Effects of Yishenjiangzhuo granules on immunity and bone metabolism in patients with stage 3-4 chronic kidney disease

Jing Zheng, Yingda Lin, Lu Huang, Caifeng Chen, Xuemin Zheng, Xinhong Wu, Ciyun Liu

Jing Zheng, Nephropathy Department, Fujian Provincial People's Hospital, Fuzhou 350004, China
Yingda Lin, Teaching and Research Section of Internal Medicine, Fujian Provincial People's Hospital, Fuzhou 350004, China
Lu Huang, Caifeng Chen, Xuemin Zheng, Xinhong Wu, Ciyun Liu, Postgraduate Department, Integration of Traditional Chinese and Western Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou 350100, China

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Correspondence to: Prof. Jing Zheng, Nephropathy Department, Fujian Provincial People's Hospital, Fuzhou 350004, China. zhengjing1964@medmail.com.cn
Telephone: +86-13799419078
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Abstract

OBJECTIVE: To determine the effects of Yishenjiangzhuo granules (YJG) on bone metabolism and to explore the changes in levels of bone Gla protein (BGP), tartrate-resistant acid phosphatase (TRAP), as well as their relationships with levels of B cells, regulatory T cells (Treg) and interleukin (IL)-17 in patients with stage 3-4 chronic kidney disease (CKD) before and after treatment.

METHODS: Fifty-three stage 3-4 CKD patients were divided randomly into two groups: YJG treatment and control. Peripheral blood was taken from two groups of CKD patients and 21 healthy subjects in the normal group. The parameters determined were the levels of CD4+, CD19+, CD19+CD69+, CD19+AV, Treg (CD4+CD25+CD127−), BGP, TRAP, IL-17, calcium, phosphate, blood urea nitrogen, serum creatinine (Scr), hemoglobin (Hb) in peripheral blood, and urinary creatinine. Calcium-phosphate products and endogenous creatinine clearance rate (CCR) were calculated according to standard protocols.

RESULTS: In YJG and control groups, Scr levels were lowered (P<0.01) after treatment, whereas CCR (P<0.05) as well as Hb and albumin levels (P<0.01) were increased. The changes in levels of CCR and Scr in the YJG group were more significant. After treatment, CD19+CD69+ and Treg levels in the two groups varied (P<0.01) compared with those of the normal group; the level of CD19+ increased but the levels of CD4+ and CD19+AV decreased (P<0.01) in both groups. Compared with the control group, the changes of CD19+ and CD19+AV in the YJG group were more apparent (P<0.05). Compared with the normal group, levels of IL-17 in both groups increased significantly (P<0.01), and the difference in the control group was more significant (P<0.05). After treatment, the TRAP level increased (P<0.05), but the difference in BGP level (P>0.05) was not significant.

CONCLUSION: In stage 3-4 CKD patients, B cells and IL-17 participated in the induction of osteoclast activation. YJG could also elevate the level of B cells and decrease their apoptosis, but showed no significant effects on active B cells, IL-17 or osteoclast activity.

Keywords: B lymphocytes; T-lymphocytes, regulatory; Osteocalcin; Acid phosphatase; Yishenjiangzhuo granules
INTRODUCTION
Renal osteodystrophy is a disease related to defects in bone metabolism. It is caused by chronic renal failure. In general, it is recognized to be related to hyperphosphatemia, decreased production of 1, 25-Dihydroxyvitamin D₃ [1α,25-(OH)₂D₃] and secondary hyperparathyroidism. Owing to the influence of urotoxin, subjects with stage 3-4 chronic kidney disease (CKD) usually have immune dysfunction. Immune cells and bone cells are closely related not only with regard to common origin, but also in mediation of the differentiation of bone cells by immune cells through the receptor activator for nuclear factor-kappa B/receptor activator for nuclear factor-kappa B ligand/osteoprotegerin (RANK/RANKL/OPG) system. Activated B cells produce RANKL, and induce osteoclast activation. Interleukin (IL)-17 can upregulate RANKL expression, increase the binding ratio of RANKL/RANK, and induce osteoclast activation. Regulatory T cells suppress osteoclast generation through cytolytic T lymphocyte-associated antigen (CTLA)-4. The Traditional Chinese Medicine (TCM) Yishenjiangzhuo granules (YJG) can strengthen the spleen and tonify the kidney, stop stasis and decrease turbidity. Hence, it could suppress osteoclast activation by invigorating the kidney and activating the blood circulation. Bone metabolism can be determined by measuring levels of bone Gla protein (BGP) and tartrate-resistant acid phosphatase (TRAP), as well as the indices of bone formation and bone resorption. In the present study, we explored whether YJG could regulate bone metabolism through immunity using flow cytometry to detect CD4⁺, CD19⁺, CD19 + CD69⁺, CD19 + AV and regulatory T cells (Treg) (CD4⁺ CD25⁺ CD127⁻) cells, and using an enzyme-linked immunosorbent assay (ELISA) to detect levels of BGP, TRAP, and IL-17 in peripheral blood.

METHODS
The study protocol was approved by the Medical Ethics Committee of Fujian Provincial People's Hospital (Fujian, China). All patients provided written informed consent.

Subjects
The 53 cases who formed the study cohort had stage 3-4 CKD and were inpatients or outpatients from the Department of Nephrology of Fujian Provincial People's Hospital from March 2008 to July 2009. They were divided randomly into two groups by random number table method. There were 31 cases [15 males; age range, 40-79 years (mean, 59 ± 11 years)] in the YJG treatment group (19 had chronic glomerulonephritis, four had diabetic nephropathy [DN] and eight had hypertension renal disease). There were 22 cases [10 males; age range, 44-74 years (mean, 60±11 years)] in the control group (12 had chronic glomerulonephritis, four had DN, and six had hypertension with renal disease). In addition, a normal group of 21 healthy volunteers [six males; age range, 20-65 years (mean, 57 ± 5 years)] was established. There were no significant differences in general demographics between the groups (P>0.05).

Diagnostic criteria
The diagnostic criteria for CKD were based on the Clinical Practice Guidelines for Chronic Kidney Disease (2002) set by the National Kidney Foundation.¹

Diagnosis and classification based on TCM
The main syndrome was spleen and kidney deficiency syndrome. The criteria for differentiating the syndrome in the literature involve having two main symptoms, or one main symptom with two secondary symptoms. The main symptoms were lumbar debility; languidness, persistent edema, anorexia and abdominal distention. The secondary symptoms were aversion to cold, cold limbs, cool and frequent nocturia, loose stools, purple tongue, and deep/slow pulse. The accompanying syndromes were damp pathogen syndrome with symptoms of nausea and vomiting; dry mouth with bitter taste; anorexia and abdominal distention; dry stools; oliguria; yellow and thick greasy tongue fur; and slippery pulse.

Inclusion and exclusion criteria
Stage 3-4 CKD was diagnosed using the criteria detailed above. The exclusion criteria were patients who had undergone surgery; who had infections or tumors in the month before study enrolment; with CKD caused by connective-tissue diseases such as systemic lupus erythematosus and Sjögren’s syndrome; with acute renal failure; undergoing treatment with anti-inflammatory drugs, antioxidants or aspirin; presenting with factors that cause reversible decline of kidney function in a short time (uncontrolled hypertension, severe infection, trauma, drugs that may cause kidney injury, and decrease in the volume of circulating blood).

Treatments
The YJG treatment group and control group were treated with caltrate (0.6 g/day; Suzhou Wyeth Nutritional Co., Ltd., Suzhou, China) and alfalcacidol (0.5 mg/day; Kunming Baker Norton Pharmaceutical Co., Ltd., Kunming, China). For the control group, the acid-base balance and electrolyte balance was adjusted. A high-quality, low-protein and high-calorie diet (containing essential amino acids) was given. Subjects with hypertensive renal disease were given an angiotensin-converting enzyme inhibitor [Angiotensin-Converting Enzyme Inhibitors (ACEI); lotensin; Beijing Novartis Pharmaceutical Co., Ltd., Beijing, China] or angiotensin receptor blocker (ARB; valsartan; Beijing Novartis Pharmaceutical Co., Ltd., Beijing, China).
and Treg (CD4+CD25+CD127)

ELISA kits were purchased from Becton Dickinson.

Assays

Blood samples were taken in the morning and analyzed within 1 h. Plasma was preserved at −20 °C for enzyme-linked immunosorbent assay (ELISA) analyses. ELISA kits were purchased from Becton Dickinson Biosciences (San Jose, CA, USA).

Statistical analyses

Data were analyzed using SPSS ver13.0 (SPSS, Chicago, IL, USA). The data of levels of CD4+, CD19+, CD19+CD69+, CD19+AV, CD4+CD25+CD127**, BGP, and TRAP did not follow a normal distribution, so rank correlation analyses were used to assess the significance of the results. Data are expressed as x ± s, and Spearman’s rank-order correlation used for correlation analyses. P<0.05 was considered statistically significant.

RESULTS

Changes in levels of BUN, albumin (Alb) and Hb as well as SCr and CCr before and after treatment

After treatment, the level of BUN showed no significant change in the YJG treatment group and control group (P>0.05). The level of SCr in both groups decreased (P<0.01), whereas levels of Hb and Alb increased (P<0.05), as did the CCr (P<0.01). Compared with the control group, the changes in SCr and CCr in the YJG treatment group were more apparent (P<0.05). Significant differences were found in the SCr and CCr as well as levels of BUN, Alb, and Hb in the normal group as compared with the YJG group and control group (Table 1).

Changes in levels of CD19+, CD19+CD69+, CD19+AV, CD4+Treg, and IL-17 before and after treatment

After treatment, the levels of CD19+ and CD19+CD69+ were decreased (P<0.01). The changes in levels of CD19+AV and Treg in the YJG treatment group were more apparent than in the control group (P<0.05). The ratio of CD19+CD69+ to CD19 was increased in the YJG group (P<0.01). The levels of CD4+ and CD4+Treg were decreased (P<0.01). The level of IL-17 in the YJG group was increased (P<0.01).

Table 1 Changes in levels of BUN, Alb and Hb as well as SCr and CCr before and after treatment (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Period</th>
<th>SCr (μmol/L)</th>
<th>BUN (mmol/L)</th>
<th>CCr (ml/min)</th>
<th>Hb (g/L)</th>
<th>Alb (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJG</td>
<td>31</td>
<td>Pre-treatment</td>
<td>310.7±92.6</td>
<td>14.8±5.6</td>
<td>25.6±8.8</td>
<td>90.9±9.1</td>
<td>35.0±6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-treatment</td>
<td>276.1±101.5</td>
<td>15.6±6.7</td>
<td>33.4±12.4</td>
<td>105.9±10.5</td>
<td>37.4±5.2</td>
</tr>
<tr>
<td>Control</td>
<td>22</td>
<td>Pre-treatment</td>
<td>317.5±71.0</td>
<td>14.6±5.4</td>
<td>25.7±6.0</td>
<td>87.7±12.7</td>
<td>35.1±6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-treatment</td>
<td>300.7±75.2</td>
<td>16.6±6.4</td>
<td>29.1±6.9</td>
<td>98.8±11.5</td>
<td>36.6±4.8</td>
</tr>
<tr>
<td>Normal</td>
<td>21</td>
<td>-</td>
<td>67.5±13.2</td>
<td>5.7±1.4</td>
<td>96.3±10.4</td>
<td>145.3±9.4</td>
<td>51.0±5.6</td>
</tr>
</tbody>
</table>

Notes: YJG group were treated with caltrate (0.6 g/day), alfacalcidol (0.5 mg/day) and Yishenjiangzhuo granules (10 g, t.d.s., p.o.). Control group were treated with caltrate (0.6 g/day), alfacalcidol (0.5 mg/day) and Symptomatic treatment. Normal group did not receive treatment. SCr: serum creatinine; Hb: hemoglobin; CCr: creatinine clearance rate; BUN: blood urea nitrogen; Alb: albumin. Compared with pre-treatment, ‘P<0.01, ‘P<0.05; compared with the YJG treatment and control groups ‘P<0.05; compared with the control group, ‘P<0.05.
in the YJG and control groups showed no significant change \((P>0.05)\). Compared with the normal group, the level of Treg in the YJG and control groups was lower \((P<0.01)\), and the level of CD19+CD69+ was higher \((P<0.01)\) (Table 2).

After treatment, in the YJG and control groups, the level of CD19+ increased, CD4+ declined, and CD19+AV decreased \((P<0.01)\). Compared with the control group, the changes in levels of CD19+ and CD19+AV were more significant in the YJG group \((P<0.05)\) (Table 2).

Compared with the normal group, the levels of IL-17 in the YJG and control groups were higher \((P<0.01)\). Compared with the control group, the level of CD19+ increased, CD4+ declined, and CD19+AV decreased \((P<0.01)\) (Table 2).

### DISCUSSION

The RANK/RANKL/OPG system is an important signal transduction pathway for osteoclast differentiation. RANKL can act on RANK on the osteoclast cell membrane and its pseudo-receptor OPG. OPG and RANKL are produced by osteoblasts. RANKL combined with RANK can induce the osteoclast differentiation. Therefore, OPG, RANK and RANKL participate together in the interaction between osteoblasts and osteoclasts, and maintain the physiological balance of bone metabolism.

BGP is secreted by matures osteoblasts. The changes of BGP levels in serum may reflect osteoblast activity. The TRAP level in osteoclasts is taken to be a specific index of osteoclast activity in renal osteodystrophy. The generation and differentiation of osteoclasts are controlled by the RANK/RANKL/OPG system. RANKL promotes the formation, differentiation and maturity of osteoclasts, and inhibits their apoptosis.

B-lymphocytes differentiate from lymphoid stem cells in the bone marrow, and regulate humoral immunity by generating antibodies. Activated B-lymphocytes promote the differentiation and activation of osteoclasts, secrete RANKL, IL-6 and tumor necrosis factor...
(TNF)-α, and promote osteoclast activity. Activated B-lymphocytes also express macrophage inflammatory protein-1α and human monocyte chemoattractant protein-3, which promote the migration and differentiation of osteoclasts. IL-17 is a cytokine secreted by active CD4+ T lymphocytes upon development of inflammation and autoimmune responses. It can up-regulate the expression of RANKL in osteoclasts. The combination of RANK and RANKL can activate osteoclasts by various signal transduction pathways, and promote bone resorption. Treg are a group of dedicated immunosuppression cells. Wang et al. reported that Treg can inhibit B-lymphocytes by direct cell-to-cell contact, with transforming growth factor-β1 and CTLA-4 being involved in this process. In addition, Treg can also inhibit osteoclast formation through cell contact mediated by CTLA-4.

According to TCM theory, the pathogenesis of CKD is due to dysfunction of the lung, spleen and kidney as well as the failure in transportation of Qi through the triple energizer (TE), with spleen and kidney deficiency presented throughout the entire process. The kidney governs bone and produces marrow, whereas the spleen is the source of Qi and blood. Spleen and kidney deficiency syndrome can cause Qi deficiency, leading to blood stasis, which obstructs the microcirculation of organs. This phenomenon is unfavorable for cells to transport substances, and eventually induces disorders of the growth and development of bone. Among the ingredients of YJG, Huangqi (Radix Astragali Mongolici), Taizishen (Radix Pseudostellariae), Fuling (Poria) and Baizhu (Rhizoma Atractylodis Macrocephalae) can strengthen the spleen and replenish Qi. Also, Sangjisheng (Herba Taxilli Chinensis) and Sangshen (Fructus Mori) can nourish the kidney, Liuyuexue (Herba Serisae Japonicae), Dahuang (Radix et Rhizoma Rhei Palm) and Cheqianzi (Semen Plantaginis) are used for resolving turbidity and eliminating dampness. Danshen (Radix Salviae Miltiorrhizae), Danggui (Radix Angelicae Sinensis) and Huainiuxi (Radix Achyranthis Bidentatae) promote blood circulation. Chenpi (Pericarpium Citri Reticulatae) strengthens the spleen and regulates Qi so as to promote transportation of substances.

It has been reported that the separate use of Sangjisheng (Herba Taxilli Chinensis), Sangshen (Fructus Mori), Huangqi (Radix Astragali Mongolici) and Dahuang (Radix et Rhizoma Rhei Palm) can inhibit osteoclast activity. In the present study, a significant reduction in SCr in CKD patients was found after YJG treatment (P<0.01). The CCr in the YJG group was higher than that in the control group (P<0.05). The levels of CD19+ and CD19+CD69+ in patients with stage 3-4 CKD were obviously higher than those in the normal group (P<0.01). The level of CD19+CD69+ in the YJG and control groups showed no significant change after treatment (P>0.05). After treatment, in the YJG and control groups, the level of CD19+ increased (P<0.01), and the level of CD19+ -AV decreased (P<0.05), and the level of CD19+ -AV in the YJG group declined more obviously (P<0.05). These results suggested that YJG could delay the progression of CKD and provide protection for uremic patients; and that the humoral immune system of patients with stage 3-4 CKD is persistently activated, even after treatment. YJG could more obviously enhance the levels of B cells and inhibit apoptosis in CKD patients than in the control group.

Non-immune factors, i.e., increases in levels of parathyroid hormone and decreases in levels of 1α,25-(OH)2D, working through osteoblasts, may also influence osteoclast activity, resulting in renal osteosclerosis. Treg are a group of dedicated immunosuppression cells. Wang et al. reported that Treg can inhibit B-lymphocytes by direct cell-to-cell contact, with transforming growth factor-β1 and CTLA-4 being involved in this process. In addition, Treg can also inhibit osteoclast formation through cell contact mediated by CTLA-4.

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No significant change was found in CD19+CD69+ levels in the YJG and control groups after treatment. Although YJG could increase the number of B cells in CKD patients and inhibit apoptosis, it had no significant influence on active B cells, and humoral immunity in CKD patients continued to be activated after treatment. The TRAP level in the YJG group increased after treatment (P<0.05), suggesting that YJG could not inhibit the activity of osteoclasts by invigorating the spleen and kidney.

Levels of inflammatory factors such as high-sensitivity C-reactive protein (hs-CRP) and IL-6 in CKD patients are obviously higher than in healthy subjects, a fact that is closely related to the progression of CKD. Levels of CD4+ T cells in CKD patients in YJG and control groups continued to decrease after treatment (P<0.01), whereas the levels of IL-17 in YJG and control groups were obviously higher than those in the normal group (P<0.01), and did not decline after treatment. Additionally, the number and levels of Treg in YJG and control groups were lower than those of the normal group, and were still lower than the normal group after treatment (P<0.05). These data suggested that CD4+ T cells in stage 3-4 CKD patients were differentiating into Th17 cells and promoted the progression of micro-inflammation. YJG could not prevent the number of IL-17 cells from increasing, and the immune system was being activated persistently. Immune function seems to be overactive with the progression of micro-inflammation. Before treatment, the following parameters were correlated: CCr with CD19+AV (r=0.380, P<0.01); Ccr with SCr (r=-0.787, P<0.01); CD19+ AV with IL-17 (r=0.316, P<0.05); and CD19+ with TRAP (r=0.333, P<0.05). These data were consistent with the fact that the CCr in stage 3-4 CKD patients decreased, inflammation factors increased, and the immune system was persistently activated, thereby giving rise to bone loss.

The level of bone metabolism is dependent on the equilibrium between the formation and resorption of bone. In stage 3-4 CKD patients, osteoclasts can be activated by hyperphosphatemia, secondary hyperparathyroidism caused by a decrease in 1α, 25-(OH)2D, levels, and activation of the immune system due to persistent micro-inflammation. Non-immune factors, i.e., increases in levels of parathyroid hormone and decreases in levels of 1α,25-(OH)2D, working through osteoblasts, may also influence osteoclast activity, resulting in renal osteosclerosis.
osteodystrophy. The level of BGP in YJG and control groups showed no significant change after treatment ($P>0.05$), indicating that osteoblast activity had no significant change on osteoclast activation in stage 3-4 CKD patients. However, the level of TRAP increased ($P<0.05$) in both groups after treatment. This finding suggested that, with micro-inflammation in the immune system of stage 3-4 CKD patients, the decrease in the number of Treg cells downregulated the inhibition of B cells and TH17 cells, leading to an increase in B-cell activation and a decrease of their apoptosis, and considerable secretion of IL-17. The osteoblast activation pathway was then activated by active B cells and IL-17 cells, and the level of TRAP increased. The difference in TRAP levels between YJG and control groups was significant after treatment, suggesting that multiple factors of bone destruction may exist in stage 3-4 CKD patients. The fact that YJG could not delay activation of osteoclasts might be related to persistent activation of osteoclasts by micro-inflammation in CKD patients.

REFERENCES