

Combination of Ginsenoside RgI and Astragaloside IV reduces oxidative stress and inhibits TGF- β 1/Smads signaling cascade on renal fibrosis in rats with diabetic nephropathy

Na Du
Zhiping Xu
Mingyue Gao
Peng Liu
Bo Sun
Xia Cao

Department of Pharmacology, Jilin University, Jilin, Changchun, China

Introduction: Anti-oxidative stress and inhibition of TGF- β 1/Smads signaling cascade are essential therapeutic strategies for diabetic nephropathy (DN). In this study, we aimed to explore the effect of combination of Ginsenoside RgI and Astragaloside IV on oxidative stress and TGF- β 1/Smads signaling in DN rats.

Materials and methods: Wistar rats were divided into five groups: N group, M group (streptozotocin [STZ], intraperitoneally), G group (STZ rats with Ginsenoside RgI, intragastrically [ig]), A group (STZ rats with Astragaloside IV, ig) and C group (STZ rats with Ginsenoside RgI and Astragaloside IV, ig). The levels of methane dicarboxylic aldehyde (MDA), catalase (CAT), glutathione peroxidase (GSH-PX), total anti-oxidative capacity (T-AOC), blood urea nitrogen (BUN), β_2 -microglobulin (β_2 -MG), serum creatinine (SCr) and urinary creatinine (UCr) were detected in all the groups. The left kidneys of the rats were harvested to detect the expression of TGF- β 1, Smad2/3, Smad7 and CTGF by immunohistochemical staining, while the right kidneys were used to detect the mRNA expression of TGF- β 1, Smad7 and CTGF by real-time PCR.

Results: Rats in G group, A group and C group had lower level of MDA but higher levels of CAT, GSH-PX and T-AOC compared with rats in M group. Rats in C group showed the best anti-oxidative stress level. G group, A group and C group treatments significantly decreased the levels of BUN, SCr, β_2 -MG and UCr. In addition, C group treatment showed the best kidney protective effect. G group, A group and C group treatments significantly diminish ED both factor and mRNA overexpression of TGF- β 1 and CTGF but increase Smad7 expression in kidney tissue.

Conclusion: The combination of Ginsenoside RgI and Astragaloside IV may potentially protect against DN by reducing oxidative stress and inhibiting TGF- β 1/Smads signaling cascade.

Keywords: Ginsenoside RgI, Astragaloside IV, oxidative stress, TGF- β 1/Smads, diabetic nephropathy

Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus (DM) which leads to end-stage renal failure in 30%–40% of DM patients.^{1–3} The incidence of DN will rapidly increase with increase in the incidence of DM in China in the decades to come.^{4,5} Oxidative stress injury and TGF- β /Smads signal transduction are vital to the development and progression of DN. The supererogatory advanced glycation end products (AGEs) and ROS act as pivotal mediators of microvascular injury when

Correspondence: Xia Cao
Department of Pharmacology, School of Pharmacy, Jilin University, Jilin, Changchun 130021, China
Email caoxia@jlu.edu.cn

induced by DM and trigger various cell signaling pathways involved in the development of DN. Oxidative stress injury caused by ROS and AGEs can activate TGF- β /Smads signaling which is involved in the development of fibrosis in renal tubular epithelial cells.^{6,7} Many results have shown that TGF- β expression is upregulated in animal renal fibrosis models as well as human counterparts.^{8,9} The transition of renal tubular epithelial cells to myofibroblasts which synthesize excessive amounts of extracellular matrix thus leading to renal fibrosis is modulated by TGF- β through TGF- β /Smads signaling pathway.² Furthermore, oxidative stress and TGF- β /Smads signaling together with other disease processes interactively and rapidly promote DN. Therefore, agents with anti-oxidative or TGF- β 1/Smads signaling prohibitive competence are likely to possess a therapeutic effect on DN.

Ginsenoside Rg1 is one of the main active ingredients of *Panax ginseng* C.A. Mey. Its proportion is the main criterion which determines the quality of *P. ginseng*.¹⁰ Studies have reported that Ginsenoside Rg1 has a strong anti-oxidative and anti-fibrotic effect.^{11–14} Astragaloside IV, a characteristic and active constituent of *Radix Astragali*, possesses many biological effects.¹⁵ Many studies have shown that Astragaloside IV has an excellent renal protective effect.¹⁶ Only a specific amount of *P. ginseng* and *Radix Astragali* and other frequently prescribed traditional Chinese medicines such as Shenqi Jiangtang Granules should be applied to avoid complications in DM patients. Some evidences show that a mixture of herbal medicines has a better effect than a single medicine in clinical practice. In this study, we aimed to determine whether the combination of Ginsenoside Rg1 and Astragaloside IV has a therapeutic effect on DN and its underlying mechanism.

Materials and methods

Chemicals

Ginsenoside Rg1 (purity $\geq 98\%$) and Astragaloside IV (purity $\geq 98\%$) were purchased from ChengDu ConBon Biotech Co., Ltd. (Chengdu, China). The methane dicarboxylic aldehyde (MDA), catalase (CAT), glutathione peroxidase (GSH-PX) and total anti-oxidative capacity (T-AOC) assay kits for rats were bought from Jiancheng Bioengineering Institute (Nanjing, China). Rat blood urea nitrogen (BUN), serum

creatinine (SCr), β_2 -microglobulin (β_2 -MG) and urinary creatinine (UCr) ELISA kits were also purchased from Jiancheng Bioengineering Institute. Streptozotocin (STZ) was bought from Sigma (St Louis, MO, USA). Polyclonal antibodies used for immunohistochemical analysis included TGF- β 1 (Santa Cruz Biotechnology, Dallas, TX, USA), Smad2/3 (Cell Signaling, Danvers, MA, China), Smad7 (Bioss, Woburn, MA, USA) and CTGF (Bioss). Rat SP immunohistochemistry kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The primer sequences of TGF- β 1, Smad7, CTGF and ACTB (listed in Table 1) used for real-time PCR analysis were bought from Sangon Biotech Co., Ltd (Shanghai, China). Real-Time PCR Reagents & Kits and Total RNA Extractor (Trizol) were bought from Thermo Fisher Scientific. The other reagents used were of analytical grade.

Animals

A total of 50 male adult Wistar rats (180–220 g) were purchased from Laboratory Animal Center of Jilin University. All the experiments were approved by the Animal Care Committee of Jilin University Pharmaceutical College (in accordance with the Animal Experimental Ethical guidelines of Jilin University; permit number: 2017050802). The rats were kept at a temperature of $22^\circ\text{C} \pm 2^\circ\text{C}$ on a 12-hour light/dark cycle and fed on a normal laboratory diet and water ad libitum.

In vivo experiments

The rats were marked after acclimating to the facilities for 7 days. Then, eight rats were randomly chosen and designated as N group, and the rest were intraperitoneally administered 60 mg/kg of STZ with 0.1 mol/L sodium citrate solution (pH 4.50).^{17–19} The blood glucose levels of all the rats, except N group, were measured at 72 hours after STZ injection (first experimental day). Only the rats with a blood glucose concentration higher than 13.8 mmol/L were chosen as model rats and used further in our study.

Model rats were randomly divided into four groups (n=8): M group, G group, A group and C group. Rats in N group and M group were intragastrically (ig) administered vehicle daily, rats in G group were ig administered 50 mg/kg/day of Ginsenoside Rg1, rats in A group were ig administered

Table 1 The primer sequences used for mRNA real-time PCR analysis

Factors	Gene ID	Forward (5'→3')	Reverse (5'→3')
TGF- β 1	59086	ATTCCTGGCGTTACCTTGG	AGCCCTGTATTCCGTCTCCT
Smad7	81516	GGGGGAACGAATTATCTGGC	CGCCATCCACTTCCCTTGT
CTGF	64032	CATTTGTTCCGAACAGTGATT	TAGCCTCAAACCTCAAACACCA
ACTB	81822	TGTCACCAACTGGGACGATA	GGGGTGTGAAGGTCTCAA

16 mg/kg/day of Astragaloside IV and rats in C group were ig administered 50 mg/kg/day of Ginsenoside RgI and 16 mg/kg/day of Astragaloside IV on the first experimental day.

Detection of the levels of MDA, CAT, GSH-PX, T-AOC, BUN, SCr, β_2 -MG and UCr

At the end of 8 weeks (56th experimental day), the rats were housed in individual metabolic cages (ZS Dichuang Co., Ltd., Shanghai, China) to collect 24-hour urine samples. The plasma samples were used to detect MDA, CAT, GSH-PX, T-AOC, BUN and SCr levels, while urine samples were used to measure β_2 -MG and UCr levels using assay kits according to the manufacturer's instructions.

Immunohistochemical staining and assessment in each group

After euthanization (57th experimental day), the kidneys (left) were harvested and rinsed free from blood with PBS. After fixing in 10% neutral formalin and embedding in paraffin, the antigen of kidney tissue slides was exposed by treatment with boiling citrate buffer (0.01 mol/L, pH 6.0). Then, the slides were respectively incubated with TGF- β 1, Smad2/3, Smad7 and CTGF antibodies for immunohistochemical analysis and examined using a light microscope (Nikon Ti). The analyses were performed to test for the gray value of the immunohistochemical stainings using Motic Images Advanced 3.2.

Real-time PCR analysis in rats' kidney tissue

The kidneys (right) were harvested and kept in liquid nitrogen for real-time PCR analysis. Total RNA was extracted from rats' kidney tissue and reverse transcribed to cDNA

according to the manufacturer's protocol. Then, real-time PCR was proceeded with SYBRTM Select Master Mix, and expression was detected using iCycler iQ (Bio-Rad). ACTB was employed as the internal standard in our study. The ratio of TGF- β 1, Smad7 and CTGF was normalized with ACTB. We took each factor of N group as 1.0000 to calculate and compare the homologous factors in the other groups.

Statistical analysis

Statistical analyses were performed utilizing SPSS 20.0 program. Results were expressed as mean \pm SD and analyzed using one-way ANOVA and post hoc least square difference test. Statistical significance was determined at $P < 0.05$.

Results

Combination of Ginsenoside RgI and Astragaloside IV increases DN rats' anti-oxidative capacity

The results of oxidative stress levels in each group are shown in Figure 1. STZ injection obviously increased MDA level, but decreased the levels of CAT, GSH-PX and T-AOC as compared with N group ($P < 0.05$). As shown in Figure 1A, G group and C group had lower level of MDA ($P < 0.05$ vs M group). The rats in C group had the lowest MDA level, although there was no significant difference between G group and C group.

G group, A group and C group showed apparent increase in CAT level ($P < 0.05$ vs M group; Figure 1B). Compared with G group, A group had lower CAT level ($P < 0.05$) while C group had higher CAT level ($P > 0.05$).

The treatment of G group and C group inhibited GSH-PX level decline in plasma ($P < 0.05$ vs M group; Figure 1C). Compared with G group, C group had better GSH-PX level ($P < 0.05$ vs G group).

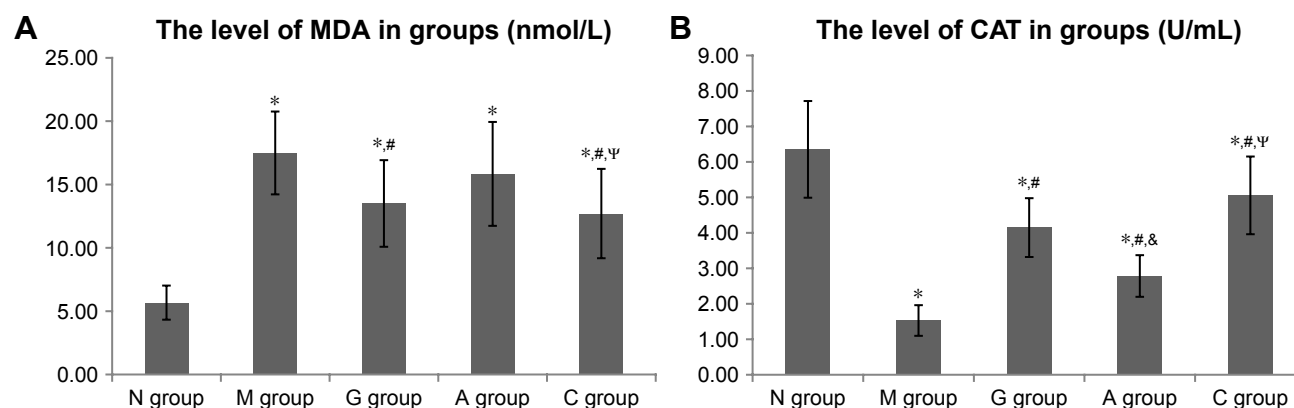


Figure 1 (Continued)

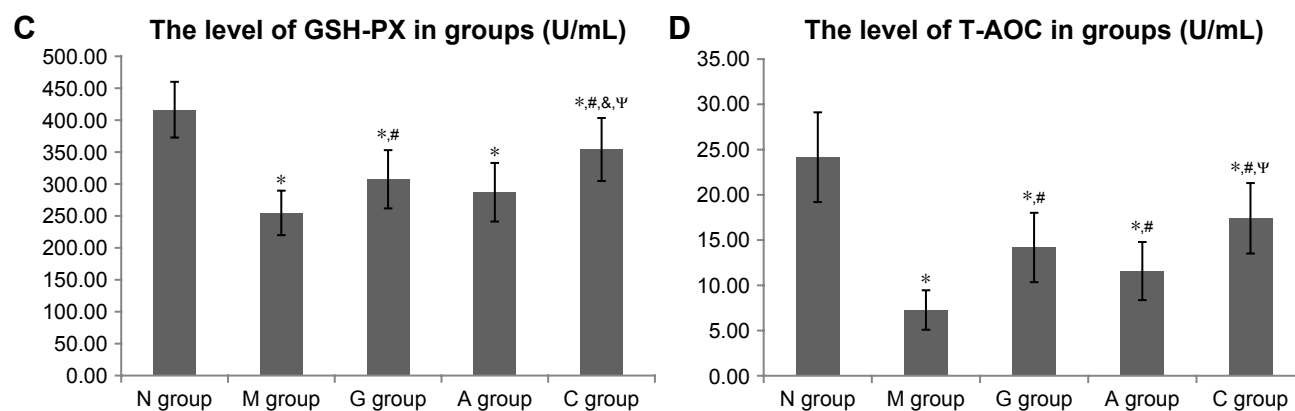


Figure 1 The plasma levels of MDA, CAT, GSH-PX and T-AOC in groups.

Notes: (A) The level of MDA. (B) The level of CAT. (C) The level of GSH-PX. (D) The level of T-AOC. * $P < 0.05$ vs N group; # $P < 0.05$ vs M group; & $P < 0.05$ vs G group; Ψ $P < 0.05$ vs A group.

Abbreviations: CAT, catalase; GSH-PX, glutathione peroxidase; MDA, methane dicarboxylic aldehyde; T-AOC, total anti-oxidative capacity.

In Figure 1D, we could find that rats in G group, A group and C group had improved T-AOC after STZ injection ($P < 0.05$ vs M group). The rats in C group had better T-AOC than the other two groups.

Combination of Ginsenoside RgI and Astragaloside IV improves DN rats' renal function

The tests showed that STZ injection induced significant increase in the levels of BUN, SCr, β_2 -MG and UCr as compared with N group ($P < 0.05$; Figure 2). We also found that G group, A group and C group treatments significantly decreased the levels of BUN, SCr, β_2 -MG and UCr ($P < 0.05$ vs M group; Figure 2).

Compared with G group and A group, rats in C group had lower BUN levels ($P > 0.05$ vs G group and $P < 0.05$ vs A group; Figure 2A). As shown in Figure 2B, C group had the lowest level of SCr among the three groups ($P < 0.05$ vs G group and $P < 0.05$ vs A group). The rats in G group had lower SCr level than rats in A group ($P < 0.05$). As shown in Figure 2C, we found that C group had lower β_2 -MG level than the other two groups ($P < 0.05$ vs G group and $P > 0.05$ vs A group). However, there was no significant difference between G group and A group. As shown in Figure 2D, C group had the lowest UCr level after 8 weeks of treatment ($P < 0.05$ vs G group or A group). However, there was no significant difference between G group and A group ($P > 0.05$).

Combination of Ginsenoside RgI and Astragaloside IV inhibits TGF- β 1/Smads signaling cascade

In order to explore the effect of combination of Ginsenoside RgI and Astragaloside IV on TGF- β 1/Smads signaling in

DN rats' kidneys, immunohistochemical staining and real-time PCR were performed. Compared with N group, rats in M group showed severe immunopositivity for TGF- β 1, Smad2/3 and CTGF, but no immunopositivity for Smad7. However, G group, A group and C group treatments diminished immunopositivity for TGF- β 1, Smad2/3 and CTGF and elevated immunopositivity for Smad7 (Figure 3). More importantly, C group had the lowest levels of TGF- β 1, Smad2/3 and CTGF and the highest level of Smad7 among the three groups as revealed by the gray value of immunohistochemical stainings tested using Motic Images Advanced 3.2 (Figure 4).

Real-time PCR analysis showed that STZ injection induced overexpression of TGF- β 1 ($P < 0.05$) and CTGF ($P < 0.05$), but decreased the expression of Smad7 ($P < 0.05$) as compared with N group. The results demonstrated that G group, A group and C group had attenuated expression of TGF- β 1 and CTGF but increased expression of Smad7 ($P < 0.05$ vs M group). In addition, C group showed better effect than the other two groups (Figure 5).

Discussion

Hyperglycemic microenvironment, which leads to tubulointerstitial fibrosis and glomerulosclerosis, induces structural and functional abnormalities in the kidney at a very early stage of DM.^{20,21} Tubulointerstitial injury actually occurs earlier than glomerular injury in the development of DN.^{16,22}

Oxidative stress injury and overactivation of TGF- β 1/Smads signaling are the major culprits in DN.²³⁻²⁶ There is some causal link between oxidative stress and TGF- β 1/Smads signal transduction in the development and progression of DN. As we know, renal cells exhibit enhanced glucose

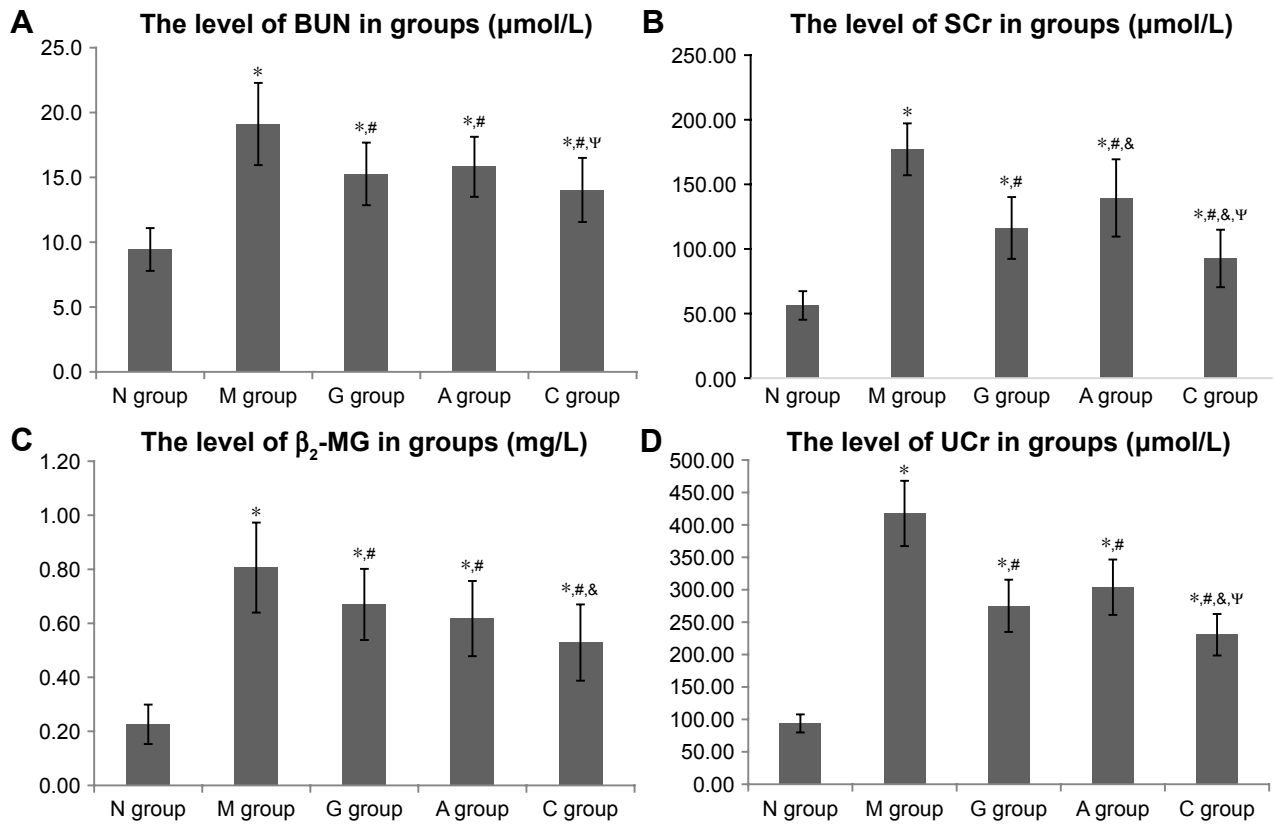


Figure 2 The levels of BUN, SCr, β_2 -MG and UCr in each group.
Notes: (A) The plasma level of BUN. (B) The plasma level of SCr. (C) The urine level of β_2 -MG. (D) The urine level of UCr. * $P < 0.05$ vs N group; # $P < 0.05$ vs M group; $\Psi P < 0.05$ vs G group; $\Psi P < 0.05$ vs A group.
Abbreviations: β_2 -MG, β_2 -microglobulin; BUN, blood urea nitrogen; SCr, serum creatinine; UCr, urinary creatinine.

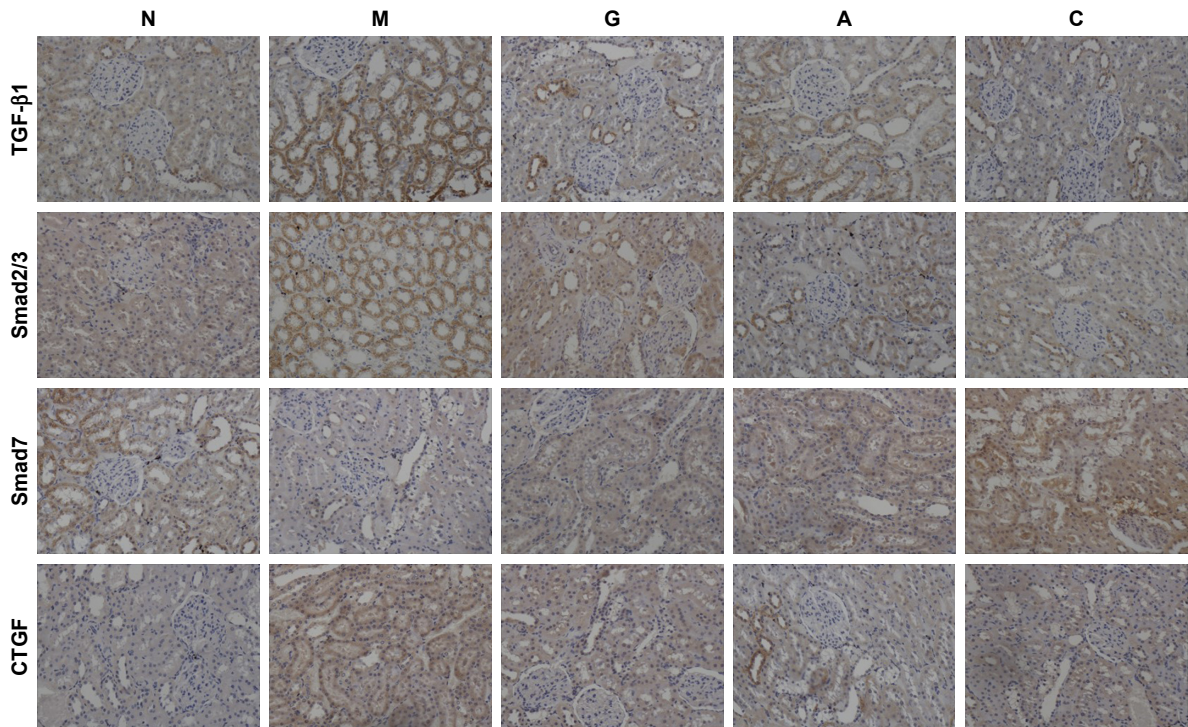


Figure 3 The photomicrographs of representative immunohistochemical staining sections of the kidney ($\times 200$ magnification).

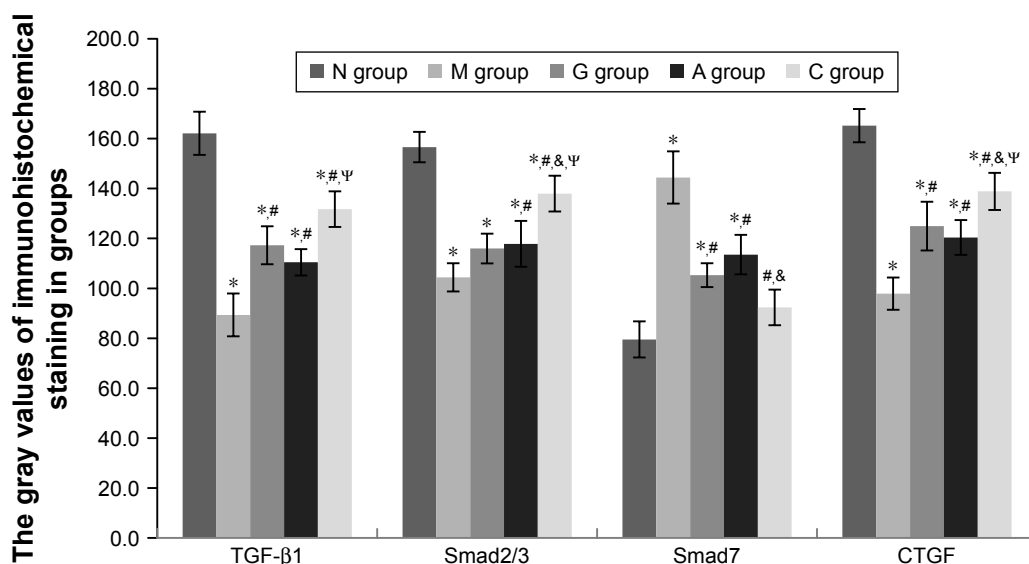


Figure 4 The gray values of TGF-β1, Smad2/3, Smad7 and CTGF in groups.
Notes: *P<0.05 vs N group; #P<0.05 vs M group; §P<0.05 vs G group; ΨP<0.05 vs A group. The severer the immunopositivity was, the lower the gray value tested.

uptake in DM, but some cell populations such as glomerular mesangial cells cannot decrease glucose transport rates efficiently to maintain intracellular glucose homeostasis.²⁷ As a result, cytosol and mitochondria in these cells will produce more ROS beyond local antioxidant capacity. Oxidative stress induced by the redundant ROS in renal cells such as podocytes and mesangial cells induces the development and progression of DN.²⁸ AGEs are the other momentous cell metabolic products produced in DM patients. Oxidative stress may hasten AGEs formation.²⁹ Superfluous ROS are also produced along with AGEs, inducing ROS/AGEs

self-perpetuating cycle in DM.²⁴ Since most AGEs are cleared by the kidneys, they are likely to accumulate and interact with renal cells. Studies have shown that AGEs correlate with the expression of TGF-β1 and CTGF.³⁰ TGF-β is a polypeptide with three isoforms in mammals namely TGF-β1, TGF-β2 and TGF-β3. The isoforms share the same conserved regions in structure and signaling pathway, but differ in distribution. TGF-β1 is expressed in all kinds of renal cells, while TGF-β2 is expressed in cells of nervous system and TGF-β3 is expressed in rhabdomyosarcoma cells.³¹ Many experiments have found that the transition of

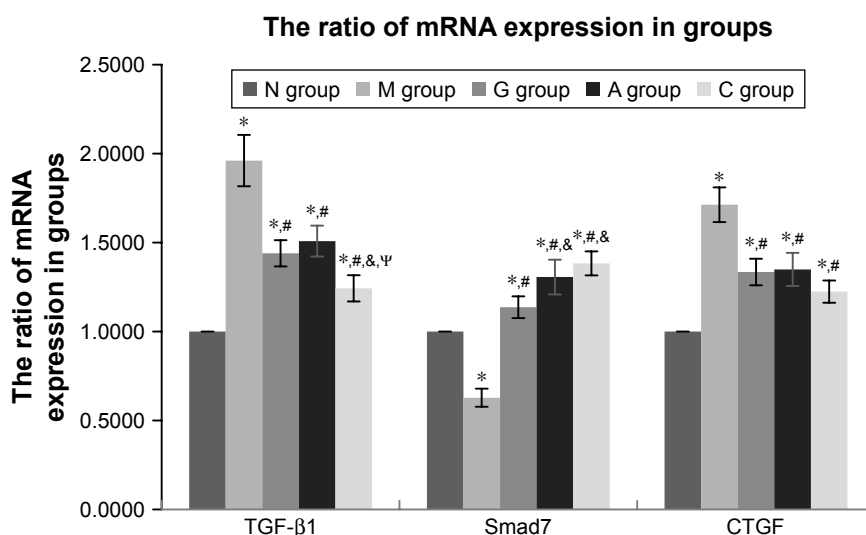


Figure 5 The ratio of TGF-β1, Smad7 and CTGF mRNA expression in each group.
Notes: *P<0.05 vs N group; #P<0.05 vs M group; §P<0.05 vs G group; ΨP<0.05 vs A group.

tubular epithelial cells to myofibroblasts leading to renal fibrosis is modulated by TGF- β 1/Smads signaling.^{32,33} In addition, CTGF, one of the main downstream products of TGF- β 1/Smad signaling pathway, contributes to renal fibrosis and exerts positive feedback on the signaling. Smad7 as a negative factor can downregulate TGF- β 1/Smads signal transduction. Hence, anti-oxidative stress and inhibition of TGF- β 1/Smads signaling cascade may be vital targets for drugs used to treat DN.

P. ginseng and Radix Astragali are together compatible with other herbs used in traditional Chinese medicine for clinical treatment of diabetes. Ginsenoside Rg1 and Astragaloside IV are the two main bioactive components present in *P. ginseng* and Radix Astragali, respectively. Many experimental studies have indicated that both Ginsenoside Rg1 and Astragaloside IV exhibit some anti-oxidative ability and suppressive activity on TGF- β 1/Smads signaling,³⁴⁻⁴⁰ but the application of combination of Ginsenoside Rg1 and Astragaloside IV and its benefits in DN have not been reported.

In our study, we found that the treatment groups (G group, A group and C group) showed different levels of improvement in DN. We found that rats in C group (treated with the combination of Ginsenoside Rg1 and Astragaloside IV) had the lowest MDA level but the highest CAT, GSH-PX and T-AOC levels among the three treatment groups. In addition, G group had lower MDA level but higher CAT, GSH-PX and T-AOC levels than A group. We can conclude that Ginsenoside Rg1 (50 mg/kg/day) has better anti-oxidative stress ability than Astragaloside IV (16 mg/kg/day). However, Astragaloside IV can strengthen the anti-oxidative stress effect of Ginsenoside Rg1 in DN. The rats in C group had better renal function than G group or A group rats with lower levels of BUN, SCr, β_2 -MG and UCr. There was almost no difference between G group and A group (merely in SCr, $P < 0.05$). We may conclude that Ginsenoside Rg1 and Astragaloside IV interact with each other to improve renal function in DN. The inhibiting effect of the three treatments on TGF- β 1/Smads signaling was detected in our experiments. G group, A group and C group treatments all significantly decreased both factor and mRNA overexpression of TGF- β 1 and CTGF but increased Smad7 expression in the kidneys of DN rats. We also conclude that Ginsenoside Rg1 has a better inhibiting effect on TGF- β 1 overexpression, while Astragaloside IV has a better activating effect on Smad7 expression. Thus, combination of Ginsenoside Rg1 and Astragaloside IV may exert excellent inhibiting effect on TGF- β 1/Smads signaling cascade.

Conclusion

Our findings provide some evidence for the protective effect of the combination of Ginsenoside Rg1 and Astragaloside IV on DN rats. We conclude that combination of Ginsenoside Rg1 and Astragaloside IV can benefit DN patients by attenuating oxidative stress, improving renal function and inhibiting TGF- β 1/Smads signaling cascade. Therefore, our study provides laboratory evidence for the beneficial effect of the combination of Ginsenoside Rg1 and Astragaloside IV on the treatment of DN. However, more investigations are required to study the underlying mechanism in detail.

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Disclosure

The authors report no conflicts of interest in this work.

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