ORIGINAL ARTICLES

Department of Pharmacy[1], JiLin University, Changchun, Department of Pharmacy[2], Yantai Yuhuangding Hospital, Qingdao University, Yantai, Tibet University Medical College[3], lhasa, China

Expression analysis of microRNAs and their target genes during experimental diabetic renal lesions in rats administered with ginsenoside Rb1 and trigonelline

Xiaona Shao[1,2,#], Chen Chen[1,#], Chunsheng Miao[1], Xiaoyan Yu[1], Xiangjun Li[1], Jianan Geng[1], Dongyan Fan[3], Xueyuan Lin[1], Zhen Chen[1], Yan Shi[1,*]

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**Corresponding author: Yan Shi, Department of Pharmacy, Jilin University, Chaoyang District, Changchun City 130021, Jilin Province, China*
shiyan@jlu.edu.cn
#Co-first authors

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Purpose: To appraise the curative effect of ginsenoside Rb1 and trigonelline in diabetic nephropathy and to analyze the expression analysis of microRNAs and their target genes during experimental diabetic renal lesions in rats. *Methods*: Wistar rats were made diabetic by intraperitoneal injection of 55 mg/kg streptozotocin. According to their fasting blood glucose values and initial body weight, diabetic rats were assigned to specific groups and treated as follows: DN group (tap water, n = 10), A group (ginsenoside Rb1, 40 mg/kg, n = 10), B group (trigonelline, 20 mg/kg, n = 10) and the C group (ginsenoside Rb1 and trigonelline, 60 mg/kg, m(ginsenoside Rb1) : m (trigonelline) = 2:1, n = 10). The control group was treated with tap water (n = 10). All rats were gavaged with drugs or tap water once daily for 12 weeks. *Results*: Renal dysfunction, oxidative stress, and pathological alteration were significantly alleviated by a combination of ginsenoside Rb1 and trigonellin (C group). Some miRNAs were expressed differentially in Con, DN, A and C groups. Results of immunohistochemistry and western blotting showed that Wnt and β-catenin were expressed differentially in Con, DN, and C groups. *Conclusion*: Ginsenoside Rb1 and trigonelline could prevent the development of diabetic renal lesions by regulating the expression of miR-3550 and further associating with the Wnt/β-catenin signaling.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease with a high incidence in society currently (Hu et al. 2013). Diabetic nephropathy (DN) is one of the most serious chronic microvascular complications of DM and the major cause of end-stage renal disease (ESRD) (Ziyadeh and Sharma 2003). *Panax ginseng* belongs to the family *Araliaceae*, is a perennial herbaceous plant, and is a rare traditional Chinese medicine (Twigg 2010). Ginsenoside Rb1 polysaccharides, and polypeptides are the main active ingredients in this medicine, and they can effectively reduce blood glucose. Therefore, they can be used to treat diabetes and complications associated with it. *Trigonella foenum-graecum L.* is an annual leguminous herbaceous plant, the main active ingredients of the plant are trigonelline, saponins, and galactomannan (Srinivasan 2006; Meghwal and Goswami 2012; Ulbricht et al. 2007; Billaud and Adrian 2001). Trigonelline has functions of reducing blood glucose, lowering cholesterol, promoting the regeneration of nerve tissue, anti-cancer, anti-oxidation, sedative and so on. So far, several studies have proved that trigonelline lowers blood glucose and protects from diabetic renal lesions (Shang et al. 2002; Eidi et al. 2007).

MiRNAs are endogenous non-coding single stranded RNAs, composed of 21~25 nucleotides. MiRNAs are shown to be associated with a variety of diseases, such as radiation-induced late central nervous system damage (Shi et al. 2012), essential hypertension (Chen et al. 2015), lung cancer (Czubak et al. 2015), breast cancer (Erbes et al. 2015), gallbladder cancer (Peng et al. 2015), and diabetic nephropathy (Chien et al. 2016; Wu et al. 2016). The results of a number of studies show that miR-192, miR-200, miR-21, miR-29, miR-377, let-7c, and other miRNAs may be involved in the occurrence and development of DN (Wang et al. 2008, 2010; Brennan et al. 2013; Zhong et al. 2011; Wang et al. 2012).

2. Investigations and results

2.1. General status, glucose levels, and lipid metabolism of rats

Compared with the Con group, the DN group rats increased urine output, thirst, weight loss, weakness, and cataract. Compared with the DN group, rats from the treatment groups (A: ginsenoside Rb1; B: trigonelline; C: ginsenoside Rb1 and trigonelline) showed a higher body weight, and fewer cataract symptoms.

The results of the blood glucose and blood lipid metabolism showed that as compared with Con group rats, the blood glucose, TCHO, LDL-C, and TG levels were significantly higher in DN group rats ($p < 0.01$). Compared with DN group rats, the blood glucose and the TCHO, LDL-C, and TG levels of treatment groups rats were significantly decreased ($p < 0.01$), and HDL-C blood level was significantly increased ($p < 0.05$) (Fig. 1A). HDL-C content in the blood of rats from group C was higher than that in other treatment groups, and their blood content of LDL-C, TG was significantly lower (Fig. 1C).

In the glucose tolerance test, compared with Con group, the blood glucose levels of DN group rats were significantly increased in 0 – 120 min ($p < 0.01$), while the area under the curve for blood glucose was significantly greater than that for the Con group ($p < 0.01$). Compared with the DN group, the treatment group levels were significantly lower than that between 0 – 120 min ($p < 0.01$) (Fig. 1B).

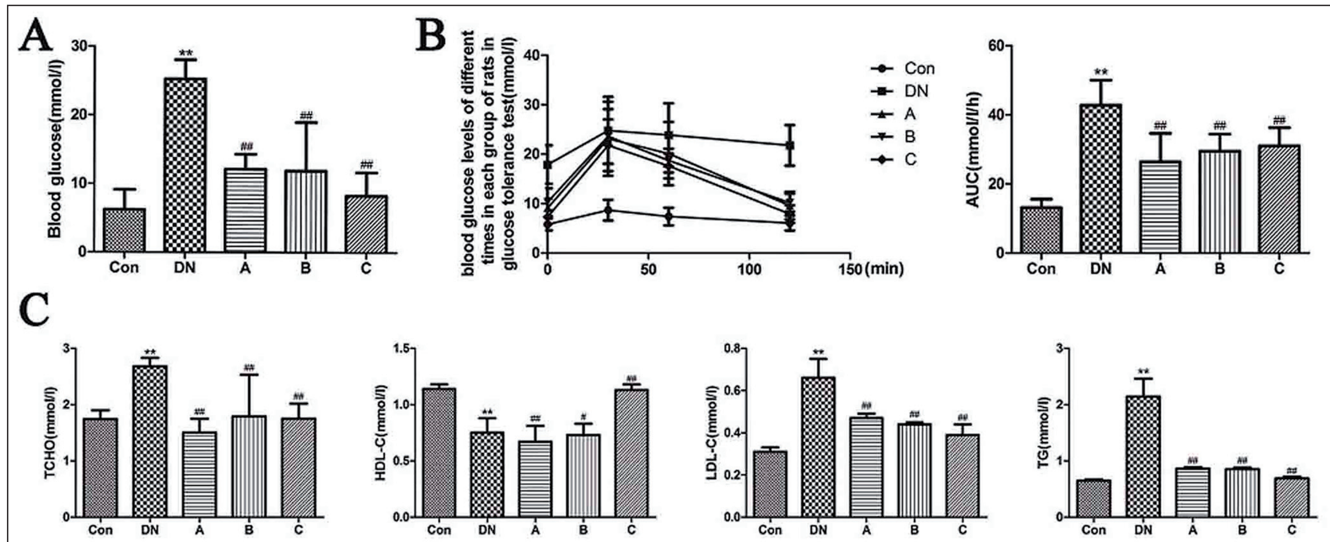

Fig. 1: Effects of ginsenoside Rb1, trigonelline, and ginsenoside Rb1 and trigonelline on glucose and lipid metabolism. (A) Blood glucose. (B) Glucose tolerance. (C) Lipid metabolism. Data represent the mean±SEM for 10 rats per group. * $p < 0.05$, ** $p < 0.01$ as compared with the Con group; # $p < 0.05$, ## $p < 0.01$ as compared with the DN group.

2.2. Renal function

The 24 h urine volume and the kidney weight/body weight ratio of rats from each group were determined. As compared with rats from the Con group, the 24 h urine volume and kidney/body weight ratio were significantly higher in rats from the DN group ($p < 0.01$). As compared with rats from the DN group, the 24 h urine volume and kidney/body weight ratio were significantly decreased ($p < 0.01$) in rats from the treatment group. In particular, the improvement of rats from the C group among the treatment groups was the most evident (Fig. 2A-B). Indexes of renal function test results showed that as compared with the serum BUN, serum CRE and MTP, and MAZ values of rats from the Con group, those of rats from the DN group increased significantly ($p < 0.01$). As compared with the lipid metabolism of rats from the DN group, which were significantly reduced in rats from the treatment groups ($p < 0.01$). Specifically, the lipid metabolism, urine BUN and urine CRE values decreased most evidently in the C groups among the treatment groups. (Fig. 2C).

2.3. Oxidative stress

The oxidative stress kit detected that the indicators in rat serum, the vitality of CAT, SOD, and GSH-PX in DN group were significantly decreased ($p < 0.01$) as compared with the Con group. The content of MDA in blood samples from rats of the DN group was significantly higher than that in samples from the Con group ($p < 0.01$). Compared with the DN group, the activity of SOD, GSH-PX activity in rats from the treatment groups was significantly increased ($p < 0.01$), the content of MDA was significantly decreased ($p < 0.01$). At the same time, the content of MDA in rats from the C group was close to those from the Con group (Fig. 3A-B).

2.4. Renal pathological morphology alterations

HE staining showed that as compared with the Con group, hypertrophy of the kidney, diffuse mesangial expansion, accumulation of extracellular matrix and infiltration of inflammatory cells were detected in rats from the DN group. As compared with rats from the DN group, these symptoms in rats from the treatment groups were significantly reduced. Renal pathological changes in rats from the C group were the most evident, and the effect was better than that in rats from A and B groups respectively (Fig. 4A).

PAS staining was used to examine the glomerular structure, to assess the protective effect of ginsenoside Rb1 and trigonelline on

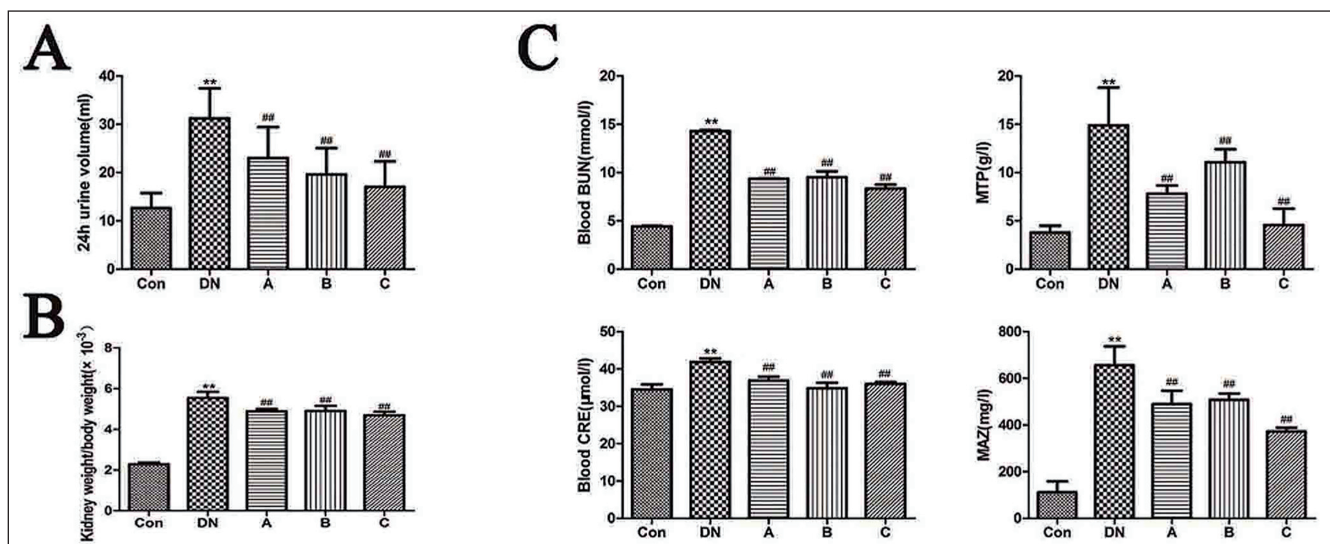

Fig. 2: Effects of ginsenoside Rb1, trigonelline, and ginsenoside Rb1 and trigonelline on renal function. (A) 24 h urine volume. (B) Kidney/body weight ratio. (C) Renal function. Data represents the mean±SEM for 10 rats per group. * $p < 0.05$, ** $p < 0.01$ as compared with the Con group; # $p < 0.05$, ## $p < 0.01$ as compared with the DN group.

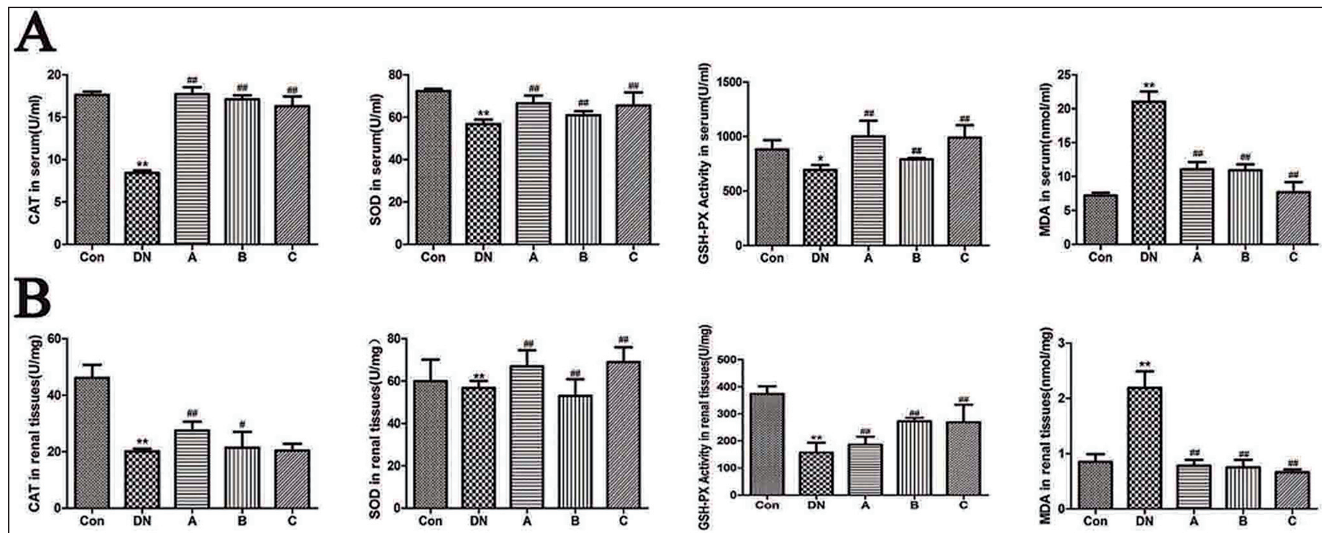

Fig. 3: Effects of ginsenoside Rb1, trigonelline and ginsenoside Rb1 and trigonelline on oxidative stress. (A) Oxidative stress in serum. (B) Oxidative stress in renal tissues. Data represents the mean±SEM for 10 rats per group. *$p < 0.05$, **$p < 0.01$ as compared with the Con group; #$p < 0.05$, ##$p < 0.01$ as compared with the DN group.

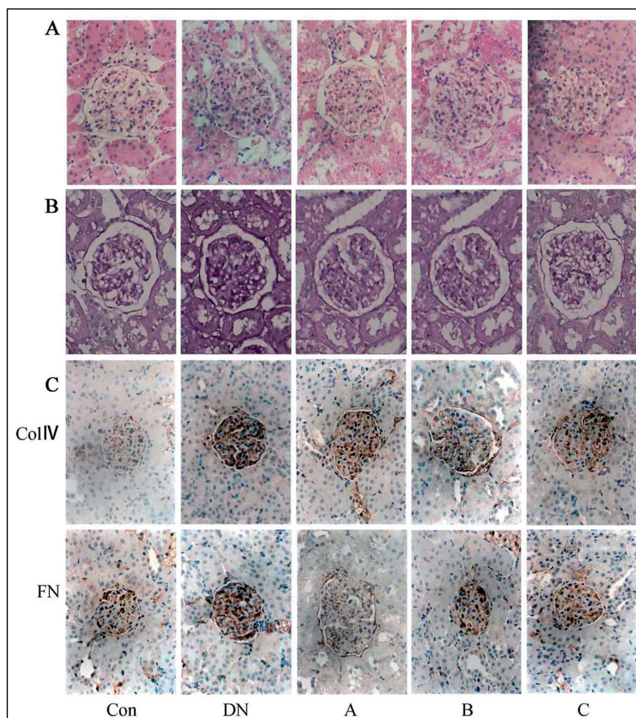

Fig. 4: Effects of ginsenoside Rb1, trigonelline and ginsenoside Rb1 and trigonelline on renal pathological morphology alterations. (A) HE staining. (B) PAS staining. (C) Immunohistochemical staining.

the experimental diabetic renal lesion rats. Using light microscopy, increase in the positively stained part (polysaccharide, the presence of protein polysaccharide), thickened basement membranes, and mesangial matrix expansion were detected in rats from the DN group. As compared with the DN group, the positive substance (polysaccharide, the presence of protein polysaccharide) in the treatment groups was significantly decreased and the basement membrane was thinner. Especially, the renal pathological changes in rats from the C group were the most significant, and the effect (less positive substances, no obvious proliferation of mesangial cells) was better than that of A group and B group (Fig. 4B).

Immunohistochemistry results showed that the expression of FN and Col IV in rats from the DN group was greater than that in rats from the Con group, which was mainly distributed in the extracellular matrix of mesangial cells. As compared with the DN group, the treatment groups had different degrees of improvement. The expression of FN in rats from the A group and Col IV in rats from B group was significantly decreased, and the expressions of FN and Col IV in rats from the C group was decreased, and the effect was clear (Fig. 4C).

2.5. Expression of miRNAs in rat renal cortical tissue

The result of miRNAs microarray showed that there were 35 miRNAs expressed differently in rats from the DN group as compared with those from the Con group ($p < 0.05$). Twelve miRNAs were upregulated and 23 miRNAs were downregulated in the DN group, the fold change was above 0.8. Twenty one miRNAs were expressed differently in the C group as compared with those in the DN group ($p < 0.05$). Six miRNAs were upregulated and 15 miRNAs were downregulated in the C group, the fold change was above 0.8 (Fig. 5A).

Differentially expressed miRNAs shortlisted from the microarray. The expression of miR-92b-5p, miR-1306-3p, and miR-3550 was found to be upregulated in rats from the DN group, whereas, they were significantly downregulated in rats from the C group and the Con group .The expression of miR-30d-5p in rats from the DN group was downregulated, whereas, it was significantly upregulated in rats from the C group, and it was close to that in the Con rats.

In order to verify the protective effects of ginsenoside Rb1 and trigonelline on experimental diabetic renal lesion rats, we detected the differential expression of miR-30d-5p, miR-92b-5p, miR-1306-3p, and miR-3550 in rats from the Con, DN and C groups by real-time PCR. The results showed that the expression of miR-92b-5p, miR-1306-3p, and miR-3550 was consistent with the microarray. However, the expression of miR-30d-5p in rats from DN and C group was significantly increased, contrary to the results of the microarray, miR-30d-5p was a false positive result in the microarray results. (Fig. 5B).

2.6. Prediction and validation of target genes

MiRNAs target prediction was performed using TargetScan, miRanda, and PicTar software. The results showed that the target genes of miR-3550 were CTNNB1 (NM_001098209, catenin, beta 1, 88kDa), EFCAB4A (NM_173584, EF-hand calcium binding domain 4A), LOC3422918 (NM_001195076, hypothetical protein LOC342918), RNF220 (NM_018150, ring finger protein 220), and PACSIN1 (NM_001199583, protein kinase C and casein kinase substrate in neurons 1), however, target genes of miR-30d-5p, miR-92b-5p, and miR-1306-3p were not present in our database.

In Con, DN, and C groups, the relative expression of Wnt and β-catenin in renal tissues were detected by immunohistochem-

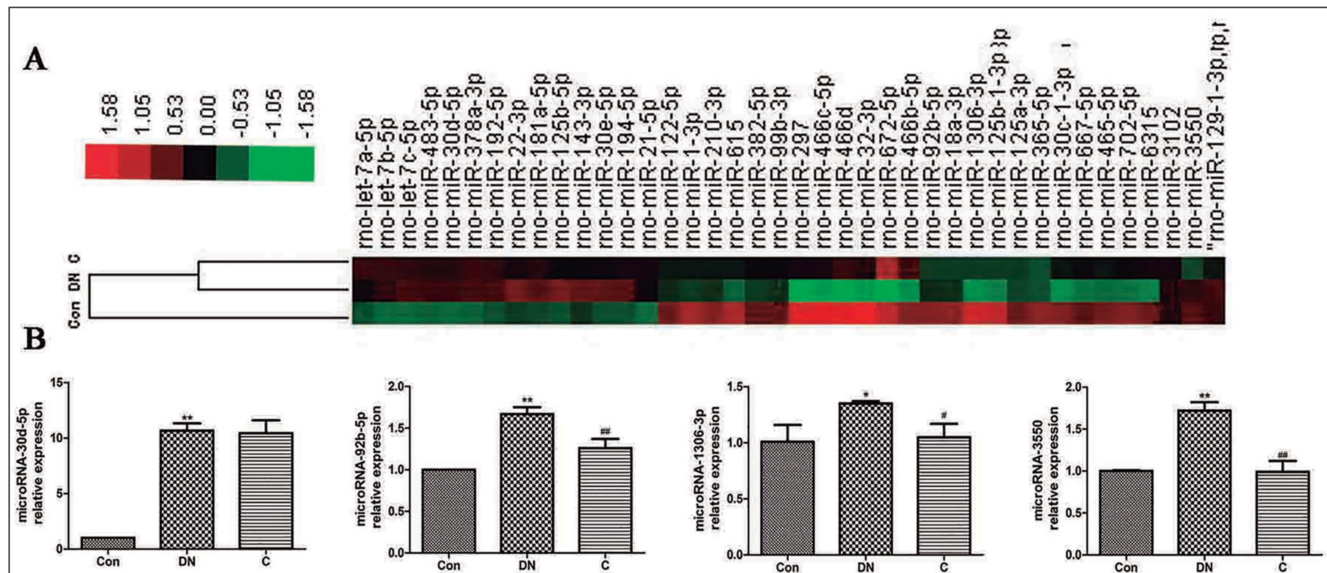

Fig. 5: Expression analysis of miRNAs and its target genes during the period of experimental diabetic renal lesion rats administered with ginsenoside Rb1 and trigonelline. MiRNAs microarray assay. (B) Real-time qPCR analysis of miR-30d-5p, miR-92b-5p, miR-1306-3p, and miR-3550, respectively. Data represent the mean±SEM. *$p < 0.05$, **$p < 0.01$ compared with the Con group; #$p < 0.05$, ##$p < 0.01$ compared with the DN group.

istry and western blotting. The result of immunohistochemistry revealed that Wnt and β-catenin were expressed in the cytoplasm and nucleus. Compared with Con group, the expression of Wnt and β-catenin in the DN group was significantly higher. Compared with the DN group, the expression of Wnt and β-catenin in C group was significantly lower (Fig. 6A).

The result of western blotting revealed that the expression of Wnt and β-catenin in the DN group was significantly more compared with that in the Con group ($p < 0.01$). At the same time, they were decreased obviously than that in DN group ($p<0.01$) (Fig. 6B).

3. Discussion

Diabetic nephropathy is one of the most serious chronic microvascular complications in diabetic patients. Its early clinical symptoms include kidney hypertrophy, proteinuria, and renal dysfunction. At present, the clinical treatment of diabetic nephropathy is mainly aimed at controlling blood glucose/pressure and reducing the excretion of urinary proteins; there are no special drugs for its treatment. Both trigonelline and ginsenoside Rb1 significantly reduce the blood glucose level and ameliorate the renal lesions in diabetic rats. Both of them are traditional Chinese medicines, however, their action against nephropathy was stable but slow.

In this study, Wistar rats received intraperitoneal injection with 55 mg/kg STZ in citrate buffer, and the rats from the DN group showed clear symptoms, including increased urine output, thirst, weight loss, weakness, and cataract. Blood glucose, blood lipid and renal function indexes were significantly increased in rats from the DN group, thus it can be considered that the rats from the DN group showed renal dysfunction. Persistent hyperglycemia is the leading cause of diabetic nephropathy. Therefore, regulating blood glucose levels is one of the main methods of prevention and treatment of diabetic nephropathy. Diabetic patients are prone to disorders of lipid metabolism. Excessive deposition and oxidation of lipoproteins in the glomeruli would lead to glomerular sclerosis. Therefore, the regulation of lipid metabolism could be used as adjuvant treatment of diabetic nephropathy. Blood glucose levels of diabetic rats treated with ginsenoside Rb1 and trigonelline were significantly lower and were similar to that of Con rats. Their serum T-CHO, LDL-C, and TG levels were decreased, renal function was significantly improved as compared with that of rats from the DN group. Blood glucose, blood lipid, and renal function of the rats from A and B groups was also improved.

It is reported that both trigonelline and ginsenoside Rb1 show antioxidant activity (Hwang et al. 2014). The results of this experiment showed that the activity of CAT, SOD, and GSH-PX in rats from the DN group were significantly decreased, while MDA levels were significantly increased. The results of experiments for oxidative stress indicated that the antioxidant defense system of rats from the DN group was damaged, and the degree of lipid peroxidation was increased, which resulted in the damage of cells and tissues. As compared with the DN group, CAT, SOD, and GSH-PX activity in serum and renal tissues of rats treated with ginsenoside Rb1 and trigonelline was generally higher than that of the DN group, and the MDA levels also decreased significantly, with an effect better better than that of A and B groups. This suggested that ginsenoside Rb1 and trigonelline can enhance the antioxidant defense system in diabetic rats and protect their kidneys.

We further observed pathological changes in kidneys from each group. The results of HE, PAS staining and immunohistochemistry showed that the glomerular volume of rats from the DN group had increased, mesangial cell proliferation, extracellular matrix accumulation, inflammatory cell infiltration, the main components of the extracellular matrix FN, and Col IV expression was increased. However, the renal pathological changes in the treatment groups were significantly improved, the glomerular volume was decreased, and mesangial cells had no evident hyperplasia, Col IV and FN expression was significantly decreased. In particular, the renal pathological changes of diabetic rats treated with ginsenoside Rb1 and trigonelline were better than that of other groups. In a word, ginsenoside Rb1 and trigonelline can effectively reduce the accumulation of extracellular matrix in the kidney tissue of diabetic rats and protect kidneys of diabetic rats.

The pathogenesis of diabetic nephropathy mainly includes genetic factors, glucose/lipid metabolism disorders, oxidative stress, cytokines and related signaling pathways. In recent years, the discovery of miRNAs has provided new ideas for the pathogenesis of diabetic nephropathy. The present study found that ginsenoside Rb1 and trigonelline has an ameliorative effect on renal lesions in diabetic rats, and its possible mechanism was examined using analysis of miRNAs expression. With advances in molecular biology, some researchers have found that miRNAs are involved in the development of diabetic nephropathy at the cellular level in animals (Kato et al. 2013). In the early diabetic nephropathy model, let-7a-3, miR-451, miR-21, miR-27a, miR-223, miR-340, and miR-574-3p were specifically expressed (Peng et al. 2015; Wang et al. 2014; Bijkerk et al. 2015). Our study found that miR-30d-5p, miR-92b-5p, miR-1306-3p, and miR-3550 expressed in renal tissues of rats from Con, DN and C groups were specifically expressed. Microarray showed that the expression of

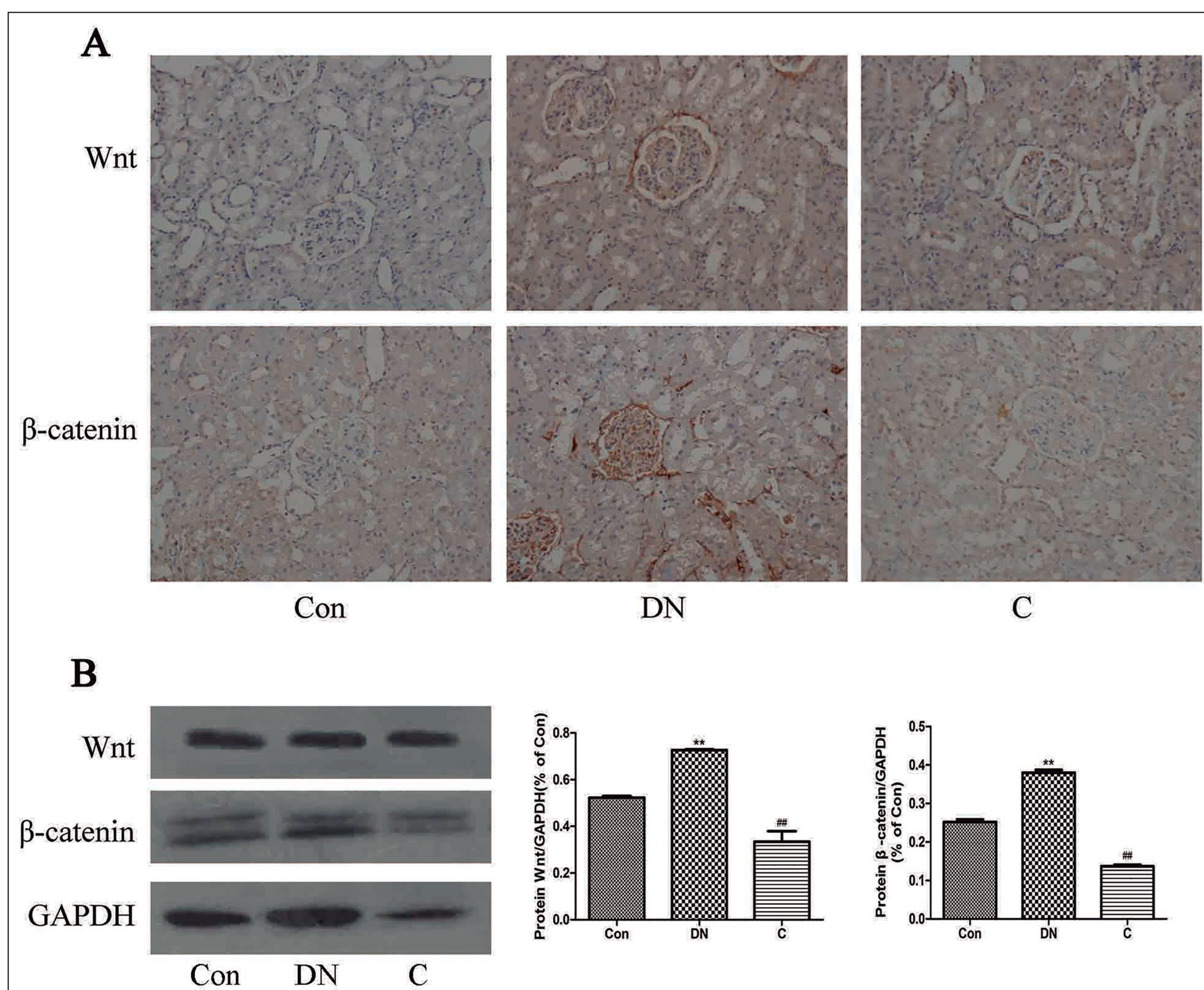

Fig. 6: Data represent the mean±SEM. $*p < 0.05$, $**p < 0.01$ compared with the Con group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the DN group.

miR-30d-5p was significantly decreased in the DN group, and the expression levels were significantly increased after treatment with ginsenoside Rb1 and trigonelline. In addition, the expression of miR-92b-5p, miR-1306-3p, and miR-3550 in rats from the DN group was significantly increased, and was significantly lower after treatment with ginsenoside Rb1 and trigonelline treatment. This shows that miR-30d-5p, miR-92b-5p, miR-1306-3p and miR-3550 are involved in the development of diabetic nephropathy, and ginsenoside Rb1 and trigonelline might regulate these miRNAs to ameliorate the renal lesion in diabetic rats. To further verify the mechanism underlying the effect of ginsenoside Rb1 and trigonelline on the renal function of diabetic rats, we demonstrated this at the gene level using real-time qPCR. The results of real-time qPCR showed that the relative expression of miR-92b-5p, miR-1306-3p, and miR-3550 was consistent with the results of microarray, but the relative expression of miR-30d-5p in Con, DN, and C groups was opposite to that seen in microarray. Han et al. found that the miR-30d expression in rats from the diabetic group was 1.64-fold of that in rats from the non-diabetic group, the relative expression of miR-30d in rats from the non-diabetic group was defined as 1 ($P < 0.05$) (Han et al. 2016). Tong found that levels of miR-106b, miR-27a, and miR-30d were significantly elevated in the skeletal muscle tissue of diabetic rats compared with those of rats in the control group. And they further suggested that miR-106b, miR-27a, and miR-30d play crucial roles in the regulation of glucose metabolism by targeting the GLUT4 signaling pathway in L6 cells (Zhou et al. 2016). Combined with the characteristics of microarray technology, we speculated that miR-30d-5p was a negative result. The prediction and functional analysis results of target genes showed that miR-92b-5p and miR-1306-3p were not found in our miRNAs database, and one of the target genes of miR-3550 is CTNNB1 (β-catenin), which is associated with the development of diabetic nephropathy.

In addition, β-catenin is the key to the Wnt/β-catenin signaling pathway, which is a sign of the activation of this pathway (Bienz et al. 2005). In the absence of Wnt, β-catenin can form complexes with many proteins such as AXIN1, AXIN2, APC, CSNK1A1 and GSK3B and this promotes the phosphorylation of β-catenin residues on N-terminal Ser and Thr residues and the ubiquitination of β-catenin *via* BTRC and its subsequent degradation by the proteasome. In the presence of Wnt ligand, β-catenin accumulates in the nucleus, where it acts as a coactivator for transcription factors of the TCF/LEF family, leading to the activation of Wnt responsive genes, involved in the regulation of cell adhesion. In insulin internalization, β-catenin acts as a negative regulator in the regulation of CDK2/PTPN6/CTNNB1/CEACAM1 pathway. The Wnt/β-catenin signaling pathway is involved in the development of renal fibrosis, acute kidney injury and diabetic nephropathy (Pulkkinen et al. 2008; Zhou et al. 2012).

Therefore, we hypothesize that ginsenoside Rb1 and trigonelline could protect the experimental diabetic rats from renal lesions by regulating the expression of miR-3550 and further associating with

the Wnt/β-catenin signaling pathway. Immunohistochemistry and western blotting results showed that the expression of Wnt and β-catenin were significantly increased in rats from the the DN group, while they were significantly decreased after treatment with ginsenoside Rb1 and trigonelline, similar to results of the Con group. Furthermore, our experiments proved that the Wnt/β-catenin signaling pathway in the kidney tissue of diabetic rats was abnormally activated, while ginsenoside Rb1 and trigonelline may regulate this signaling pathway to protect the renal tissues of diabetic rats.

In conclusion, the present study demonstrates that diabetic rats treated with ginsenoside Rb1 and trigonelline showed lower blood glucose, improved renal function, increased antioxidant capacity, less extracellular matrix, and fewer renal pathological changes. This suggests that ginsenoside Rb1 and trigonelline have a protective effect on the experimental diabetic renal lesion rats. The results of miRNAs microarray, real-time PCR, prediction and functional analysis of target genes, immunohistochemistry, and western blotting showed that ginsenoside Rb1 and trigonelline could protect the experimental diabetic rats from renal lesions by regulating the expression of miR-3550 and further associating with Wnt/β-catenin signaling pathway.

4. Experimental

4.1. Experimental design

Wistar rats (male, 50 rats, 10 weeks old; 180-200 g) were obtained from the Experimental Animal Center of Jilin University. After acclimatization for 7 days, the rats were fasted for 12 h, and then received intraperitoneal injections containing 55 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO, USA) prepared in citrate buffer solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.2). At the same time, the control group (Con group) rats were injected with citrate buffer solution of the corresponding volume. Three days later, their blood glucose levels were determined by cutting the tail tip (Yicheng type JPS-5 blood glucose meter, Beijing, China), and detecting glucose levels from urine. (When the enzyme test paper and urine meet, it is orange yellow, with three plus signs (+++), 1-2 % sugar in urine.) Rats with blood glucose concentrations above 16.7 mmol/L and urine glucose above +++ were considered as diabetic and were used for subsequent experiments. According to their fasting blood glucose values and initial body weight, diabetic rats were assigned to specific groups and treated as follows: DN group (tap water, n = 10), A group (ginsenoside Rb1, weikeqi-biotech, Sichuan, China, 40 mg/kg, n = 10), B group (trigonelline, weikeqi-biotech, Sichuan, China, 20 mg/kg, n = 10) and the C group (ginsenoside Rb1 and trigonelline, 60 mg/kg, m (ginsenoside Rb1) : m (trigonelline) = 2:1, n = 10). The control group was treated as follows: (tap water, n = 10). All rats were gavaged corresponding drugs or tap water once daily for 12 weeks.

4.2. Determination of biochemical indicators

In the sixth week after treatment, we measured glucose tolerance of the rats in each group. The area under the curve (AUC) for glucose was calculated using the following formula.

$$AUC = (BG0 + BG30) \times 0.5 / 2 + (BG30 + BG60) \times 0.5 / 2 + (BG60 + BG120) \times 1 / 2$$

BG0, BG30, BG60, BG120 represent the blood glucose levels at 0, 30, 60, and 120 min respectively.

After grouping of rats, their blood glucose levels were determined using blood samples from the tail veins of the rats every week, at the same time, body weight was also measured. At the end of the experiment, the rats were fasted for 12 h, and their urine was collected for 24 h. We calculated the kidney weight / body weight ratio after fasting the rats for 12 hours of. Blood lipid indexes : total cholesterol (T-CHO), high-density lipoprotein-cholesterol (HDL-C) , low-density lipoprotein-cholesterol (LDL-C), glycerol triester (TG) were determined using the AU5800 Beckman Coulter automatic biochemical analyzer (Beckman Couter, USA). The renal function indexes (blood urine BUN, blood urine CRE, MTP, MAZ) were determined using the DXC-800 Beckman Coulter automatic biochemical analyzer (Beckman Couter, USA).

4.3. Determination of oxidative stress

Oxidative stress parameters were determined according to the instructions of each test kit (njjcbio, Nanjing, China) for the serum and kidney tissues of each group of rats. The activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and malondialdehyde (MDA) content were detected.

4.4. Histological examinations

Rat kidney tissues were fixed by immersion in 10 % buffered formalin and embedded in paraffin for light microscopy studies. Sections of 4 μm thickness were stained with Periodic Acid-Shiff (PAS) and Hematoxylin-Eosin (HE) stains to evaluate the degree of mesangial matrix expansion in different groups of rats. Protein levels of fibronectin (FN) and type IV collagen (Col IV) in renal tissue sections were examined using immunohischemistry. Briefly, renal tissue sections were treated with the polyclonal rabbit anti-rat fibronectin antibody (1:100 dilution, Santa Cruz Biotechnology, CA, USA) and the polyclonal rabbit anti-rat Col IV antibody (1:100 dilution, Santa Cruz Biotechnology, CA, USA). The bound antibodies were detected with the HRP-anti-rabbit antibody (ZSGB-BIO, Beijing, China) and diaminobenzidine (DAB), followed by counterstaining with hematoxylin. The percentages of positive staining areas in the glomerulus were determined semiquantitatively using SPSS22.0 computer imaging analysis system.

4.5. MiRNAs microarray assay

The renal cortex tissues (weight 80 mg) of the Con, DN, and C group were used for RNA extraction using 1 ml Trizol (Invitrogen, USA). Small RNA (<200 bp) fragments were enriched (Pall Corporation, USA) from 2.5 μg total RNA using NanoSep 100K (Pall Corporation, USA). Fluorescent targets were prepared using miRNA the ULSTM labeling kit (Kreatech Diagnostics, The Netherlands). Labeled fluorescent targets were hybridized to pre-hybridized Mouse miRNA OneArray® v5 (Phalanx Biotech Group, Hsinchu, Taiwan). The slides were dried by centrifugation and scanned using an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensity of each spot was analyzed using the GenePix 4.1 software (Molecular Devices).

4.6. Quantitative PCR

Renal cortex tissues (40 mg) of the Con, DN, and C groups were used for RNA extraction using 1 ml Trizol (Invitrogen, USA). cDNA was synthesized using the TransScript miRNAs First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). MiRNAs expression was quantified using the TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). All primers were obtained from GeneCopoeia (USA), the sequences of primers are listed as follows: miR-30d-5p (5′-UGUAAACAUCCCCGACUGGAAG-3′), miR-1306-3p (5′-GACGUUGGCUCUGGUGGUGAUG-3′), miR-92b-5p (5′-AGGGACGGGAC-GCGGUGCAGUGUU-3′), miR-3550 (5′-CCGAGCUCCCUGCCCUAG -3′). U6 served as an internal control. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001) and normalized to the expression levels of U6 RNA. All real-time qPCRs were performed at least three separate times in triplicates and data are presented as mean±SD. miRNA-specific reverse transcription primers were procured from GeneCopoeia, USA.

4.7. Prediction and validation of target genes

Target genes of miRNAs we selected were predicted using TargetScan, miRanda, and PicTar. We analyzed functions of target genes using Uniprot (http://www.uniprot.org/) and KEGG databases (http://www.kegg.jp) and looked for their associated signaling pathways. Expression of related proteins from kidney tissues was further verified using immunohistochemistry and western blotting.

4.8. Immunohistochemistry

The protein levels of Wnt and CTNNB1 in renal tissue sections were examined using immunohistochemistry. Briefly, renal tissue sections were treated with the polyclonal rabbit anti-rat Wnt antibody (1:300 dilution, BBI Life Science Products & Services, Shanghai, China) and the polyclonal rabbit anti-rat CTNNB1 antibody (1:2000 dilution, Abclonal, USA). The bound antibodies were detected with the HRP-anti-rabbit antibody and diaminobenzidine (DAB), followed by counterstaining with hematoxylin. Negative controls were incubated with PBS. The percentages of positive staining areas in the glomerulus were determined semiquantitatively using computer imaging analysis system.

4.9. Western blotting

Protein levels of Wnt and CTNNB1 in renal tissue sections were examined using western blotting. The renal cortical tissues of rats in Con, DN and, C groups were homogenized in the lysis buffer and centrifuged. The supernatants were collected. Following quantification of protein concentrations, tissue lysates (100 μg protein/lane) were separated using SDS-PAGE on a 7.5 % polyacrylamide gel, followed by transfer onto nitrocellulose membranes (GE Healthcare, Beijing, China). Subsequently, the blots were blocked in 5 % skimmed dry milk in PBS and incubated with the polyclonal rabbit anti-rat Wnt antibody (1:300 dilution, BBI Life Science Products & Services, Shanghai, China), and the polyclonal rabbit anti-rat CTNNB1 antibody (1:2000 dilution, Abclonal, USA), and the anti-rat GAPDH antibody (1:1000 dilution, ZSGB-BIO, Beijing, China) at 4 °C overnight. Bound antibodies were detected with the horseradish peroxidase-(HRP-)conjugated anti-rabbit antibody and visualized using the enhanced chemiluminescence kit (Thermo Scientific Pierce, USA), according to the manufacturers' instructions. The levels of target proteins were normalized to those of control (GAPDH) were determined using densitometry.

4.10. Statistical analyses

Data are presented as mean±SD. The difference among the groups was analyzed using one-way ANOVA and *post hoc* analysis using the Bonferroni correction. The difference between two groups was analyzed using the SPSS 19.0 software. Sex difference was also accounted for in the statistical analysis. A P-value of < 0.05 was considered statistically significant.

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