Original Article

Protective effects of She Jing Xiao Bai capsule on diabetic nephropathy in a diabetic rat model

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Abstract: Objective: To investigate the protective effects and mechanism of She Jing Xiao Bai capsule, a traditional Chinese medicine, on diabetic nephropathy (DN) in a rat diabetes model. Methods: Diabetes was induced in 32 male Sprague-Dawley rats by intraperitoneal injection of streptozotocin (STZ). Rats were randomly allocated to a control group, and groups given She Jing Xiao Bai capsule, irbesartan, or She Jing Xiao Bai capsule plus irbesartan. Serum creatinine and 24 h urine protein were assayed, and renal pathology was evaluated at 2, 4, 6 and 8 weeks after treatment. Transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) were assayed by immunohistochemistry. Spleen CD4+Foxp3+T cells were assayed by flow cytometry. Results: Urine protein levels were significantly lower in rats treated with She Jing Xiao Bai capsule, irbesartan, or She Jing Xiao Bai capsule plus irbesartan than in control rats. Renal TGF-β and CTGF expression decreased, and splenic CD4+Foxp3+T cells increased, with treatment. The strongest effect was observed following treatment with She Jing Xiao Bai capsule plus irbesartan. Conclusion: She Jing Xiao Bai capsule significantly reduced renal injury in this STZ-induced DN model. Treatment was associated with decreased TGF-β and CTGF expression and increased numbers of CD4+Foxp3+T cells.

Keywords: Diabetic nephropathy, She Jing Xiao Bai capsule, urine protein, CD4+Foxp3+T cells

Introduction

The worldwide prevalence of type 2 diabetes mellitus (T2DM) is expected to rise along with increasing urbanization and aging of the population. Diabetic nephropathy (DN) is a common microvascular complication of T2DM, and is estimated to occur in 40% of diabetes patients. The pathogenesis of DN is not fully understood, but may include innate immune responses [1]. The formation of immune complexes such as NLRP3 inflammasomes in patients with hyperuricemia leads to hyperplasia and thickening of the glomerular basement membrane and mesangial matrix [2, 3]. Regulatory T cells (Tregs), a subpopulation of T cells previously called suppressor T cells, modulate the immune system, maintaining tolerance to self-antigens, and preventing autoimmune disease [4]. Tregs suppress or downregulate the stimulation and proliferation of effector T cells. Lower Treg counts have been observed in T2DM patients than in healthy controls, with significant reductions associated with disease progression [5].

She Jing Xiao Bai capsule used in this study were prepared at our hospital from purified Chinese medicines and contained black soybean, atractylodes, astragalus, Chinese yam, ant powder, and hirudo powder. We previously reported that treatment with She Jing Xiao Bai capsule plus irbesartan significantly reduced urine protein levels in a diabetes model [6]. However, the efficacy of She Jing Xiao Bai capsule monotherapy in a DN model, especially the effect on immune function is largely unknown. In this study, the protective effect of She Jing Xiao Bai capsules was investigated in an animal T2DM model with the objective of providing experimental data supporting clinical application in DN patients.

Materials and methods

Animal model and treatments

Diabetes was induced in 50 male Sprague-Dawley rats by intraperitoneal injection of streptozotocin (STZ, 1% in 0.1 mmol/L citric acid-
sodium citrate buffer, pH 4.2). The rats weighed approximately 220 g and were fasted for 12 h before treatment, but had free access to water. After 72 h, blood was collected from the tail vein to measure fasting blood glucose (FBG). FBG concentrations >16.7 mM for 10 consecutive days indicated successful development of the diabetes model. A 24 h urine protein concentration >30 mg indicated the presence of DN. Thirty-two rats developed DN and were randomly allocated in equal numbers (n=8) to a model control group and experimental groups given She Jing Xiao Bai capsule, irbesartan, or She Jing Xiao Bai capsule plus irbesartan. All treatments were administered intragastrically. She Jing Xiao Bai was given at a dose of 2 g/kg/d; each capsule contained black soybean (0.04 g), atractylodes (0.04 g), astragalus (0.04 g), Chinese yam (0.04 g), ant powder (0.04 g), and hirudo powder (0.02 g). Irbesartan was administered at 90 mg/kg/d as monotherapy, and the rats given combined therapy received 2 g/kg/d She Jing Xiao Bai capsule plus 90 mg/kg/d Irbesartan. The model control rats were given saline. Treatment continued for 8 weeks. Urine samples were collected at 2, 4, 6, and 8 weeks, and blood was collected from the tail vein at 2, 4, 6 weeks. At 8 weeks, animals were sacrificed by decapitation. At that time, blood was collected from the heart, and kidney tissue was obtained and either fixed in 10% formalin for hematoxylin-eosin (HE) staining and immunohistochemistry or frozen in liquid nitrogen for western blotting and quantitative real-time polymerase chain reaction (qRT-PCR). Rats were not given insulin to control the blood glucose level. This study was approved by Hospital of Changshou City New District.

**Biochemical assays**

A metabolic cage (Taichang Co, Ltd, China) was used to collect urine. Urine protein level was determined by the biuret method and serum creatinine (SCr) was assayed by the saturated picric acid method following the kit manufacturer’s instructions (Sichuang Maike Technology, China).

**Immunohistochemistry**

Fixed renal tissue was embedded in optimum cutting temperature compound (OCT) and sectioned at 5 μm for staining. The immunohistochemistry procedure was conducted following the staining kit manufacturer’s instructions (Beijing Zhongshan Biotech, Co, Ltd, China).

**Western blotting**

A 100 mg sample of renal column tissue taken from frozen kidney tissue was homogenized in proteolysis buffer and the total protein was isolated by centrifugation. The proteins in aliquots (50 μL) prepared from tissue of rats in each experimental group were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for staining. The membranes were incubated overnight with anti-transforming growth factor beta (TGF-β, 1:1000; Beijing Zhongshan Biotech, Co, Ltd, China) and anti-connective tissue growth factor (CTGF, 1:1000; Beijing Zhongshan Biotech, Co, Ltd, China). After washing, secondary antibody (1:5000) was applied for 1 h. The optical density of the blots was normalized against actin.

**Electron microscope**

Fresh tissue was fixated in 2.5% glutaraldehyde. After washed with 0.01 mol/L PBS buffer, the tissues were dehydrated in alcohol, embedded in EPOR812, microtomed, stained with acetic acid uranium and citromalic acid and observed by an electron microscope (Hitachi, Japan) with the magnification of 10000×.

**qRT-PCR**

mRNA was isolated from kidney tissue of each group using a trizol agent kit (Beijing Zhongshan Biotech, Co, Ltd, China) and the RNA content was assayed by ultraviolet spectrophotometry (Puruisi Instrument Co, Ltd, China) (A260/A230>1.8). Reverse transcription was carried out using random primer and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). The PCR assay of TGF-β and CTGF was performed with a quantitative ther-

| Table 1. Effects of treatment on FBG level |
|-----------------|--|----------------|----------------|
| Group | n | Before treatment | At 8 weeks |
| G1    | 8 | 15.35±4.73       | 16.45±2.34  |
| G2    | 8 | 16.05±2.68       | 16.04±2.29  |
| G3    | 8 | 16.76±3.80       | 14.87±2.80  |
| G4    | 8 | 15.76±2.24       | 14.65±2.60  |

G1, model control; G2, She Jing Xiao Bai capsule; G3, Irbesartan; G4, She Jing Xiao Bai capsule plus Irbesartan.
Effects of She Jing Xiao Bai capsule on DN mal cycler (Mastercycler ep realplex, Eppendorf, Germany) using the following procedure: 50°C 20 min (×1), 95°C 10 min (×1), 94°C 15 s, 46.4°C 20 s, 72°C 30 s (×45). Relative expression was calculated as the ratio of target cDNA to GAPDH. The primers used were TGF-β (449 bp) sense 5'-TGAACCAAGGAGACGGATA-3' and antisense 5'-CACGCAGCACGGTGATG-3'; CTGF (533 bp) sense 5'-CCGCCAACCGCAGGATT-3' and antisense 5'-CTTGGCAATTTAGGCTCC-3'; and GAPDH (115 bp) sense 5'-ATCGTGGAAGGGCTAATG-3' and antisense 5'-ATC GTGGAAGGGCTAATG-3'.

**CD4^+Foxp3^+T in spleen**

Fresh spleen cell suspensions were prepared, and red blood cells were hemolyzed using lysis buffer. The CD4^+Foxp3^+T cells were counted in suspensions containing at least 10^6 cells labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD25, anti-CD16/23 and phycoerythrin (PE)-conjugated anti-Foxp3 antibodies.

**Statistical analysis**

All data were reported as means ± standard deviation (SD). The statistical analysis was carried out by ANOVA with post hoc Turkey test using SPSS 12 (SPSS, Chicago, USA) *P*<0.05 was considered statistically significant.

**Results**

FBG level was not affected by the experimental treatments

FBG levels were determined in each group before and at 8 weeks after treatment. There were no significant differences in FBG levels among the groups of rats with DN before the start of treatment or after 8 weeks of treatment. Moreover, the experimental treatments did not affect the FBG levels within each group (*Table 1*).

**She Jing Xiao Bai capsule decreases urine protein in DN rats**

Urine protein levels were measured in each group at 2, 4, 6 and 8 weeks. In the model control group (given only saline) the urine protein level increased gradually, and was significantly higher at weeks 4, 6, and 8 than it was at week 2. Urine protein levels increased in rats treated with She Jing Xiao Bai capsule, but the rate of increase was much slower than that observed in the model control group. At week 8, urine protein levels in the She Jing Xiao Bai capsule, irbesartan, and She Jing Xiao Bai capsule plus irbesartan groups were significantly lower than that in the model control group (*Table 2*).

**She Jing Xiao Bai capsule decreased Scr in DN rats**

Scr levels were measured in each group at 2, 4, 6 and 8 weeks. In the model control group, Scr levels increased gradually, and were significantly higher at weeks 4, 6, and 8 than at week 2. At week 8, Scr levels in the She Jing Xiao Bai capsule, irbesartan, and She Jing Xiao Bai capsule plus irbesartan groups were significantly lower than that in the model control group (*Table 3*).

**Kidney pathology after She Jing Xiao Bai capsule treatment**

Morphological changes in kidney tissue were monitored by evaluation of HE-stained sections. Compared with normal tissue, glomeru

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### Table 2. The effects of treatment on 24 h urine protein levels in DN rats

<table>
<thead>
<tr>
<th>Week</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>63.4±17.5</td>
<td>59.4±15.2</td>
<td>43.4±12.5</td>
<td>39.2±11.4</td>
</tr>
<tr>
<td>4</td>
<td>73.4±16.7</td>
<td>62.3±16.3</td>
<td>52.1±13.5</td>
<td>44.3±14.4*</td>
</tr>
<tr>
<td>6</td>
<td>123.2±20.2</td>
<td>64.4±19.2</td>
<td>63.1±13.3</td>
<td>60.4±13.3*</td>
</tr>
<tr>
<td>8</td>
<td>145.2±12.3</td>
<td>73.1±15.4</td>
<td>70.4±14.3</td>
<td>62.7±19.5*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. G1 at the same time; *P<0.05 vs. the same group at week 2. G1, model control; G2, She Jing Xiao Bai capsule; G3, irbesartan; G4, She Jing Xiao Bai capsule plus irbesartan.

### Table 3. The effects of treatment on Scr level (mg) in DN rats

<table>
<thead>
<tr>
<th>Week</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>173.4±32.4</td>
<td>129.3±25.5</td>
<td>143.3±22.5</td>
<td>139.3±31.0*</td>
</tr>
<tr>
<td>4</td>
<td>214.3±36.2*</td>
<td>132.1±29.2</td>
<td>132.2±33.3</td>
<td>128.3±23.5*</td>
</tr>
<tr>
<td>6</td>
<td>227.3±19.4*</td>
<td>140.3±39.4*</td>
<td>149.1±19.3</td>
<td>145.2±33.9*</td>
</tr>
<tr>
<td>8</td>
<td>240.3±27.4*</td>
<td>137.4±35.3*</td>
<td>150.3±24.5*</td>
<td>142.4±21.4*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. G1 at the same time; *P<0.05 vs. the same group at week 2. G1, model control; G2, She Jing Xiao Bai capsule; G3, irbesartan; G4, She Jing Xiao Bai capsule plus irbesartan.
Effects of She Jing Xiao Bai capsule on DN

of DN rats had an increased diameter with expansion of capillary loops, increased cellularity and expansion of the matrix in the glomerular mesangial area, and lymphocyte and mononuclear cell infiltration. The epithelium of the renal tubules had begun to degenerate, and glomerular sclerosis was observed. In contrast, the pathological changes in the She Jing Xiao Bai capsule, irbesartan group, and She Jing Xiao Bai capsule plus irbesartan group were less frequent and less severe (Figure 1A).

Electron microscopy showed that in DN model control rats, the glomerular basement membrane was thickened and matrix was expanded. Other changes consistent with DN included fusion of epithelium podocyte feet, amplification of the mesangial region, and deposition of highly electron-dense substance. In contrast, those characteristics were attenuated by treatment with She Jing Xiao Bai capsule, irbesartan, and She Jing Xiao Bai capsule plus irbesartan (Figure 1B).

She Jing Xiao Bai capsule decreased TGF-β and CTGF mRNA expression in DN rats

The expression of TGF-β and CTGF mRNA was assayed by qRT-PCR. As shown in Figure 2A, She Jing Xiao Bai capsule, irbesartan, and She Jing Xiao Bai capsule plus irbesartan significantly down-regulated TGF-β and CTGF mRNA expression.

Immunohistochemical staining revealed that TGF-β was expressed primarily in the cytoplasm and matrix of hypertrophic glomeruli and rarely in renal tubules. Compared with the model control group, treatment with She Jing Xiao Bai capsule, irbesartan, and She Jing Xiao Bai capsule plus irbesartan significantly decreased TGF-β and CTGF expression (Figure 2B). These staining results were consistent with the results of Western blotting (Figure 2C).

She Jing Xiao Bai capsule increased the number of CD4^+Foxp3^+T cells in DN rats

Flow cytometric analysis of spleen cell populations labeled with fluorescent antibodies revealed that the numbers of Treg cells in each of the three experimental groups was significantly higher than in the model control group (Figure 3A, 3B).

Discussion

DN is one of the most severe complications of DM, and is also an important step in the pathogenesis of more advanced renal disease [7]. Because damage to glomerular vessels is a major contributor to death in end-stage renal disease, effective treatments are urgently needed. The clinical manifestations of DN include proteinuria, edema, hypertension and decreased renal function. Although the pathogenesis of DN is not fully known, recent studies point to metabolic and endocrine disorders together with microvascular hemodynamic and structural changes [8]. Glomerular hypertrophy and sclerosis that occur in DN lead to alteration of glomerular function, increased synthesis and decreased degradation of ECM [9].
Effects of She Jing Xiao Bai capsule on DN

There is evidence for a role of cytokines and growth factors in the development of DN, [10], and one of the candidates is TGF-β [11]. Released by autocrine or paracrine secretion, TGF-β induces cell hypertrophy or stimulates the accumulation of extracellular matrix (ECM). The binding of TGF-β to its receptor is followed by a number of effects including: (1) regulation of cell proliferation and differentiation; (2) promotion of protein synthesis and cell hypertrophy; (3) stimulation of ECM and platelet-derived growth factor (PDGF) by glomerular and mesenchymal cells; and (4) induction of the transformation of renal tubular epithelial cells to mesenchymal cells.

Figure 2. The effects of treatment on TGF-β and CTGF expression in DN rats. A. mRNA expression; B. Protein expression detected by immunohistochemical staining; C. Protein expression detected by Western blotting. #P<0.05 vs. G1 at the same time; *P<0.05 vs. the same group at week 2. G1, model control; G2, She Jing Xiao Bai capsule; G3, irbesartan; G4, She Jing Xiao Bai capsule plus irbesartan.
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In this study, the potential role of TGF-β in DN was investigated in an experimental T2DM rat model. We found that TGF-β was upregulated in model control DN rats, and that the increase in TGF-β was attenuated by treatment with She Jing Xiao Bai capsule, irbesartan, and a combination of the two agents.

In a mouse model, glomerular CTGF expression was found to have increased at 7 days after development of DM and to increase 28-fold after 3.5 months [13]. Concurrent decreases observed in matrix metalloproteinase (MMP) might contribute to strong expression of proteoglycan, collagen type I and collagen type IV [14]. The increased synthesis and decreased degradation of ECM expected under those conditions would be expected to result in glomerular sclerosis and fibrosis [15]. CTGF has thus emerged as a key mediator of renal fibrosis. The increase of CTGF expression observed in our DN rat model is thus consistent with the findings of previous studies, and it was attenuated by She Jing Xiao Bai capsule and/or irbesartan treatment.

Tregs are a subtype of T cells that can suppress activation of the immune system and inhibit inappropriate reactions leading to autoimmune diseases. They also restrict the area, extent and reaction time of immune responses. Treg cells thus have a key role in regulating the stability of immune reactions including those relevant to type 1 diabetes mellitus (T1DM) [16]. Indeed, Treg cell counts were found to decrease before the appearance of T1DM and non-obese diabetes (NOD) [16]. Dysfunctional Tregs lose expression of CD25 during cell proliferation [17], and in T2DM, Tregs counts were found to decrease [17]. Moreover, a continuing Treg decrease has been related to disease progression. Thus development of DN is related to immune dysfunction. In this study, She Jing Xiao Bai capsule with irbesartan, and alone increased Treg cell populations.

In traditional Chinese medicine theory, symptoms of DN are “Xiao ke”, “hydroncus”, and “asthenia”, and the pathogenesis of DN is due to long-duration “Xiao ke”, which leads to consumption of “Jing” and “Qi”, and weakness of “Qi” and “Yin”. Cumulative damage of the nephron leads to blood stasis. She Jing Xiao Bai capsule consists of black soybean, atractylodes, astragalus, Chinese yam, ant powder, and hirudo powder. Based on its prescription, She Jing Xiao Bai capsule acts to supplement Qi and
nourish Yin, invigorate the spleen, prevent blood stasis, and alleviate water retention. It should therefore be beneficial for the treatment of DN.

In this study, She Jing Xiao Bai capsule significantly decreased SCR and urine protein levels and mitigated renal pathology. She Jing Xiao Bai treatment increased the Treg count in this DN model, but this experimental effect was seen in a context that differs from clinical practice. In clinical practice, most patients are in an early stage of DN, whereas here the treatment was at a later stage [6]. Finally, She Jing Xiao Bai capsule reduced the extent of renal pathology possibly through multiple pathways including TGF-β, CTGF and Tregs. Although the active chemical compounds are not known, this capsule has been used in our hospital for more than 20 years. Future studies should be conducted to identify the active compounds.

In conclusion, She Jing Xiao Bai capsule clearly reduced renal injury in this STZ-induced DN model. The mechanism of action might involve decrease of TGF-β and CTGF expression and increase in the numbers of CD4+Foxp3+ T cells. Inflammatory factors such as IL-10 might also be involved. The study results warrant further study of its effectiveness and mechanism.

Disclosure of conflict of interest

None.

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References


