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Paraquat promotes the epithelial-mesenchymal transition in alveolar epithelial cells through regulating the Wnt/β-catenin signal pathway

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Abstract. – OBJECTIVE: To investigate whether paraquat (PQ)-induced rat alveolar type II cells (RLE-6TN) epithelial-mesenchymal transition (EMT) and to explore the underlying molecular mechanism.

MATERIALS AND METHODS: In the present study, RLE-6TN cells were treated by 20 μmol/L PQ, and then the morphology was observed under an inverted microscope; RT-PCR and Western blot were performed to detect the expression level of EMT related markers, E-cadherin, and vimentin, as well as Wnt/β-catenin signaling pathway. In addition, we performed the transwell invasion assay to detect the ability of cell invasion.

RESULTS: We demonstrated that PQ was able to induce the transition of RLE-6TN cells from epithelial morphology to fibroblast-like morphology, associated with the acquisition of migratory properties. Phenotypically, PQ induced-EMT was characterized by loss of epithelial cell markers including E-cadherin, while upregulation of mesenchymal cell markers including vimentin, was concurrent with the activation of Wnt/β-catenin signaling pathway. Furthermore, knockdown of β-catenin by using specific siRNA could reverse PQ triggered EMT process and attenuated cell migration ability.

CONCLUSIONS: PQ promotes RLE-6TN epithelial-mesenchymal transition by upregulating the expression of Wnt/β-catenin.

Key Words:
Paraquat, Pulmonary fibrosis, Wnt/β-catenin, Epithelial-mesenchymal transition.

Introduction

Paraquat (PQ) or N,N'-dimethyl-4,4'-bipyridinium dichloride, is a kind of heterocyclic organic fast-acting herbicide that has been widely applied in agriculture. PQ is highly toxic to human beings and animals. However, there remains no effective antidote in clinical treatment yet, and the lethal dosage of PQ is only 5 to 10 mL in oral administration. Among patients who are poisoned by PQ, the mortality rate can be as high as 90% or above, and those who have survived after rescue suffer from the pulmonary interstitial fibrosis, which severely affects the living quality of patients[1,2]. Currently, the pathogenesis of PQ poisoning remains unclear yet; thus, to investigate the pathogenesis of pulmonary interstitial fibrosis caused by PQ poisoning and search for the effective treatment method have become a hotspot and challenge in clinical research. New studies have shown that epithelial-mesenchymal transition (EMT) plays a substantial effect in the pathogenesis of diseases in respiratory system, such as chronic obstructive pulmonary disease (COPD)[3] and pulmonary fibrosis[4,5]. In type II alveolar epithelial cells, EMT can facilitate the transition of alveolar epithelial cells from the epithelial phenotype to the interstitial phenotype, thereby promoting the occurrence and progression of pulmonary fibrosis, but the relevant pathogenesis remains unknown. In addition, some literatures reported that EMT is co-regulated by multiple signal pathways, in which Wnt/β-catenin signal pathway, as the key link in the initiation of EMT, is also involved in the occurrence and progression of pulmonary fibrosis[6,7]. In this study, we aimed at discovering how PQ induced the occurrence of pulmonary fibrosis to provide new theoretical evidence and therapeutic target for clinical diagnosis and treatment of pulmonary fibrosis.

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Materials and Methods

Materials

Rat alveolar type II epithelial cell line RTE-6TN was purchased from Cell Bank (ATCC, Denver, CO, USA); PQ solution in mass fraction of 42% was purchased from Pilarquim Co., Ltd (Shanghai, China); TRIzol RNA extraction kit was purchased from TaKaRa (Tokyo, Japan); RT-PCR primer sequences were synthesized by Tianyi Huiyuan Biotechnology Co., Ltd (Beijing, China); rabbit anti-human polyclonal antibodies of E-cadherin and Vimentin were purchased from Abcam (Cambridge, MA, USA), mouse anti-human polyclonal antibodies of β-catenin and glyceraldheyde 3-phosphate dehydrogenase (GAPDH) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and horseradish peroxidase (HRP)-labeled goat anti-mouse or -rabbit immunoglobulin G from Boster Biological Technology Co., Ltd (Wuhan, Hubei, China); medium-molecular-weight protein marker was purchased from Thermo Fisher Scientific (Waltham, MA, USA); ECL kit was purchased from Pierce (Harrisburg, PA, USA); fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd; high-glucose Dulbecco's modified eagle Medium (DMEM) was purchased from HyClone (South Logan, UT, USA); radio-immunoprecipitation assay (RIPA) kits for cells and tissues and BCA protein quantification kit were purchased from Beyotime Biotechnology Institute (Shanghai, China); 0.45 mm polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA); 6-well plate, 25 cm^2 plastic culture bottle and 24-well transwell chamber (8 μm) were purchased from Beaver Biosciences Inc. (Suzhou, China); Matrigel used for invasion experiment was purchased from BD Biosciences (Franklin Lakes, NJ, USA); inverted phase contrast microscope (Olympus, Tokyo, Japan); horizontal, vertical and transfer electrophoresis tanks (Beijing Liuyi Biotechnology Co., Ltd, Beijing, China).

Cell Culture

In an incubator (37°C, 5% CO_2 and saturated humidity), RLE-6TN cell line was cultured in the high-glucose DMEM culture medium containing 10% endotoxin-free fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin solutions. When 90 to 95% of cells adhered to the wall, we used 0.25% trypsin to digest the cells for passage, in which cells in the logarithmic phase (2nd or 3rd generation) were collected for following experiments. After 70 to 80% of cells were merged in culture, they were starved in the serum-free culture medium overnight followed by the treatment of cells according to the requirement of experiment.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Primer sequences of β-actin: upstream sequence 5'-CGGAGTCAACGGATTTGGTCGTAT-3', downstream sequence 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. Primer sequences of β-catenin: upstream sequence 5'-AGCTTCCAGACACGCTATCAT-3′, downstream sequence 5'-CGGTACAACGAGCTGTTTCTAC-3′. Primer sequences of E-cadherin: upstream sequence 5'-ATTTTTCCCTCGACACCCGAT-3', downstream sequence 5'-TCCCAGGCGTAGACCAAGA-3'. Primer sequences of Vimentin: upstream sequence 5'-AGTCCACTGAGTACCGGAGAC-3', downstream sequence 5'-CATTTCACGCATCTGGCGTTC-3'. PCR reaction conditions were set as follows: initial denaturation at 94°C for 2 min; denaturation at 94°C for 20 s, annealing at 56°C for 1 min, extension at 72°C for 30 s, for a total of 32 cycles; extension at 72°C for 5 min; amplified product was preserved at 4°C. Statistical analysis of the RT-PCR results was performed.

Western Blotting Assay

RLE-6TN cells were immersed into the 20 *μmol*/L PQ for 24 h, and then cells on 6-well plate were washed on ice using the pre-cooled phosphate buffered saline (PBS) for three times followed by cell lysis using RIPA on ice for 20 min. Then, cells that were sufficiently lysed were collected gently using a cell scraper, and transferred into 1.5 mL Eppendorf tube (Hamburg, Germany) which was centrifuged centrifugation at 12000 rpm for 1 min with the sediment being discarded. The collected supernatant was preserved at -20°C. Prior to the sample loading, the protein concentration was assayed using bicinchoninic acid (BCA) method, in which proteins were accumulated through electrophoresis at 80 V, isolated under 120 V, and transferred onto the membrane followed by blocking using 5% skimmed milk for 1 h. Rabbit anti-human E-cadherin and vimentin polyclonal antibodies and mouse anti-human of β-catenin and GAPDH polyclonal antibodies were added onto the membrane for incubation at 4°*C* overnight. The membrane was washed using Tris-buffered saline and Tween (TBST) on the decoloring shaker for 3 times (5 min/time); then horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG was added onto the membrane for

incubation at room temperature for 1 h. TBST was used to wash the membrane for 3 times (5 min/time). Enhanced chemiluminescence (ECL) reagent was dropped onto the membrane for color development, and the images were scanned and analyzed using Image J analytic software, by which the gray value of specific stripes was digitalized. The digitalized gray value was used to calculate the relative value of the experiment in comparison with the gray value of internal reference.

Cell Invasion Experiment

Matrigel was melted at 4°C in advance, and then 40 µL Matrigel diluted using the serum-free medium (1:3) was spread on the polycarbonate microporous membrane. The membrane was coagulated in an incubator for 4 h for later use. RLE-6TN cells in logarithmic phase were starved on a serum-free high-glucose DMEM medium for 24 hours, digested using 0.25% EDTA, and used to prepare the single-cell suspension using the serum-free high-glucose DMEM medium, in which the cell density was adjusted to 4×10^5/mL. In each chamber, 200 µL serum-free cell suspension were added into the upper chamber, and grouping was carried out according to the requirement of experiment with three replicative wells being set in each group; in each lower chamber, 600 µL high-glucose DMEM medium containing 20% FBS were added into each well. The membrane was delivered into an incubator for 48 h of culture. Chambers were taken out for removing the residual medium by washing the chambers using pre-cooled phosphate-buffered saline (PBS) for three times. In the upper chamber, cells that failed to pass through the membrane were scraped using a wet cotton swab, whilst remaining cells were fixed in 4% paraformaldehyde for 20 min. Thereafter, cells were dried at room temperature for crystal violet staining for 20 min. Subsequently, pre-cooled PBS was used to wash the chambers for 3 times, and the chambers immersed in the PBS were placed onto the inverted phase contrast microscope to perform cell count for those cells succeeded in passing through the membrane in five visions (upper, lower, left, right and middle visions, ×400). The results of cell count were averaged.

Statistical Analysis

All values were expressed as ±s. SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used to perform one-way analysis of variance in statistical analysis. Tukey's HSD (honestly significant difference) test was used in conjunction with ANOVA to find means that were significantly different each other. $p<0.05$ suggested that the difference had statistical significance.

Results

PQ Induces the Morphological Variations of rat Alveolar Type II Epithelial Cell RLE-6TN

In the negative control group, RLE-6TN cells were in typical epithelioid polygon, and those cells were closely arranged with mutual connection. After 24 h of treatment using 20 µmol/L PQ, polarity among the RLE-6TN cells disappeared, and the flattened epithelium-shaped cells in close connection were transformed to those loosely connected cells in long fusiform and cambiform. Cells were in disperse arrangement and in migration (Figure 1).

PQ Induces the Occurrence of EMT in Rat Alveolar Type II Epithelial Cell RLE-6TN

After 24 h of treatment using 20 µmol/L PQ, the results of RT-PCR and Western blotting assay showed that compared with the negative control group, the mRNA and protein expressions of E-cadherin in those RLE-6TN cells of epithelial phenotype in the PQ group were significantly decreased, while the expressions of Vimentin in the mesenchymal cells were increased with the activation of Wnt/β-catenin signal pathway (Figure 2).

PQ Enhances the in vitro Invasion Capability of rat Alveolar Type II Epithelial cell RLE-6TN

After 24 hours of treatment using 20 µmol/L PQ, the results showed that in comparison with the negative control group, the invasion capability of RLE-6TN cells in the PQ group was remarkably augmented (Figure 3).

EMT mediated by PQ is Suppressed by Silencing the Wnt/β-catenin Signal Pathway

We used the siRNA to specifically silence the expression of β-catenin to discover the role of β-catenin in PQ-induced EMT. Results of RT-PCR and Western blotting assay showed that, after the expression of β-catenin was specifically silenced, the EMT induced by PQ was reversed with an increase in expression of E-cadherin and a decrease in expression of Vimentin (Figure 4).

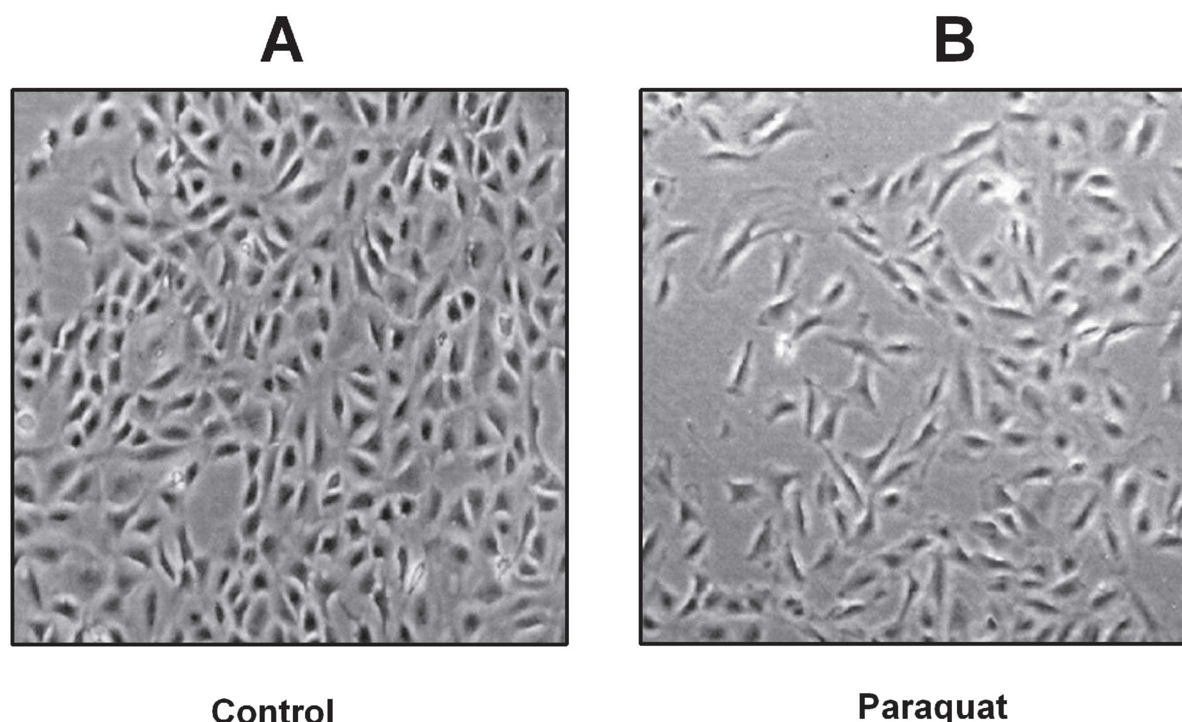

Figure 1. Morphological changes of the RLE-6TN cells under light microscope (×400). ***A,*** RLE-6TN cells in negative control group; ***B,*** RLE-6TN cells in paraquat treated group.

Silencing the expression of β-catenin Weakens in in vitro Invasion Capability Induced by PQ

We further verified whether Wnt/β-catenin signaling pathway could affect the cell invasion induced by PQ. In cell invasion using transwell chamber, we found that after the expression of β-catenin was specifically silenced by siRNA, the invasion capability of RLE-6TN cells induced by PQ was significantly weakened (Figure 5).

Discussion

PQ poisoning can affect multi-organ system of human, such as lung, kidney, liver, heart and adrenal gland, and patients with high-dosage intake of PQ die of multi-organ failure in short-term[8,9]. Enriching the most of PQ in the body, lung is not only the organ that can easily absorb the PQ, but also the major target organ of PQ with the most prominent clinical symptoms[10]. Acute lung injury is manifested as the early-stage clinical feature of lung injuries caused by PQ, mainly the pulmonary edema and hemorrhage in 24 hours after poisoning. With the progression of the disease, it will be developed into irreversible pulmonary interstitial fibrosis in varying degrees in several days and most of the patients die of pulmonary failure in the advanced stage[11].

As a highly conservative process, EMT can result in the disappearance of polarity and loss of close intercellular connection of epithelial cells in adhesive form, and break through the basal membrane to facilitate the transition of cells into the mesenchymal cells, during which cells can gain migration and invasion capability[12,13]. In addition to the involvement in the formation of embryo morphology and development of multiple organs and tissues, EMT also plays an important role in a variety of fibrotic diseases[14,15]. The occurrence of EMT is always accompanied by the downregulation in expression of E-cadherin, the phenotypic biomarker of epithelial cells, and the upregulation of Vimentin, the phenotypic biomarker of mesenchymal cells. Recent studies have confirmed that alveolar type II epithelial cells can be transformed into the mesenchymal cells, thereby facilitating the occurrence and development of pulmonary fibrosis[16,17]. Nevertheless, in clinical practice, whether the pulmonary fibrosis induced by PQ poisoning is associated

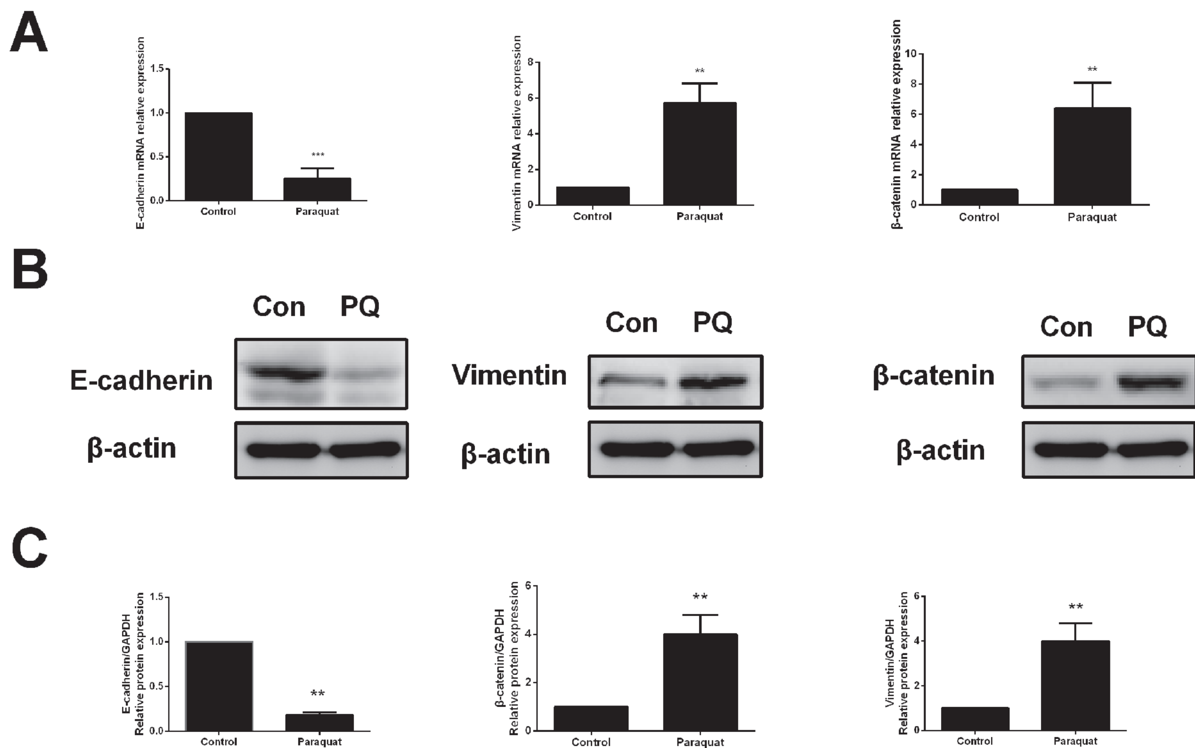

Figure 2. EMT of RLE-6TN cell triggered by paraquat. ***A,*** RT-PCR analysis of E-cadherin, Vimentin, β-catenin mRNA; ***B,*** Western blot analysis of E-cadherin, Vimentin, β-catenin protein; ***C,*** Quantification of Western blot results

with the EMT, the relevant regulation mechanism remains unknown yet.

The important roles of Wnt/β-catenin signal pathway have been identified in the development of a variety of tissue embryos, such as lung, kidney, and neurological tissues. Besides, it is also one of the key regulatory factors in EMT[18,19]. Classical Wnt signal pathway can block the degradation of β-catenin in cytoplasm through inhibiting the GSK3β-mediated phosphorylation, resulting in mass accumulation of β-catenin that passes into the nucleus and regulates the transcription and expression of downstream target genes through binding with the TCF/LCF3 compound (downstream promoter), thereby inducing the initiation of EMT[20,21]. Previous investigations have shown that the aberrantly high expression of β-catenin exists in various respiratory system diseases, including pulmonary fibrosis and pulmonary tumors. Also, some researches indicated that PQ poisoning can regulate the occurrence of pulmonary fibrosis at an early stage through acting on the snail/β-catenin signal pathway. However, there have been few papers reporting whether EMT is involved in the pulmonary fibrosis caused by PQ, and the role as well as the regulation mechanism of Wnt/β-catenin in this process. In this work, rat alveolar type II epithelial cell RLE-6TN was immersed in 20 μmol/L PQ for 24 h, and then placed under the inverted phase contrast microscope to observe the morphological changes in cells. Thereafter, Western blotting assay and RT-PCR were utilized to verify whether PQ could induce the occurrence of EMT in RLE-6TN cells in *in vitro* experiment. The results showed that, compared with the blank control group, after RLE-6TN cells were immersed into the PQ for 24 h, morphological changes from the initial polygon shape to the fibrotic cells in long fusiform and cambiform. Meanwhile, the morphological changes were accompanied with the decrease in expression of E-cadherin (epithelial phenotype) and the increase in expression of vimentin (mesenchymal phenotype). The result of transwell invasion experiment also showed that RLE-6TN cells with EMT, cultured in hypoxic environment, gained an increase in invasion capability. To further discover the molecular mechanism how PQ initiated the EMT, we simultaneously detected Wnt/β-catenin signal pathway, the key regulation pathway in EMT. The results showed that, compared with the blank control group, after RLE-

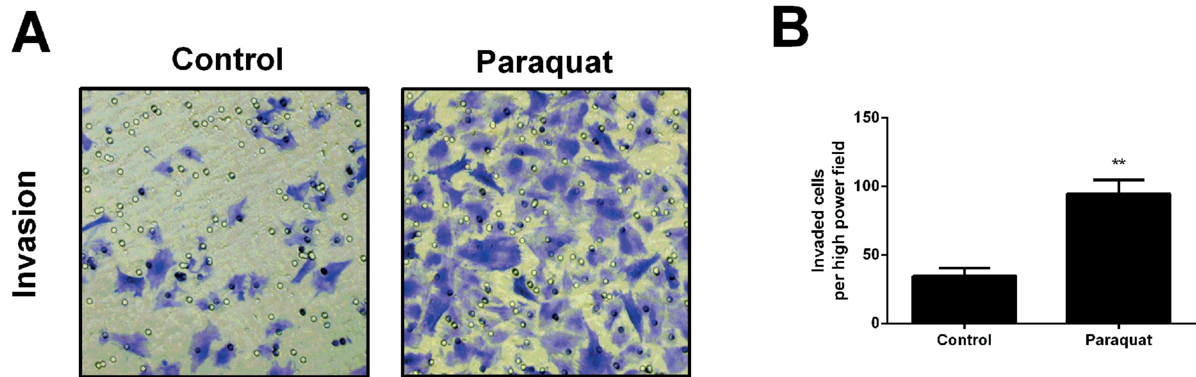

Figure 3. The invasion potentiality of RLE-6TN enhanced by paraquat (crystal violet staining ×200). ***A*** and ***B,*** Transwell assay detect the invasion abilities of RLE-6TN cells and quantification analysis of the results.

6TN cells were treated using PQ for 24 hours, β-catenin was significantly activated, suggesting that β-catenin may be involved in the EMT mediated by PQ. To clarify the specific mechanism of Wnt/β-catenin signal pathway in EMT, β-catenin-specific siRNA was used to silence the expression of β-catenin, and the effect of β-catenin on the initiation of EMT and cell invasion capability was further detected. The results revealed that, after the β-catenin was specifically silenced, the initia-

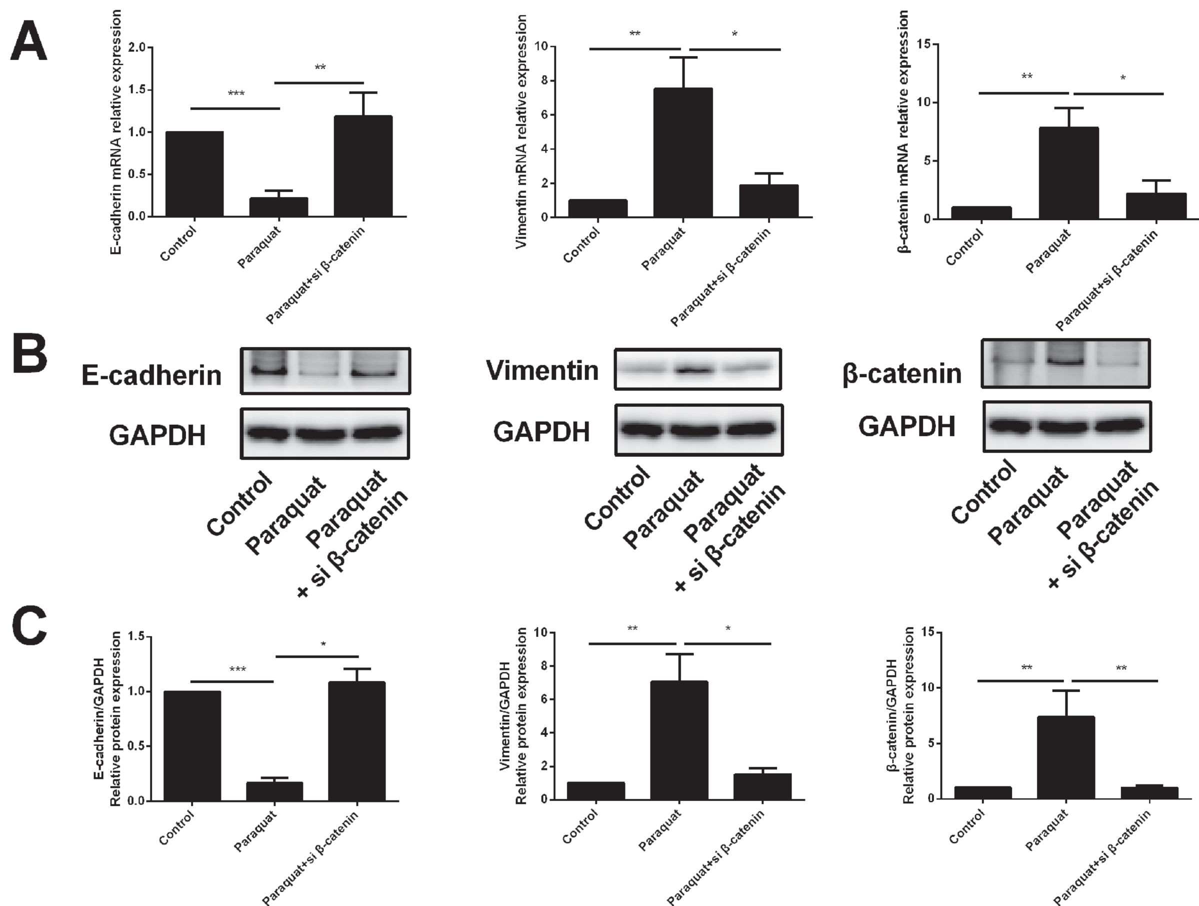

Figure 4. Knockdown of β-catenin inhibits paraquat triggered EMT. ***A,*** RT-PCR analysis of E-cadherin, Vimentin, β-catenin mRNA; ***B,*** Western blot analysis of E-cadherin, Vimentin, β-catenin protein; ***C,*** Quantification analysis of Western blot results.

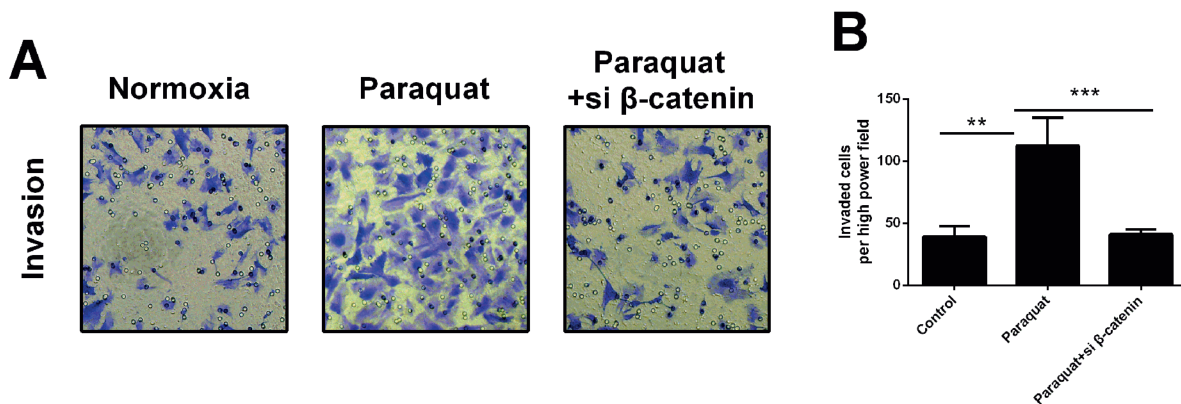

Figure 5. β-catenin specific siRNA attenuates paraquat triggered RLE-6TN cell invasion (crystal violet staining× 200). ***A*** and ***B*,** Transwell assay detect the invasion abilities of RLE-6TN cells and quantification of the results.

tion of EMT and cell invasion in RLE-6TN cells mediated by PQ were reversed, indicating that β-catenin plays a key role in this process. Based on the results of above work, we suggested that PQ can initiate the EMT process through activating the Wnt/β-catenin signal pathway, which can enhance the invasion capability of RLE-6TN cells, and facilitate the occurrence and development of pulmonary fibrosis.

Conclusions

In this study, we preliminarily confirmed that in rat alveolar type II epithelial cell RLE-6TN, PQ can trigger the EMT process through the activation of Wnt/β-catenin to facilitate the occurrence and development of pulmonary fibrosis. These results can deepen the understanding on pathogenesis of pulmonary fibrosis. Therefore, in-depth studies are required to figure out the role and specific action mechanism of Wnt/β-catenin signal pathway in EMT process of pulmonary fibrosis. Through specifically blocking the expression of β-catenin, EMT process can be reversed and the pulmonary fibrosis can also be inhibited. With a great clinical significance and promising application prospect, the results in this study will suggest new ideas and theoretical evidence for developing precaution strategies and targeted therapies of pulmonary fibrosis caused by PQ poisoning.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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