$.

Results

Live status and renal function assay of rats

Living status, kidney/body weight ratio and renal function index were observed. Results showed good mental status, shining furs, normal diet, drinking and motility, and normal urea volume. However, DN model rats showed aggravated mental status, fur detachment, increased food/water intake, larger urea volume, plus lower body weight, kidney/body weight ratio, and significantly elevated levels of Scr, BUN

Table 2. General condition and kidney function index

Index	Control	DN	Tripterygium Wilfordii polyglycoside	Catalpol	Combined treatment
Body weight (g)	491.4±52.7	227.9±36.1*	302.8±31.2*,#	298.7±29.5*,#	417.2±26.4*,##
Blood glucose (mmol/L)	6.3±0.5	27.6±2.3	17.1±2.5*,#	16.3±2.9*,#	11.1±3.1*,##
Kidney/body (mg/g)	2.5±0.3	5.7±0.9*	4.6±0.7*,#	4.8±0.5*,#	3.2±0.1*,##
Scr (μmol/L)	89.2±12.3	1577.6±41.5*	982±81.2*,#	891±89.7*,#	582±71.2*,##
BUN (mmol/L)	7.1±0.8	13.2±1.1*	10.7±1.7*,#	10.1±1.5*,#	9.2±1.5*,##
UAlb (mg/24 h)	0.3±0.1	1.5±0.2*	0.8±0.1*,#	0.9±0.2*,#	0.6±0.2*,##

Note: *, P<0.05 compared to control group; #, P<0.05, ##, P<0.01 compared to DN group.

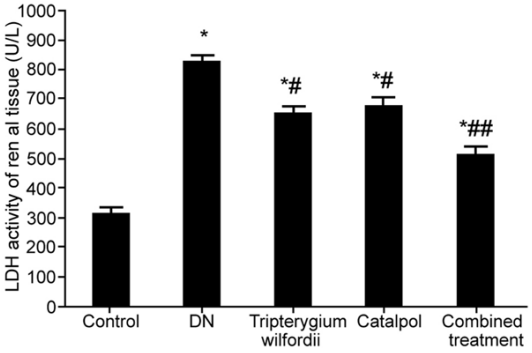

Figure 1. Combined effects of Tripterygium Wilfordii polyglycoside and catalpol on LDH activity. *, P<0.05 compared with control group; #, P<0.05, ##, P<0.01 compared with DN group.

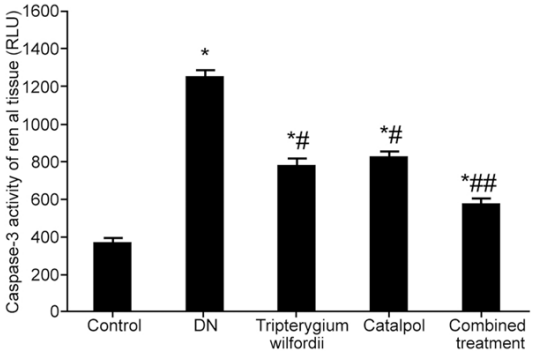

Figure 3. Effects of Tripterygium Wilfordii polyglycoside and catalpol on rat renal caspase 3 activity. *, P<0.05 compared with control group; #, P<0.05, ##, P<0.01 compared with DN group.

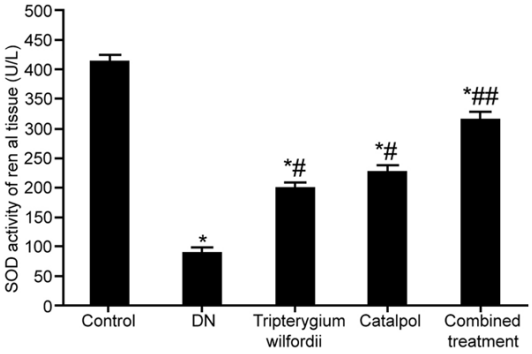

Figure 2. Effects of Tripterygium Wilfordii polyglycoside and catalpol on rat SOD activity. *, P<0.05 compared with control group; #, P<0.05, ##, P<0.01 compared with DN group.

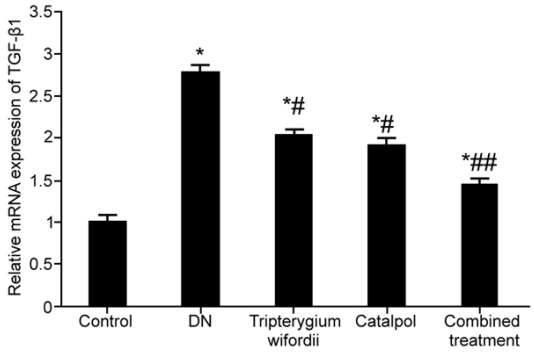

Figure 4. Effects of Tripterygium Wilfordii polyglycoside and catalpol on rat renal mRNA expression of TGF-β1. *, P<0.05 compared with control group; #, P<0.05, ##, P<0.01 compared with DN group.

and UAlb (P<0.05 compared with control group). Single or combined treatment of Tripterygium Wilfordii polyglycoside or catalpol treatment improved the general condition of rats, with improved fur color, body weight, and decreased levels of Scr, BUN or UAlb (P<0.05 compared with DN group). However, better effects were obtained when using combined treatment (P<0.01, **Table 2**).

Combined effects of Tripterygium Wilfordii polyglycoside and catalpol on LDH of DN rats

We analyzed the change of LDH activity in renal tissues after single or combined treatment using Tripterygium Wilfordii polyglycoside and/or catalpol. Results demonstrated elevated LDH activity in DN rats (P<0.05 compared with control group). After single or combined treat-

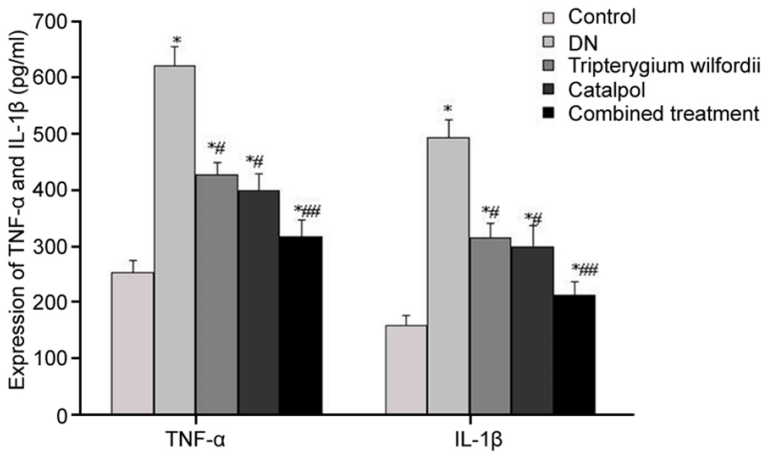

Figure 5. Effects of Tripterygium Wilfordii polyglycoside and catalpol on inflammatory factor expression in DN rat serum. *, P<0.05 compared with control group; #, P<0.05, ##, P<0.01 compared with DN group.

ment, LDH activity was significantly decreased (P<0.05 compared with DN group). However, combined treatment had strong effects (P< 0.01, **Figure 1**).

Effects of Tripterygium Wilfordii polyglycoside and catalpol on rat SOD activity

We analyzed the change of SOD activity in renal tissues after single or combined treatment. Results showed suppressed SOD activity in DN rats (P<0.05 compared with control group). After single or combined treatment, SOD activity was significantly increased (P<0.05 compared with DN group) with combined treatment having more potent effects (P<0.01, **Figure 2**).

Effects of Tripterygium Wilfordii polyglycoside and catalpol on rat renal caspase 3 activity

We analyzed the effect of single or combined treatment on renal Caspase 3 activity. Results showed elevated Caspase 3 activity in DN rats (P<0.05 compared with control group). After single or combined treatment, Caspase 3 activity was significantly suppressed (P<0.05 compared with DN group) with combined treatment having stronger effects (P<0.01, **Figure 3**).

Effects of tripterygium wilfordii polyglycoside and catalpol on rat renal mRNA expression of TGF-β1

Real-time PCR was used to test the effect of single or combined treatment on renal TGF-β1 mRNA expression. Results showed elevated TGF-β1 mRNA in DN rats (P< 0.05 compared with control group). However, after single or combined treatment, TGF-β1 mRNA was significantly suppressed (P<0.05 compared with DN group) with stronger effects observed after combined treatment (P<0.01, **Figure 4**).

Effects of Tripterygium Wilfordii polyglycoside and catalpol on inflammatory factor expression in DN rat serum

ELISA was used to test the effect of single or combined treatment on serum levels of inflammatory factors including TNF-α and IL-1β. Results showed significantly elevated serum TNF-α and IL-1β in DN rats (P<0.05 compared with control group). After single or combined treatment, TNF-α and IL-1β levels were significantly suppressed (P<0.05 compared with DN group). However, combined treatment had more potent effects (P<0.01, **Figure 5**).

Discussion

Diabetes can induce microvascular disease, leading to protein urea, accompanied with progressive renal dysfunction, eventually leading to the development of DN [8]. DN can cause irregular renal hemodynamic, and thickening of glomerular basal membrane, kidney hypertrophy and enhanced ECM, which results in endothelial cell dysfunction and further irreversible kidney function failure [18]. Currently major treatments for DN include blood glucose management, and blood pressure control by angiotensin transferase inhibitor (ACEI) but with unsatisfactory efficiency [19]. Therefore, the illustration of DN pathogenesis, and the identification of effective treatment medication, can retard the occurrence and progression of DN.

Recent studies showed an important role of inflammation and immune injury in DN pathogenesis [20]. Body oxidative stress injury is also closely correlated with DN occurrence [21]. Tripterygium Wilfordii polyglycoside plays an important role in regulating inflammatory res ponse via enhancing vascular permeability and inhibiting platelet aggregation [13]. On the

other hand, catalpol, extracted from traditional Chinese medicine, plays an important role in anti-inflammation and regulating oxidation-reduction balance [16]. The combined effect of Tripterygium Wilfordii polyglycoside and catalpol in treating DN, however, has not been reported. This study aimed to investigate the effect of Tripterygium Wilfordii polyglycoside and/or catalpol treatment on DN. Results showed significant improvement of DN symptoms, decrease of blood glucose, protein urea, Scr and BUN by single usage of Tripterygium Wilfordii polyglycoside or catalpol. However, combined treatment had more potent effects in ameliorating DN symptoms.

During DN development, inflammation and oxidative stress disrupt body oxidation/anti-oxidation balance, leading to production abundant reactive oxygen species (ROS). Due to the decrease of important anti-oxidation enzyme SOD for clearing body ROS, and higher LDH activity during cell damage or death, glomerular mesenchymal cells proliferation was then stimulated, plus injury of vascular endothelial cells by inflammation factors and decreased release of nitric oxide (NO), thus facilitating urea trace protein production and subsequent accelerating DN progression [22, 23]. This study demonstrated elevated expression of TGF-β1, TNF-α and IL-1β during DN development, plus increased Caspase 3 and LDH activity, and decreased SOD. TGF-β1, which exists as a pre-propeptide, can be prominently activated during diabetes and thus participating in DN pathology. As important inflammatory factors, TNF-α and IL-1β can stimulate inflammation, and induce DN-related vascular endothelial injury, leading to increased Caspase 3 activity and renal tissue apoptosis [24, 25]. This study found that Tripterygium Wilfordii polyglycoside and catalpol administration inhibited Caspase 3 activity, decreased TGF-β1, TNF-α and IL-1β expression, suppressed LDH activity, and increased SOD activity. However, combined usage of both drugs exerted more potent modulatory effects on inflammation, oxidative stress and apoptosis. This study further plans to investigate the functional mechanism of Tripterygium Wilfordii polyglycoside and catalpol in the treatment of DN.

Conclusion

Tripterygium Wilfordii polyglycoside orcatalpol can regulate oxidation/anti-oxidation balance, inhibit apoptosis or inflammation progression during DN development. However, the combined treatment using both drugs has better treatment efficiency.

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Disclosure of conflict of interest

None.

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