EFFECT OF QUERCETIN ADMINISTRATION ON TRANSDIFFERENTIATION OF GLOMERULAR PODOCYTES TO MESENCHYMAL CELLS INDUCED BY TRANSFORMING GROWTH FACTOR

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ABSTRACT

Objective: *The inhibitory effect of quercetin (Qu) on transforming growth factor - β 1(TGF-β1)induced glomerular podocyte transdifferentiation (EMT) was analyzed.*

Methods: *The in vitro primary cultured podocytes were divided into five groups using the random method. The control group was cultured in the normal mode. The model group was induced by TGF-β1 for 48 hours. Experimental group A was induced by quercetin for 24 hours, and subsequently induced by TGF-β1. At 24 hours, experimental group B was induced by quercetin and TGF-β1 for 48 hours. Experimental group C was induced by TGF-β1 for 24 hours, and subsequently treated with quercetin for 24 hours. The morphological changes of podocytes were observed under confocal laser microscopy. The expression of TGF-β1, ncphrin, WT-1, vimentin and α-SMA in podocytes were measured by reverse transcription polymerase chain reaction (RT-PCR) and double staining flow cytometry.*

Results: *Compared with the control group, the mRNA expression of TGF-β1 increased in the model group, when compared to the control group (P<0.05). Compared with the model group, the mRNA expression levels of TGF-β1 decreased in experimental groups A, B and C, when compared to the model group (P<0.05). Compared to the control group, the podocyte index growth slowed down, and the total cell apoptosis rate increased. However, when the podocyte index increased, the total cell apoptosis rate decreased in experimental groups A, B and C (P<0.05). Compared to the model group, the expression levels of podocyte marker proteins ncphrin and WT-1 increased, the expression levels of EMT marker proteins vimentin and α-SMA decreased (P<0.05), and the protein of experimental group A was tested. The expression is optimally improved.*

Conclusion: *Quercetin can reduce the mRNA expression of TGF-β1 induced by TGF-β1 and the expression levels of EMT marker proteins α-SMA and vimentin, increase the expression of podocyte marker proteins ncphrin and WT-1, and inhibit podocyte orientation. Furthermore, mesenchymal cells are transdifferentiated.*

Keywords: *Quercetin, TGF-β1, podocyte, mesenchymal cell transdifferentiation.*

DOI: 10.19193/0393-6384_2020_4_402

Received *November 30, 2019;* ***Accepted*** *January 20, 2020*

Introduction

In the latest global kidney disease health report in 2017, the incidence of kidney disease in 125 countries around the world was counted, showing that the prevalence of kidney disease is 10%, and that most people do not know about kidney disease[(1)]. Among these, chronic kidney disease (CKD) accounts for a large proportion of kidney diseases, and the mortality rate is extremely high, which has become a global public health problem[(2)].

A large number of studies have suggested that podocyte injury has an important influence on the pathogenesis and progression of CKD, and that epithelial-mesenchymal transition (EMT) is an important initial factor in end-stage renal disease and glomerular sclerosis[(3-5)]. The medical community considers that a comprehensive understanding of podiatric EMT, an effective treatment to ensure the integrity of the podiatric cell structure and function, and preventing the occurrence of EMT in podiatric cells are the key points that should be given attention in the diagnosis and treatment of CKD in the future[(6)]. Studies have indicated that transforming growth factor-beta (TGF-β1) is an important mediator that affects the progression of CKD. TGF-β1 would mediate the fibrosis, proliferation and apoptosis of podocytes, which could produce EMT under

the stimulation of TGF-β1[7]. However, resveratrol, triptolide and quercetin (Que) may interfere with EMT in podocytes. Therefore, in the present study, a model of TGF-β1-induced injury of mouse glomerular podocytes was established, and the inhibitory effect of Que on podocyte EMT was analyzed through the intervention of Que concentration[8].

Methods

Podocytes was cultured in vitro

A specific pathogen-free (SPF) grade healthy, adult, clean, male breast rats(10g), were provided by the Experimental Animal Center of Wenzhou Medical University (Animal production license no.: SCXK [Zhejiang, China] 2015-0001; Animal experiment certificate no.: X1504128).

Then, rats were washed with sterile distilled water, and immersed in a beaker containing 75% ethanol for five minutes or more. These were sacrificed by decapitation, and the kidneys were removed by laparotomy. Then, these rat kidneys were quickly washed three times with PBS containing 10% cyan-streptomycin double-antibody.

Afterwards, the kidney pedicle, fascia, fat, blood vessels and other tissues were carefully removed, the renal cortical tissue was cut by standard operation, and these were broken for culture and digestion. Then, these cell tissues were filtered, the filtrate was collected and subjected to high-speed centrifugation, the supernatant was discarded, and the precipitate was added to 5% FBS complete medium and seeded in a cell culture flask containing rat tail collagen.

Podocyte digestion, passage, cryopreservation and resuscitation

The glomerular implanted cells were cultured at constant temperature and humidity for four days. Then, these cells were exchanged on the 5th day and digested and passaged on the 9th day, and subsequently changed every three days. Next, an even cell suspension was blown into the centrifuge tube for high-speed centrifugation, the supernatant was discarded, 1 ml of the frozen solution was added to the centrifuge tube, this was mixed by pipetting, and transferred to the cryotube and sealed, and the frozen information were recorded. This was stored in a refrigerator at 4°C for 30 minutes, frozen in a refrigerator at -20°C for 30 minutes, and finally stored in a refrigerator at -80°C for use. At the time of use, the frozen rat podocytes were taken out from the -80°C refrigerator and fully dissolved into a liquid. After disinfection with 75% spray, these cells were transferred from the cryotube to the centrifuge tube, and serum-free DMEM/F12 was completely added.

Then, the medium was centrifuged at high speed, the supernatant was discarded, and 5% FBS complete medium was added, mixed by pipetting, transferred to a cell culture flask, and cultured at a constant temperature and humidity.

Podocyte identification

The morphology of podocytes after hematoxylin and eosin (H&E) staining: The pod cells that grew for three days in the first subculture were used for climbing.

The cell growth density was approximately 60%, and the medium was washed with PBS and fixed with 4% paraformaldehyde for 30 minutes. Then, the cell samples were sequentially subjected to paraffin embedding, slicing, alcohol dehydration, hematoxylin staining, blue returning, color separation, bluing, eosin staining, alcohol dehydration, transparency, resin sealing, and the like. Afterwards, these were observed and photographed through a confocal microscope. Morphological observation of podocytes by scanning electron microscopy: Pod cells that grew for three days in the second subculture were used for climbing.

These cells were washed with PBS at a cell growth density of approximatley 50%, and pre-fixed, washed and alcohol dehydrated. The morphology of these cells was observed using a scanning electron microscope. Immunohistochemical staining of podocytes after SP staining: The 3rdsubcultured podocytes were cultured for three days to observe the cell growth density.

When the cell growth density was approximately 50%, the medium was washed with PBS, and 4% paraformaldehyde was fixed at room temperature for 30 minutes. Then, the cell samples were sequentially subjected to paraffin embedding, sectioning, dewaxing and blocking, added with the primary antibody for secondary incubation, prevented from light and color development, alcohol dehydration, transparent and resin sealing, and the cell morphology was observed by laser confocal microscopy.

Grouping and intervention methods

The podocytes with good growth state, uniform density and logarithmic growth phase were randomly divided into five groups: control group, model group, experimental group A, experimental group B and

experimental group C. The normal model culture was not included in the control group. All groups received drug intervention. The optimal concentration of Que was 50 μmol/L by MTT assay. The model group was induced with TGF-β1 at a concentration of 5 ng/mL for 48 hours. Experimental group A was given 50 μmol/L of Que for 24 hours, and induced by TGF-β1 for 24 hours.

Experimental group B was given the same dose of Que and TGF-β1 for 48 hours at the same time. Experimental group C was given 5 ng/mL of TGF-β1 for 24 hours, and subsequently continued with 50 μmol/L of Que for 24 hours.

Detection indicator

Observation of cell morphology after Que intervention

The podocytes were cultured for the first time, and the cell growth density was observed to be approximately 60%. These cells were randomly divided into five groups, and treated with drug concentration for 48 hours. The changes in podocyte density and morphology were observed under an inverted microscope.

Detection of renal TGF-β1 mRNA levels

Fluorescence quantitative reverse transcription-polymerase chain reaction (RT-PCR) was established to detect the mRNA expression of TGF-β1 in the kidney. A pair of primers and a TaqMan-MGB probe were designed between exons 1 and 2 of the TGF-β1 gene.

The 102-bp product fragment of TGF-β1 was amplified by conventional polymerase chain reaction (PCR). Then, the product was purified and ligated into the pUC-T vector to construct the TGF-β1 recombinant plasmid clone. Afterwards, the plasmid was completely linearized, and the cRNA was synthesized by T7 RNA polymerase in vitro. After further manipulation, the mRNA expression level of TGF-β1 was detected, and the method was evaluated by methodology.

Apoptosis detection of podocytes

In order to ensure the smoothness and accuracy of the experiment, three groups of repeats were set up in each group of podocyte drug intervention experiment. After intervention for 48 hours, 0.25% trypsinized cells were washed with PBS and centrifuged at high speed for five minutes, and the supernatant was discarded. Then, 500 uL of apoptosis positive control solution was added to each cell sample and resuspended on ice for 30 minutes. After centrifugation with PBS, this was centrifuged at high speed, and the supernatant was discard.

Then, this was resuspend in 500 μL of 1× binding buffer, added with Annexin V-FITC (phospholipid binding protein) and propidium iodide (PI), and mixed and incubated at room temperature in the dark. After five minutes, the apoptosis of each group was detected by double staining flow cytometry.

Marker protein detection

After 48 hours of induction, podocyte marker proteins ncphrin and WT-1, and EMT marker proteins vimentin and α-SMA were added to each cell culture well, and the expression levels of each protein were detected by laser confocal microscopy.

Statistical processing

The data analysis was performed using SPSS 20.0 statistical software. The measurement data were expressed by ($\bar{x}\pm s$). The two groups were compared by t-test. The comparison of multiple groups was analyzed by one-way ANOVA. The variance was analyzed by LSD-t test. $P<0.05$ was considered statistically significant.

Detection of target genes

There were targeted binding sites between SNHG7 and miR-34a through starBase3.0 gene prediction website. The SNHG7-3'UTR wild type (Wt) and SNHG7-3'UTR mutant (Mut), miR-34a-mimics and miR-NC were transferred into BC cells using the Lipofectamine™ 2000 kit.

After 48 hours, the luciferase activity in the cells was detected using the dual luciferase reporter gene assay kit (Promega, USA).

Results

Analysis of podocyte digestion, passage and identification results

The podocytes were observed under a microscope. After the first fluid exchange for 24 hours, the podocytes were cobblestone-like. Furthermore, most of the podocytes did not protrude from the foot. After the first passage of the third passage, the podocytes and somatic cells significantly increased, and the podocytes were mostly mononuclear.

On the 9th day after passage, the podocyte cell body protruded a lot of slender fluff, and the adjacent podocytes were tightly connected by dendritic bulg-

es. However, as the number of passages increased, the proliferative capacity of the podocytes gradually decreased, and the cell growth rate slowed down (Figure 1).

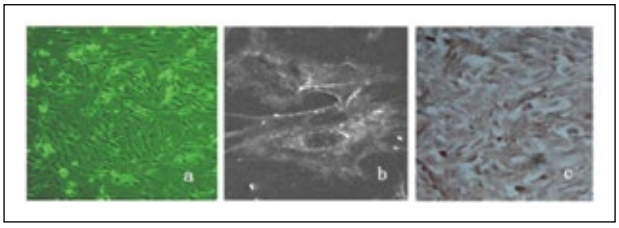

Figure 1: Analysis of podocyte identification results.
a: H&E staining (×400), the cell volume of podocytes expanded, and most cells were mononuclear and interconnected; b: Scanning electron microscopy (×800), the podocyte bodies had several protruding elongated fluff, and typically, there are dendritic bulges; c: Immunohistochemical SP method (×200), the stained granules with a positive protein expression of WT-1 were distributed on the nucleus and around the nucleus.

Morphological analysis of podocytes after Que intervention

It was observed that the podocytes and podocytes were the same after 48 hours of culture in the control group, while those in the model group were induced by TGF-β1, in which cell adherence decreased, and the podocytes were obviously atrophied. In experimental groups A, B and C, the adherence of cells increased after the administration of Que. The characteristic "dendritic" structure of the podocytes was visible under a microscope, and the morphology of these podocytes in experimental group A was the best(Figure 2).

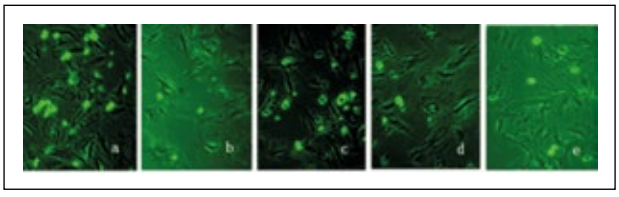

Figure 2: Analysis of podocyte morphology after Que intervention.
a: Control group; b: Model group; c: Experimental group A; d: Experimental group B; e: Experimental group C.

Analysis of TGF-β1 mRNA expression level

Compared with the control group, the mRNA expression of TGF-β1 increased in the model group (P<0.05). However, the mRNA expression of TGF-β1 decreased in experimental groups A, B and C (P<0.05) (Figure 3 and Figure 4).

Podocyte growth index and apoptosis analysis

The podocyte index in the control group significantly increased, the podocyte index in the model group slowly increased, and the podocyte index in experimental groups A, B and C increased to varying degrees. With the prolongation of time, the growth rate of the cell index in each group also differed.

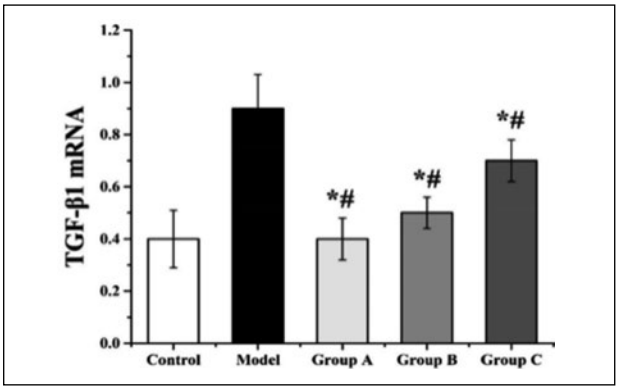

Figure 3: Analysis of the TGF-β1 mRNA expression levels.
*Compared with the control group, *P<0.05; compared with the model group,*#P<0.05.*

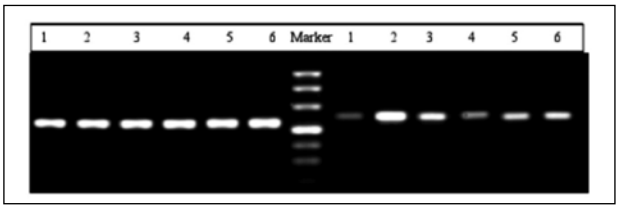

Figure 4: The TGF-β1 mRNA expression in each group.
The left side of Marker is the β-actin, the right side is the TGF-β1 mRNA, and 1-5 represent the control group, model group, experimental group A, experimental group B and experimental group C, respectively.

In general, this rapidly grew in the control group, followed by experiment group A. The cell growth rate in experiment group B and experiment group C was relatively slow (P<0.05). Through double staining flow cytometry, the total apoptosis rate in the control group was the lowest, while the rate in the model group was the highest. Compared with the model group, the total apoptosis rate decreased in the experimental groups, and the difference was statistically significant (P<0.05, Table 1).

Groups	Sample size (*n*)	CI (0-120 hours)	Total apoptosis rate
Control group	3	1.34±0.28	2.26±0.88
Model group	3	0.47±0.06	22.8±3.14
Experiment A	3	1.06±0.17*#	9.6±1.25*#
Experiment B	3	0.78±0.11*#	11.4±0.82*#
Experiment C	3	0.71±0.09*#	12.8±1.05*#

Table 1: Podocyte growth index and apoptosis analysis.
*Compared with the control group, *P<0.05; compared with the model group, *#P<0.05.*

Analysis of the expression levels of marker proteins in each group

The expression of the EMT marker protein was detected by laser confocal microscopy. The expression of ncphrin, WT-1, vimentin and α-SMA in po-

docytes increased at the perinuclear level, but was less expressed in the nucleus.

The expression levels of podocyte marker proteins ncphrin and WT-1 decreased in the model group, while the expression levels of EMT marker proteins vimentin and α-SMA increased. Compared with the control group, the difference in marker proteins was statistically significant ($P<0.05$). Compared with the model group, the expression levels of ncphrin and WT-1 in podocytes in experimental groups A, B and C increased to some extent, while the protein expression levels of vimentin and α-SMA decreased to some extent, and the difference was statistically significant ($P<0.05$). Among this, in experimental group A, the protein expression level of WT-1 in podocytes was the highest, while the protein expression level of vimentin and α-SMA was the lowest (Figure 5).

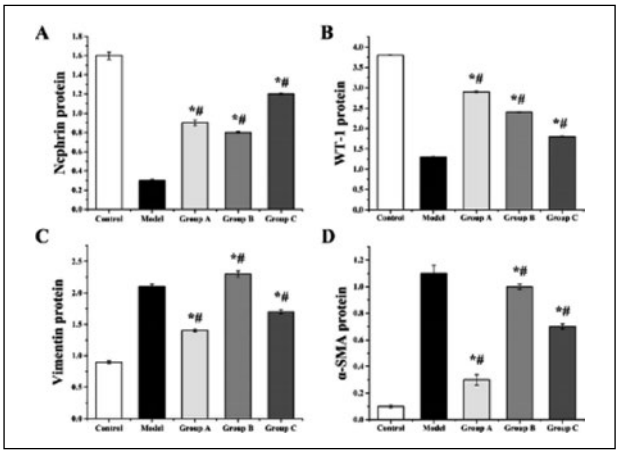

Figure 5: Analysis of the expression levels of marker proteins.
Podocyte marker proteins ncphrin (A) and WT-1 (B); EMT marker proteins vimentin (C) and α-SMA (D). $^{*}P<0.05$; compared with the model group, $^{*\#}P<0.05$.

Discussion

The medical community considers that podocyte injury plays an important role in the development of CKD, while podocytes are transdifferentiated into mesenchymal cells (EMT), and in various kidney diseases, such as diabetic nephropathy, secondary nephropathy and glomerular sclerosis. Hence, its occurrence and development are closely correlated[9]. Therefore, the study of podocyte EMT has become the present focus of medical community. TGF-β1 is a cluster of functional family proteins that promote profibrosis, and mediate cell proliferation and apoptosis. Foot cells can undergo EMT under TGF-β1 stimulation[10]. Therefore, the downregulation of TGF-β1 expression would help inhibit EMT in podocyte damage, thereby reducing kidney damage. Que, as a natural flavonoid, has anti-oxidation, hypoglycemic and hypolipidemic effects. It is presently considered that Que can inhibit renal tubulointerstitial fibrosis and glomerular basement membrane thickening. Therefore, the present study was combined with the literature analysis of TGF-β1-induced podocytes in different Que administration modes, and the impact of EMT was determined.

It was found that after the podocytes were induced by TGF-β1, cells adhered to the wall and atrophied. Then, the dendritic structure of these podocytes gradually atrophied and disappeared. However, after the administration of Que, these podocytes exhibited a better growth state. Hence, the podocyte growth density was higher, and the growth morphology of podocytes in experimental group A was the best. From the analysis of the cell growth index and total cell apoptosis rate, experimental group A presented with the highest cell growth index and the lowest apoptosis rate, which was significantly different from the TGF-β1-induced model group.

The expression levels of podocyte marker proteins ncphrin and WT-1 in the TGF-β1-induced model group significantly decreased, TGF-β1 mRNA expression level increased, and the expression levels of EMT marker proteins vimentin and α-SMA increased. After the treatment with Que, the expression levels of ncphrin and WT-1 increased, and the expression levels of vimentin and α-SMA, and mRNA expression of TGF-β1 decreased, suggesting that the Que administration inhibited the EMT at different time points. The expression of marker proteins vimentin and α-SMA, the mRNA expression of TGF-β1, and the experimental group A administered with Que first inhibited the transdifferentiation of podocytes to mesenchymal cells.

In conclusion, the pre-administration of Que can maintain the integrity of the podocyte structure and protect the glomerular filtration barrier, thereby exerting a strong inhibitory effect on TGF-β1-induced podocyte EMT.

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Acknowledgement:
This work was supported by Scientific Research Project of Hubei Provincial Health and Family Planning Commission (WJ2015Z064).

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