[Frontiers in Bioscience, Landmark, 22, 258-267, January 1, 2017]

Linking expression of aquaporin 3 to activation of JNK pathway

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1. ABSTRACT

Aquaporin 3 (AQP3) has been shown to be low in the amnion and chorion tissues of patients with oligohydramnios and that *S. miltiorrhiza,* a Chinese herbal medicine, results in increased AQP3 in human amniotic epithelial cells (hAECs). Here, we provide evidence for the involvement of the JNK pathway in AQP3 regulation in isolated oligohydramnios tissues *in vitro,* in hAECs derived from normal amniotic fluid and fluid from patients with isolated oligohydramnios. Phosphorylation of JNK was suppressed by pretreatment of cells with JNK-specific inhibitor (SP600125) and was up-regulated by *S. miltiorrhiza*; *S. miltiorrhiza* combined with SP600125 prevented SP600125-induced down-regulation of phospho-JNK both in normal amniotic fluid volume and in isolated oligohydramnios. In isolated oligohydramnios, AQP3 expression was significantly suppressed by SP600125 in a concentration- and time-dependent mannner, while its expression was up-regulated by *S. miltiorrhiza*. *S. miltiorrhiza* combined with SP600125 inhibited the increased expression of AQP3 relative to the *S. miltiorrhiza* treated group. Together, the data suggest that c-jun N-terminal kinase (JNK) pathway unerlies the regulation of AQP3 by *S. miltiorrhiza* amnion and chorion tissues.

2. INTRODUCTION

Amniotic fluid (AF) provides an ideal environment for fetal growth and development. Oligohydramnios is a condition defined by amniotic fluid volume (AFV) smaller than 300 mL (1). This condition is clearly linked to pathologies that can lead to significant perinatal mortality and long-term morbidity (2). Oligohydramnios is associated with an increased risk of umbilical cord occlusion, fetal growth restriction, operative deliveries, and stillbirth at term (3). The prevention and treatment of oligohydramnios present a considerable challenge because of an incomplete understanding of the pathological and physiological mechanisms that underlie this condition. Recent studies suggest that the regulation

of placental water transfer and intramembranous resorption may be critical for AFV modulation, and aquaporins (AQPs) may play an important role in this process (4-6).

AQPs are a family of small hydrophobic integral membrane proteins. They function as water-selective pores and regulate the flow of water across a variety of biological membranes (7, 8). Expression of the *AQP*3 gene and the presence of the AQP3 protein have been documented in human placenta and fetal membranes (9-12). We found that AQP3 protein levels are lower in oligohydramnios patient amnion than in normal AFV (9). Previous work from others and from our laboratory has indicated that alteration in the expression of AQP3 in the fetal membrane may be important to the pathophysiology of isolated oligohydramnios.

In Chinese herbal medicine, *Salvia miltiorrhiza* extract has been widely used for the treatment of cardiovascular and neurodegenerative diseases (13, 14). Multiple pharmacological activities of *S. miltiorrhiza* have been identified, including anti-oxidant and anti-inflammatory activities, and the extract is used to treat miscarriage, edema, and platelet aggregation (15-18). In recent years, clinical research has confirmed that *S. miltiorrhiza* effectively improves AFV in oligohydramnios, with approximately 70% cases were found AFV increased (19). In a previous study, we demonstrated that *S. miltiorrhiza* extract increased AQP3 protein levels in human primary culture amnion epithelium cells (hAECs) obtained from normal AFV and from patients with isolated oligohydramnios (20). However, the mechanism underpinning the modulation of AQP3 levels by *S. miltiorrhiza* is poorly understood.

Mitogen-activated protein kinases (MAPKs) are responsible for many cellular events, from complex developmental programs, such as embryogenesis, cell growth, inhibition, and apoptosis, to short-term changes required for stress and acute hormonal responses (21, 22). One MAPK subgroup, c-jun N-terminal kinases (JNKs), is ubiquitous; JNKs are known as stress-activated protein kinases (23-25). Studies of cultured human retinal pigment epithelial (RPE) cells showed that chemical hypoxia increased *AQP*3 gene expression through JNK activation (26). Furthermore, Zhang *et al*. (27) reported that *S. miltiorrhiza* inhibited the growth of A549 non-small cell lung cancer cells via the JNK pathway. Thus, we hypothesized that the JNK pathway may also mediate *S. miltiorrhiza*-regulated AQP3 expression in hAECs.

The purpose of this study was to investigate whether AQP3 protein levels are regulated by JNK pathway activation in cultured hAECs derived from normal AFV and from patients with isolated oligohydramnios. We also examined whether *S. miltiorrhiza*-induced AQP3 levels in cultured hAECs are mediated by activation of the JNK pathway.

3. MATERIALS AND METHODS

3.1. Patients

3.1.1. Selection of patients

Patients (n=120) who requested and had undergone cesarean deliveries between January 2011 and November 2012 in the Second Affiliated Hospital of Wenzhou Medical University were recruited for this study. The cohort included 60 individuals with normal AFV and 60 individuals with isolated oligohydramnios. The age of the patients ranged from 20 to 32 years. The patients had single pregnancy. They were at the 37- to 41-week gestation age, and had no history of illegal drug use, oxytocin pregnancy induction, or drug abuse. No intrauterine growth restriction or other pregnancy complications, such as gestational or pre-gestational diabetes, hypertensive disorders, or cardiovascular diseases were noted. Fresh placental tissues were obtained immediately after the patients gave birth; the samples were randomly divided into groups and processed (see Section 3.2.1.).

The study protocol was approved by the Research Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University and met the standards of the Declaration of Helsinki. Informed written consent was obtained from patients before tissue collection.

3.1.2. Diagnosis criteria for oligohydramnios

AFV was assessed before cesarean delivery with ultrasound imaging. The uterus was divided into four quadrants during the assessment, with the linea nigra and umbilicus as landmarks. The AF index (AFI, cm) is the sum of the largest vertical pocket of amniotic fluid, free of umbilical cord, within each uterus quadrant. For every patient, three AFI measurements were performed and averaged. A diagnosis of oligohydramnios was made when AFI < 5 cm, with 8–18 cm AFI being considered normal AF (28). Oligohydramnios was also defined as AFV < 300 mL, while AFV 300–2,000 mL at the time of cesarean section was considered normal (1). Patients in the oligohydramnios group were excluded from the study if AFV measured at cesarean delivery was inconsistent with pre-operative AFI, as described by Horsager *et al.* (29).

3.2. Methods

3.2.1. Cell culture

Cells (hAECs) were isolated from freshly collected placenta tissues. Tissues were cut and thoroughly washed to remove blood. Thereafter, they were rinsed with phosphate-buffered saline (PBS). The isolated amnion was then incubated with trypsin (Gibco, USA) under sterile conditions for 60 min at 37 °C in a water bath, with continuous shaking to dissociate amnion epithelial cells. Dispersed cells were centrifuged at 400 g for 20 min, 37 °C. The resultant deposit was

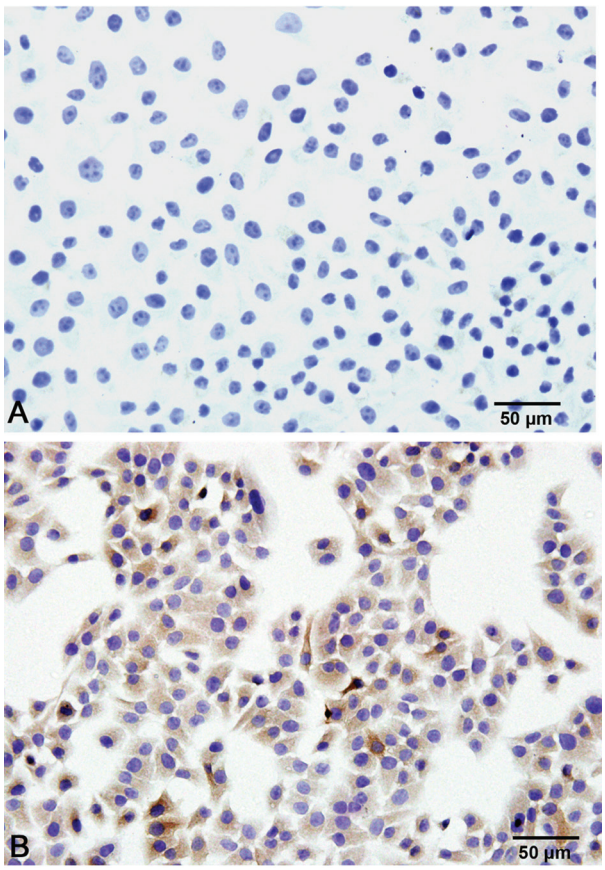

Figure 1. AQP3 protein expression in the hAECs. A: AQP3 immunocytochemistry (yellow labeling) was performed on the hAECs. Cell nuclei were visualized by blue staining. B: Primary antibody-free incubations are presented as negative controls. Magnification: x200.

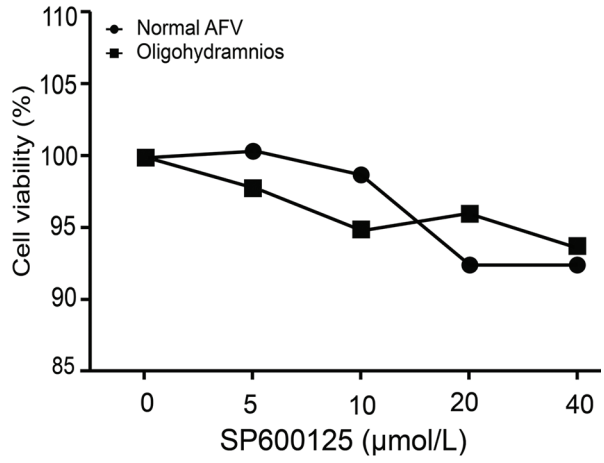

Figure 2. Cell viability of primary cultured hAECs from normal AFV or isolated oligohydramnios treated with different doses of SP600125 measured by CCK-8 technique. The hAECs were treated with different concentrations of SP600125 (0, 5, 10, 20 and 40μmol/L). Controls consisted of cells treated with 10% fetal bovine serum (control). Data are representative of 6 experiments made in duplicate. Results are expressed as percentage of controls. Statistical analyses were done by using one-way analysis of variance followed by the LSD multiple comparison tests.

transferred to a fresh tube and subjected to additional centrifugation, at 300 g for 5 min, 37 °C. Cells were plated at a density of 1 × 10^5 cells per 60 mm dish (Corning, USA), and incubated in 5% CO_2 atmosphere at 37 °C, in a complete culture medium comprising Dulbecco's Modified Eagle Medium (Gibco, USA), 10% fetal bovine serum (Hangzhou Sijiqing Bioengineering Material Co., Ltd., China), 100 IU/mL penicillin G, and 100 μg/mL streptomycin. The culture medium was changed every other day. Cells were passaged at a 1:2 split ratio every 5 to 7 days.

For each patient group, samples from 20 patients were corrected and used in preliminary experiments to establish the optimal *S. miltiorrhiza* dosing and time point. The samples from remaining 40 patients were divided into four different treatment groups: control group, JNK inhibitor SP600125 (Cell Signaling Technology, USA) group, *S. miltiorrhiza* (Shanghai New Asia Pharmaceutical Gaoyou Co. Ltd., China, batch number: 101227-3, specifications: 2 mL) group, *S. miltiorrhiza* and SP600125 co-treatment group. Cells were starved for 24 h in a serum-free medium after reaching 60–80% confluence, and incubated in the complete culture medium with different SP600125 concentrations (0, 5, 10, 20, and 40 μM, in 0.1% (v/v) dimethyl sulfoxide) for 2 h. Once the optimal SP600125 concentration was established, exposure time was varied (0, 1, 2, 4, and 8 h) to further optimize the treatment conditions. Dimethyl sulfoxide (0.1% v/v) was used as a solvent control. The cells were then incubated in the presence or absence of 5 mg/mL *S. miltiorrhiza* for 12 h. The dosage of *S. miltiorrhiza* was as established in our previous study (20).

3.2.2. Cell counting kit-8 (CCK-8) assay

Cells (hAECs) were seeded in 96-well plates, 1 × 10^4 cells/well, and incubated at 37 °C, in 5% CO_2 atmosphere. The culture medium was removed after 24 h and cells treated as follows: CCK-8 (10 μL) (Dojindo, Japan) was added to each well and incubated for additional 1 to 4 h. Spectrophotometric quantification, λ = 450 nm, followed, and percentage viability was calculated as (absorbance of treated cells/absorbance of non-treated cells) × 100. The dimethyl sulfoxide: culture medium ratio was 1.6: 1000.

3.2.3. Immunocytochemistry

Cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 15 min, extensively washed with PBS, and permeabilized in 0.2% Triton X-100 in PBS for 15 min. They were then blocked in a serum-free blocking solution for 15 min. Cells were incubated with primary anti-AQP3 antibody (anti-human, produced in rabbits; 1:100 dilution; product no. BA1559, Boster, China), overnight, at 4 °C. After extensive washing with PBS, cells were incubated with biotinylated rabbit anti-human IgG secondary antibody, 30 min, at 37°C. The

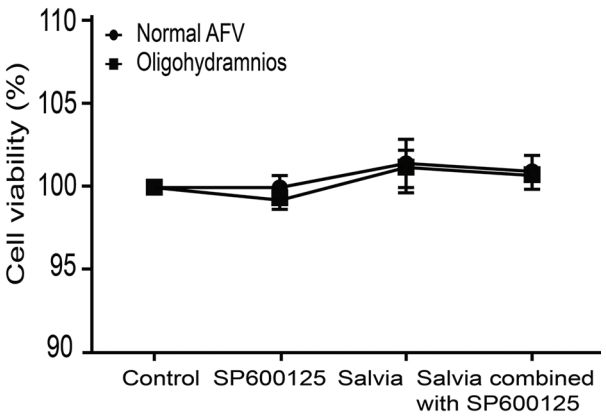

Figure 3. Effect of Salvia miltiorrhiza injection on the viability of hAECs. Cell viability of primary cultured hAECs with normal AFV and isolated oligohydramnios treated with SP600125 in the presence or absence of 5mg/mL Salvia miltiorrhiza injection measured by CCK-8 assay. Controls consisted of cells treated with 10% fetal bovine serum (control). Data are presented as the mean ± standard of 6 independent experiments. Results are expressed as percentage of controls. Statistical analyses were done by using one-way analysis of variance followed by the LSD multiple comparison tests.

staining was developed using 3,3′-diaminobenzidine as the substrate. Finally, nuclei were stained with hematoxylin. In the negative controls, PBS was used instead of the primary antibody.

3.2.4. Cell lysate preparation and Western blot analysis

Whole-cell lysates were prepared using ice-cold cell lysis buffer (50mM Tris(pH 7.4), 150mM NaCl, 1% NP-40, 0.5 % sodium deoxycholate, 0.1% SDS). Proteins were collected from supernatants cells lysates by centrifugation (14,000 g for 20 min, 4 °C). Proteins were quantified by bicinchoninic acid (Beyotime, China) method, as described (20). Proteins (8 μg) were solubilized in sample buffer (4% SDS, 30 mM dithiotreitol, 0.25 M sucrose, 0.01 M EDTA-Na_2, 0.075% bromophenol blue), and heated at 100 °C for 5 min. Lysates were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels and then electroblotted onto PVDF membranes (Millipore, USA) in the cold for 45 min at 300 mA using a Mini-Wet blot transfer system (Bio-Rad Laboratories, CA, USA). Membranes were blocked for 2 h at room temperature (20 °C to 25 °C) in 0.05 M Tris-buffered saline with 0.5% Triton X-100 (TBS-T, pH 7.4) and 5% skimmed milk, and then incubated with the appropriate primary antibody raised against human antigens, in TBS-T, at 4 °C overnight: anti-phospho-JNK (anti-human, produced in rabbits; 1:1,000 dilution; product no. 9668, CST, USA); anti-JNK (anti-human, produced in rabbits; 1:1,000 dilution; product no. 9258, CST, USA); anti-AQP3 (anti-human, produced in rabbits; 1:300 dilution); or anti-GAPDH (anti-human, produced in mouse;1:2,000 dilution; product no. AG019-1, Beyotime, China). After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution; anti-rabbit-IgG or anti-mouse-IgG, both produced in goat; Hangzhou Sijiqing Bioengineering Material Co., Ltd., China), 1 h at room temperature. Blots were developed by enhanced chemiluminescence. Bands were quantified using Quantity One software (BioRad, USA). Protein band intensities were analyzed with Labworks software. The GAPDH protein signal was used as an internal control to normalize protein loading.

3.2.5. Statistical methods

Statistical analysis was performed using SPSS 17.0 software. For normal data distribution and homogeneity of variance, quantitative data were presented as mean ± standard deviation, differences between two groups were analyzed by Student's t-test, and differences among multiple groups were analyzed by one-way ANOVA. If the variances were homogeneous, least significance difference (LSD) method was used for two-group comparisons. If the variances were nonhomogeneous, Dunnett's T3 method was used. $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1. AQP3 localization is unchanged in oligohydramnios-derived hAECs and is not affected by SP600125

Immunocytochemistry with anti-AQP3 antibody was performed to determine cellular localization and levels of AQP3 protein in hAECs. AQ3-specific signals were detected in both the cytoplasm and cell membrane, with the strongest staining in cytoplasm (Figure 1). No obvious difference in AQP3 localization was detected between hAECs derived from normal AFV and those derived from isolated oligohydramnios tissues. Furthermore, no significant differences were observed in AQP3 localization between normal AVF- and oligohydramnios-derived hAECs submitted to different SP600125 dosing regimes.

4.2. JNK inhibitor SP600125 treatment results in a time- and dose-dependent decrease in hAEC p-JNK levels

CCK-8 assay was used to assess SP600125 toxicity against primary hAECs. As shown in Figure 2, the tested SP600125 concentrations (0 to 40 μM) were not toxic to hAECs over the 2-h treatment period, thus excluding possible nonspecific cytotoxicity effects on JNK levels. Similarly, SP600125 (10 or 20 μM), alone or in combination with *S. miltiorrhiza* (5 mg/mL), was not toxic to hAECs (Figure 3), thus excluding nonspecific cytotoxicity effects on protein levels.

To determine whether the JNK signaling pathway may be implicated in regulating AQP3 levels in hAECs, cells were subjected to various SP600125 dosing

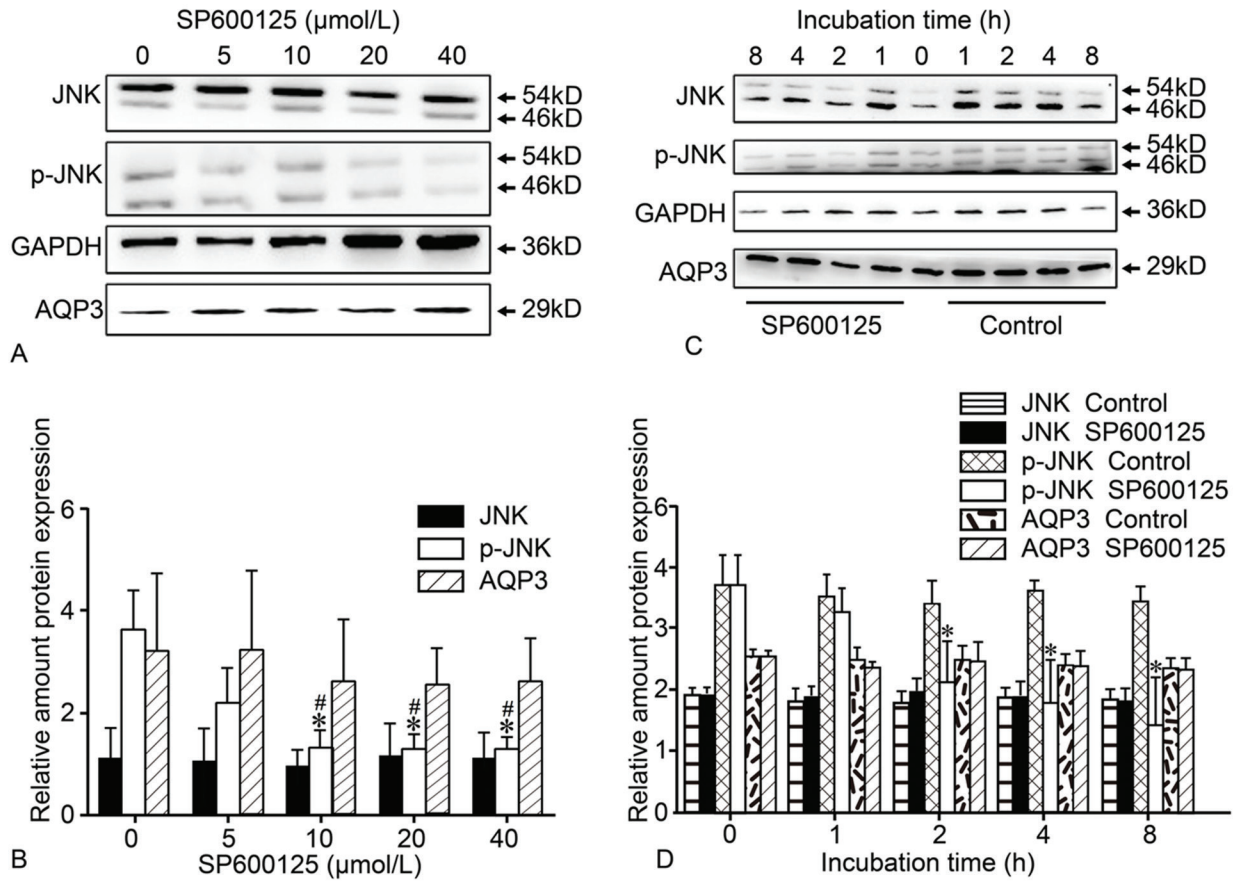

Figure 4. Effect of SP600125 on JNK/p-JNK/AQP3 protein expression in primary cultured the hAECs from normal AFV. The hAECs were treated with 0, 5, 10, 20 and 40μmol/L SP600125 for 2 h. Then cells were submitted to 10μM SP600125 at different time points (0, 1, 2, 4 and 8 hours). Protein expression (p-JNK, JNK and AQP3) were detected by Western blot analysis. GAPDH level was used as internal controls. A and C: Effects of SP600125 on the expression of total JNK1/2, p-JNK1/2 and AQP3 in the hAECs by Western blot. B and D: The relative expression level of total JNK1/2, p-JNK1/2 and AQP3. Data are shown as the mean ± standard from at least 3 independent experiments. Statistical analysis was conducted using ANOVA with a LSD post test. A *P* value less than 0.05 was considered as statistically significant. (*indicates that $P < 0.05$ vs. control; #indicates that $P < 0.05$ vs. 5 μmol/L).

regimes, as described in the Materials and Methods. JNK, p-JNK, and AQP3 protein levels were analyzed by western blotting. The optimal SP600125 dosing regimes were found to be 10 μM.

In normal AFV-derived hAECs, no significant differences in total JNK levels were observed between groups (Figure 4 A&B). However, JNK phosphorylation was considerably lower in 5–40 μM SP600125-treated cells than in untreated controls (2 h treatment), while the lowest level was at 10 μM SP600125 concentration. Phosphorylated JNK (p-JNK) levels significantly decreased 2 h after 10 μM SP600125 exposure and the decrease continued up to 8 h (Figure 4 C&D).

Total JNK levels in isolated oligohydramnios-derived cells did not significantly differ from those in the normal AFV group (Figure 5 A&B). At least 10 μM SP600125 was required to inhibit p-JNK. Pronounced p-JNK inhibition was observed after exposure to 20 μM SP600125. A substantial and significant decline in p-JNK levels was observed in cells treated with 20 μM SP600125 for 1 h, and the decrease continued up to 8 h (Figure 5 C&D).

In a negative control group (no SP600125 treatment), no changes in JNK and p-JNK protein levels were observed with prolonged incubation times. Collectively, our data indicate that SP600125 inhibits p-JNK levels in a concentration- and time-dependent manner in both normal term AFV- and isolated oligohydramnios-derived hAECs.

4.3. JNK inhibitor SP600125 suppresses AQP3 levels in isolated oligohydramnios-derived hAECs

In normal AFV-derived hAECs, 2 h SP600125 (0 to 40 μM) treatment or varying-time exposure to 10 μM SP600125 did not lead to significant differences in AQP3 protein levels from those in the untreated control

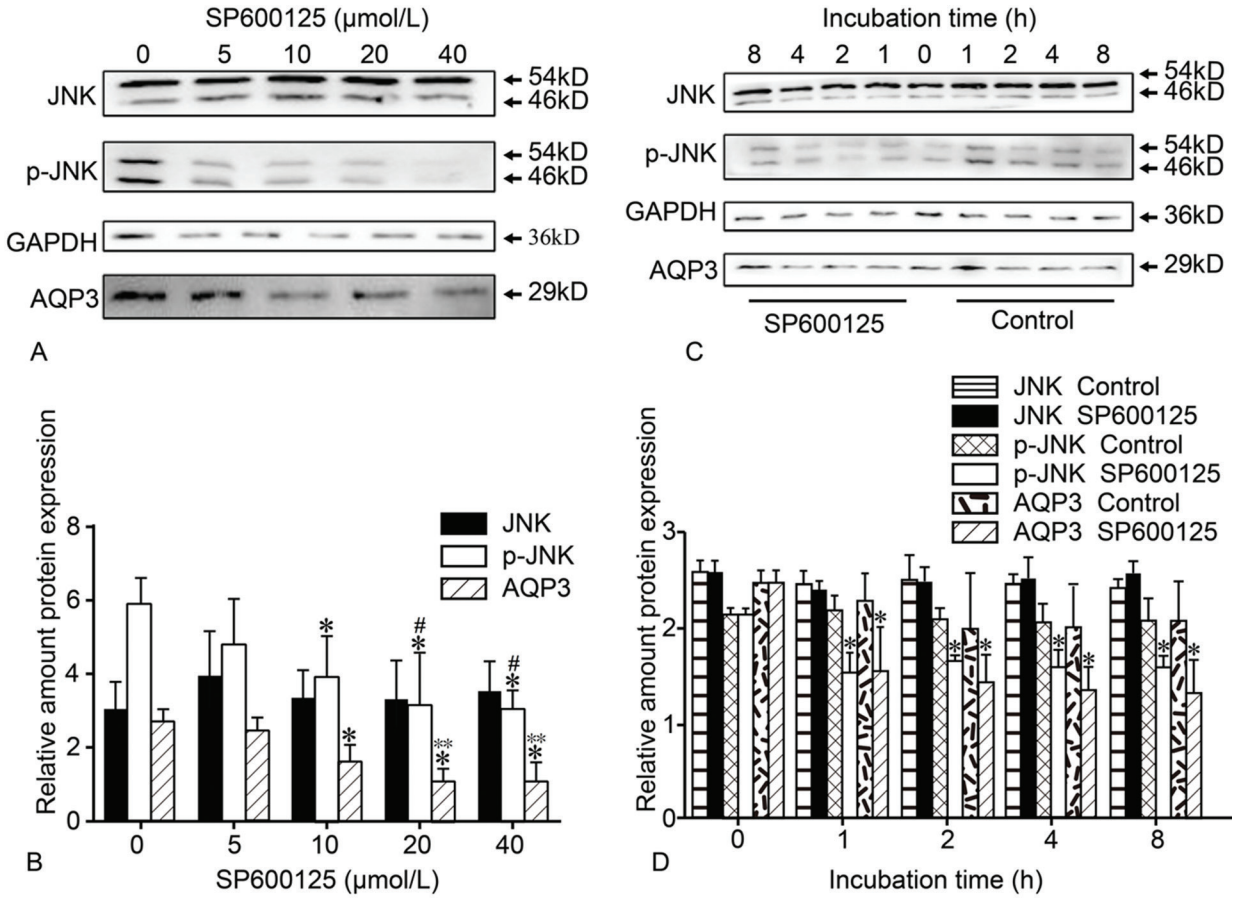

Figure 5. Effect of SP600125 on JNK/p-JNK/AQP3 protein expression in primary cultured hAECs from isolated oligohydramnios. The hAECs were treated with 0, 5, 10, 20 and 40μmol/L SP600125 for 2 h. Then cells were submitted to 20μM SP600125 at different time points (0, 1, 2, 4 and 8 hours). Protein expression (p-JNK, JNK and AQP3) were detected by Western blot analysis. A and C: Effects of SP600125 on the expression of total JNK1/2, p-JNK1/2 and AQP3 in the hAECs by Western blot. B and D: The relative expression level of total JNK1/2, p-JNK1/2 and AQP3. Control represents cells submitted to fetal bovine serum at different time points but not stimulated with SP600125. The expression levels were expressed as the ratios between JNK/p-JNK/AQP3 and GADPH protein expression levels, and were compared with those at time zero. Data are shown as the mean ± standard from at least 3 independent experiments. Statistical analysis was conducted using ANOVA with a LSD post test. A *P* value less than 0.05 was considered as statistically significant. (*indicates that $P < 0.05$ vs. control; #indicates that $P < 0.05$ vs. 5 μmol/L; **indicates that $P < 0.05$ vs. 10 μmol/L).

(Figure 4), indicating that the JNK pathway may not be involved in regulating AQP3 levels in normal AFV.

In isolated oligohydramnios-derived hAECs, 10 μM SP600125 was required to decrease AQP3 levels, while the lowest AQP3 levels was observed after 20 μM SP600125 treatment (Figure 5). A significant decline in AQP3 protein levels was observed 1 h in cells treated with 20 μM SP600125, and the decrease continued up to 8 h. SP600125 affected AQP3 levels in a concentration- and time-dependent manner, indicating that the JNK pathway may be involved in activating AQP3 in isolated oligohydramnios-derived hAECs.

In a negative control group, not submitted to SP600125 treatment, no changes in AQP3 protein levels were apparent with changing incubation times. Together, these results indicate that SP600125 suppresses intracellular AQP3 levels via JNK pathway in isolated oligohydramnios-derived hAECs.

4.4. *S. miltiorrhiza* treatment results in increased hAEC p-JNK levels but does not affect hAEC JNK levels

To assess the involvement of JNK signaling pathway in *S. miltiorrhiza* regulation of AQP3 levels, hAECs were incubated in the presence or absence of SP600125 (normal AFV material: 10 μM SP600125, 2 h; isolated oligohydramnios material: 20 M SP600125, 1 h), with and without *S. miltiorrhiza* supplementation (5 mg/mL for 12 h after SP600125 used in the co-incubation experiments).

Total JNK levels did not change in response to the treatment in normal AFV-derived material, as shown in Figure 6 A&B. Phosphorylated JNK levels were

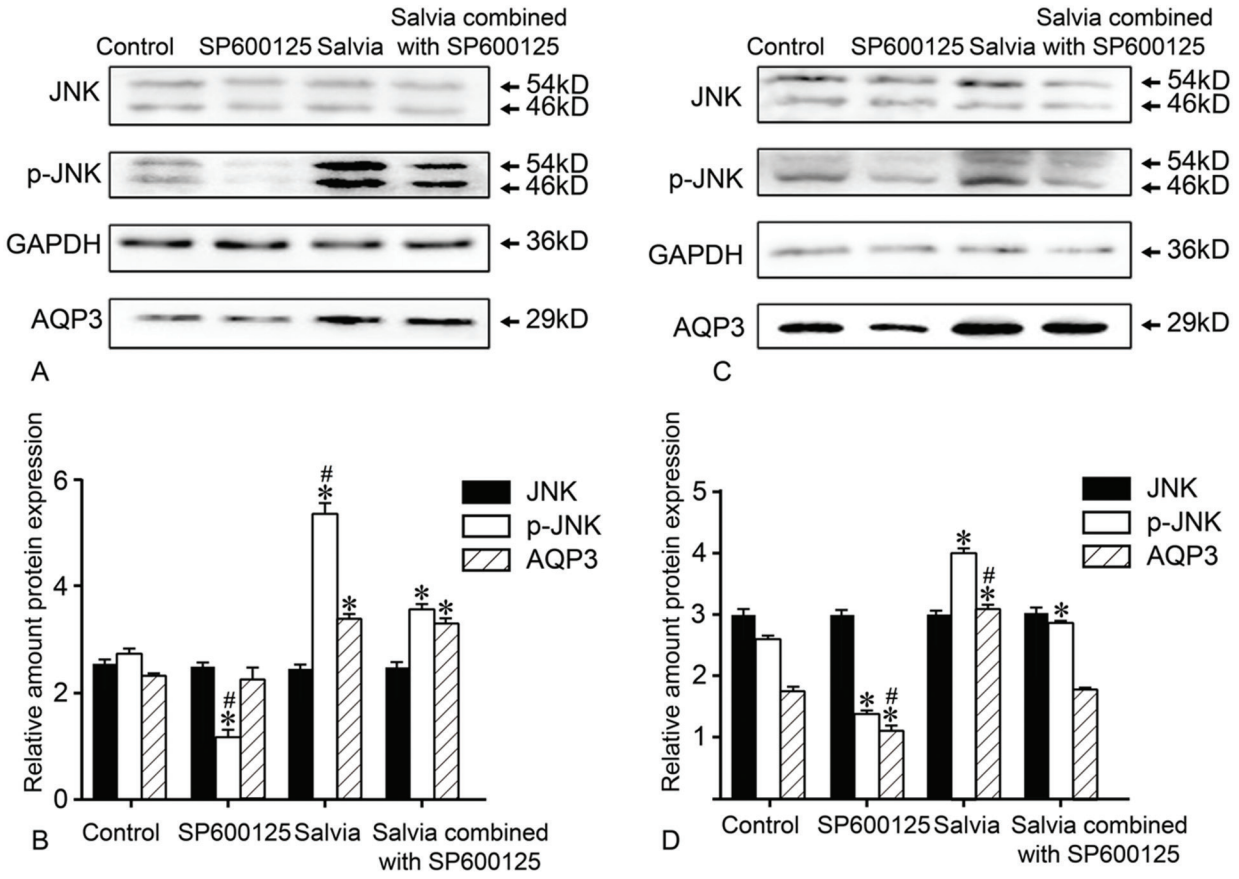

Figure 6. Effects of Salvia miltiorrhiza and/or SP600125 on the JNK activation and the expression of AQP3 in the hAECs from normal amniotic fluid volume or isolated oligohydramnios. The cells were treated with SP600125 in the presence or absence of 5mg/mL Salvia miltiorrhiza measured by Western blot. The expression profiles of JNK, p-JNK and AQP3 proteins in the hAECs were analyzed as described in the text. The expression levels of these proteins were normalized to that of GAPDH. Data are shown as the mean ± standard from at least 3 independent experiments. Statistical analysis was conducted using ANOVA with a LSD post test. A: Effects of Salvia miltiorrhiza and/or SP600125 on the expression of total JNK1/2, p-JNK1/2 and AQP3 in the hAECs from normal amniotic fluid volume by Western blot; B: The relative expression level of total JNK1/2, p-JNK1/2 and AQP3 in the hAECs from normal amniotic fluid volume; C: Effects of Salvia miltiorrhiza and/or SP600125 on the expression of total JNK1/2, p-JNK1/2 and AQP3 in the hAECs from isolated oligohydramnios by Western blot; D: The relative expression level of total JNK1/2, p-JNK1/2 and AQP3 in the hAECs from isolated oligohydramnios. (*indicates that $P < 0.05$ vs. control cultures; #indicates that $P < 0.05$ vs. Salvia combined with SP600125-treated cultures).

significantly increased in hAECs after 12 h-incubation with 5 mg/mL *S. miltiorrhiza* ($P < 0.05$), but the increase was not as pronounced when cells were co-treated with SP600125 and *S. miltiorrhiza* ($P < 0.05$). Meanwhile, p-JNK levels were higher in co-treated cells than in SP600125-only treated cells and the control group ($P < 0.05$).

Similar results were obtained in hAECs from isolated oligohydramnios tissues (Figure 6 C&D).

4.5. *S. miltiorrhiza* and SP600125 exert different effects on AQP3 protein levels in normal AFV- and isolated oligohydramnios-derived hAECs

In normal AFV-derived hAECs, as shown in Figure 6 A, AQP3 protein levels were higher upon *S. miltiorrhiza* treatment for 12 h than with SP600125 ($P < 0.05$). Co-treatment with SP600125 did not significantly alter AQP3 protein levels from those observed in cells treated with *S. miltiorrhiza* alone. This indicated that *S. miltiorrhiza* regulation of AQP3 levels may not proceed via JNK signaling pathway in normal AFV hAECs. However, in isolated oligohydramnios-derived cells, AQP3 protein levels increased after 12 h *S. miltiorrhiza* when compared with SP600125 ($P < 0.05$), and co-treatment with SP600125 attenuated *S. miltiorrhiza*-induced up-regulation of AQP3 (Figure 6 B).

Taken together, these results suggest that JNK signal transduction pathway may be involved in *S. miltiorrhiza*-induced AQP3 regulation in hAECs derived from isolated oligohydramnios tissues.

5. DISCUSSION

The JNK pathway is a central component of intracellular signaling networks that regulate cell stress, apoptosis, and the cell cycle (25, 30). This pathway can be activated by various stress factors, while the same protein kinase signal transduction cascade ultimately modulates gene expression and protein synthesis. JNK antagonist SP600125 has been reported to down-regulate the expression of *AQP1* mRNA in C3H/HeN mice (31). Furthermore, it has been suggested that hypoxic expression of *AQP3* in cultured human RPE cells is mediated by JNK signal transduction cascade activation (26). Activation of the JNK pathway has also been associated with AQP up-regulation in various cell systems and in response to various stimuli (32, 33). In the present study, we demonstrated that SP600125, a specific JNK antagonist, effectively inhibited phosphorylation of JNK in hAECs, while the overall JNK levels were unaffected. The data presented here revealed that SP600125 prevented AQP3 synthesis in isolated oligohydramnios-derived hAECs, suggesting that a decrease in amnion AQP3 levels in these cells occurs via JNK pathway. In normal-term AFV-derived hAECs, no statistically significant difference was observed in AQP3 levels after SP600125 treatment. Our findings thus suggest that the regulation of normal amnion AQP3 levels occurs via a JNK-independent pathway. The JNK pathway may be an important therapeutic target in the treatment of isolated oligohydramnios. Furthermore, our CCK-8 analysis showed that the JNK pathway inhibitor SP600125, alone or in combination with *S. miltiorrhiza,* was not toxic toward hAECs. This highlights the notion that *S. miltiorrhiza* may be safely explored for selective treatment of oligohydramnios.

The exact mechanism of the regulation of AQP3 level by *S. miltiorrhiza* is unclear. In this study, we evaluated AQP3 levels in hAECs exposed to *S. miltiorrhiza* during a simultaneous JNK inhibition. We found that *S. miltiorrhiza* treatment resulted in significantly elevated AQP3 levels in a concentration- and time-dependent manner. Further, we showed that in hAECs exposed to *S. miltiorrhiza* for 12 h, p-JNK and AQP3 protein levels significantly increased. This effect was partly inhibited by SP600125 in isolated oligohydramnios-derived cells. In normal AFV-derived cells, JNK phosphorylation inhibition did not prevent AQP3 protein production, indicating that *S. miltiorrhiza*-induced AQP3 regulation may be modulated by JNK-independent factors. The second-messenger cyclic adenosine monophosphate (cAMP) has been implicated in the up-regulation of *AQP3* expression in the amnion (10) and might be a candidate modulator of physiological effects of *S. miltiorrhiza*. However, the exact mechanisms involved in *S. miltiorrhiza*-regulated AQP3 production in hAECs require further investigation.

6. CONCLUSIONS

In conclusion, the present study demonstrates for the first time SP600125 regulation of hAEC AQP3 protein levels and a regulatory link between the JNK pathway and AQP3 in isolated oligohydramnios material *in vitro*. Furthermore, this is also the first report of the involvement of the JNK pathway in the up-regulation of AQP3 by *S. miltiorrhiza* in hAECs derived from isolated oligohydramnios tissues. Other mechanisms may be implicated in *S. miltiorrhiza*-associated alteration of AQP3 levels in hAECs and they require further study.

These results encourage the continued evaluation of JNK pathway activation as a potential therapeutic target for oligohydramnios.

7. ACKNOWLEDGEMENTS

Qi Shen and Jingjing Wang contributed equally to this work and they are the first co-authors. This work was supported by grants from Zhejiang Province Administration of Traditional Chinese Medicine Key Projects (No. 2010ZZ009) and Zhejiang Provincial Program for the Cultivation of High-Level Innovative Health Talents. The study sponsors had no involvement in study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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Abbreviations: AF: Amniotic fluid; AFV: amniotic fluid volume; AQPs: aquaporins; hAECs: human primary culture amnion epithelium cells; MAPKs: Mitogen-activated protein kinases; JNK: C-jun N-terminal kinase; AFI: AF index; PBS: phosphate buffered saline; CCK-8: cell counting kit-8.

Key Words: Isolated Oligohydramnios, Human Amnion Epithelium Cells, Aquaporin3, Salvia Miltiorrhiza, JNK Signal Transduction Pathway

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