Research on mechanism of MAPK signal pathway induced by BMSCs for the proteinuria of rat's kidney, glomerulosclerosis and activity of RAS

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Abstract. – **OBJECTIVE:** The aim of the article was to explore the mechanism of MAPK (Mitogen-activated protein kinase) signal pathway induced by BMSCs (Bone marrow mesenchymal stem cells) for the proteinuria of rat's kidney, glomerulosclerosis and activity of RAS (Renin angiotensin) system.

PATIENTS AND METHODS: Thirty rats were divided into sham group, FSGS (Focal Segmental Glomerular Sclerosis) group and BMSCs group. The variation of biochemical criterion and protein of rats in the three groups was compared. The variation condition of rats' kidney and GSI (Glomerular sclerosis index), ECM/GA (Extracellular matrix/glomerular area) was compared. The activity of RAS was analyzed. Finally, the p38 MAPK and p-p38 MAPK protein was compared.

RESULTS: Compared with sham group rats, the SCr, BUN and proteinuria after twenty-four hours in FSGS group was improved. The blood albumin was notably reduced. At the same time, there was evident deterioration in the pathology of nephridial tissue ($p<0.05$). The biochemical criterion in transplanted BMSCs group was significantly reduced. At the same time, the blood albumin and pathology of nephridial tissue was also improved ($p<0.05$). The glomerulus in sham group was normal. There was abundant induration for the glomerulus in FSGS group compared with sham group. The relative value of GSI and ECM/GA was higher than in sham group ($p<0.05$). The relative value of GSI and ECM/GA in BMSCs group was reduced notably compared with FSGS group ($p<0.05$). The activity of RAS in FSGS group was enhanced. But activity of RAS in BMSCs group was remarkably restrained. The p38 MAPK and p-p38 MAPK protein in FSGS group was significantly increased compared with the other groups ($p<0.05$). The protein expression in BMSCs group and inhibitor group was restrained ($p<0.05$).

CONCLUSIONS: The BMSCs could restrain the proteinuria of rat's kidney and activity of RAS and they were related with the expression of MAPK signal pathway closely.

Key Words:
  BMSCs, MAPK signal pathway, FSGS, Proteinuria, RAS.

Introduction

There was the focal segmental glomerulosclerosis (FSGS) lesion at the early stage of glomerulosclerosis. In recent years, the incidence of FSGS was increased gradually. It was accounting for nine percent of primary glomerular diseases. Its pathological feature was focal and segmental commonly[1]. The clinical performance was the elevation of proteinuria in the patients with FSGS generally. It was along with renal hypofunction and damage of renal tubular function[2]. The glomerular disease was still at the first place in the alternative therapeutic end stage of renal disease in China. The FSGS was one of the most common pathological patterns in all kinds of kidney diseases. Its treatment was relatively difficult. There was progressive damage in the renal function of patients when the stat of patients' disease could not be released. Then, patients' disease was developed into the terminal stage. So, it was important for FSGS to improve the therapeutic. In recent years, domestic and foreign scholars have reported that when abnormal activation of RAS occurs in kidney tissue, pathological changes of the kidney appear. RAS was composed of a series of angiotensin and related enzyme. Not only it could prompt the activity of blood vessel, but also it could adjust the blood pressure efficiently. At the same time, RAS could control blood volume and resistance brought from periphery. It could make the blood pressure, water and electrolytes in body maintaining balance[3]. At the same time there was RAS in blood and part tissue. It could adjust most of physiological and pathological process-

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es[4]. The MAPK was mainly composed of JNK, ERK, BMK1/ERK5 and p38. It could transmit the extracellular signals. Moreover, it could adjust the gene expression in body. Besides, it could react with the immune inflammation in body so as to affect the biological activity of cells[5,6]. The plasmin and RAS could prompt the expression of TGF-β1 and activate the MAPK signal which could make it phosphorylated so as to there being abnormal proliferation from related study. It may cause injury and apoptosis in Sertoli cells[7]. The BMSCs was one kind of adult stem cells. It was able to replicate itself along with self-differentiation[8]. The BMSCs was mainly obtained from myeloid tissue. It could be differentiated into different cells in the procession of self-updating. The BMSCs could be confirmed to treat the damage condition in renal tubule efficiently in recent years. It could differentiate the renal tubular epithelial cell. Kidney cells could be proliferated largely so as to change the growing environment of cytokines[9]. Whether FGS could be performed for interventional treatment through the MAPK signal pathway was still unclear. So the model of FSGS rats was set as research object in our study to discuss the inhibiting effect of BMSCS on the proteinuria and RAS in FSGS rats. Whether it could develop inhibiting action through MAPK signal pathway was analyzed.

Materials and Methods

Experimental Animals

The forty rats were adopted in our study. They were purchased from Changchun Yisi Experimental Animal Technology Co. LTD (Wuhan, China). The forty rats were healthy and disease-free. The weight of rats was two hundred to two hundred and twenty grams. The rats were fed normally and freely. The rats were adopted for experiments after three days.

The study protocol was approved by the Research Ethics Committee of Affiliated Hospital of Southwest Medical University.

Reagent and Apparatus

All reagents and apparatus were adopted from Sigma Co. Ltd (Aldrich, St. Louis, MO, USA) Adriamycin purchased from Hualian Pharmaceutical Co. Ltd (Shanghai, China), slicer purchased from Lyo Technology instrument Co., Ltd, and ultra-low temperature refrigerator were purchased from Sanyo Corporation of (Tokyo, Japan).

Isolation, Culture, Identification and Mark of BMSCs

The rats' thigh bone and tibial marrow was put out respectively. It was isolated. The cell was suspended with fetal calf serum. Then, it was put into incubator for culture. The no-adherent cells were removed after two days. It was performed for turn digest generation and amplification culture when the cell grew up to eight percent. The cells in the third generation as SD34, SD44 and SD166 was detected by FCM. Moreover, the cell activity was detected. The BMSCs of rats in the third generation and sixth generation was put out. Then, sterile DAPI was added in. The final concentration was 50 mg/ml. Then, the cell was put into incubator for culture. It was centrifuged after thirty minutes to remove incompletely blended DAPI. The centrifuged cells were collected and diluted. Then, it was observed under fluorescence microscope. It was reserved in ice bath when the DAPI was shown as blue fluorescence. Finally, the cell was injected into rats after one hour.

Animal Grouping and Modeling

The urine of all rats after twenty-four hours was collected from the beginning of experiment. The quantitation of urine protein after twenty-four hours was found to be negative. All rats were divided into ten rats in control group and thirty rats in model group. The adriamycin nephropathy model was prepared according to the method of intravenous doxorubicin of Yang Wei-Na[10]. The rats were fixed in supine position. It was injected into rats' bilateral caudal vein according to the dosage as four micrograms with every kilogram of rat. The initial angle of inserting needle was ten degree. The angle of inserting needle was adjusted flatly when frustrated feeling was felt. The depth of inserting needle was about one centimeter. The injector was removed when the injection finished. The injected position was suppressed with cotton ball for hemostasis. The rats' vital sign and variation condition of tail color was observed. The normal saline was injected in control group at the dosage of two milliliter normal saline with every kilogram of rat. The above experimental procedure was repeated at the 14th day for all rats. The urine protein quantitation after twenty-four hours was detected at the first and second week of rats respectively after it was injected into rats' tail vein at the second time. The model was successful when it was more than one hundred micrograms. The second tail intravenous injection was performed for the rats in two groups. One rat

was selected in model group after eight weeks. The rat's glomerulus was stained with PAS, there was visible and slight change of FSGS in the rat. At the same time, the urine protein quantitation in the rat was more than one hundred micrograms. The modeling of six rats failed in model group when modeling. Four rats died when there was ulceration in tail. The remaining twenty rats were adopted in experiment entirely.

The ten rats in normal group was set as sham group (sham-operated group). The twenty successfully-modeling rats were divided into FSGS group (focal segmental glomerulosclerosis rat's model group) and BMSCs group (focal segmental glomerulosclerosis rat with intervention of BMSCs transplantation group) averagely. The 4×10^6 of BMSCs was prepared into one milliliter of cell suspension liquid when modeling was finished. The suspension liquid of BMSCs was injected into tail vein of the rats. The same amount of normal saline was injected in sham group and IRI group at the same position. The follow-up experiment was conducted after the rats in the three groups were injected for eight weeks continuously.

Specimen Collection

The urine of twenty-four hours in the rats of the three groups were collected with metabolism cage respectively after four weeks and eight weeks of administration. The post cava of rats in the three groups was collected. The serum was isolated and reserved at the refrigerator with below twenty degree temperature. The rats were executed after eight weeks. The renal tissue of part rats was collected. Then, it was reserved at the refrigerator with below eighty-degree temperature.

Detection on Biochemical Criterion

The rats' blood and urine after four weeks and eight weeks of administration were collected. The content of SCr, BUN, and urine protein of twenty-four hours and ALB in the rats of three groups was compared respectively (Cell Signaling Technology; Danvers, MA, USA). The content of SCr was detected with Jaffe's assay. The BUN level of rats was detected with enzymic method. The content of urine protein was detected with pyrogallol red. The content of ALB was detected with bromocresol green method.

Observation on the Pathological Morphology of Renal Tissue

All rats were executed after the biochemical criterion in the rats of three groups was detect-

ed. Then, the renal tissue was put out. The infarction tissue was fixed with formaldehyde for twenty-four hours. Then, the tissue was cut into paraffin sections. The thickness of ever section was four micrometer. The section was stained with HE after dewaxing procession. Finally, glomerular and tubular interstitial changes were observed.

Index on Glomerulosclerosis and Detection on ECM/GA

The two clear view was collected from upper, lower, left, right, center position randomly from HE stained section of rats in three groups respectively. The degree of glomerulosclerosis was graded according to the class. It was divided into zero grade to four grades entirely. International classification of the glomerular diseases for the FSGS were graded semi-quantitatively, based on the affected glomerulosclerosis area, as a percentage of the total glomerulosclerosis area, 0, 0-25%, 25%-50%, 50%-75% and 75%-100%, respectively[11]. The renal tissue was stained with PAS staining method. The two clear view was collected from upper, lower, left, right, center position randomly with the same method. The ECM and area ratio of glomerulus (ECM/GA) was observed. It was analyzed with image analytic system. The area of positive signal and its area in glomerulus was measured. Finally, the degree of stromal proliferation was analyzed.

Detection on the RNA of Components of RAS (AGT, ACE, AT1R, AT2R) by RT-PCR

The renal tissue in the rats of three groups was collected respectively. The total RNA was extracted with TRIzol dissociation method. The obtained cDNA from reversion was performed for fluorescence reaction experiment. The reaction was conducted for amplification according to reactive condition, followed by 40 cycles at four minutes of degeneration at ninety-eight-degree, one minute of degeneration at ninety-four-degree, one minute of degeneration at fifty-five degree, two minutes of degeneration at seventy-two degree, five minutes of extension at seventy-two degree. The Eastep Super Total RNA Extraction Kit, Easy Script First-Strand cDNA Synthesis Super Mix Kit, and TransStarat Tip Green qPCR Super Mix Kit were all from Promega (Madison, WI, USA). The Ct value was obtained from the average. The $2^{\Delta\Delta Ct}$ method was adopted as calculated method for analysis (Table I).

Table I. Primer sequences.

mRNA	Gene	Primer sequence	Primer length
AGT	F	5'-GCACGACTTCCTGACTTGGA -3'	265bp
	R	5'-GGTAGACAGCTTGGCCTGAG-3'	
ACE	F	5'-CAGCTATAACTCGAGTGCCGA-3'	383bp
	R	5'-CGCATTCTCCTCCGTGATGT-3'	
AT1R	F	5'-GCTTCAACCTCTACGCCAGT -3'	445bp
	R	5'-AGGCGAGACTTCATTGGGTG -3'	
AT2R	F	5'-TGCTCTGACCTGGATGGGTA-3'	275bp
	R	5'-AGCTGTTGGTGAATCCCAGG-3'	
GAPHD	F	5'-CGCTAACATCAAATGGGGTG-3'	617bp
	R	5'-TTGCTGACAATCTTGAGGGAG-3'	

Detection on the rats' p38 MAPK and p-p38 MAPK protein by Western Blot

The SB203580 as the inhibitor of p38 MAPK was injected intrathecally into the three rats in FSGS group for intervention. It was set as inhibitor group. The one hundred micrograms of renal tissue of rat in every group was collected for dissociation. The total nucleoprotein was extracted. The concentration of nucleoprotein was detected. It was reserved at the environment of below twenty degree after subpackage. The extracted protein solution was mixed with buffer solution according to the ratio as four to one. The protein solution was boiled for protein denaturation. The fifty microgram of protein sample was injected onto the electrophoresis plate. Then, the sample was transferred on the PVDF membrane. The skimmed milk powder was added. The primary antibody MAPK were from Cell Signaling Technology (Danvers, MA, USA) and it was added in after one hour of fixation. Finally, the second antibody was added in for dilution. Then, it was stained with DAB after one hour of fixation and it was observed.

Statistical Analysis

The SPSS 19.0 software was adopted for analysis (IBM Corp., Armonk, NY, USA). The biochemical index of rats in sham group, FSGS group and BMSCs group was detected. The pathological change of renal tissue and activity of RAS was detected. The expression of p38 MAPK and p-p38 MAPK protein in renal tissue of rats in three groups was compared. The t-test method was adopted for comparison on the results of every group. The one-way ANOVA statistical analysis was performed for the data among three groups (Sham, FSGS, BMSCs). The comparison on results was represented as x±s. There was significant difference ($p<0.05$).

Results

Comparison on Biochemical Criterion of Rats in Three Groups

The content of SCr, BUN and urine protein in twenty-four hours in FSGS group was higher than sham group equally ($p<0.05$). After transplantation, the biochemical criterion of rats in BMSCs group was remarkably decreased ($p<0.05$). There was hypoproteinemia in FSGS group. In addition, the content of blood albumin was lower than in sham group ($p<0.05$). The content of albumin in

Table II. Comparison on the SCr and BUN of rats in three group at four weeks and eight weeks (x̄± s).

| Group	n	SCr μmol/L (4w)	SCr μmol/L (8w)	BUN mmol/L (4w)	BUN mmol/L (8w)
Sham group	10	38.62±1.46	39.29±1.74	7.50±0.62	7.65±0.83
FSGS group	10	58.14±2.92*	83.06±2.87*	16.91±2.01*	20.92±2.95*
BMSCs group	10	44.78±1.67*#	66.57±2.26*#	8.56±1.01*#	9.12±1.31*#
F		222.2	895.4	146.4	142.9
p		<0.0001	<0.0001	<0.0001	<0.0001

Noted: * represented as compared with sham group, $p <0.05$; #represented as compared with FSGS group, $p < 0.05$.

Table III. Comparison on urine protein of twenty-four hours and blood albumin of rats in three groups ($\bar{x} \pm s$).

Group	n	Urine protein of twenty-four hours 24h (four weeks)	Urine protein of twenty-four hours 24h (eight weeks)	Blood albumin g/L (four weeks)	Blood albumin g/L (eight weeks)
Sham group	10	10.16±1.02	10.92±0.98	39.68±0.65	40.63±0.58
FSGS group	10	73.64±5.16*	123.14±7.96*	22.54±1.87*	12.46±1.39*
BMSCs group	10	56.77±3.21*#	76.28±6.71*#	27.66±2.39*#	23.57±1.26*#
F		854.2	871.6	241.1	1566
p		<0.0001	<0.0001	<0.0001	<0.0001

Noted: *represented as compared with sham group, $p < 0.05$; #represented as compared with FSGS group, $p < 0.05$.
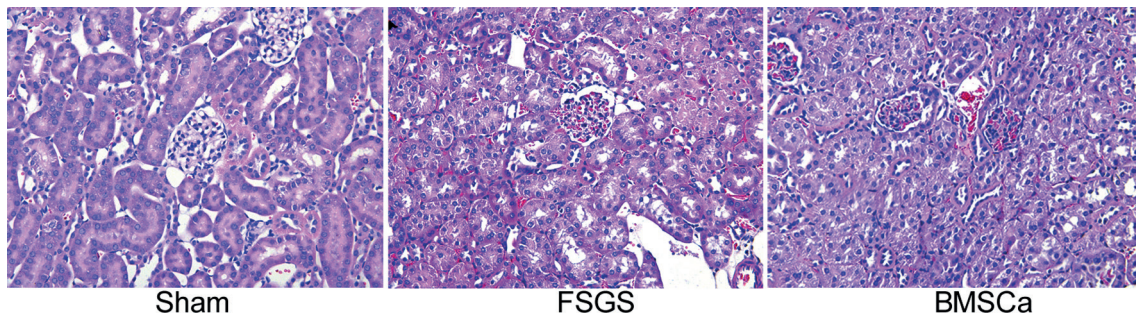
Figure 1. Renal tissue of rats in three groups with HE staining method (magnification, ×200).
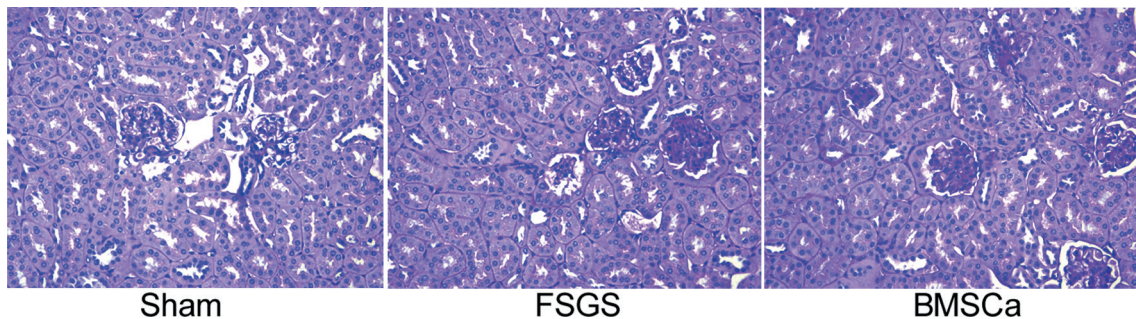
Figure 2. Renal tissue of rats in three groups with PAS staining method (magnification, ×200).

the transplanted BMSCs group was higher than in FSGS group ($p<0.05$). (Table II and Table III).

Comparison on the Pathological Morphology of Renal Tissue of Rats in Three Groups

Compared with sham group, there was evident segmental sclerosis in glomerulus, abundant-narrow in the lumen of blood capillary, blocking in most of blood capillary, evident focal and segmental distribution on glomerulosclerosis, abundant fibration in renal interstitium and inflamma-

tory infiltration in FSGS group. The condition of renal tissue in BMSCs group was significantly improved compared with FSGS group (Figure 1 and Figure 2).

Comparison on the Hardness Index of Glomerulus and ECM/GA of Rats in Three Groups

The glomerulus in Sham group was normal. There was evident induration on the glomerulus in FSGS group. The GSI and ECM/GA in FSGS group was also higher than in sham group

Table IV. Comparison on GSI and ECM/GA of rats in three groups ($\bar{x} \pm s$).

Group	n	GSI (grade)	ECM/GA (%)
Sham group	10	0	2.86±1.14
FSGS group	10	2.82±0.28*	48.41±23.89*
BMSCs group	10	1.68±0.32*#	7.11±2.46*#
F		333.9	32.85
p		<0.0001	<0.0001

Noted: * represented as compared with sham group, p <0.05; # represented as compared with FSGS group, *p* <0.05.

Table V. Comparison on mRNA expression of AGT, ACE, AT1R, AT2R of rats in three groups ($\bar{x} \pm s$).

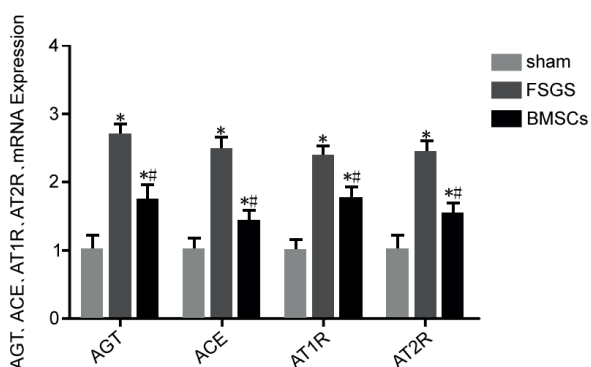| Group	n	AGT	ACE	AT1R	AT2R
Sham group	10	1.0±0.11	1.0±0.12	1.0±0.11	1.0±0.13
FSGS group	10	2.89±1.13*	2.48±1.01*	2.37±0.85*	2.42±0.91*
BMSCs group	10	1.73±0.59*#	1.42±0.41*#	1.76±0.61*#	1.52±0.43*#
F		16.65	14.51	12.77	15.03
p		<0.0001	<0.0001	0.0001	<0.0001

Noted: * represented as compared with sham group, p <0.05; # represented as compared with FSGS group, *p* < 0.05.

($p<0.05$). The GSI and ECM/GA in BMSCs group was lower than in FSGS group ($p<0.05$). (Table IV)

Comparison on the mRNA Expression of Components of RAS (AGT, ACE, AT1R, AT2R) with RT-PCR

The mRNA expression of AGT, ACE, AT1R, AT2R in FSGS group was elevated equally compared with sham group ($p<0.05$). The mRNA expression of AGT, ACE, AT1R, AT2R in transplanted BMSCs group was reduced notably. It was less than in FSGS group ($p<0.05$) (Table V and Figure 3).


Figure 3. Comparison on the mRNA of AGT, ACE, AT1R, AT2R of rats in three groups. Noted: * represented as compared with sham group, *p*<0.05; # represented as compared with FSGS group, *p*<0.05.

Comparison on the Expression of p38 MAPK and p-p38 MAPK Protein of Rats Detected by Western Blot

The expression of p38 MAPK and p-p38 MAPK protein in FSGS was notably higher than in sham group ($p<0.05$). The expression of p38 MAPK and p-p38 MAPK protein in BMSCs group and inhibitor group was significantly reduced compared with FSGS group ($p<0.05$). Moreover, there was no evident change on the protein between the BMSCs group and inhibitor group ($p>0.05$) (Figure 4 and Figure 5).

Discussion

The FSGS was the most common pathological type in all glomerular diseases. The most common clinical performance of FSGS was proteinuria and nephrotic syndrome[12-13]. In recent years the morbidity of FSGS was gradually increased, so the treatment on FSGS was also very difficult. The worse condition of prognosis could lead to the occurrence of end stage renal disease. FSGS was treated with hormones and cytotoxic drugs normally. Although the therapeutic effect with hormones and cytotoxic drugs was very evident, the long-term and abundant use with this kind of drugs could lead to serious adverse reaction and the patients would terminate treatment for recur-
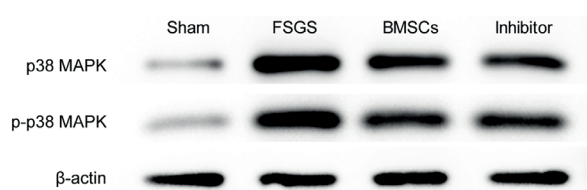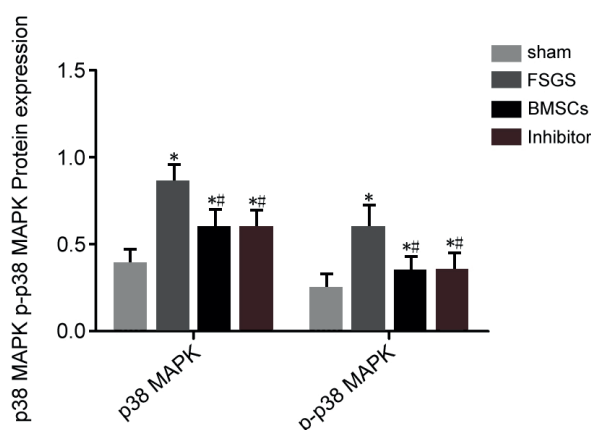
Figure 4. Comparison on the p38 MAPK and p-p38 MAPK protein of rats in four groups.

rent proteinuria[14]. At recent years, stem cell treatment had been the hotspot for the clinical study on kidney disease. The mesenchymal stem cells were adopted widely for its feature as convent extraction, strong amplified ability and easy reservation. Its immunogenicity was relatively low. At the present, it was also confirmed that mesenchymal stem cells could protect the tissue damage efficiently[15]. The FSGS rat model was established in our study. It was interfered with BMSCs. The effect of BMSCs on pathological change and activity of RAS activity were analyzed.

There was important correlation between conditions of prognosis of FSGS and dropping degree of proteinuria in twenty-four hours, BUN and renal tubule[16]. The condition of proteinuria in twenty-four hours was the most significant factor in the prognosis of FSGS. The biochemical index in FSGS was remarkably elevated along with hypoproteinemia from our study. There was also deterioration on the pathology of renal tissue. The biochemical index in transplanted BMSCs group was notably reduced. Hypoproteinemia was remarkably improved. It indicated that the transplanted BMSCs could alleviate the damage of renal function efficiently and improve the filtration action of glomerulus. The weight of rats model with chronic renal insufficiency was remarkably increased while the proteinuria in twenty-four hours was reduced notably from the administration with BMSCs as reported by Yang et al[17]. In addition, the degree of glomerulosclerosis was efficiently improved. Therefore, it was predicted that the BMSCs could secret abundant vascular endothelial growth factor and restrain the proteinuria in twenty-four hours for rats with chronic renal insufficiency. It was consistent with the results from our study. The BMSCs could recover tissue efficiently, differentiate the renal parenchymal cell and recover the acute injury of cells from literature[18]. However, the cell proliferation was responsible for glomerulosclerosis. Therefore, it is indicated that hyperplastic from lesion of chronic kidney diseases maybe come from bone marrow

hepatocyte or part bone marrow stem cells[19]. The damage of model rats with mesangial proliferative nephritis injury was recovered partly with the intervention treatment with injection of BMSCs. Its mechanism was probably related with TGF-β1 inhibited with BMSCs as reported by Liao et al[20].

The renin-angiotensin system (RAS) was mainly composed of renin, Ang II, AEC and ATR. The Ang II was mainly effector molecule. It could adjust the equilibrium for fluid and electrolyte efficiently. It could induce the growth and inflammatory reaction of cell[21]. There were four subtypes for ATR. The AT1R and AT2R could induce the most effect of Ang II. The expression of part RAS and abnormal activity in renal tissue was the important influence factor for the gradual development on the pathological change of renal diseases under physiological status. The expression level of AGT and ACE gene in FSGS group was notably elevated. The expression of mass-produced Ang II and AT1R and AT2R was also elevated. So the Ang II was found to prompt the occurrence of renal failure by enhancing the action of blood vessel. In addition, the cell damage was aggravated further. The expression level of AGT, ACE, AT1R and AT2R in the transplanted BMSCs group was reduced. The visible BMSCs could restrain the activity of RAS efficiently so as to protect renal tissue of FSGS rats. The MAPK signal transduction had its biopotency through tertiary kinase cascade mode. The p38 MAPK was one kind of subtype in MAPK. Not only it could transmit extracellular signal, but also it could adjust the gene expression. The p38 MAPK had important action on the occurrence and development on the

Figure 5. Expression on the p38 MAPK and p-p38 MAPK protein of rats in four groups. Noted: *represented as compared with sham group, $p<0.05$; #represented as compared with FSGS group, $p<0.05$.

renal diseases from literatures. Nevertheless, the p38 MAPK could be changed into p-p38 MAPK through phosphorylation with the effect of lots of factors. So some certain genes in cell could be activated by p-p38 MAPK so as to cause the occurrence of FSGS. The expression level of p38 MAPK and p-p38 MAPK protein in FSGS group was elevated notably from our study ($p<0.05$). Moreover, the expression level of p38 MAPK and p-p38 MAPK protein in BMSCs group and inhibitor group was restrained ($p<0.05$). However, there was no evident change on the protein between the BMSCs group and inhibitor group. It indicated that the p38 MAPK and p-p38 MAPK protein could restrain through BMSCs to restrain the occurrence of FSGS. The TGF-β1 could interact with the p38 MAPK from the research on glomerulosclerosis[22]. The activation of p387 MAPK signal pathway could lead to the abundant secretion and precipitation of ECM. In addition, the secondary reaction could be developed in cell for glomerulosclerosis. TGF-β1 could lead to the continuous activation the expression of p38, finally, phosphorylation of p38 also activates the expression of TGF-β1 and exacerbates glomerular sclerosis.

Conclusions

BMSCs could restrain the occurrence of proteinuria of FSGS rats. At the same time, it could restrain the activity of RAS. There was close correlation between the action of BMSCs and inhibiting the MAPK signal pathway, and the present study provides evidence that BMSCs could restrain the proteinuria of rat's kidney and activity of RAS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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