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Sericin suppresses high glucose-induced EMT in mouse podocytes via miR-30a-5p and its target Snai1

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Abstract: The effect of sericin in high glucose (HG)-induced podocyte injury and the mechanisms involving Snai1 and miR-30a-5p were investigated. Bioinformatics and dual-luciferase reporter assay evaluated the relationship of Snai1 with miR-31a-5p. Podocyte injury mouse induced by HG were randomly divided into control (5.5mmol/L D-glucose), HG (30mmol/L D-glucose), HG + Sericin (30mmol/L D-glucose+600μg/ml sericin), miR-30a-5p inhibitor NC (sericin+30mmol/L D-glucose+miR-30a-5p inhibitor negative control) and miR-30a-5p inhibitor groups (sericin+30mmol/L D-glucose+miR-30a-5p inhibitor). The migration ability of podocytes was detected by Transwell assay. The expressions of Snai1, podocin, E-cadherin, FSP-1, ZO-1, α-SMA, Desmin, and miR-30a-5p were assessed with RT-qPCR and Western blot. Snai1 was one direct target of miR-30a-5p. HG group had significantly larger number of migrated podocytes and higher levels of Snai1, FSP-1, α-SMA and Desmin, but significantly lower levels of podocin, ZO-1 and E-cadherin than control and HG + Sericin group. These effects of sericin were reversed by miR-30a-5p inhibitor, as evidenced by increased podocyte migration and increased expressions of Snai1, α-SMA, FSP-1 and Desmin, whereas decreased expressions of podocin, ZO-1 and E-cadherin. Sericin may protect podocytes from damage caused by HG via up-regulating epithelial phenotype markers, down-regulating mesenchymal phenotype markers, and reducing migration of podocytes. The mechanism may be through targeting miR-30a-5p and its target Snai1.

Keywords: Sericin, diabetic nephropathy, podocytes, epithelial-mesenchymal transdifferentiation, miR-30a-5p.

INTRODUCTION

Diabetic nephropathy (DN) is the foremost reason of chronic kidney injury. Glomerular podocytes are the main target cells of kidney injury in the DN early stage (Meng *et al.,* 2022). Under the induction of high glucose (HG), a series of alterations occur in podocytes, such as epithelial mesenchymal transition (EMT), cell hypertrophy, and apoptosis (Cui, 2019; Jia *et al.,* 2023). Among them, EMT of podocytes has been confirmed to be an important pathophysiological mechanism for the occurrence and progression of DN (Jin *et al.,* 2020; Shi *et al.,* 2020; Wu *et al.,* 2019).

In addition to many classical signaling pathways, epigenetic regulations such as non-coding RNAs, chromatin remodeling, and DNA methylation are also involved in the pathogenesis of DN (Gu *et al.,* 2020). MicroRNAs (miRNAs) can regulate the expression of more than 60% of protein-encoding genes, and abnormal changes in their expression profiles can lead to the occurrence of many diseases (Yarahmadi *et al.,* 2021), including kidney disease (Zhang *et al.,* 2021). MiR-30a-5p is differentially expressed in the plasma and urine of patients with nephrotic syndrome (Wan *et al.,* 2021). The miR-30a-5p is widely and stably expressed in podocytes, and can regulate the physiological and pathological functions of podocytes (Yuan *et al.,* 2022). HG-induced renal tubular epithelial cell injury has down-regulated miR-30a-5p (Wang *et al.,* 2021) and this injury can be improved by miR-30a-5p overexpression (Wang *et al.,* 2021). EMT is one of the important forms of podocyte injury in DN (Qin *et al.,* 2023; Zhang *et al.,* 2020c). MiR-30a-5p overexpression can suppress the nuclear transport of NFATc3 in activated T lymphocytes, and can delay EMT during podocyte injury by lowering the NFATc3 level (Peng *et al.,* 2015). In addition, the zinc finger transcription factor Snai1 is also a key regulator of podocyte EMT (Liu & Liu, 2022). Whether miR-30a-5p can participate in podocyte EMT by targeting Snai1 remains to be further explored.

Sericin has high biocompatibility and biodegradability (Muhammad Tahir *et al.,* 2020). We previously found that sericin could improve kidney damage in diabetic rats (Zhang *et al.,* 2020a). However, the role and mechanism of sericin on podocyte damage induced by HG has not been fully elucidated.

Herein, we explored the role of sericin on HG-induced podocyte injury. The immortalized mouse podocytes

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were used to establish a model of HG-induced podocyte injury. The mechanisms involving Snai1and miR-30a-5p were further analyzed and discussed. Our findings may facilitate further understanding the pathogenesis of DN.

MATERIALS AND METHODS

Cell culture

MPC-5 (Guangzhou Jennio Biotech Co., Ltd, China) were cultured in PRMI-1640 medium with IFN-γ at 33°C. When the cell confluence reached 50%, IFN-γ was removed from the medium, and cells were cultured at 37°C. Podocyte morphology was observed using an inverted microscope.

Indirect immunofluorescence

Podocyte maturation was identified by indirect immunofluorescence. In detail, cells were seeded on the coverslips. On the 3rd and 14th days of culture, cells were fixed and permeabilized. After blocking with goat serum (cat# C2530-0500; Shanghai XP Biomed Ltd., China), cells were probed with 1:100 diluted rabbit anti-Nephrin antibody (cat# DF7501; Affinity Biosciences, USA) at 4°C overnight. Following washing, incubation with 1:100 diluted DyLight™488 fluorescently labeled goat anti-rabbit IgG (cat#611-141-002; Rockland, USA) was performed. DAPI stained the cell nuclei.

Prediction of miR-30a-5p target genes

The TargetScanHuman (http://www.targetscan.org/), miRBase (http://www.mirbas e.org/), and miRDB (http://mirdb.org/) predicted miR-30a-5p target genes.

Dual-luciferase reporter assay

The pmir-Glo/wild-type Snai1 plasmids, pmir-Glo/mutated Snai1 plasmids and negative control (NC) plasmids as well as mmu-miR-30a-5p mimics (sense strand UGUAAACAU CCUCGACUGGAAG, antisense strand: UCCAGUCGAGGAUGUUUAC AUU) and mmu-miR-30a-5p mimics NC (sense strand UUGUACUACACAAAAAGUACUG, antisense strand: GUACUUUUGUGUAGUACAAUU) were constructed. According to different treatments, 293T cells were grouped. The reaction intensity of Renilla luciferase was detected with E1910 dual-luciferase reporter gene detection kit (Promega, Wisconsin, Madison, USA).

Determination of the optimal concentration of miR-30a-5p inhibitor

Mature podocytes were treated miR-30a-5p inhibitor (0nM, 100nM, 150nM and 200nM; Guangzhou Ruibo Biotechnology Co., Ltd., China). We determined the optimal concentration of miR-30a-5p inhibitor via detecting miR-30a-5p with RT-qPCR and Snai1 protein with Western blot. Fluorescence was observed under an inverted fluorescent microscope to evaluate the transfection efficacy.

Podocyte grouping

Mature podocytes in control group were intervened with 5.5mmol/L D-glucose while those in HG group were cultured with 30mmol/L D-glucose for 48h. HG+Sericin group was intervened with 600μg/ml sericin (Sigma, USA) and 30mmol/L D-glucose for 48h. The miR-30a-5p inhibitor NC group was first stimulated for 4h with miR-30a-5p inhibitor NC (150nM) and then with 30mmol/L D-glucose and 600μg/ml sericin for 48h. The miR-30a-5p inhibitor group was first treated for 4h with miR-30a-5p inhibitor (150nM) and then with D-glucose (30mmol/L) and sericin (600μg/ml) for 48h.

Transwell assay

Podocytes in each group were cultured in the upper chamber (3×10^5 cells/well, 200μl per well). Meanwhile, the lower chamber was dealed with 20% FBS-containing RPMI-1640 medium (600μl) for 48h. Then 0.1% crystal violet was used for staining. Cells were counted under three random fields (100×).

Western blot

After separation, proteins were transferred to PVDF membranes. After 2h of blocking, overnight incubations with anti-Snai1 (1:1000) (Cat#: ER1706-22; Hangzhou Huaan Biotechnology Co., Ltd., China), podocin (1:200) (Santa Cruz, USA; Cat#: sc-518088), E-cadherin (1:500) (Hangzhou Huaan; Cat#: 0407-25), ZO-1 (1:1000) (GeneTex; Cat#: GTX108613), FSP-1 (1:1000) (Abcam; Cat#: ab197896), α-SMA (1:500) (Hangzhou Huaan; Cat#: ET1607-43), Desmin (1:1000) (Hangzhou Huaan; Cat#: ET1606-30) and α-tubulin (1:1000) (Hangzhou Huaan; Cat#: ER130905) were performed. After washing, 1h incubation with the secondary antibody (Sino Biological Inc.; Beijing, China; Cat#: SSA021) was conducted. The protein bands were visualized.

Real-time quantitative PCR (RT-qPCR)

Total RNAs or miRNAs were extracted and then reversed to cDNA. Using the cDNA as the template, RT-qPCR was performed on Mx3000P/Mx3005P real-time PCR instrument (Agilent, USA). table 1 listed the primer sequences. The primers of *Snai1* and *β-actin* were synthesized by Guangzhou Funeng Gene Co., Ltd (China). U6 and miR-30a-5p were from Tiangen Biochemical Technology (Beijing) Co., Ltd. (China). Other primers were provided by Invitrogen (Carlsbad, CA, USA). The internal references were β-actin and U6.

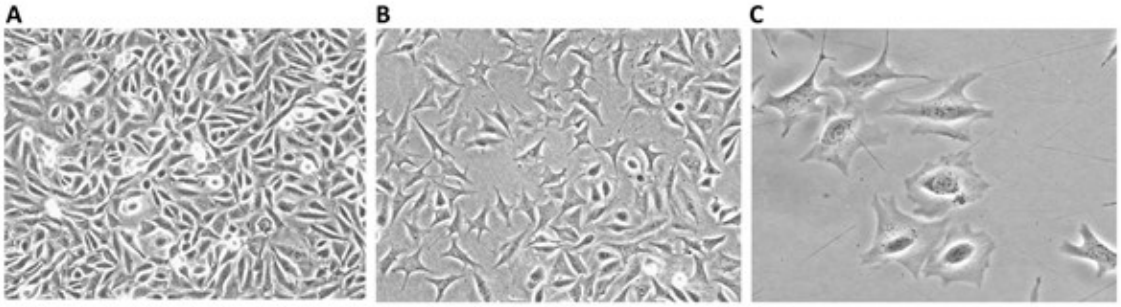

Fig. 1: Morphology of podocytes at different culture temperatures.
A: 33°C (×100). B: 37°C on the 7th day (×100). C. 37°C on the 14th day (×200).

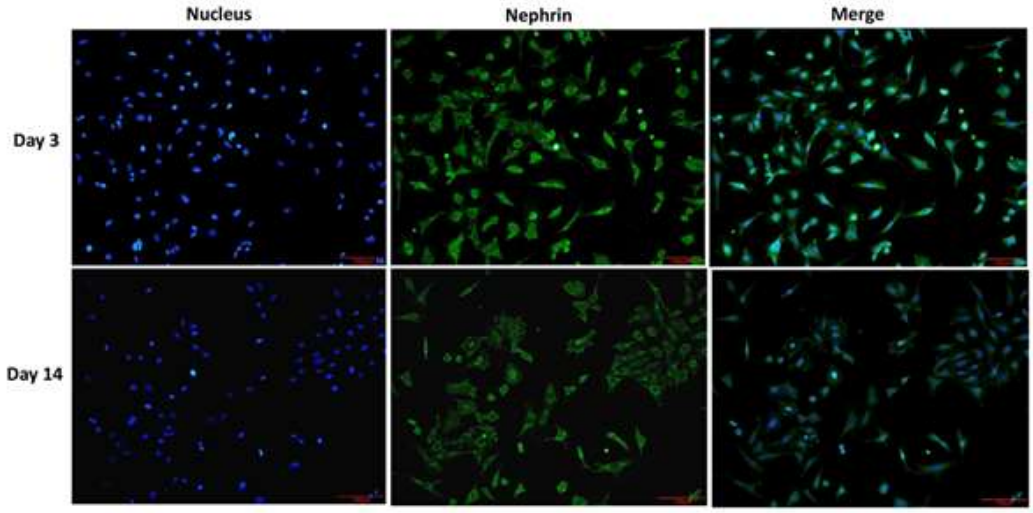

Fig. 2: Localization and expression of Nephrin protein in podocytes.
Nephrin protein expression and distribution at 37°C on the 3rd day and on the 14th day.

STATISTICAL ANALYSIS

Data are presented as mean ± SD and were compared with one-way ANOVA and LSD-t test. SPSS 22.0 analyzed data. $P<0.05$ suggests significant difference.

RESULTS

Morphology of podocytes at different culture temperatures

The morphology of podocytes was observed. At 33°C (fig. 1A), the actively growing dedifferentiated podocytes were of medium volume, polygonal shape, and without protrusions. The cells rapidly grew to a confluent state, showing a paving stone-like appearance. At 37°C (fig. 1B and 1C), the cell body and nucleus of the podocytes were significantly larger than those in the proliferative state, and there were obvious branch-like protrusions extending from the cell body.

Localized expression of Nephrin protein in podocytes

Nephrin protein expression in podocytes was evaluated with immunofluorescence (fig. 2). On the 3rd day, Nephrin protein in podocytes was distributed in the perinuclear area and cytoplasm. On the 14th day, Nephrin protein in podocytes was distributed in the perinuclear area and cell membrane. This indicates the maturation of podocytes on day 14.

Results of dual luciferase reporter assay

Bioinformatics analysis revealed that one target gene of miR-30a-5p was Snai1. Their binding sequence was TGTTTACA (fig. 3A). The Snai1-3'-UTR-WT+miR-30a-5p mimics group had significantly lower luciferase activity than other groups ($P<0.05$) (fig. 3B).

Results of network meta-analysis

Determination of the optimal concentration of miR-30a-5p inhibitor

After miR-30a-5p inhibitor treatment, the level of miR-30a-5p and Snai1 protein was assessed with real-time quantitative PCR and Western blot. The miR-30a-5p level in podocytes in the 100nM and 150nM groups was significantly decreased (fig. 4A), while Snai1 protein was evidently elevated (fig. 4B) ($P<0.05$), compared to the 0nM group. Snai1 and miR-30a-5p were not significantly changed in the 200nM group ($P>0.05$).

A

MicroRNA and Target Gene Description:

miRNA Name	mmu-miR-30a-5p	**miRNA Sequence**	UGUAAACAUCCUCGACUGGAAG
Previous Name	mmu-miR-30a-5p;mmu-miR-30a		
Target Score	90	**Seed Location**	630
NCBI Gene ID	20613	**GenBank Accession**	NM_011427
Gene Symbol	Snai1	**3' UTR Length**	743
Gene Description	snail family zinc finger 1		

3' UTR Sequence

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   1 ccctgctacc tccccatcct cgctggcatc ttcccggagc tcaccctcct cctcactgcc
  61 aggactcctt ccagccttgg tccggggacc tgtggcgtcc atgtctggac ctggttcctg
 121 cttggctctc ttggtggcct ttgccgcagg tggctgatgg agtgcctttg tacccgccca
 181 gagcctccta cccctcagta ttcatgaggt gtagcctctg gacacagctg cttcgagcca
 241 tagaactaaa gccaacccac tggctgggaa gcttgaaccc cgctcagggg accccacttc
 301 cctacctccc tcaaggaccc ttcaggccac cttctttgag gtacaacaga ctatgcaata
 361 gttcccctcc cccccacccc gtccagctgt aaccatgcct cagcagggtg gttactggac
 421 acatgtccag gtgccccttgg gcctgggcaa ctgtttcagc ccccgccccc atttgtcctg
 481 gtgacacctg tttcacagca gtttaactgt ctcagaaggg accatgaata atggccatca
 541 cttgttaggg gccaagtggg gtgcttcagc ctggccaatg tgtctcccag aactattttg
 601 gggcccaaca ggtggccccg ggagaaagat gtttacattt taaaggtatt tatattgtaa
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 721 tatactaaaa aaaaaaaaaa aaa
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B

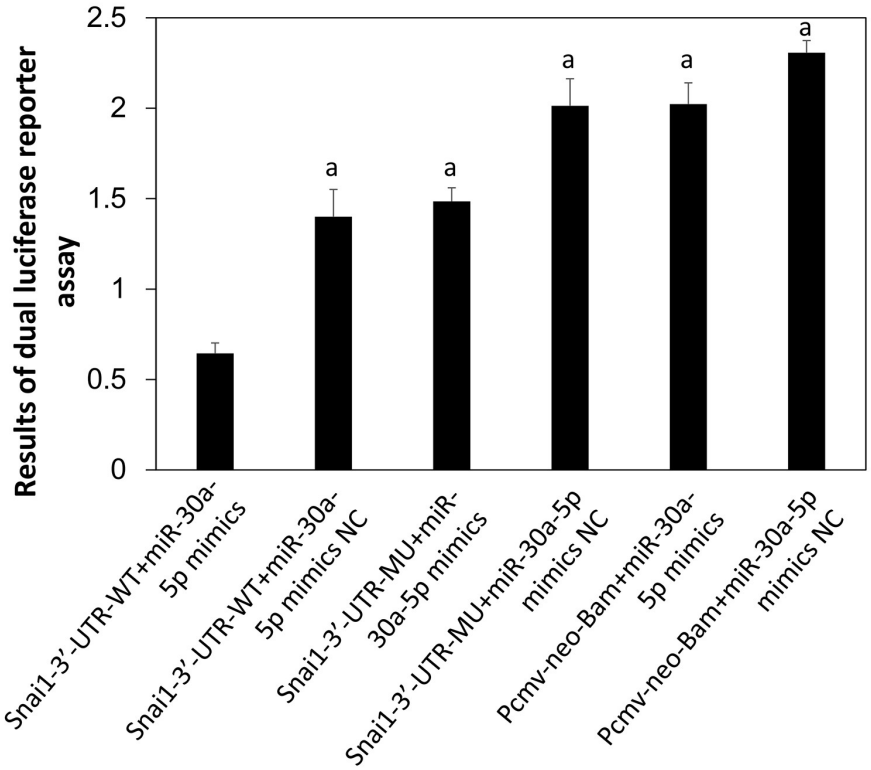

Fig. 3: Results of dual luciferase reporter assay.
A: Bioinformatics analysis of the targets of miR-30a-5p. B: Relative luciferase activity of each group. Compared with Snai1-3'-UTR-WT+miR-30a-5p mimics group, aP<0.05.

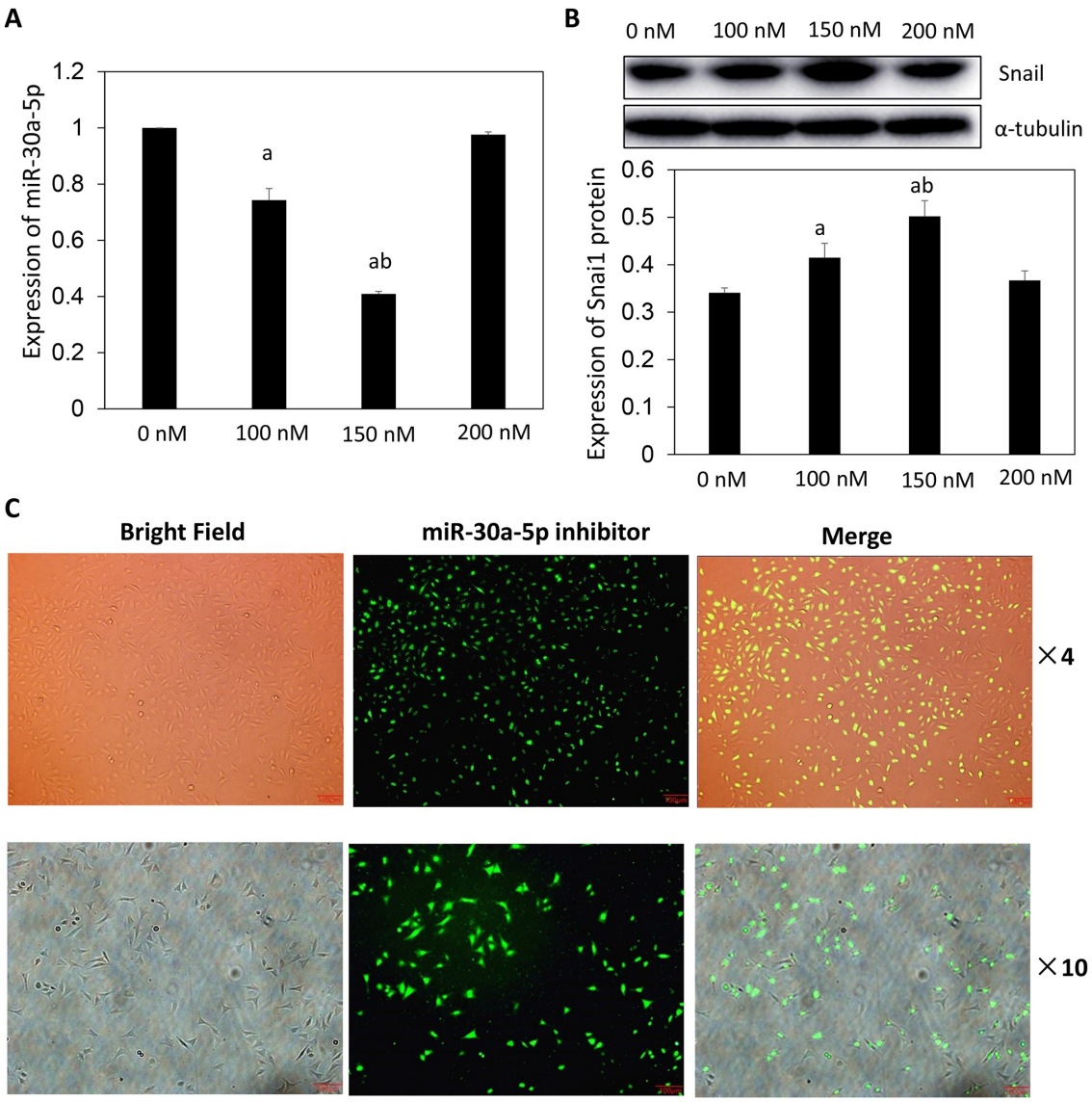

Fig. 4: Determination of optimal concentration of miR-30a-5p inhibitor.

Podocytes were transfected with 0, 100, 150, and 200nm of miR-30a-5p. A: Expression of miR-30a-5p in podocytes in each group evaluated by real-time quantitative PCR. Compared with 0nM group: aP<0.05; compared with 100nM group: bP<0.05. B: Expression of Snai1 protein in podocytes in each group measured by Western blot. Compared with 0nM group: aP <0.05; compared with 100nM group: bP<0.05. C: Transfection efficacy of 150nM miR-30a-5p inhibitor at 4h. Magnification: ×4; ×10.

The miR-30a-5p was significantly decreased, whereas the Snai1 was significantly increased (P<0.05) in the 150nM group than in the 100nM group. Therefore, the optimal concentration of miR-30a-5p inhibitor to transfect podocytes was determined to be 150nM. At 4h after transfection, the podocytes were counted (fig. 4C). The transfection efficiency was about 85%.

Effect of sericin on migration of podocytes

Transwell assay found that compared with control, the HG group had significantly increased number of migrated podocytes (P<0.05) (fig. 5A and 5B).

The HG+Sericin group had significantly lower number of migrated podocytes than HG group (P<0.05). The miR-30a-5p inhibitor significantly inhibited the effect of sericin, as evidenced by significantly higher migrated podocytes in the miR-30a-5p inhibitor group than in the HG+Sericin group (P<0.05) (fig. 5A and 5B).

Effect of sericin on Snai1

As shown in fig. 6A and 6B, the HG group had significantly increased expressions of Snai1 protein and mRNA than the control group (P<0.05). However, in comparison to the HG group, Snai1 protein and mRNA in HG+Sericin group was lower (P<0.05). The miR-30a-5p inhibitor group had up-regulated Snai1 than HG+Sericin group (P<0.05).

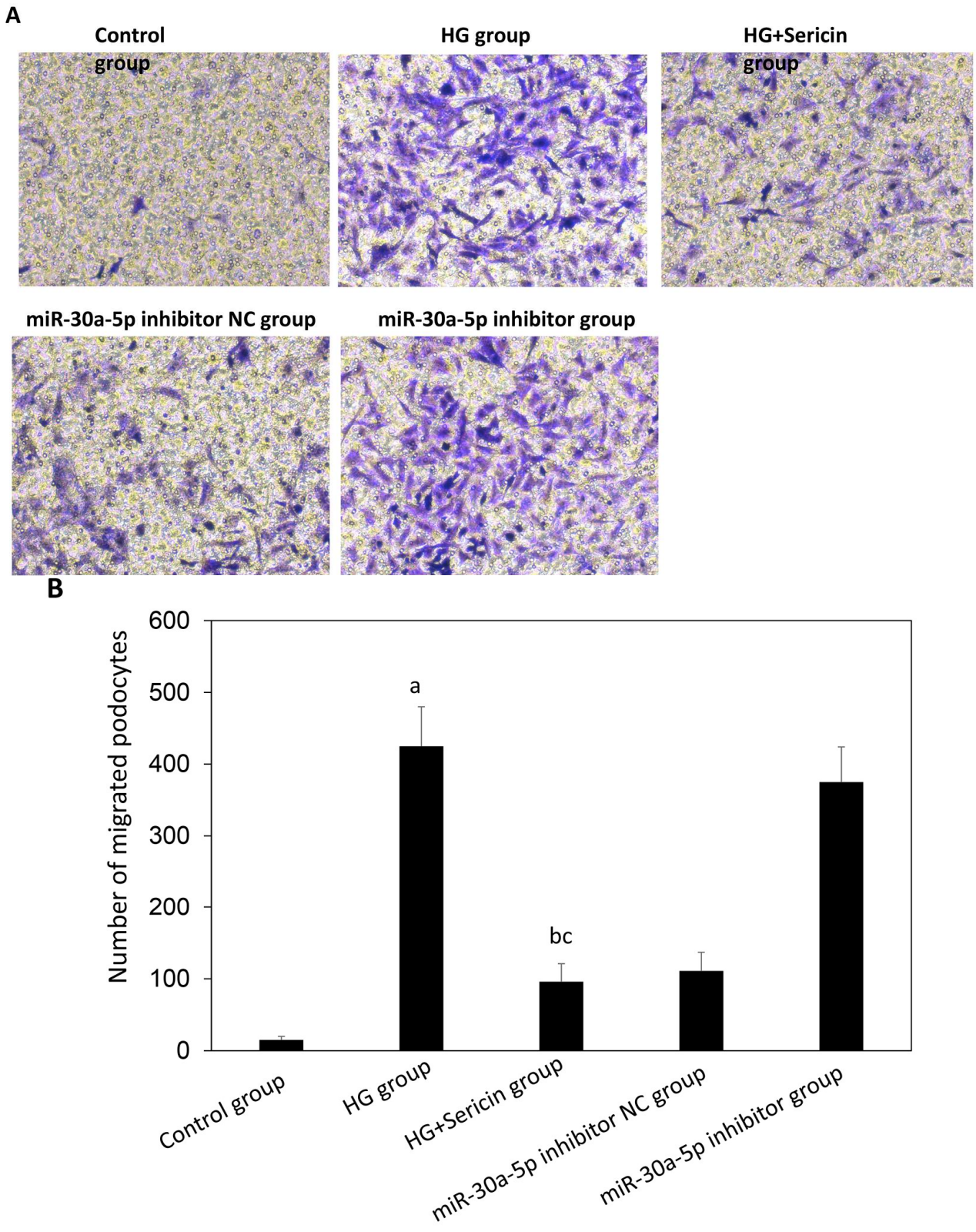

Fig. 5: Transwell assay results.

The migration of podocytes was evaluated with Transwell assay. A: Representative images of migrated cells in control group, HG group, HG+Sericin group, miR-30a-5p inhibitor NC group and miR-30a-5p inhibitor group. B: Number of migrated podocytes in each group. Compared with control group: aP <0.05; compared with HG group: bP<0.05; Compared with miR-30a-5p inhibitor group, cP<0.05.

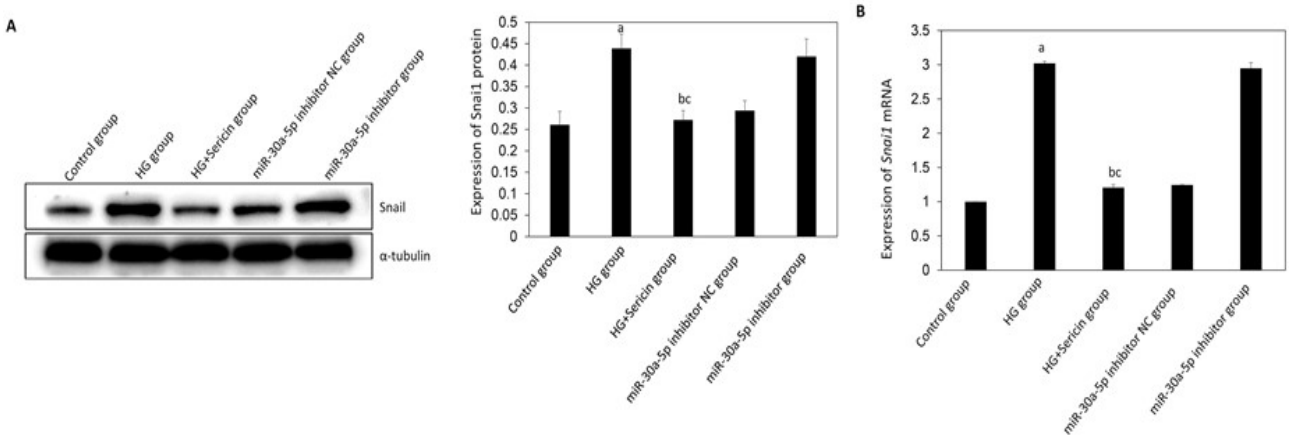

Fig. 6: Effect of sericin on protein and mRNA expression of Snai1.
A: The expression of Snai1 protein in podocytes in each group. B: The expression of *Snai1* mRNA in podocytes in each group. Compared with control group, aP<0.05; compared with HG group, bP<0.05; compared with miR-30a-5p inhibitor group, cP<0.05.

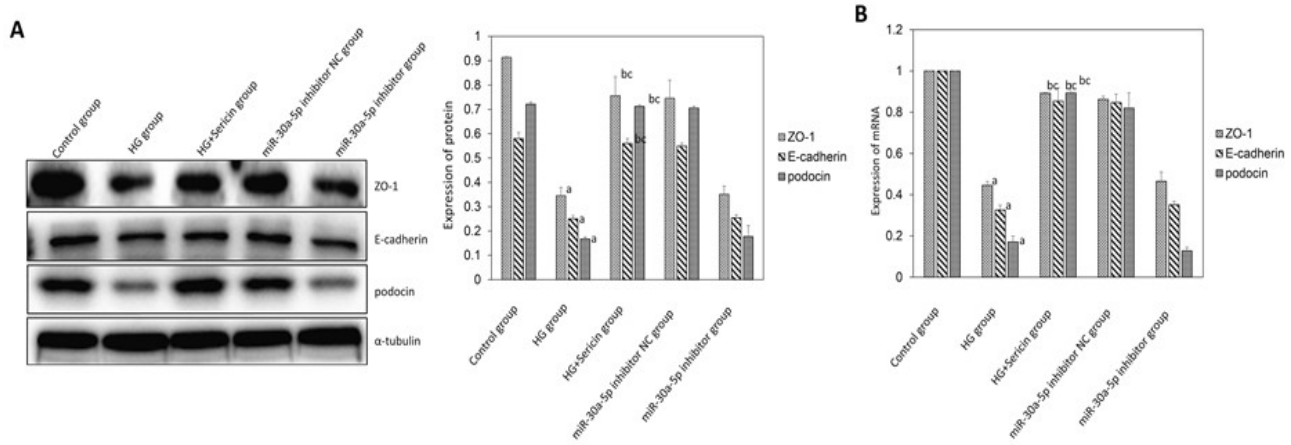

Fig. 7: Effect of sericin on protein and mRNA expression of podocin, E-cadherin and ZO-1.
A: Expression of podocin, E-cadherin and ZO-1 proteins in podocytes in each group. B: Expression of podocin, E-cadherin and ZO-1 mRNAs in podocytes in each group. Compared with control group: aP<0.05; compared with HG group: bP<0.05; compared with miR-30a-5p inhibitor group: cP<0.05.

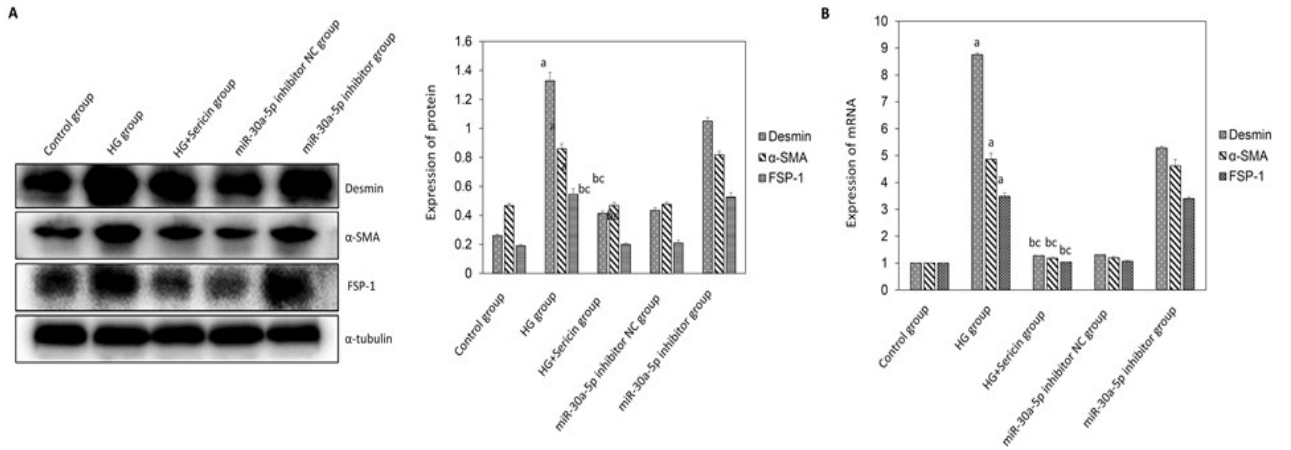

Fig. 8: Effect of sericin on protein and mRNA expression of interstitial phenotype markers FSP-1, α-SMA and Desmin.
A: Expression of FSP-1, α-SMA and Desmin proteins in podocytes in each group. B: Expression of FSP-1, α-SMA and Desmin mRNAs in podocytes in each group. Compared with control group: aP<0.05; compared with HG group: bP<0.05; compared with miR-

Effect of sericin on podocin, E-cadherin and ZO-1

Comparing to the control group, the HG group had significantly decreased protein and mRNA expressions of podocin, ZO-1, and E-cadherin ($P<0.05$) (fig. 7A and 7B). HG+Sericin group had significantly higher podocin, E-cadherin, and ZO-1 levels than HG group. This effect was reversed by miR-30a-5p inhibitor ($P<0.05$). Their levels reduced significantly in miR-30a-5p inhibitor group than in HG+Sericin group ($P<0.05$) (fig. 7A and 7B).

Effect of sericin on FSP-1, α-SMA and Desmin

As shown in fig. 8A and 8B, HG group had significantly higher levels of FSP-1, α-SMA and Desmin than control group ($P<0.05$). In comparison to the HG group, their levels reduced significantly in HG +Sericin group ($P<0.05$). Interestingly, these changes were reversed in miR-30a-5p inhibitor group ($P<0.05$).

DISCUSSION

Sericin has the functions of improving immunity, promoting cell proliferation, resisting oxidation, protecting liver, and inhibiting bacterial infection (Li *et al.,* 2023; Zhang *et al.,* 2020d; Zhao *et al.,* 2020; Zhu, 2022). Furthermore, sericin is non-immunogenic and has no toxic side effects (Tariq *et al.,* 2021). Our previous study found that sericin effectively reduced blood glucose and protected kidney damage induced by diabetes (Zhang *et al.,* 2020a). However, the underlying mechanism is unclear.

Podocytes are important for the glomerular filtration barrier, and their damage is an important factor leading to proteinuria (Eftekhari *et al.,* 2020). In diabetes, high blood glucose, metabolic disturbances, oxidative stress damage and inflammation can all lead to podocyte damage, thereby disrupting the glomerular filtration barrier and aggravating diabetic kidney damage (Kang *et al.,* 2023). Therefore, alleviating podocyte damage is crucial for DN treatment. Therefore, we investigated the mechanism of sericin in protecting podocytes by using mouse podocyte model with HG-induced injury.

Podocyte EMT and DN

Initially, podocyte apoptosis is considered as the main cause of podocyte loss (Chen *et al.,* 2020b). Subsequent studies have shown that in addition to apoptosis, the podocyte EMT can also mediate podocyte loss (Warren *et al.,* 2019; Wu *et al.,* 2022; Zhang *et al.,* 2020b). Podocyte EMT, as an intermediate state between apoptosis, can be triggered by high blood glucose and oxidative stress, and, is closely associated with proteinuria and basement membrane thickening during DN (Chen *et al.,* 2020a; Kang *et al.,* 2020). During EMT, the epithelial characteristics are lost and the expression of epithelial phenotype proteins is down-regulated. Meanwhile, they acquire the mesenchymal characteristics of migration and invasion, accompanied with up-regulated expression of mesenchymal phenotype proteins (such as FSP-1, Desmin, etc.). The EMT of podocytes can enhance the migration ability of podocytes and impair the barrier function of the filtration membrane (Chen *et al.,* 2016; Yu *et al.,* 2021). Here, we demonstrated that in HG group, the levels of podocin, E-cadherin and ZO-1 in damaged podocytes were significantly reduced, while Desmin, α-SMA and FSP-1 significantly increased. The migration capability of podocyte significantly elevated. These results confirmed that there was EMT in HG-induced podocytes and the migration ability of podocytes was enhanced.

miR-30a-5p, Snai1 and EMT

We observed that Snai1 increased significantly and miR-30a-5p decreased significantly under HG condition, suggesting that podocyte injury during DN is possibly due to miR-30a-5p and Snai1.

Some studies have found that various miRNAs are abnormally expressed in DN, such as the miR-30 family (Long *et al.,* 2020), miR-638 (Han *et al.,* 2020), miR-16-5p (Song & Lv, 2021), miR-124 (Petrica *et al.,* 2020), etc. Among the miR-30 family, miR-30a-5p is mainly expressed in glomerular podocytes (Chen, 2019), which is critical for the regulation of podocyte EMT (Wu *et al.,* 2014). Snai1 is involved in EMT by regulating erythrocyte membrane protein band 4.1-like protein 3 (Yuan *et al.,* 2020), lncRNA PCGEM1 (Zhang *et al.,* 2019) and miR-30b-5p (Wang *et al.,* 2017).

Snai1 targeted miR-30a, and miR-30a-5p negatively regulated Snai1. miR-30a-5p inhibitor elevated Snai1, FSP-1, Desmin and α-SMA expressions, reduced podocin, E-cadherin and ZO-1 expressions, and, enhanced the migration ability. Therefore, inhibiting miR-30a-5p may boost the podocyte EMT and podocyte shedding by up-regulating Snai1.

The possible mechanism of sericin in protecting podocyte injury

The previous work of our group has shown that sericin could significantly reduce blood glucose and had protective effect on kidney damage in diabetes (Chen *et al.,* 2010). The underlying mechanism was explored herein.

We revealed that in podocytes, 600μg/ml sericin significantly up-regulated miR-30a-5p, podocin, ZO-1, and E-cadherin but significantly down-regulated Desmin, Snai1, α-SMA, and FSP-1. Additionally, the podocyte migration ability was significantly weakened. Interestingly, these effects of sericin were reversed by further treatment with miR-30a-5p inhibitor. Thereby, we suppose that sericin may protect podocytes from HG-induced injury by up-regulating miR-30a-5p and inhibiting Snai1, thereby affecting the EMT progression and migration capacity of podocytes.

CONCLUSION

In summary, sericin may exert protective effect on podocytes with HG-induced injury. The mechanism may be through up-regulating miR-30a-5p and suppressing Snai1, thereby delaying the EMT and migration ability of podocytes. However, the specific signaling pathway underlying this effect still needs to be further explored. Our data may deepen the understanding of DN pathogenesis, and may facilitate DN treatment.

ACKNOWLEDGMENTS

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