

Enhanced expression levels of aquaporin-1 and aquaporin-4 in A549 cells exposed to silicon dioxide

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Abstract. Aquaporins (AQPs), water channel proteins in the cell membranes of mammals, have been reported to be important in maintaining the water balance of the respiratory system. However, little is known regarding the role of AQP in occupational pulmonary diseases such as silicosis. The present study investigated the expression of AQP1 and AQP4 in the human A549 alveolar epithelial cell line stimulated by silica (SiO₂). A549 cells were cultured and divided into four groups: Control, SiO₂-stimulated, AQP1 inhibitor and AQP4 inhibitor. The cells of the SiO₂-stimulated group were stimulated with SiO₂ dispersed suspension (50 mg/ml). The cells of the inhibitor group were pretreated with mercury (II) chloride (HgCl₂; a specific channel inhibitor of AQP1) and 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020; a specific channel inhibitor of AQP4) and stimulated with SiO₂. The mRNA expression levels of AQP1 and AQP4 were detected by reverse transcription-quantitative polymerase chain reaction, and the protein expression levels of AQP1 and AQP4 were detected by western blotting and immunocytochemistry. Compared with the control group, the expression levels of AQP1 and AQP4 mRNA and protein in SiO₂-stimulated groups increased and subsequently decreased (AQP1 peaked at 2 h and AQP4 at 1h; both P<0.001 compared with control group). In the inhibitor group, expression levels were increased compared with controls; however, they were significantly decreased compared with the SiO₂-stimulated group at 2 h (AQP1; P<0.001) and 1 h (AQP4; P<0.001). The expression of AQP1 and AQP4 increased when exposed to SiO₂, and this was inhibited by HgCl₂ and TGN-020, suggesting that AQP1 and AQP4 may contribute to

A549 cell damage induced by SiO₂. AQP1 and AQP4 may thus be involved in the initiation and development of silicosis.

Introduction

Silicosis is a fibrotic lung disease caused by inhalation of free crystalline silicon dioxide or silica. Occupational exposure to respirable crystalline silica dust particles occurs in many industries (1). To date, there is no general therapy for the treatment of this disease. In 1997, the International Agency for Research on Cancer upgraded crystalline silica to a Group 1 human carcinogen (2). Despite extensive research efforts, the exact mechanism of silicosis remains to be fully elucidated (3).

Silica (SiO₂) dust is the critical pathogenic factor in the initiation of silicosis (4), and the alveolar epithelial cells of the lung are the primary target cells, particularly in the early stages of the disease. However, the underlying molecular mechanism of epithelial cell damage induced by SiO₂ remains to be elucidated (5). Aquaporins (AQPs), of which there are 13 subtypes (AQP0-AQP12), are cell membrane transport proteins associated with water permeability. AQPs are widely distributed in animals and plants and their important role in water transport has been extensively investigated (6-8). AQP1 was first reported in 1992 (9), and has been demonstrated to be ubiquitously involved in the water balance of the respiratory system (10-13). In addition, AQP4 regulates the exchange of fluid between the alveolar space and alveolar epithelium barrier and has a significant compensational role in pulmonary liquid clearance in the event of sodium transport damage in acute lung injury (14,15). However, a limited number of studies have investigated the involvement of AQP1 and AQP4 in occupational pulmonary diseases, such as silicosis (16,17). Therefore, in the present study, human A549 type II alveolar epithelial cells cultured *in vitro* were stimulated with SiO₂ dust to investigate possible mechanisms of AQP1 and AQP4 in the pathological process of silicosis. This may provide an experimental basis to identify effective targets for the prevention and control of silicosis.

Materials and methods

Reagents. Crystalline silica (MIN-U-SIL5®; SiO₂ purity, 99.2%; median particle diameter, 1.6 µm) was purchased from

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U.S. Silica (Frederick, MD, USA). RPMI-1640 culture medium was supplied by HyClone; GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) and 0.25% trypsin were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). TRIzol[®] Reagent for total RNA extraction was purchased from Invitrogen; Thermo Fisher Scientific, Inc. The Moloney Murine Leukemia Virus Reverse Transcriptase RNase H Minus Point Mutant kit and SYBR Green quantitative polymerase chain reaction (qPCR) reagent kit were products of Promega Corporation (Madison, WI, USA). Anti- β -actin rabbit polyclonal antibody (ab8227) was purchased from Abcam (Cambridge, MA, USA) and anti-AQP1 (sc-20810) and anti-AQP4 (sc-20812) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase-conjugated sheep anti-rabbit IgG secondary antibody (A0208) was obtained from Beyotime Institute of Biotechnology (Haimen, China). SP Immunohistochemical kit (SP-9000) was a product of Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Enhanced chemiluminescence (ECL) substrate kit was obtained from PerkinElmer, Inc. (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was obtained from Beyotime Institute of Biotechnology. Triton[™] X-100 was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental cells and design. Human A549 type II alveolar epithelial cell lines (purchased from the Cell Center of the Institute of Basic Medical Sciences, Peking Union Medical College (Beijing, China) were cultured in RPMI-1640 medium containing 10% FBS in a 37°C and 5% CO₂ incubator. The cultured cells were divided into four groups: Control (untreated A549 cells); SiO₂-stimulated (50 mg/ml SiO₂ composite suspension-treated A549 cells); AQP1 inhibitor [A549 cells were treated with 0.2 mmol/l mercury (II) chloride (HgCl₂) for 3 min and then SiO₂-stimulated]; and AQP4 inhibitor [A549 cells were treated with 100 μ mol/l 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) for 2 h and then SiO₂-stimulated] (13).

Detection of SiO₂ influence on the growth of A549 cells by CCK-8 assay. A549 cells were transferred into 96-well plates at 5,000 cells/well. Following acclimatization in 100 μ l RPMI-1640 containing 0.4% FBS for 24 h, cells were stimulated with mixed suspensions of SiO₂ particles for 24 h (10, 25, 50 or 100 mg/ml in RPMI-1640 containing 10% FBS). Cell counting kit-8 reaction solutions (20 μ l) were subsequently added to the wells (6 wells/group). Cell growth was measured by absorbance at 450 nm on a microplate reader 1 h later. The dose of 50 mg/ml SiO₂ was thus selected as the stimulus in subsequent experiments.

Reverse transcription (RT)-qPCR analysis. A549 cells were transferred to 6-well plates at 20,000 cells/well for 24 h. Cells were treated as described in the previous section (3 wells/group). Total cellular RNA extraction was performed using TRIzol[®] reagent according to the manufacturer's instructions. RNA was reverse transcribed to complementary (c) DNA by incubation with reverse transcriptase at 42°C for 50 min. qPCR analysis was performed in a Roter-Gene 3000 Sequence Detection System (Qiagen Pty Ltd., Melbourne, Australia) using SYBR Green PCR Master Mix. PCR amplification was performed

on 0.5 μ l of cDNA using the following gene-specific primers: AQP1 forward, 5'-TGACCCGCTCGGACTTACT-3' and reverse, 3'-CTTCTGGACCCATGCTGTG-5'; AQP4 forward, 5'-CATCTCCCTTTGCTTTGGACTC-3' and reverse, 3'-CAG ATAGAGGATTCTGCTCCAA-5'; and β -actin forward, 5'-CACCCCACTGAAAAAGATGA-3' and reverse, 5'-CAT CTTCAAACCTCCATGACG-3'. The following conditions were used: A 1 min pre-denaturing step at 95°C; and cycles of 15 sec at 95°C and 20 sec at 59°C. A total of 40 cycles were performed to prevent saturation of the reaction. Melting curve analysis revealed a single sharp peak with the correct melting temperature. GAPDH was used as an internal control, and AQP1 and AQP4 mRNA expression was normalized against GAPDH expression. Relative quantification by the 2^{- $\Delta\Delta C_q$} method was performed by comparing to control groups (18).

Western blotting. A549 cells were transferred to 6-well plates at 20,000 cells/well for 24 h. Cells were treated as described in the previous section (3 wells/group). Proteins were extracted from cells using radioimmunoprecipitation assay buffer (catalog no. P0013; Beyotime Institute of Biotechnology, Haimen, China), and quantified by a bicinchoninic acid assay (catalog no. P0012; Beyotime Institute of Biotechnology). A total of 30 μ g extracted protein was loaded onto a 12% SDS-PAGE gel, subjected to electrophoresis at 120 V for 90 min and transferred to a nitrocellulose membrane (Whatman; GE Healthcare Life Sciences). Following blocking with 5% skim milk in Tris-buffered saline with 0.1% Tween (TBST) at room temperature for 2 h, the membranes were incubated with primary antibodies at 4°C overnight (anti-rat AQP1 and AQP4; 1:800). Membranes were washed in TBST three times, and incubated with the HRP-conjugated secondary antibody at room temperature for 2 h (anti-rat antibodies; 1:1,000). Protein bands were visualized using ECL. All western blotting densitometry data were normalized to β -actin using Image Lab software version 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunocytochemical staining. A549 cells were cultured for 24 h in 24-well plates at 5,000 cells/well; sterile coverslips were placed in each well in advance to allow cells to adhere during growth. The cells were fixed in 1% paraformaldehyde for 15 min and rinsed three times by phosphate-buffered saline, and incubated with 0.1% Triton X-100 in phosphate-buffered saline at room temperature for 5 min. Normal goat serum blocking fluid (taken from the SP-9000 Immunohistochemical kit; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) was added for 15 min, and cells were subsequently incubated with primary antibodies recognizing AQP1 and AQP4 (1:100) overnight, followed by the biotinylated secondary antibody and finally the S-A/HRP reagent taken from the SP-9000 Immunohistochemical kit; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 15 min. Immunoreactivity was visualized with DAB (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.). Brown staining was considered to indicate a positive result. Staining was visualized using an optical microscope at magnification, x20 (Olympus Corporation, Tokyo, Japan). Cells that were stained a brown color were considered positive. Immunocytochemistry was quantified using Image-Pro Plus analysis software version 5.0 (Media

Cybernetics, Inc., Rockville, MD, USA) to detect positive cells and automatically determine the optical density and percentage per field, which were used to calculate the expression levels of AQP1 and AQP4 protein in A549 cells. All sections were incubated under identical conditions with the same concentration of antibodies therefore the immunostaining was comparable among the various experimental groups.

Statistical analysis. All experiments were performed three times and all statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons were performed using a Student's *t*-test for unpaired samples and a one-way analysis of variance for multiple comparisons. Post hoc comparisons were performed using the Least Significant Difference test when equal variances were assumed or with Dunnett's test when equal variances were not assumed. Data are expressed as the mean \pm standard error. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Influence of various concentrations of SiO₂ on the growth of A549 cells. CCK-8 assay absorbance of A549 cells reduced following stimulation with various concentrations of SiO₂, suggesting cell growth suppression (Fig. 1). The suppression was similar for SiO₂ concentrations of 25 and 50 mg/ml, at 78 and 76% of the control, respectively. Although 100 mg/ml SiO₂ produced the greatest suppression of cell activity, it led to increased cell death and influenced the detection of mRNA and protein of AQP1 and AQP4. Therefore 50 mg/ml was selected as the stimulus concentration for the subsequent experiments.

The expression levels of AQP1 and AQP4 mRNA in A549 cells exposed to SiO₂. Following SiO₂ stimulation, the AQP1 mRNA expression levels increased rapidly compared with the control group (0.5 and 1 h, both $P < 0.001$ vs. control group), and peaked at 2 h ($P < 0.001$ vs. control group) post SiO₂ treatment (Fig. 2). From 2 h onwards there was a gradual decrease, and this difference (2 vs. 4 and 8 h) was statistically significant (both $P < 0.001$). Following exposure to SiO₂ for 8 h, the mRNA expression levels of AQP1 returned to those of the control group. Pretreatment of cells with AQP1 inhibitor prior to stimulation with SiO₂ for 2 h resulted in significantly decreased AQP1 mRNA expression levels compared with the SiO₂-stimulated group at 2 h ($P < 0.001$). The AQP4 mRNA expression levels in A549 cells followed a similar pattern (0.5, 1 and 2 h, $P < 0.001$ vs. control group; 1 vs. 2 h $P = 0.023$; 1 vs. 4 h, $P < 0.001$; 1 vs. 8 h, $P < 0.001$; Fig. 2).

Western blot analysis of AQP1 and AQP4 protein expression levels in A549 cells exposed to SiO₂. Western blotting (Fig. 3) revealed that SiO₂ treatment led to an increase in the expression levels of AQP1 protein in A549 cells. The increase was observed at 3 h of stimulation and peaked at 6 h. Protein expression levels declined from 12 h; however, at 24 h they remained greater than in the control group ($P = 0.021$). Following pretreatment with inhibitor, AQP1 protein expression levels were increased compared with those of the control group ($P < 0.001$); however, they were reduced compared with

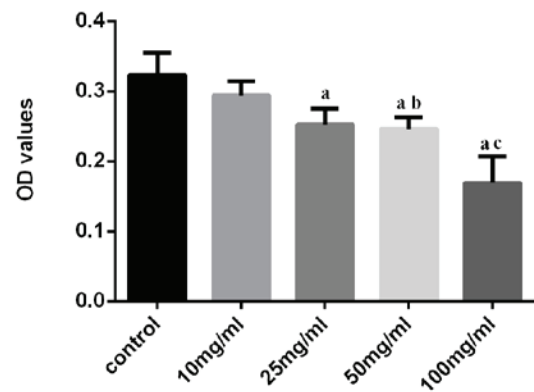


Figure 1. Selection of SiO₂ concentration for stimulation of A549 cells investigated by CCK-8 assay. A549 cells were stimulated with mixed suspensions of SiO₂ particles (10, 25, 50 and 100 mg/ml). CCK-8 assay absorbance for A549 cells was reduced following SiO₂ stimulation, suggesting that cell growth was suppressed, and the suppression rate for the cells following treatment with 25 and 50 mg/ml SiO₂ was similar at 78 and 76%, respectively. Therefore, 50 mg/ml SiO₂ was selected as the stimulus concentration for subsequent experiments. OD, optical density; CCK-8, cell counting Kit-8. ^a $P < 0.01$ vs. control; ^b $P < 0.05$ vs. 10 mg/ml; and ^c $P < 0.01$ vs. 50 mg/ml.

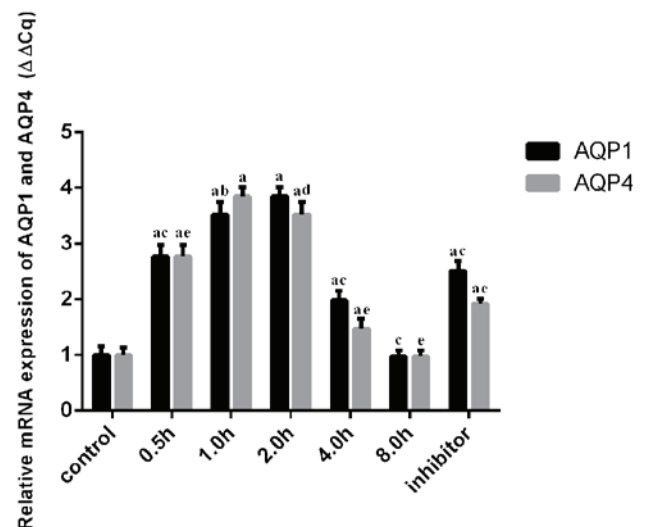


Figure 2. Expression levels of AQP1 and AQP4 mRNA in A549 cells exposed to SiO₂. AQP1 mRNA expression levels in SiO₂-stimulated groups increased rapidly and peaked at 2 h following SiO₂ treatment, then gradually decreased. By 8 h, the mRNA expression level of AQP1 had returned to that of the control group. AQP1 inhibitor, used at 0 h and detected at 2 h, decreased the mRNA expression levels of AQP1. The AQP4 mRNA expression levels in A549 cells followed a similar pattern; however, the peak was observed at 1 h. ^a $P < 0.01$ vs. control; ^b $P < 0.05$ and ^c $P < 0.01$ vs. 2 h SiO₂ stimulation; and ^d $P < 0.05$ and ^e $P < 0.01$ vs. 1 h SiO₂ stimulation. AQP, aquaporin; SiO₂, silicon dioxide.

the SiO₂-stimulated group at 6 h ($P < 0.001$). AQP4 protein expression levels were increased in the SiO₂-stimulated groups compared with the control group (3 h, $P = 0.001$), and peaked at 6 h ($P < 0.001$). Following pretreatment with inhibitor, AQP4 protein expression levels remained increased compared with those of the control group ($P = 0.014$); however, they were reduced compared with the SiO₂-stimulated group at 6 h ($P < 0.001$).

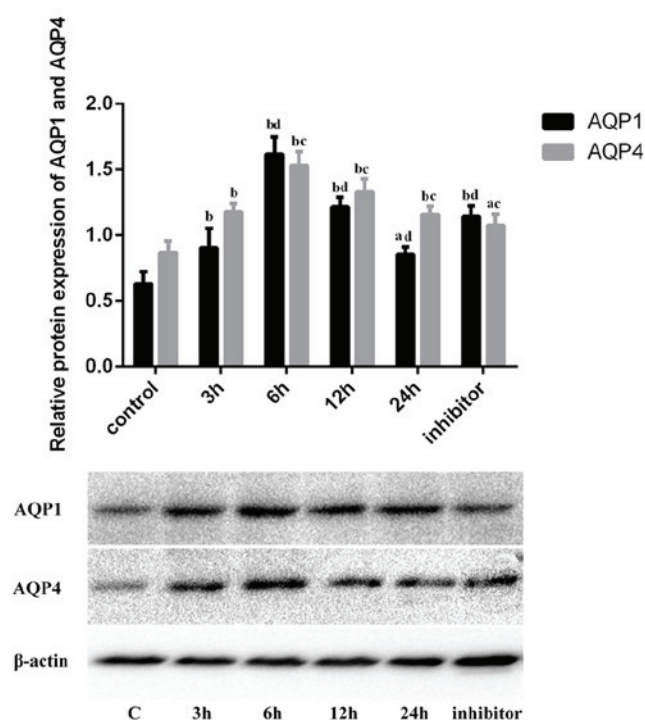


Figure 3. Expression levels of AQP1 and AQP4 protein in A549 cells exposed to SiO₂. Western blotting revealed that SiO₂ treatment increased protein expression levels of AQP1 and AQP4 in A549 cells. This increase was observed following 3 h of stimulation and peaked at 6 h, declining from 12 h. Following pretreatment with inhibitors (used at 0 h and detected at 6 h), AQP1 and AQP4 protein expression levels were increased compared with the control group; however, they were reduced compared with the SiO₂-stimulated group at 6 h. ^aP<0.05 and ^bP<0.01 vs. control; and ^cP<0.05 and ^dP<0.01 vs. 6 h SiO₂ stimulation. AQP, aquaporin; C, control.

Immunocytochemistry staining evaluation. AQP1 and AQP4 immunocytochemistry staining products appeared brown (Fig. 4) and were expressed in the cytoplasm and nucleus. The immunocytochemistry quantitative analysis revealed that AQP1 protein expression levels were increased compared with untreated cells at 3 ($P=0.004$), 6 ($P<0.001$) and 12 h ($P<0.001$) following SiO₂ exposure and gradually declined from 6 h until similar to control levels at 24 h ($P=0.153$). Following pretreatment with inhibitor, AQP1 expression levels decreased ~29% compared with the SiO₂-stimulated group at 6 h ($P<0.001$). The AQP4 protein expression levels in A549 cells followed a similar pattern (Fig. 4).

Discussion

The water balance of the body is important for the maintenance of health. The AQP family is crucial in this balance and is closely associated with the development of various diseases. Therefore, AQPs have attracted attention as targets for the treatment of numerous diseases (19). To date, 13 AQP isoforms (AQP0-AQP12) have been identified in mammals and are expressed in diverse tissues (20-22), and 6 AQPs (AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9) are expressed in the lung and airways (23,24). AQP1 is expressed in the microvascular endothelia, AQP3 and AQP4 in the airway epithelia and AQP5 in type I alveolar epithelial cells, submucosal gland acini and a subset of airway

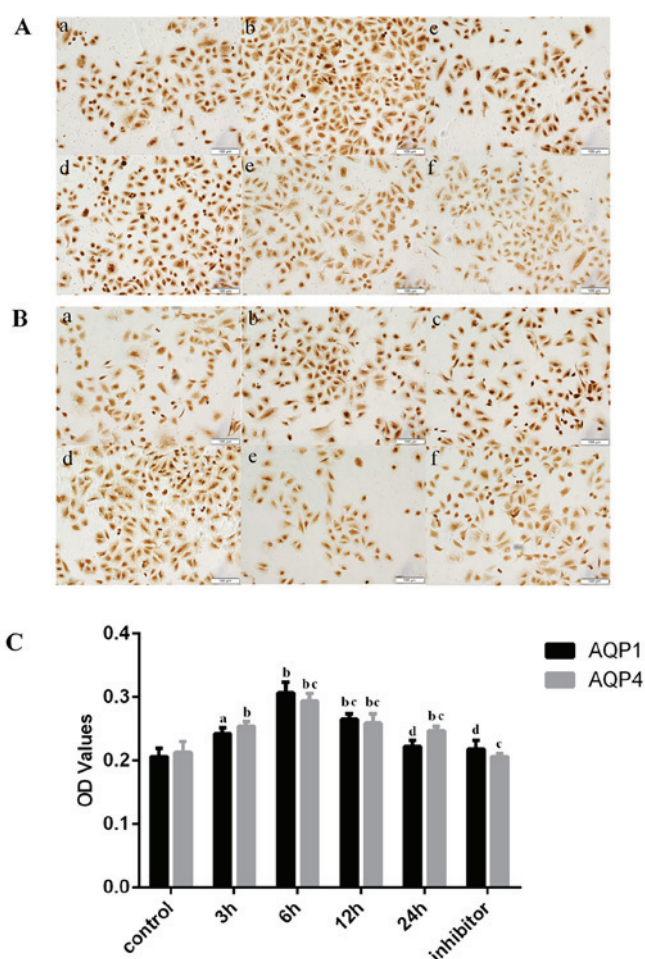


Figure 4. Immunocytochemistry staining and evaluation (A) AQP1 and (B) AQP4 immunocytochemistry staining products were brown and expressed in the cytoplasm and nucleus of: A, control cells; b, cells stimulated by SiO₂ for 3 h; c, cells stimulated by SiO₂ for 6 h; d, cells stimulated by SiO₂ for 12 h; e, cells stimulated by SiO₂ for 24 h; and f, cells pretreated with inhibitor. (C) AQP1 and AQP4 expression levels in A549 cells followed similar patterns, increasing at 3, 6 and 12 h following SiO₂ exposure compared with the control and gradually declining from 6 h until similar to control levels at 24 h. Following pretreatment with inhibitor (at 0 h and detected at 6 h), expression levels declined ~29% compared with the SiO₂-stimulated group at 6 h. ^aP<0.05 and ^bP<0.01 vs. control; and ^cP<0.05 and ^dP<0.01 vs. 6 h SiO₂ stimulation. AQP, aquaporin; OD, optical density.

epithelial cells (10). Although AQP8 and AQP9 protein expression has been observed in the lung, immunolocalization of these proteins has not yet been demonstrated (25,26). AQP1 is the primary water channel responsible for water transport through numerous epithelia and endothelia (10). In addition, a previous study suggested important roles for this protein in gas permeation, angiogenesis, cell proliferation and migration (27). The expression of AQP1 protein may be mechanistically involved in the airway inflammation and pathogenesis of chronic obstructive pulmonary disease (COPD) (28), and expression of AQP1 increased markedly in pulmonary fibrosis, suggesting that AQP1 may be important in the progression of this disease (12). However, in our previous study it was demonstrated that the expression of AQP1 increased first and then declined in the lung tissue of silicosis rats, suggesting that AQP1 may potentially be involved in the pathogenesis of silicosis (17).

This phenomenon also existed in rat lung infection and endotoxin-induced lung injury, and a study observed mouse lung AQP-1 and AQP-5 were downregulated (29). This may be due to the disorder of water balance caused by silica dust. AQP4 is primarily distributed in the airway epithelium, alveolar endothelial and epithelial cells (29). A previous study revealed that AQP4 expression was significantly increased in cells from patients with asthma (30). However, in other respiratory diseases, including COPD, patients also demonstrated decline of AQP4 mRNA expression (31). This varying expression of AQP4 may be due to the nature and different stages of these diseases. In addition, AQP4 has been associated with the tumorigenesis and migration of lung adenocarcinoma, potentially promoting lung adenocarcinoma cancer cell migration via adjustment of E-cadherin protein expression levels (32).

In pulmonary diseases, lung epithelial cells are highly susceptible to become targets for damage (33). In the development of silicosis, SiO₂ is the initiating factor, and upon entering the body it affects alveolar epithelial cells, particularly in the acute phase of lung injury. Notably, at this stage the inflammatory response is pronounced and increased vascular permeability and pulmonary edema follow (29). The pathway for the transport of water across the endothelial and epithelial lung barrier remains to be fully elucidated; however, AQPs have been described as critical in water removal from the lung extracellular space (30). The present study was designed to observe the alteration of AQP1 and AQP4 expression levels in A549 cells exposed to SiO₂, to indicate whether a water imbalance within lung epithelial cells exists in acute lung injury resulting from SiO₂ stimulation.

The results of the present study demonstrated that the expression levels of AQP1 and AQP4 mRNA increased in A549 cells following exposure to SiO₂, and that this increased expression was inhibited by pretreatment with specific inhibitors of AQP1 and AQP4, HgCl₂ and TGN-020, respectively. Notably, by immunocytochemistry, inhibitor reduced AQP protein expression to control levels, whereas by western blot analysis, it did not. This may be due to differential sensitivity of various methods and measurement software. The alterations in AQP1 and AQP4 expression levels may therefore be associated with SiO₂ stimulation and these proteins may contribute to A549 cell damage. Alterations in AQP1 and AQP4 expression levels may induce the imbalance of lung epithelial water transportation. This may exacerbate acute inflammatory damage of lung tissue, which may then gradually develop into chronic hyperplastic lesions, ultimately resulting in silicosis fibrosis. However, the specific underlying mechanism remains to be elucidated.

The acute inflammatory damage stage induced by SiO₂ may be crucial for the early occurrence and development of silicosis (1). Early application of specific inhibitors of water channel proteins may thus reverse the imbalance of lung epithelial water transportation, and decrease or even prevent the inflammatory reaction caused by SiO₂ stimulation and so reduce the degree of silicosis fibrosis (34). Therefore, AQP1 and AQP4 may become novel targets for silicosis prevention and early treatment (35-37).

Although HgCl₂ and TGN-020 act as specific inhibitors for AQP1 and AQP4 and effectively inhibit their expression (35,38),

the results of the present study suggest that they have specific cell toxicity. The identification and development of novel water channel protein inhibitors is therefore required for use in the prevention and treatment of silicosis.

There are numerous AQPs in lung tissue, and in the present study only the expression of AQP1 and AQP4 was investigated; therefore, the expression of other AQPs and their roles in silicosis requires additional investigation. Research into the role of AQPs in the pathogenesis of silicosis is at an early stage; however, novel therapeutic agents targeting AQP have been identified (39-41). Additional studies are necessary to clarify the causal association between AQP1 and AQP4 upregulation and the development of silicosis, and to determine whether AQP1 and AQP4 are suitable targets for silicosis treatment or prevention.

In conclusion, the results of the present study demonstrated that the expression of AQP1 and AQP4 increased when exposed to SiO₂, and that the specific inhibitors HgCl₂ and TGN-020 inhibited this SiO₂-stimulated increase. This suggests that AQP1 and AQP4 may contribute to A549 cell damage induced by SiO₂. AQP1 and AQP4 may thus be involved in the initiation and development of silicosis.

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