Effectiveness of Qingre Lishi Yishen decoction on the glomerular fibrosis of immunoglobulin A nephropathy in a rat's model

Dang Wanyu, Hou Linyi, Wu Huimin, Yan Xiaoming, Zhen Xiaofang, Hao Jing

Abstract

OBJECTIVE: To investigate the effect of the clinical effective prescription of Qingre Lishi Yishen decoction (QRLS) on the activation of mesangial cells in immunoglobulin A nephropathy (IgAN) rats.

METHODS: IgAN rat's model was established by combine with intragastric administration of bovine serum albumin (BSA) + intravenous injection of lipopolysaccharide (LPS) by + subcutaneous injection of carbon tetrachloride (CCL4). Then the animals were randomly divided into four groups: control group, IgAN model group, IgAN model with valsartan (Val) treatment group and IgAN model with QRLS treatment group. To observe the indexes of 24-h urine protein, renal function, deposition of immune complexes, expression of activation factor, fibrosis marker and inflammatory cytokines in four different groups.

RESULTS: The Val or QRLS treatment group: (a) it reduced the immune complexes deposition of IgA in glomerular mesangial and inhibited mesangial cell proliferation; (b) it decreased the expression of smooth muscle actin (α-SMA), fibronectin (FN) and tumor necrosis factor alpha (TNF-α).

CONCLUSION: The study suggested that QRLS ameliorate renal structure and function in IgAN rat's model. Furthermore, we also observed that QRLS alleviated mesangial cells activation and matrix accumulation partly by decreasing the α-SMA, then to downregulated the expression of FN and TNF-α.

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Keywords: Glomerulonephritis, IgA; Mesangial cells; Fibronectins; Tumor necrosis factor-alpha; Qingre Lishi Yishen decoction

INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is the most frequent glomerular disease that is characterized by extensive and pronounced IgA deposits in the glomerular mesangium. It is the most common glomerulonephritis worldwide. IgAN was first reported by Berger and Hinglaisin France in 1968, so it was also called Berger disease. In addition, they were also called IgA mesangial deposition glomerulonephritis and IgA mesangial nephritis. The clinical manifestation of IgAN is recurrent attack of gross hematuria or microscopic hematuria, accompanied by varying degrees of proteinuria, and some patients may have severe hypertension or renal insufficiency. IgAN can occur at any age, but 80% of patients develop between the ages of 16-35. The disease is mostly in chronic and progressive development, the pathogenesis of IgAN has not been fully understood, the current...
application of hormone, immunosuppressant, angioten-
sin converting enzyme inhibitor (ACEI) and angioten-
sin receptor blocker (ARB) were used to reduce renal
pathological lesions and improved the clinical manifesta-
tions of IgAN.44 But there is still no effective western
medicine to control the diseases.45 So far, therefore,
about 20%-40% of patients induced to end-stage renal
failure in 20 years,5 so dialysis treatment and kidney
transplantation are required. As a common primary
glomerular disease in children, IgAN seriously endan-
gers the health of children. Therefore, the study of
pathogenesis of IgAN and research of the effective pre-
vention and treatment drugs has become an urgent re-
search in the field of pediatrics.
A large number of clinical studies have shown that Chi-
nese medicine treatment of IgAN has good clinical effi-
cacy, but the mechanism of the action and drugs are
still unclear. The pathogenesis of IgAN has not yet
been fully elucidated. The present study found that cir-
culating galactose deficient IgA (Gd-IgA1) level in-
creased and the formation of IgA immune complex de-
position in the glomerular mesangial area in IgAN dis-
eases,6 activated mesangial cells, induced mesangial cell
proliferation and excessive secretion of extracellular ma-
trix, cytokines, chemokines, causing kidney damage
may be important mechanism of IgAN.10-12
In this study, we aimed to investigate the effectiveness
of QRLS decoction in the treatment of IgAN patients,
and the possible mechanism behind the action.

METHODS AND MATERIALS

Ethics statement
The animal study protocol was approved by the Ethics
Review Committee for Animal Experimentation of the
Capital Medical University. Animal procedures were
conducted in compliance with Institutional Animal
Care and Use Committee of Capital Medical University.
The ethical code is AEEI-2016-084.

Animals
The 6 to 8 week-old male Sprague-Dawley (SD) rats,
specific pathogen-free, weighing (180 ± 20) g, were
purchased from the Experimental Animal Center of
Capital Medical University, Beijing, China, rearing en-
vironment [(22 ± 1) °C temperature, 40%-50% humidity
environment 12 h light/dark cycle period]. The studied animals were given free access to clean water
and a standard laboratory diet. Before the start of the experiments, all the animals were healthy, active and
had glossy hair.

Drugs
Valsartan (Val): Valsartan capsules were purchased
from Novartis Pharma Schweiz AG (7239473 CN-b,
Beijing, China).
Qingre Lishi Yishen decoction (QRLS), also called
heat-clearing dampness-disinhibiting Chinese herb for-
ma, the recipe consists of: Fengweicao (Herba Pteri-
dis Multiflora) 15 g, Yiyiren (Semen Coicis) 30 g, Kush-
en (Radix Sophorae Flavescentis) 10 g, Shuweicao (Her-
ba Salviae Japonicae) 12 g, Daokoucao (Herba Achyran-
this Asperae) 15 g, Baijiangcao (Herba Patriniae Scabio-
saefoliae) 10 g, Lianqiao (Fructus Forsythiae Suspensae)
10 g, Chishao (Radix Paeoniae Rubra) 10 g, Huangqi
(Radix Astragali Mongolicis) 10 g, Shanyao (Rhizoma Di-
ocorae Oppositae) 15 g, Pulhuang (Pollen Typhae) 15 g,
Lianxu (Stamen Nelumbinis) 10 g, Dandouchi (Semen Sojae Preparatum) 12 g, Chixiaodou (Semen Vignae An-
gularis) 30 g, and Qianshi (Semen Euryales) 20 g. The
Chinese herbs were purchased by the pharmaceutical
department, Beijing Children’s Hospital.

Experimental Design
The rats (n = 32) were randomly distributed into four
groups, each group consists of 8 rats: control, IgAN
model, IgAN model with Valsartan (IgAN + Val) treat-
ment and IgAN model with QRLS (IgAN + QRLS)
treatment. Four rats were sacrificed at the end 12
weeks to detect IgA deposition in mesangial cell. No
rats died for anyther reasons in the study.
At the end of 12 weeks, IgAN rat’s model was estab-
lished. Valsartan (10 mg·kg⁻¹·d⁻¹; Novartis Pharma
Schweiz AG, China) and QRLS (22 g·kg⁻¹·d⁻¹; Bei-
jing Children’s Hospital, China) was dosed by intragas-
tric administration from 13 to 20 weeks. This dose is
based on conversion equation of drug dose between an-
imals and human. Rat dose (mg/kg) = Adult dose (mg/
kg) × conversion factor (6.25). Two months after con-
cluding the QRLS treatment (at 20 weeks), the rats
were anesthetized by chloral hydrate subcutaneous in-
jection and kidney and blood tissue samples were har-
vested and stored in −80 °C. The rats were weighted
weekly, 24-h urine collection was collected by using
metabolic cages at the end of 0, 12 and 20 weeks. All
rats were free to eat normal laboratory animal diet and
drink clean water for 5 months.

Establishment of the IgAN model
IgAN rat model was established by intragastric adminis-
tration of bovine serum albumin (BSA) together with
lipopolysaccharide (LPS) by intravenous injection and
subcutaneous injection of the mixture of castor oil and
carbon etachloride (CCL4) mixture.
The experimental rats group were intragastric adminis-
trated with BSA (Sigma, St. Louis, MO, USA) at dos-
age of 400 mg/kg every other day, continuously for 12
weeks. At the 6, 8, 10, 12th week, the rats received
LPS (Sigma, St. Louis, MO, USA) by intravenous in-
jection, at a dose of 0.25 mg/kg. The rats were received
subcutaneous injection of the mixture of 0.3 mL castor
oil and 0.1 mL CCL4 once a week, continuously for 12
weeks.13-15
In this study, for the control group, distilled water
replaced the BSA and the CCL4 mixture, saline replaced
the LPS. At the end of 12 weeks. The IgAN rat’s model was established.

Renal function examination
The serum and urinary examination were used to tested the renal function of the studies animals. Rat urinary were collected from the method of metabolizable cage. Rat Blood samples were harvested from the tail-cue method. The serum and urine supernatant samples were stored at −80 °C refrigerator to use. The levels of serum creatinine, urea nitrogen, albumin and urine protein were measured by radioimmunoassay (GC-2010, Gamma radioimmunoassay counter ICS, Clinical testing center of capital medical university, Beijing, China).

Histopathological examination
The kidney tissue was collected in Formalin-fixed and Parrifin-Embedded blocks. The tissue blocks were cut at 3-4 μm thickness sections, and stained with hematoxylin-eosin (HE) and Periodic acid-Schiff (PAS). Morphologic alterations, like mesangial cell proliferation, cellular infiltration and mesangial matrix changed in the renal tissues were examined for each stained section at 400× magnification vision by light microscopy and documented by each photographs.

Transmission electron microscope (TEM)
TEM was used to observe the morphology of podocytes and mesangial cells of kidney tissue, the fresh kidney tissues were divided in Less than 1 cubic millimeter blocks, quickly fixed in 2% phosphate-buffered glutaraldehyde for 2 h or more time, washed three times with PBS, fixed in 1% osmium tetroxide for 2 h, and then embedded in acetone wrap after dehydration. The specimens were sliced in 50-60 nm ultrathin sections, double-stained with 3% uranyl acetate and lead citrate, and then examined under a transmission electron microscope (JEOL-JEM-1400Plus, Japan Electron Optics Laboratory, Japan).

Immunofluorescence examination (IF)
The fresh renal tissue was embedded in OCT and stored at −80 °C refrigerator, proceed frozen sections for immunofluorescence staining. We use direct immunofluorescence (FITC-conjugated rabbit anti-rats IgA antibody from Abcam) to detect whether the IgA deposited in the glomeruli section. At least seven pictures were taken randomly for each stained section at 400× magnification vision, and were semi-quantitative analyzed by microscopic image analyzer (Image-Pro Plus 6.0 Software). The integrated optical density (IOD) value was used to represent the density of IgA deposition in tissues.

Immunohistochemistry examination (IHC)
The renal tissues were collected and fixed in 10% phosphate-buffer-formalin and then embedded in paraffin. The renal tissue blocks were then cut into 3-4 um thickness sections, transferred to glass slides and subjected to the Immunohistochemical ABC staining method. The tissue sections were incubated with the primary antibodies of mouse polyclonal to alpha smooth muscle actin (α-SMA) antibody (1∶200; Abcam) and rabbit polyclonal to Fibronectin (FN) antibody (1∶100; Abcam), antigen retrieval, 4 °C overnight. Then renal slices were incubated with species-specific secondary antibodies. Color was harvested by incubated with diaminobenzidine (DAB kit) and counterstaining with hematoxylin. Similarly, we choose seven pictures for each stained section and analyzed by microscopic image analyzer (Image-Pro Plus 6.0 Software). The mean density of IOD value of α-SMA and FN were used to represent the density of the actors in tissue.

RNA preparation and real-time PCR analysis
Total RNA extraction of the renal cortex tissues was done using Trizol reagent (Invitrogen, USA). c-DNA was reverse-transcribed from total RNA using Prime Script™ RT reagent Kit with gDNA Eraser (Takara, RR047A). Real-time quantitative PCR were performed with SYBR Premix Ex Taq (GoTag™ qPCR Master Mix, A6001). Specific primers for TNF-α were identified in Gen Bank to designed. Primers: forward: 5‘-GCCCTTGATACCAACTATTGCTTCA-3’, reverse: 5‘-CAGAAGTTGGCATGGT-3’. The Size (bp) of the genes is 155. The reaction conditions of PCR was: 95 °C × 10 min, 1 cycle; then 40 cycles of denaturation at 95 °C × 15 s, annealing at 60 °C × 30 s and extension at 72 °C × 30 s. Each experimental sample was measured in triplicate, and the average of Ct was taken. The relative expressions of the results were measured by using 2-ΔΔCt method.

Western blot analysis
Renal cortex tissues were lysed in Protein lysis buffer (Solarbio Biotech, Beijing, China) using a Grinding tool; Lysates was centrifuged at 12 000 rpm for 15 min at 4 °C. Take the supernatant for the protein concentrations tested by using a BCA assay kit (wbiootech, 02912E). Samples containing 40 μg of protein were separated on 10% SDS-polyacrylamide gels electrophoresis (SDS-PAGE) (Sigma, St. Louis, MO, USA) and then transferred topolyvinylidene difluoride (PVDF, 0.45 um, Millipore, Billerica, MA, USA) membranes. After blocked with 5% skim milk in PBS + 0.05% Tween-20 (Amresco0777),the membranes were incubated at 4 °C with primary antibodies (TNF-α, Abcam) at a 1∶2000 dilution, overnight. On the second day, washed, and membranes were hybridized for 2 h at 37 °C with a horseradish, peroxidase-conjugated secondary antibody [goat anti rabbit IgG (H + L), HRP Jackson,111-035-003] at a 1∶10000 dilution. Then washed three times, specific bands were visualized with enhanced chemiluminescence (ECL, Millipore, Billerica, MA, USA) and then film exposure and photograph-
ic fixing. The relative densitometry was detected by ImageJ. Western blot analyses of each specimen were at least in triplicate.

Statistical analysis
The data were analyzed by using SPSS 23.0 Software (IBM Corp. Released 2015, IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA). Differences between groups were tested with one-way analysis of variance followed by Tukey’s test. Data were expressed as the mean ± standard deviation. $P < 0.05$ was as the significant level.

RESULTS

Renal function parameters in IgAN rats after treatment with Val and QRLS
In IgAN rats, 24-h urine protein was observed markedly increasing to (62.7 ± 11.0) mg/d at the end of the experiment (20 th week), however there was a low level of urine protein (10.0 ± 2.9) mg/d in the control group. After the treatment with Val and QRLS, the final 24-h urine protein decreased respectively to (28.3 ± 6.4) and (30.0 ± 8.0) mg/d, which was significantly lower than the IgAN group (both $P < 0.05$), but still a little higher compared to the control group (both $P < 0.05$). In addition, the two treatment groups showed no statistical significance.

Although IgAN rats had significant 24-h urine protein levels, there were no obvious changes in blood creatinine and urea nitrogen of renal functional damage index during the whole study period serum creatinine levels slightly increased in IgAN rats [(36.2 ± 9.2) μmol/L] compared to control rats [(31.7 ± 7.6) μmol/L], there was not statistically significance between Val group and QRLS group. Similarly, the results of urea nitrogen showed consistent trend with Creatinine [control: (7.3 ± 1.7) vs IgAN: (8.0 ± 1.3) vs IgAN + Val: (7.8 ± 1.3) vs IgAN + QRLS: (7.7 ± 1.6) mol/L]. The data show no statistically significances among the groups.

QRLS reduced IgA deposition on glomerular mesangial
Immunofluorescent staining of IgA deposition showed that in control group rats had very little IgA deposition in the glomeruli regions, but in contrast, IgAN rats had increased highly up to large amounts of IgA immune complex deposition in glomeruli at the end of the study. After treatment with Val and QRLS, IgA deposition was significantly alleviated compared to the IgAN group, but still remained a little higher than the control group, there was no statistical significance be-

![Figure 1 Immunofluorescence staining for IgA deposition in four different groups](image-url)

Control: control group; IgAN: IgA nephropathy model group; Val: IgA nephropathy model with Val treatment group; QRLS: IgA nephropathy model with QRLS treatment group. Val (10 mg·kg$^{-1}$·d$^{-1}$) and QRLS (22 g·kg$^{-1}$·d$^{-1}$) was dosed by intragastric administration from 13 to 20 weeks. Val: valsartan; QRLS: Qingre Lishi Yishen decoction; IgAN: immunoglobulin A nephropathy; IOD: integrated optical density. A: IgA immune complex deposition was visualized by direct immunofluorescence in four different groups: control, IgAN, Val and QRLS. All the sections were imaged with an immunofluorescence microscope (× 400). B: the pictures were semi-quantitative analyzed by microscopic image analyzer. The mean density of IOD value was used to represent the density of IgA deposition in renal tissues. $\cdot P < 0.01$, $\cdot P < 0.05$ vs control group; $\cdot P < 0.01$ vs IgAN group.
between the two treatment groups (Figure 1A). The mean density of IOD value was used to represent the density of IgA deposition in renal tissues. As is shown in Figure 2B, the IOD value confirmed again the consistency of the results again with Figure 2B.

**Effects of QRLS on renal morphology change in IgAN model rats**

To determine whether QRLS can inhibit the proliferation of mesangial cells and matrix, the renal sections were subjected to HE, PAS staining and TEM observation in four different groups respectively. The results of HE and Masson staining were consistent in Figure 2A, 2B. It showed that the shape of glomerular, renal tubular cavity and interstitial remained normal in control group. However, in IgAN group, the number of glomerular mesangial cells (GMC) increased, mesangial matrix proliferation, capillary lummen narrowed and shape of renal tubules became irregular compared to control group. After the treatment in Val and QRLS, the deposition of renal mesangial cells and matrix decreased significantly, suggesting that both two drugs can alleviated the renal pathomorphology changes in IgAN rat’s model. There was no significant difference between the two treatment groups.

As is shown in Figure 2C, under electronic microscopic examination (TEM ×8000), the partial or full fusion of the podocytes, disruption and thicken of glomerular basement membrane (GBM), enlargement of infiltration pores and proliferation of mesangial cells and matrix processes were observed in IgAN group compared to the control, while in Val or QRLS group it attenuated the mesangial and matrix, reduced the fusion of podocytes and improved the damage of GBM compared to IgAN model group. Similarly, the effects of the both treatment groups make no significant differences.

**QRLS decreased the expression of activation factor α-SMA, fibrosis markers FN**

The IOD value of activation factor α-SMA and fibrosis markers FN were used to represent the density of the actors in tissue. As is shown in Figure 3 (A, C), α-SMA-positive staining barely detectable in GBM of control group, however, the expression of α-SMA was dramatically elevated in IgAN compared to the control group, while in Val and QRLS groups, it was obviously relieved compared to the IgAN group respectively. It was still a little increased compared to the control. There was no statistically significant difference between the two treatments Val and QRLS groups.

Similarly, relative density of FN showed the consistent results. As is shown in Figure 3 (B, D), FN staining was weakly detectable in GMC. However, in IgAN group, the elevated expression of FN was detected. Also in Val and QRLS groups, FN expression obviously decreased in both groups, the two groups makes no statistically significant differences.

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**Figure 2** Representative images of Histopathology of renal sections in four different groups
Control: control group; IgAN: IgA nephropathy model group; Val: IgA nephropathy model with Val treatment group; QRLS: IgA nephropathy model with QRLS treatment group. Val (10 mg·kg⁻¹·d⁻¹) and QRLS (22 g·kg⁻¹·d⁻¹) was dosed by intragastric administration from 13 to 20 weeks. Val: valsartan; QRLS: Qingre Lishi Yishen decoction; IgAN: immunoglobulin A nephropathy. Representative renal histological changes for hematoxylin-eosin (PAS) stain (A, ×400), Periodic acid-Schiff stain (B, ×400), and Transmission electron microscope observation respectively (C, ×8000). The black arrow head in Figure 2C represents the podocytes and the GBM of renal tissue.
There was no statistical difference between the two protein expression of TNF-α. However, after the treatment with Val and QRLS, the expressed relatively very low protein level of TNF-α, which were significantly increased in IgAN group. In addition, the mRNA levels of the inflammatory factor TNF-α showed the similar results. It was significantly elevated in IgAN compared to the control group, while the levels decreased markedly after treatment groups with Val and QRLS. Similarly, there was no significant difference between the two treatment groups.

**DISCUSSION**

This study was undertaken to evaluate a preventive effect between QRLS and Western Medicine Val in IgAN rat’s model. We focused on the important pathogenesis of IgA nephropathy that Gd-IgA1 immunological complex induced mesangial cell activation in the disease. The study was demonstrated in a laboratory experiment with SD rats. In order to explore the effects of QRLS on the glomerular fibrosis of IgAN, we carry through the intensive study of the animals as whole, organ, cell and gene levels, so as to clarify the mechanism and target of clinical effective Chinese herbs QRLS on the treatment of IgAN, then to provide the scientific basis for the prevention of IgA nephropathy with Traditional Chinese Medicine.

Large molecules such as IgA immune complexes inflow into the kidney by capillary blood circulation, under the influence of glomerular filtration pressure, through the fenestra of endothelial cell and deposited in glomerular mesangial region, induced expression of α-SMA in mesangial cells increased, also called activation mesangial cells. The activation of mesangial cell germinated excessive proliferation. At the same time, it synthesizes and secretes excessive extracellular matrix that were deposited in the glomeruli, such as FN, collagen type III and so on. Multitudinous ECM replaces the space of the functional cells of the glomerulus, destroys the organizational structure of the glomerulus, damages the glomerular function and ultimately leads to glomerulosclerosis. In addition, mesangial cell activation can also release a series of inflammatory factors.
inflammatory and profibrotic factors, such as TNF-α, interleukin-6 and platelet-activating factor. These factors can not only cause acute and chronic inflammation of kidney, but also play an important role in the sertoli cell and mesangial cells, leading to further damage to two types of renal intrinsic cells.

In the pathogenesis of IgA nephropathy, IgA and other large molecular immune complexes easily deposited in the mesangial areas thus to activated mesangial cells, caused excessive proliferation of mesangial cells and synthesized extracellular matrix (ECM) composition. The tissue released massive inflammatory cytokines and fibrosis factors, resulting in excessive accumulation of ECM, kidney inflammation, mesangial cell dysfunction and glomerulosclerosis, lead to end-stage renal disease. Although various types of cells and cytokines were involved in the process of renal fibrosis, α-SMA positive fibroblast plays the most important role in the whole process. It is the significant marker which can differentiate between ‘activated’ and resting GMCs.

Previous studies have shown that inflammatory factor cytokine TNF-α plays as a major effect or factor in glomerular mesangial proliferation. TNF is produced by activated mononuclear cells, and the appropriate amount of TNF is protective in normal body condition, while excessive TNF can cause immune damage to the body. Previous studies have shown that mononuclear macrophage infiltration into the kidney tissues and released TNF-α, induced renal vasculitis, leading to focal glomerular injury, glomerular membrane cell proliferation and aggravation of renal disease resulting in proteinuria and kidney damage. QRLS can also reduce the expression of FN and α-SMA, which can effectively inhibit the transformation of muscle fibroblasts in renal tissue of IgAN rat’s model, eliminate the production and deposition of extracellular matrix, thus to mitigate the fibrosis of renal tissue, in order to improve the proteinuria and ease kidney injury of the diseases.

The effects between the Val and QRLS in rat’s models of IgA nephropathy makes no statistically significant difference in the reduced of 24-h Urine protein, pathological changes of renal fibrosis, weaken IgA Immunofluorescence complex deposition, decreased the expression of activation factor α-SMA, fibrosis markers FN and downregulated the expression of the profibrotic growth factors and inflammatory factors TNF-α. Our findings suggest that QRLS decoction is able to mitigate mesangial cell proliferation in rats with IgAN, reduced the expression of α-SMA, FN, TNF-α in mesangial cells of IgAN rats. It also attenuated the expression of mesangial activating factor, fibrosis, inflammation markers in the kidney of IgAN rats. In conclusion, QRLS inhibits renal fibrosis by regulating the expression of activation factor α-SMA, further influence the fibrosis markers FN and the expression of the inflammatory factors TNF-α to affect the proliferation of mesangial cells matrix on IgA nephropathy. In addition, other relevant profound mechanisms underlying the effects of QRLS on IgAN still require further investigation.

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