Effects of *Tripterygium wilfordii* glycosides on regulatory T cells and Th17 in an IgA nephropathy rat model

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ABSTRACT. In this study, we examined the effects of *Tripterygium wilfordii* glycosides (TWGs) on Th17 and regulatory T cells (Tregs) in an immunoglobulin A nephropathy (IgAN) rat model. IgAN model rats were randomly divided into the model group, TWG treatment group, and prednisone group. Normal rats were included as controls. There were 6 rats in each group. The urine protein levels and the number of red blood cells in urine were analyzed at 24 h. IgA deposition in renal tissue was detected by fluorescence microscopy. The concentration of interleukin-17 in serum was detected by an enzyme-linked immunosorbent assay and the number of Tregs in blood was analyzed by flow cytometry. TWGs and prednisone significantly reduced urine protein levels and urine red blood cells at 24 h in IgAN model rats (P < 0.01), but prednisone had a greater effect than did TWGs (P < 0.05). TWGs and prednisone reduced IgA deposition in renal tissue, but prednisone had a greater effect than TWGs. *T. wilfordii* glycosides and prednisone significantly decreased the serum IL-17 level in an IgAN rat model and increased the number of Tregs in the blood (P < 0.01). There was no significant difference between prednisone and TWGs.
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(P > 0.05). In conclusion, TWGs had therapeutic effects on IgAN model rats and may regulate the immune balance of Th17 and Tregs.

Key words: Immunoglobulin A nephropathy; Regulatory T cells; Th17 cells; Tripterygium wilfordii glycoside

INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is a common primary glomerular disease. Its clinical manifestations are complex, mainly including recurrent hematuria associated with different degrees of proteinuria. Renal pathologies of IgAN include the following features, which are important causes of end-stage nephropathy: IgA granular or strip deposition in mesangial cells; varying levels of IgG, IgM, and complement C3 deposition; and cell and stromal hyperplasia. The pathophysiology of IgAN is not completely understood. IgAN is thought to be associated with immune abnormalities of T cells, causing abnormal signal transduction and resulting in an altered B cell response (Lai, 2012; Lin et al., 2012).

Th17 cells are a type of T helper cell. Their main function is to secrete interleukin 17 (IL-17); these cells play an important role in many immune diseases (Afzali et al., 2007; Li et al., 2014; Annunziato et al., 2015). Regulatory T cells (Tregs) are a type of inhibitory T cells, and forkhead box P3 protein is the main molecular marker of Tregs. Tregs inhibit T cell activation, as well as mediate the occurrence and development of immune tolerance, autoimmune diseases, tumors, and immunosuppressive diseases (Whibley and Gaffen, 2014; Kumar and Subramaniyam, 2015). Th17 and Treg cells limit each other in vivo, contributing to the maintenance of immune homeostasis (Franzese et al., 2013; Shan et al., 2015). Previous studies have shown that the number of Tregs in IgAN patients is reduced, weakening the ability to inhibit the immune response and produce immune responses to pathogens. The increase in cytokines elevates the transformation of certain molecules in the immune system, which may lead to IgAN (Huang et al., 2010). An imbalance of Treg-Th17 may result in IgAN.

The Tripterygium wilfordii glycoside (TWG) tablet is a pale yellow tablet. Its taste is a little bitter and astringent, but it has strong anti-inflammatory and immune-inhibitory effects. To further understand the mechanism of TWGs in the treatment of IgAN, the effect of TWGs on Treg and Th17 in vivo and their interactions were studied in an IgAN rat model.

MATERIAL AND METHODS

Animal models and groups

Twenty-six healthy male SD rats weighing 180-220 g (200 ± 15 g) were purchased from the experimental animal center of Henan Province, Zhengzhou, China. The rats were randomly divided into a model group (N = 20) and normal control group (N = 6). The IgAN model was established according to the method described by Tang et al. (2006). Briefly, rats in the model group were administered 400 mg/kg lipopolysaccharide, bovine serum albumin, and carbon tetrachloride solution (Amresco LLC, Solon, OH, USA) by gavage every other day for 12 weeks. Additionally, 0.05 mg lipopolysaccharide was injected into the caudal vein during the 6th and 8th weeks. Once per week, 0.1 mL carbon tetrachloride and 0.5 mL castor oil were subcutaneously injected. Injections
were administered for a total of 9 weeks. Rats in the control group were administered sterile distilled water by gavage. An equal volume of saline was injected into the caudal vein (matched with lipopolysaccharide), and carbon tetrachloride was subcutaneously injected.

The diets of all rats were composed of standard rodent chow. During the 9th week, 1 rat was randomly killed in the model group. Renal tissue from this rat was examined by immunofluorescence and light microscopy to confirm whether preparation of the IgAN model was successful. If the IgAN model was successful, subsequent studies were conducted. If it was not successful, the rats were fed according to the IgAN model preparation method. Next, 1 rat was sacrificed to determine whether model preparation was successful. In this study, the IgAN rat model was prepared successfully in the 9th week. In the 10th week, the remaining 19 rats were administered bovine serum albumin in the stomach every 2 days. These 19 rats were randomly divided into the model group (N = 6), TWGs (Zhihe Co., Zhengzhou, China), treatment group (N = 6, 6 mg/kg), and prednisone treatment group (N = 6, 10 mg/kg). The remaining rat was discarded. Rats in the model and the control groups were given an equal volume of sterile water daily for 2 consecutive weeks. Before sample collection, the rats were sacrificed using 3.5 mL/kg 10% chloral hydrate. This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Henan University Huaihe Hospital.

**Clinical observation**

The general condition of rats was observed, including diet, behavior, activities, and hair. Twenty-four-hour urine samples were collected in metabolic cages for urine protein detection. The number of urine red blood cells was counted using a red blood cell counting plate.

**Pathological examination**

Immunofluorescence and optical microscopy were used to examine renal tissue specimens. Renal tissue specimens were fixed in 10% formalin solution, dehydrated, embedded in paraffin, stained with periodic acid-Schiff stain, and examined under an optical microscope. The remaining portion of each fixed specimen was used for immunofluorescence detection of IgA. Five classification levels were used for IgA immunofluorescence: “−” indicated that they were not visible under low magnification and high magnification; “+” indicated that they were visible under low magnification and slightly visible under high magnification; “++” indicated that they were visible under low magnification and clearly visible under high magnification; “+++” indicated that they were clearly visible under low magnification and bright under high magnification; “++++” indicated that they were very bright under high magnification.

**Detection of IL-17**

For detection of IL-17, 3 mL blood was extracted from the heart of the sacrificed rat, placed in a test tube, solidified at room temperature for 10-20 min, and centrifuged at 700 g for 20 min. The supernatant was collected and preserved at -20°C. The level of serum IL-17 was detected using an IL-17 enzyme-linked immunosorbent assay kit (Jiahe Biotechnology Co., Changsha, China).
Detection of Tregs

For detection of Tregs, 2 mL blood was extracted from the heart of the sacrificed rat and collected in an anticoagulant tube. Next, 100 µL fresh blood was collected for counting of white blood cells and $10^6$ cells. A mixture of 1 µg fluorescein isothiocyanate-labeled mouse CD25 antibody (BioLegend Inc., San Diego, CA, USA) and 0.25 µg phycoerythrin-labeled rat CD4 antibody (BioLegend Inc.) was incubated in the dark for 20-30 min. Three volumes of red blood cell lysate were added. The sample was incubated for 5 min at room temperature. After complete fixation, the sample was centrifuged with 850 g for 5 min. The supernatant was discarded. The cells were washed with 1 mL phosphate-buffered saline and centrifuged at 550 g for 5 min. These processes were repeated 3 times. Finally, the cells were suspended in 300 µL phosphate-buffered saline for immediate flow cytometry analysis.

Statistical analysis

All data were analyzed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The data are reported as means ± standard deviation. P < 0.05 indicates that the difference was significant.

RESULTS

Urine protein and urine red blood cells in 24 h

The urinary protein concentration in 24 h in the TWG treatment group was lower than that in the model group (P < 0.01), but higher than that in the prednisone treatment group (P < 0.01). The number of urine red blood cells in the TWG treatment group was lower than that in the model group (P < 0.01), but higher than that in the prednisone treatment group (P < 0.01) (Table 1).

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (N = 6)</th>
<th>Model (N = 6)</th>
<th>TWG (N = 6)</th>
<th>Prednisone (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twenty-four hour urine protein (mg/day)</td>
<td>8.80 ± 1.52</td>
<td>44.34 ± 5.11</td>
<td>22.03 ± 3.24</td>
<td>11.75 ± 2.31</td>
</tr>
<tr>
<td>Erythrocytes ($10^{12}$/L)</td>
<td>8.46 ± 3.45</td>
<td>435.31 ± 21.12</td>
<td>69.18 ± 9.25</td>
<td>12.20 ± 2.36</td>
</tr>
</tbody>
</table>

Table 1. Effects of different treatment methods on urine protein and urine red blood cells in 24 h in IgAN model rats.

After IgAN rat models were treated with TWG and prednisone, the level of urine protein and the number of urine red blood cells at 24 h decreased significantly (P < 0.01). The effect of prednisone was superior to that of TWG (P < 0.05).

Pathological changes in renal tissue

The pathological changes in renal tissue are shown in Figure 1A. The TWG and prednisone groups showed the attenuated injury compared with the model group. The immunofluorescence intensity of renal tissue is shown in Figure 1B. There was a significant difference in the fluorescence intensity between the control group and the other groups. The fluorescence intensity of IgA in the TWG and prednisone treatment groups decreased. However, the degree of decrease in IgA fluorescence intensity in the prednisone treatment group was greater.
Level of serum IL-17

IL-17 in the model group was significantly higher than that in the control, prednisone, and TWG groups (P < 0.01). Serum IL-17 in the prednisone treatment group was significantly lower than levels in the model group and TWG groups (P < 0.01), but was significantly higher than that in the control group (P < 0.01). Compared with the prednisone treatment group, the level of IL-17 in the TWG group was significantly lower than that in the model group (P < 0.01) and prednisone treatment group (P > 0.05), but higher than that in the control group (P > 0.05) (Table 2).

Table 2. Effects of different treatment methods on IL-17 and Tregs.

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (N = 6)</th>
<th>Model (N = 6)</th>
<th>TWG (N = 6)</th>
<th>Prednisone (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17 (pg/mL)</td>
<td>36.25 ± 2.58</td>
<td>61.52 ± 4.10</td>
<td>38.25 ± 3.25</td>
<td>42.05 ± 2.84</td>
</tr>
<tr>
<td>Tregs (%)</td>
<td>5.58 ± 1.23</td>
<td>1.28 ± 0.52</td>
<td>4.78 ± 0.32</td>
<td>3.72 ± 0.64</td>
</tr>
</tbody>
</table>

Treg levels

The level of Treg in the blood of the model group was significantly lower than that in the control, prednisone, and TWG groups (P < 0.01). The level of Tregs in the TWG treatment group was higher than that in the prednisone group (P > 0.05), but showed no significant difference compared with treatment group and prednisone treatment group (Figure 2).
DISCUSSION

In recent years, the immune balance between Tregs and Th17 in autoimmune diseases has gained increasing attention. This balance is also important in many kidney diseases. Shao et al. (2009) showed that the number of Th17 cells in children with renal syndrome increased, but the number of Tregs decreased, and expression of IL-17 was upregulated in renal tissue, indicating the occurrence of Th17 and Treg disorders. Increasing evidence has demonstrated a relationship between immune disorders of T cells and IgAN (Holdsworth et al., 1999; Nogaki et al., 2000; Toyabe et al., 2001). Huang et al. (2010) found that the immune suppression of IgAN patients was weakened because of reduced Treg levels; thus, the immune response to pathogens was initiated, the production of inflammatory cytokine was increased, and mutual conversion between immune molecules was improved (for example, from IgM to IgA), leading to IgAN. Subsequent studies have confirmed this hypothesis (Huang et al., 2010). Recent studies reported Treg-Th17 immune imbalance in IgAN patients (Lin et al., 2012).

TWGs are extracted from *T. wilfordii* and belong to mixtures that include terpenoid compounds, trace diterpene, and small amount of alkaloids. It shows anti-inflammation and

Figure 2. Number of Tregs in the blood of rats (%). A. Control group. B. Model group. C. TWG treatment group. D. Prednisone treatment group.
immunosuppression effects, as well as inhibits mesangial cell and stromal proliferation (Zhao and Sheng, 2009). In animal experiments and in clinical practice, TWGs can reduce urine protein and urine red blood cells induced by different types of nephritis. It is also widely used for treating IgAN. However, the mechanism is not well understood. Our results showed that both TWGs and prednisone reduced urine protein and urine red blood cells in 24 h in IgAN model rats. However, the effect of TWGs was inferior to that of prednisone. TWGs reduced the level of serum IL-17 in IgAN model rats as immunomodulators by decreasing the number of Th17 cells or the secretion of IL-17. However, its inhibitory function was weaker than that of prednisone. In addition, compared with the model group, TWGs significantly increased the number of Tregs in the blood of IgAN model rats (P < 0.01) and showed no significant difference in the number of serum Tregs compared with the prednisone treatment group (P > 0.05). In this study, renal pathology was observed in experimental IgAN model rats. After a variety of treatments, the renal pathological changes and the fluorescence intensity of IgA showed different degrees of attenuation. The effects of prednisone and TWG treatment were clear, but the reduction in IgA deposition in the prednisone treatment group was superior to that in the TWG group. We hypothesized that treating IgAN with prednisone and TWG may have a synergistic effect, which requires further analysis.

In conclusion, we found that TWGs, like prednisone, reduced the level of urine protein and number of urine red blood cells in IgAN model rats and reduced the deposition of IgA in renal tissue. The mechanism may involve reduction of the number of Th7 cells and increase in the number of Tregs by regulating the balance between immune Treg-Th17 cells.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

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