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The Effect of Shen Qi Wan Medicated Serum on NRK-52E Cells Proliferation and Migration by Targeting Aquaporin 1 (AQP1)

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Shen Qi Wan (SQW) as a well-known formula for the amelioration of kidney yang deficiency syndrome (KYDS), and it has been widely employed in traditional Chinese medicine (TCM). This study aimed to investigate the effect and underlying mechanism of SQW medicated serum on proliferation and migration in NRK-52E cells.

Material/Methods: We employed the real-time cell analysis (RTCA) system to investigate the effect of SQW medicated serum on proliferation and migration in NRK-52E cells. In addition, the migration was further investigated by using a wound-healing assay. The mRNA and protein expression level of aquaporin 1 (AQP1) of NRK-52E cells with SQW medicated serum-treated were quantified by real-time quantitative polymerase chain reaction (q-PCR) and western blot assay, respectively. Furthermore, NRK-52E cells were transfected with lentivirus AQP1-RNAi to assess migratory cell abilities *in vitro*.

Results: The migratory abilities of NRK-52E cells were significantly increased after SQW medicated serum treatment ($P<0.05$), and no significant difference in cell proliferation. In addition, SQW medicated serum was significantly upregulated the mRNA and protein expression level of AQP1 in NRK-52E cells ($P<0.05$). Additionally, the *in vitro* metastasis test proved that knockdown of AQP1 suppressed migratory abilities according to RTCA and wound healing test while was reversed by SQW medicated serum ($P<0.05$).

Conclusions: Our study demonstrates that SQW medicated serum effectively promotes the migration of NRK-52E cells by increasing AQP1 expression, and AQP1 may be as a therapeutic target of SQW for renal injury treatment under KYDS.

MeSH Keywords: **Aquaporin 1 • Cell Migration Assays • Endothelial Cells**

Abbreviations: **KYDS** – kidney yang deficiency syndrome; **SQW** – Shen Qi Wan; **TCM** – traditional Chinese medicine; **RTCA** – real-time cell analysis; **AQP1** – Aquaporin 1; **q-PCR** – quantitative polymerase chain reaction; **SD** – Sprague Dawley; **DMEM** – Dulbecco's modified Eagle's medium; **FBS** – fetal bovine serum; **PVDF** – polyvinylidene fluoride; **NC** – negative control

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Background

Kidney yang deficiency syndrome (KYDS) is a diagnostic category in traditional Chinese medicine (TCM) which is characterized by a metabolic disorder of body fluids and warm dysfunction, with symptoms including cold back and waist, cold limbs, tinnitus, fatigue, and aches of waist and knee [1,2]. According to the theories of TCM, "kidney" yin and "kidney" yang were yuan yin and yuan yang of the body. Various factors that can cause renal dysfunction can presents as "kidney" yin deficiency and "kidney" yang deficiency in TCM. Clinically, chronic nephritis, diabetes, adrenal insufficiency, chronic bronchial asthma, menopausal syndrome, and other kidney damage signs of "kidney" yang deficiency. A growing number of herbal formulas have been successfully proven attribute to reinforce the "kidney" yin or yang.

Shen Qi Wan (SQW), which first registered in the "Synopsis of Prescriptions of the Golden Chamber", is a classical formula that has been used successfully treatment of "kidney" yang deficiency. It contains prepared *Radix Rehmanniae*, *Chinese Yam*, *Cornus officinalis*, *Alisma Orientale*, *Poria Cocos*, *Cortex Moutan*, *Cassia Twig*, and *Aconite*. We previously showed that SQW might improve the general symptoms and signs of "kidney" yang deficiency renal damage in model rats induced by adenine [1]. However, the underlying targets of the injured kidney intervened by SQW remains a mystery.

Aquaporins (AQPs), also called water channels, have been demonstrated to have an important role in the proliferation, migration, and invasion of human cells. AQP1 is the first member of the water channel protein family to be discovered, and it is ubiquitously expressed in the human body [3]. Besides, there are currently many papers suggesting that AQP1 participates in water transportation and osmotic homeostasis, and helps in cell migration in multiform cell types [4]. The study of Saadoun et al. demonstrated that the AQP of cell membranes plays an important role in cell migration, and AQP1-deficient mice had significantly inhibited tumor growth by subcutaneous injection of melanoma cells into wild-type and AQP1-deficient mice [5]. Similarly, Shu et al. showed that silencing of AQP1 inhibited cell adhesion and invasion abilities of ectopic endometrial cells [6]. AQP1 is expressed at the apical and basolateral plasma membranes in epithelial cells of the proximal tubule and the thin descending limb of Henle in the kidney [7]. *In vitro* studies have shown that AQP1 facilitates the migration of primary proximal renal tubule epithelial cells. Hara-Chikuma et al. reported remarkably greater tubule dilation and degeneration in AQP1 null versus wild-type kidneys [8]. One study using an ischemia-reperfusion injury model to investigate the potential relevance of cell migration with AQP1 deficiency *in vivo* found the F-actin protein disintegration [8], suggesting that AQP1 might be an important target for renal diseases treatment.

Modern medical research indicates that the physiological and pathological process of renal injury and repair is mainly as follows: under the stimulation of injury factors, renal tubular epithelial cells lose normal polarity characteristics and a large number of them experience necrosis and apoptosis, and detachment from the tubular basement membrane, then new epithelial cell proliferation and adhesion occurs, with recovered polarity characteristics, and fill in the injured site, and reconstruct the structure and function of the renal tubules [9]. Many studies pointed out that the migration and proliferation of innate tubular epithelial cells are the main sources of renal tubular regeneration, and some endogenous and bone marrow-derived stem cells do not or rarely participate in the repair process [10–16].

In preliminary research, we founded that SQW attenuated the downregulation of AQP1 in a renal injury rat model that was induced by adenine and improved the symptoms of "kidney" yang deficiency syndrome [1]. In the present study, we investigated the effect of SQW medicated serum on proliferation and migration of NRK-52E cells. Moreover, NRK-52E cells were stably infected with lentivirus targeting AQP1 to investigate the effects of SQW medicated serum on migration, then preliminarily evaluated the potential mechanism of renal repair under the condition of kidney yang deficiency.

Material and Methods

Preparation of rat medicated serum

We used 32 male Sprague Dawley (SD) rats, aged 6–8 weeks old, weighing 200±20 grams, that were purchased from Shanghai Super-B&K Laboratory Animal Corp., Ltd. (laboratory animal production license number: SCXK (Hu) 2013-0016). The rats were housed at 23±2°C with 50–60% relative humidity with a 12-hour light/12-hour dark cycle in the Laboratory Animal Service Center of Zhejiang Chinese Medical University; the rats had free access to water and standard diet. The experimental protocols were approved by the Ethics of Committee of Zhejiang Chinese Medical University (permit number: ZSLL-2016-054). The rats were randomly divided into 4 groups (n=8): normal control group, SQW (1.5 g/kg) group, SQW (3.0 g/kg) group, and SQW (6.0 g/kg) group. The dose groups were orally administered with SQW (Henan Wanxi Pharmaceutical Corp., Ltd., Henan, China), and the normal control group was administered suspension the normal saline (NS) with the same volume, twice a day, treated 5 days. Before the preparation of the serums, the rats fasted for 12 hours. After 2 hours of final administration, blood serums of rats were collected under sodium pentobarbital anesthesia (150 mg/kg), and then centrifuged at 3000 rpm for 15 minutes at 4°C. Finally, the serums were sterilized by 0.22 μm MILI filter filtration and stored at –20°C prior to further use.

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Cell culture

Renal tubular epithelial cell, NRK-52E cells (Shanghai Cell Library of Chinese Academy of Science, Shanghai, China) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA)) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Mediatech, USA) in a humidified atmosphere with 5% CO_2 at 37°C. When treated with SQW mediated serum or other reagents, cells were cultured in serum-free medium for 2 hours previously.

Cell proliferation and migration assay

We measured the proliferation and migration of the NRK-52E cells by using a fully automated high-throughput and real-time system (ACEA Biosciences, Hangzhou, China). The real-time cell monitoring device (xCELLigence) consists of single-use E-plates, which covered with a gold microelectrode array on the bottom of wells inserted into an real-time cell analysis (RTCA) single-plate station and located within the incubator in this study. We can measure the fluctuations of impedance when a population of cells grow, attach, and spread on the surface of electrodes. We added 100 µL cell suspension of 5×10^4 cells/well to the E-Plate 16, and the E-Plate 16 was loaded onto the RTCA analyzer inside the incubator for measurements.

Wound healing assay

Wound healing assay was performed to detect the cell migration of NRK-52E cell, which were seeded into a 6-well plate for 12 hours, and then incubated with medium containing SQW medicated serum for 24 hours and grown until 90% confluence. Sterilized 200- µL pipette tip was used to generate wounding across the cell monolayer, and the debris was washed with phosphate-buffered saline (PBS). The migration of cells into the wound was then observed at different times. Cells migrated into the wounded area from the border of the wound were visualized and photographed under the inverted microscope at 0 hours, 6 hours, and 12 hours. Wound healing was analyzed through the average linear speed of the wound edges.

Quantitative polymerase chain reaction (q-PCR) assay

Total RNA extraction of NRK-52E cells was conducted using the TaKaRa MiniBEST Universal RNA Extraction kit (TaKaRa, Japan) according to the manufacturer's instruction. Reverse transcription reactions were performed with PrimerScript™ RT Master Mix (Perfect Real Time) (TaKaRa, Japan) for complimentary DNA, according to the manufacturer's protocol. The primer sequences were as follows: forward: 5'-GCTCACCCGCAACTTCTCA-3' and reverse: 5'-CCTCTATTTGGGCTTCATCTCC-3' for AQP1; forward: 5'-GGCACAGTCAAGGCTGAGAATG-3' and reverse: 5'-ATGGTGGTGAAGACGCCAGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The q-PCR was conducted with SYBR Green qPCR Mix (TaKaRa, Japan) in a CFX Connect Real-Time System (Bio-Rad Laboratories, Inc.). The relative amount of AQP1 was normalized on the basis of the GAPDH, and analysis of relative gene expression data using the $2^{-\Delta\Delta Ct}$ method.

Western blot assay

Protein samples were extracted from cultured cells with protein lysis RIPA (radioimmunoprecipitation assay) buffer (Goodbio Technology, China) supplemented with protease inhibitor PMSF (phenylmethylsulphonyl fluoride) and phosphatase inhibitor according to the manufacturer's protocol. Protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Lot: 20140623, KeyGEN BioTECH Co., Ltd, Nanjing, China). Proteins (80 µg) from each group were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (BioRad, USA). The membranes were blocked with tris buffered saline-Tween (0.05% Tween) containing 5% non-fat milk for 2 hours at room temperature. Then the membranes were incubated with the following primary antibodies: anti-AQP1 (Abcam, ab15080, USA) and anti-GAPDH (Hua'an, China) overnight at 4°C. Membranes were washed 3 times (15 minutes per wash) with phosphate-buffered saline (PBS)-Tween and incubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase (HRP) (Invitrogen, USA) to detect primary antibodies for 1 hour at room temperature. Membranes were washed 3 times again and then scanned by an infrared laser dual-color imaging system (Odyssey). The relative intensity of the measured protein was calculated by comparing it with the housekeeping protein, GAPDH.

Lentivirus-mediated RNA interference

RNA interference-mediated knockdown of AQP1 was performed through the transfection of the lentiviral vector. Construction and packaging of AQP1 RNAi lentivirus vector were completed by Shanghai JiKai Gene Chemical Technology Co., Ltd. There were 3 kinds of lentivirus with different interfering targets. Firstly, screening the highest jamming efficiency of lentivirus to do subsequent experiments using the lentiviral (Shanghai JiKai gene Chemical Technology Co., Ltd, Shanghai, China) according to the manufacturers' instructions. The RNAi group (RNAi-AQP1 lentiviral vector) and negative control lentivirus (NC) were used in the current study. Cells were grown in a 6-well plate and were transfected at 70% confluence. The medium was changed after 12 hours, and SQW medicated serum was added to the cells. After 24 hours of culturing, protein expression, cell index, and wound healing were assessed.

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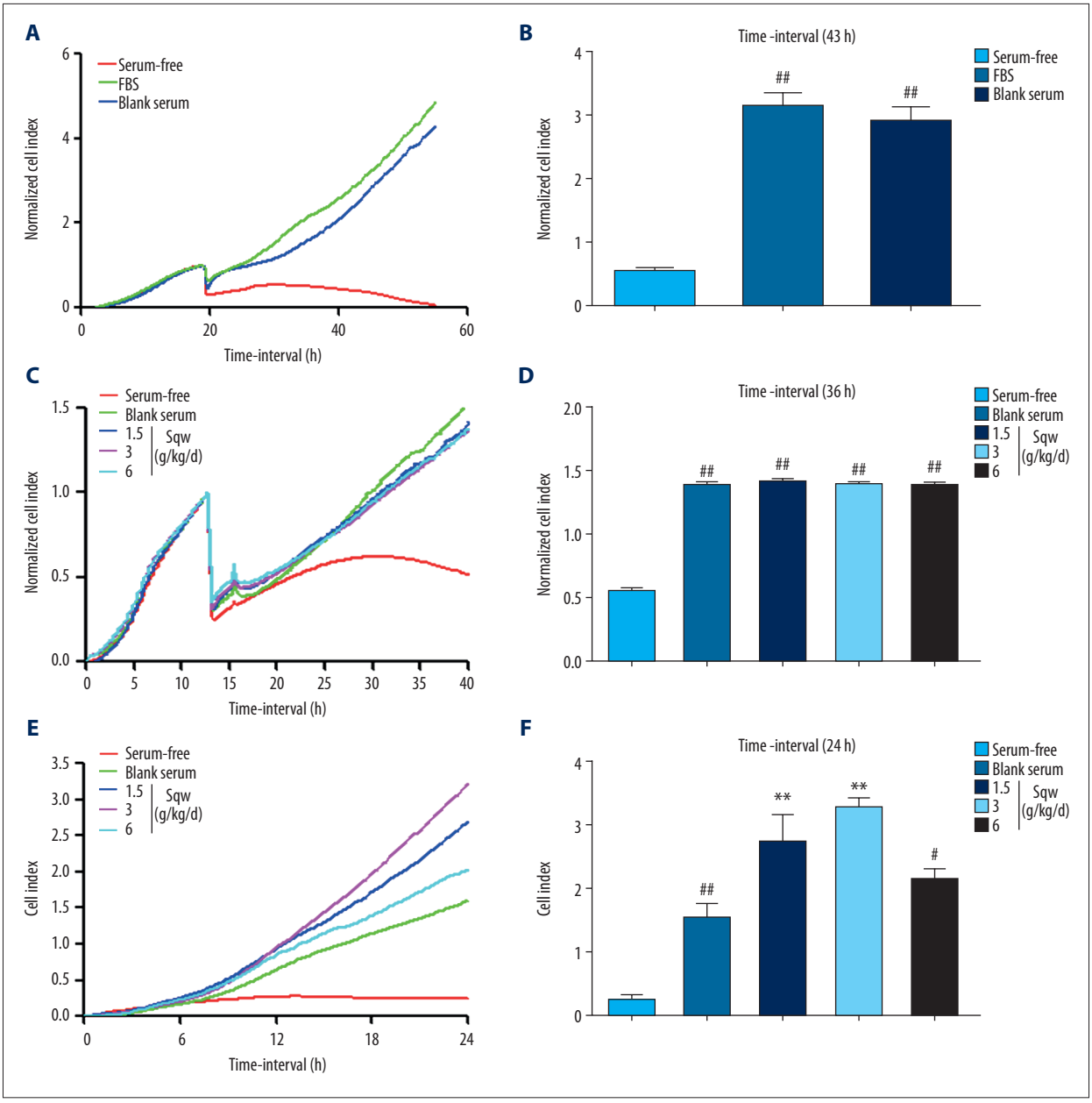

Figure 1. Effects of Shen Qi Wan (SQW) medicated serum on NRK-52E cell proliferation and migration. (**A, B**) Effects of Blank Serum and Fetal Bovine Serum on NRK-52E cell proliferation. (**C, D**) Effects of different concentrations of SQW medicated serum on NRK-52E cell proliferation. (**E, F**) Effects of different concentrations of SQW medicated serum on NRK-52E cell migration. All results represent the means±standard deviation. ## $P<0.01$ *vs.* the Serum-free group; * $P<0.05$, ** $P<0.01$ *vs.* the Blank Serum group.

Statistical analysis

Statistical analysis was performed using the SPSS17.0 software. All data are presented as mean±standard deviation (SD). One-way ANOVA was used for multiple comparisons. $P<0.05$ was considered statistically significant. Figures were obtained by using GraphPad Prism 5.

Results

Effect of SQW medicated serum on migration and proliferation of NRK-52E cells

To evaluate the difference between normal rat serum (Blank Serum) and fetal bovine serum (FBS) on NRK-52E cells, we performed a cell proliferation assay by using RTCA. The results

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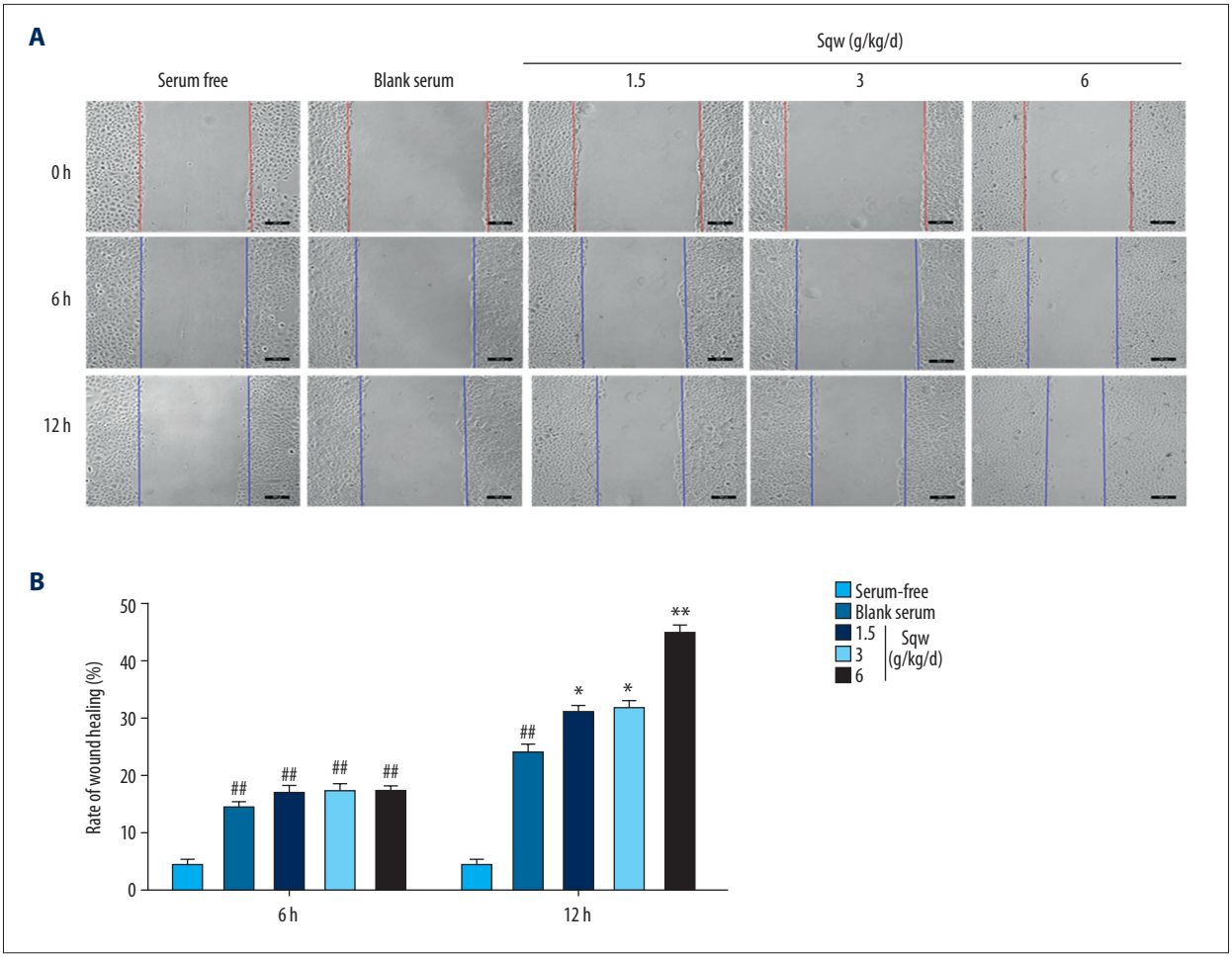

Figure 2. Effect of Shen Qi Wan medicated serum on wound healing of NRK-52E cells. (**A**) After wounding, cell migration was measured by the speed of wound healing. For each scratch, 3 different visual fields were recorded for measurement. The line on images was indicating the wound edges (red, initial; blue, after 6 hours or 12 hours). The scale bar indicates 200 μm. (**B**) Statistical results for all treatment groups in wound healing assay. ## $P<0.01$ *vs.* the Serum-free group; * $P<0.05$, ** $P<0.01$ *vs.* the Blank Serum group.

demonstrated that the cell index of the Blank Serum group and the FBS group was significantly higher than the Serum-free group but showed no difference between Blank Serum group and FBS group (Figure 1A, 1B). Therefore, we used the Blank Serum group as the control group and ignored the effects of FBS in this study. Then, we treated NRK-52E cells with various concentrations of SQW medicated serum. In this study, there were no statistical differences in proliferation between the SQW and Blank Serum groups (Figure 1C, 1D). However, different concentrations of SQW medicated serum groups resulted in substantial promotion of the migration effect on NRK-52E cells compared with the Blank Serum group in the cell migration assay (Figure 1E, 1F). These results indicated that SQW medicated serum promotes cell migration, but there was no significant difference in the proliferation of NRK-52E cells.

Effect of SQW medicated serum on wound healing in NRK-52E cell

After wounding, the rate of wound-healing was significantly elevated in the SQW groups when compared with the Blank Serum group at 12 hours (Figure 2A, 2B). This result indicated that SQW medicated serum significantly improved the rate of wound healing in the NRK-52E cells.

Effect of SQW medicated serum on the expression of AQP1 in NRK-52E cells

To investigate whether SQW medicated serum regulates the mRNA and protein expression of AQP1 in NRK-52E cells, we treated NRK-52E cells with various concentrations of SQW and assessed the expression of AQP1 by q-PCR and western blot assays. The result of q-PCR showed that the mRNA expression

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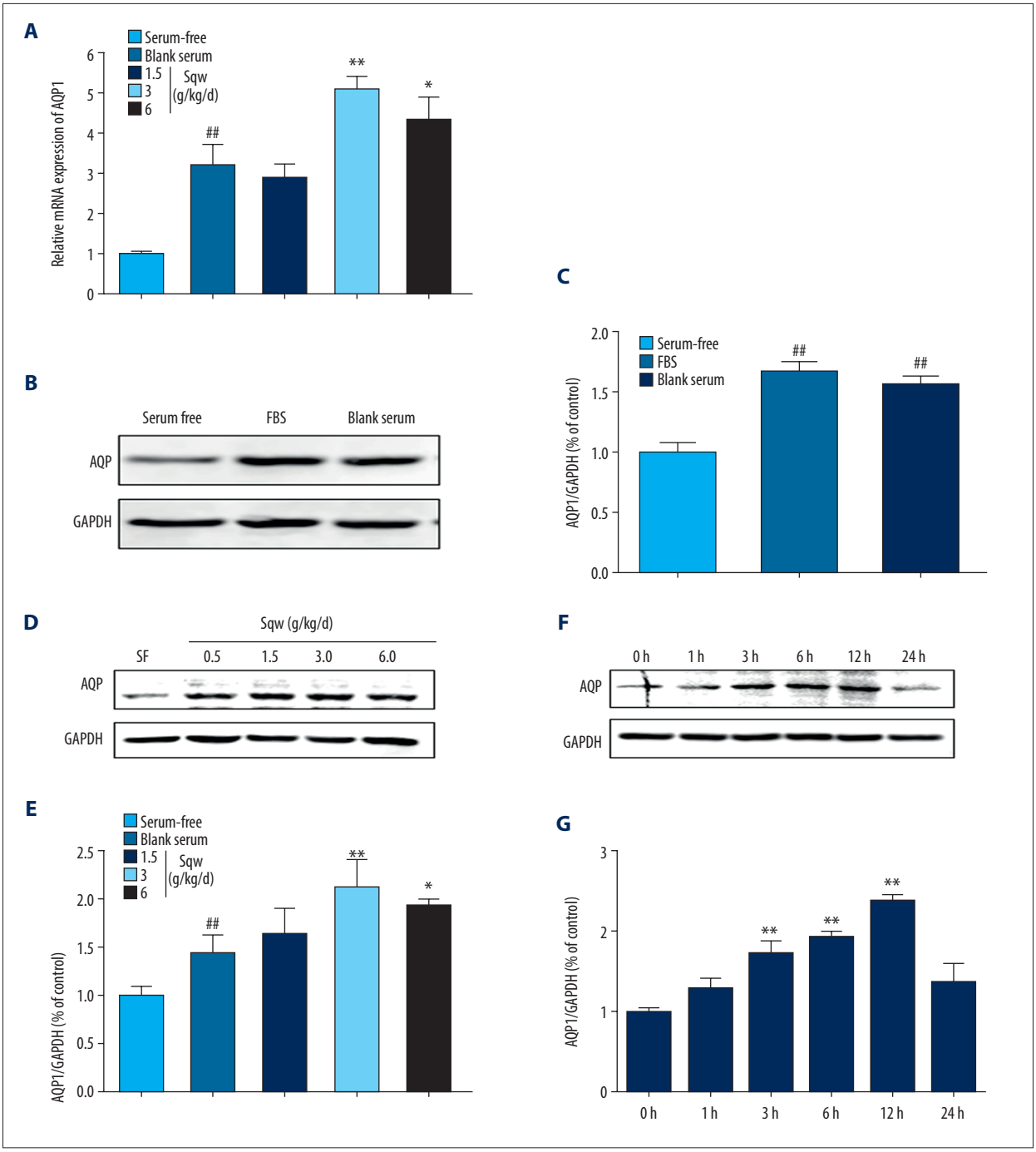

Figure 3. The effect of Shen Qi Wan (SQW) medicated serum on the expression of aquaporin 1 (AQP1) in NRK-52E cells. (**A**) Cells were cultured with the indicated concentration of SQW medicated serum for 24 hours, and the total RNA was extracted and analyzed by quantitative polymerase chain reaction. (**B, C**) Cells were cultured with Fetal Bovine Serum or Blank Serum for 24 hours, and the total protein was extracted cell extracts and analyzed by western blot. (**D, E**) Different concentrations of SQW medicated serum effects on protein expression of AQP1. (**F, G**) Cells were cultured with SQW (3.0 g/kg) medicated serum for different times, and the protein expression of AQP1 was detected by western blot. ## $P<0.01$ *vs.* the Serum-free group; * $P<0.05$, ** $P<0.01$ *vs.* the Blank Serum group.

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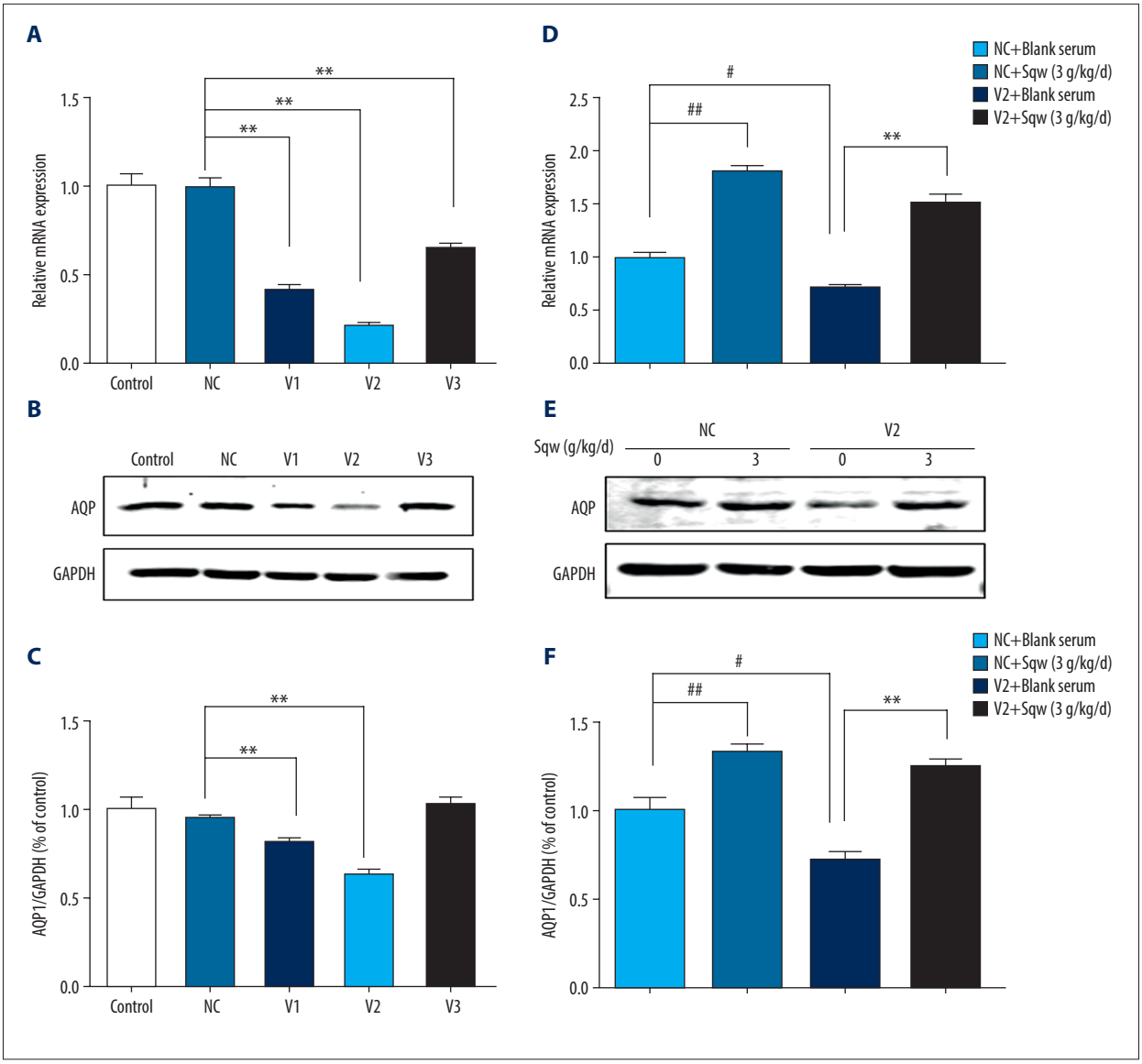

Figure 4. Effects of aquaporin 1 (AQP1) RNAi lentivirus on the expression of AQP1 in NRK-52E cells. (**A**) Effects of different interference targets of AQP1 RNAi lentivirus (V1, V2, V3) and negative control (NC) on mRNA expression of AQP1 in NRK-52E cells. (**B, C**) Effects of lentivirus V1, V2, V3, and NC on the protein expression level of AQP1 in NRK-52E cells. (**D**) With lentivirus V2 and Shen Qi Wan (SQW) (3 g/kg) medicated serum treatment, the level of AQP1 mRNA was detected by quantitative polymerase chain reaction. (**E, F**) With lentivirus V2 and SQW (3 g/kg) medicated serum treatment, the protein expression of AQP1 was detected by western blot. # $P<0.05$, ## $P<0.01$ *vs.* the NC+Blank Serum group; ** $P<0.01$ *vs.* V2+Blank Serum group.

levels of AQP1 were significantly increased with SQW medicated serum treatment (Figure 3A).

For the western blot assay, we first treated NRK-52E cells with FBS and Blank Serum and assessed the protein expression level of AQP1. The results showed no statistical differences between FBS group and Blank Serum group (Figure 3B, 3C). Then we treated NRK-52E cells with various concentrations and durations of SQW medicated serum; we found that the protein expression level of AQP1 in NRK-52E cells was significantly increased following a 24-hour treatment with SQW medicated serum (Figure 3D, 3E). In the time curve, for the cells treated with SQW (3.0 g/kg) medicated serum, the protein expression of AQP1 increased gradually and reached the highest expression at 12 hours (Figure 3F, 3G).

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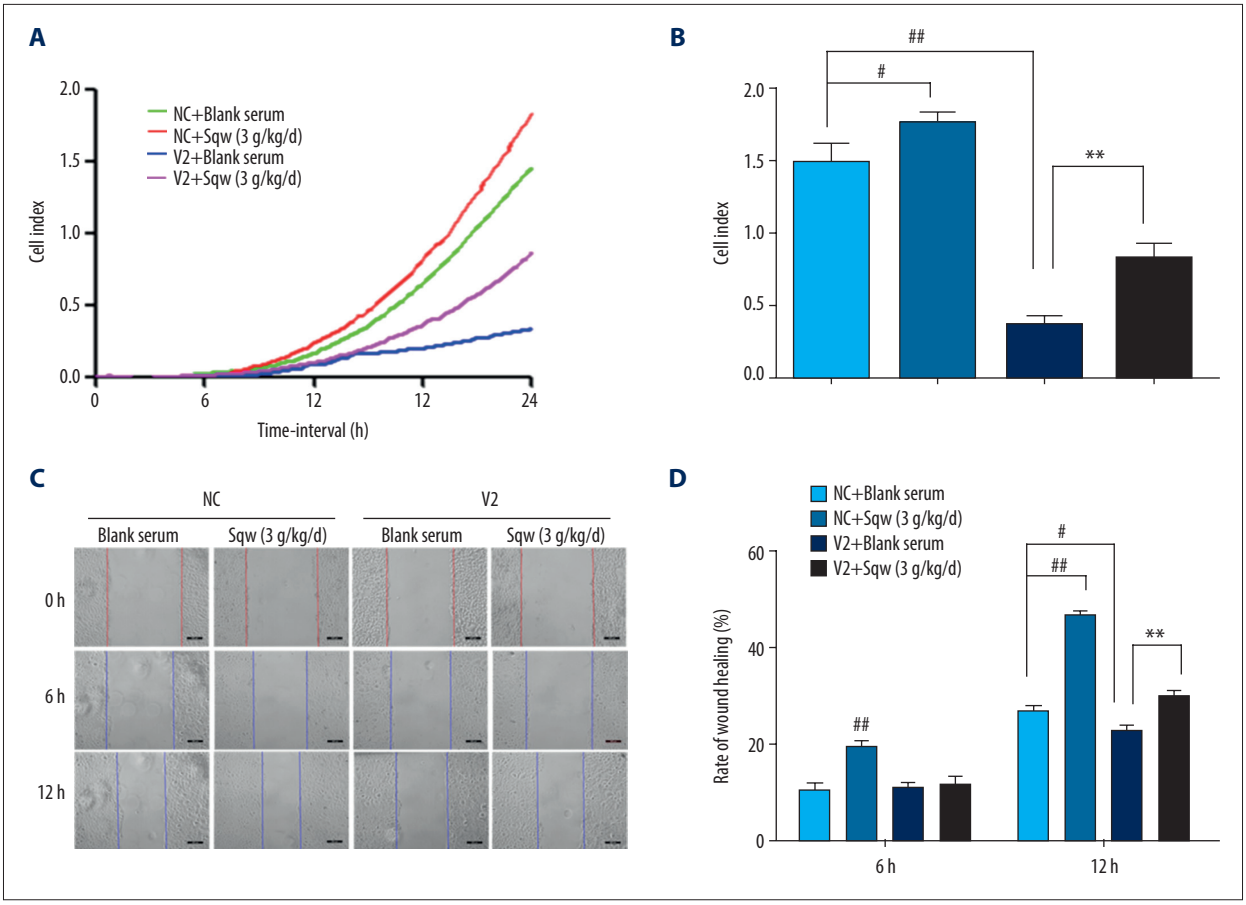

Figure 5. Effects of cell migration in NRK-52E with aquaporin 1 (AQP1) knockdown. (**A, B**) With lentivirus V2 and Shen Qi Wan (SQW) (3 g/kg) medicated serum treatment, the migration ability was detected by real-time cell analysis. (**C, D**) With lentivirus V2 and SQW (3 g/kg) medicated serum treatment, the rate of wound-healing was detected by wound healing assay. # $P<0.05$, ## $P<0.01$ *vs.* the NC+Blank Serum group; ** $P<0.01$ *vs.* V2+Blank Serum group.

Effect of AQP1 with SQW medicated serum-treated in NRK-52E cells

SQW medicated serum has been reported to promote the migration of NRK-52E cells and simultaneously augmented the mRNA and protein abundance of AQP1. To validate whether AQP1 mediates migration facilitated by SQW medicated serum, we transfected NRK-52E cells with AQP1 RNAi lentivirus. After transfection, the mRNA and protein expression levels of AQP1 successfully declined in NRK-52E cells (Figure 4A–4C). These data suggested that the lentiviral-mediated AQP1 RNAi could efficiently and stably weaken the AQP1 level in NRK-52E cells, and knockdown efficiency of the lentivirus V2 group is especially remarkable that chosen for further study. In addition, the medicated serum at a dosage of 3 g/kg SQW has significantly attenuated the down-regulation of AQP1 mRNA and protein levels of NRK-52E cells with the silencing of AQP1 (Figure 4D–4F). Furthermore, the results showed that the knockdown of AQP1 caused a marked attenuation of migration (Figures 5A–5D), and the migration of NRK-52E cells might be reversed by SQW medicated serum.

Discussion

Chinese Herbal Medicine Compound Preparation is made up of many kinds of drugs, which has the weakness of being a complex composition and containing a lot of impurities. In traditional pharmacology, crude drugs are directly added into a culture system of cells or organs *in vitro*. However, this approach could seriously interfere with the results of an experiment with many non-specific physical and chemical factors, such as pH, osmotic pressure, inorganic salt ions, tannin, and so on. Li and Wu [17], as the representative of TCM scholars, opened up a new area of "Chinese medicine serum pharmacology" research. In serum pharmacology, drugs have undergone a series of biotransformation after digestion and absorption in the gastrointestinal tract *in vivo* and result as medicated serum. Then, the medicated serum is added into a culture system of cells or organs *in vitro*. The actions of medicated serum *in vitro* are much more similar to drugs *in vivo* [18].

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SQW is representative of renal tonal prescriptions in traditional Chinese medicine and usually is applied in chronic kidney diseases. Although previous experiments demonstrating protective effect for renal damage of SQW *in vivo*, few results have been reported specifying the mechanism. In this study, we investigate the effect on SQW medicated serum on proliferation and migration in NRK-52E cells, and we explored the role of AQP1 with SQW medicated serum treatment at the cellular level to clarify the mechanism of renal damage repair. In previous research, we found that "kidney" yang deficiency has the symptom of water-fluid metabolic disorders, and the regulation of water-fluid metabolic was inseparable from the role of AQP.

AQP is a family of cell transmembrane proteins, which are distributed in multiple organs and tissues of the body, and has cytological function involved in the transport, absorption, secretion and cell migration, fat metabolism, epidermal renewal, neurotransmission, and volume regulation [19–29]. In the kidney, as the primary urinary organ of the body, AQP1 and AQP2 are involved in the formation of the original urine filtration, reabsorption, and other important physiological processes. In addition, AQP1 is the earliest discovered subtype of the AQP family, and AQP1 mainly is distributed in the apical and basolateral plasma membranes in epithelial cells of the proximal tubule and the thin descending limb of Henle. The amount of water transported by the kidney, an important part of the urinary concentrating mechanism, accounts for 80% of all water reabsorption [30,31]. Additionally, we founded that SQW can reversible the downregulation of AQP1 expression and symptoms of "kidney" yang deficiency in a rat model. In this study, we detected the effects of SQW medicated serum on mRNA and protein expression levels of AQP1 in NRK-52E cells by q-PCR and western blot. The results showed that SQW medicated serum significantly increased the mRNA and protein level of AQP1 in NRK-52E cells.

Furthermore, AQP1 was initially discovered as a channel protein for water transportation [32]. In recent years, researchers have found another function of AQP1, which facilitates cell migration, which is closely related to the occurrence, development, and outcome of multiple diseases [33–35]. Saadoun et al. [5] indicated that AQP1 plays an important role in cell migration and the regeneration of renal epithelium cells after damage. AQP1 expressed in both epithelial and endothelial cells and also detected in renal epithelium NRK-52E cells.

In the present study, we found that SQW medicated serum markedly increased AQP1 protein expression in NRK-52E cells, indicating a link between AQP1 and SQW medicated serum contribute to migration. Furthermore, we confirmed that SQW medicated serum regulated cell migration of NRK-52E cells through upregulating the expression of AQP1. In addition, transfecting NRK-52E cells with AQP1 RNAi lentivirus, the mRNA and protein expression levels of AQP1 were successfully declined and resulted in a marked attenuation of facilitation of migration. These results indicate that AQP1 might be the mediator for the facilitate migration effect of SQW medicated serum. However, there are several limitations in the current study. NRK-52E is a mouse renal tubular epithelial cell, not of human origin. Due to the SQW medicated serum obtained from SD rats, we selected the NRK-52E cells to conduct this work. Meanwhile, using human renal tubular epithelial cells in further studies is still required. In addition, a renal injury model as a control group and more tests *in vivo* in experimental and clinical trials are needed.

Conclusions

In summary, our study has shown that SQW medicated serum promotes NRK-52E cell migration in NRK-52E cells and was associated with the increase of AQP1 mRNA and protein expression levels, indicating that SQW could has a therapeutic effect on renal injury. Therefore, AQP1 might function as a potential therapeutic target of renal diseases.

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