

Danshen extract regulates the expression of aquaporin 3 in human amniotic epithelial cells

Wenwen Zhang¹, Yiwen Zhang¹, Xiaoli Hu¹, Huihui Cheng¹, Ying Hua¹, Xueqiong Zhu¹

¹Department of Obstetrics and Gynecology, the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325027, China

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Tissue specimens
 - 3.2. Culture of human amniotic epithelial cells
 - 3.3. Immunocytochemical analysis of cultured human amniotic epithelial cells
 - 3.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)
 - 3.5. Western blotting
 - 3.6. Statistical analysis
4. Results
 - 4.1. Immunocytochemical identification
 - 4.2. Maternal characteristic of two groups
 - 4.3. AQP3 mRNA and protein expression in two groups
 - 4.4. Alteration of AQP3 expression after treatment with various concentrations of Danshen extract
 - 4.5. Alteration of AQP3 expression after treatment with Danshen extract for various times
 - 4.6. Alteration of AQP3 expression after treatment with optimal concentration and time of Danshen extract
5. Discussion
6. Acknowledgments
7. References

1. ABSTRACT

Danshen extract has been used in the treatment of oligohydramnios, however, the mechanism of its action has not been elucidated. Previously, we demonstrated that down-regulation of AQP3 in fetal membranes may contribute to the development of oligohydramnios. In this study, we investigated the effects of Danshen extract on AQP3 expression in human amniotic epithelial cells from term pregnancies with oligohydramnios or those with those with (those with) normovolemic amniotic fluid. Human amniotic epithelial cells from the oligohydramnios group expressed a lower level of AQP3 mRNA and protein than those with normovolemia. Twelve hour (Twelvehours) of treatment with Danshen extract, in a dose dependent manner, significantly increased the expression of AQP3 in the two groups. However, human amniotic epithelial cells from the oligohydramnios patients showed a greater sensitivity to the treatment of Danshen extract. These data provide a molecular basis for the treatment of patients with oligohydramnios.

2. INTRODUCTION

Oligohydramnios is a condition during pregnancy characterized by a deficiency of amniotic fluid (1). It is reported that the incidence rate of oligohydramnios ranges from 1% to 5% in all full-term pregnancies (2). The risk of oligohydramnios is associated with increased perinatal morbidity and mortality caused by umbilical cord compression, fetal distress, meconium staining, and meconium aspiration syndrome (3). But the molecular mechanisms of oligohydramnios remain unclear. As a result, there is no effective method to prevent or treat oligohydramnios.

Danshen extract, a traditional Chinese herbal medicine, has been used to treat cardiovascular disease, chronic renal insufficiency, and intrahepatic cholestasis for at least 30 years all over the world (4-7). Danshen extract has been known to possess multiple pharmacological activities such as anti-inflammatory, anti-oxidative and organ protective effects (8). Danshen extract has been used for treating oligohydramnios for

decades (9). Although efficacy of Danshen extract therapy for oligohydramnios has been proven in clinical research, only effective for some patients and the molecular mechanisms responsible for the beneficial effects of Danshen extract on oligohydramnios remain poorly understood.

As a large family of membrane proteins, the aquaporins (AQPs) play a key role in the selective transportation of small neutral solutes and water with 13 homologous members, named AQP 0 to 12 in mammals (10). AQPs increase cell plasma membrane water permeability five to fifty times in comparison with membranes in which water passes through the lipid bilayer primarily (11). In mammals, the AQPs are found in endothelium, fluid-transporting epithelium and in other tissues (eg. leukocytes) (12). It was first reported by Wang *et al.* (13) that AQP3 was expressed in human fetal membranes, which was subsequently confirmed by our study (14). In our previous study (14), it was also found that AQP3 expression in amnion from oligohydramnios was downregulated as compared to that in amnion from normal pregnancies, suggesting that decreased expression of AQP3 in fetal membrane may contribute to oligohydramnios. Based on this observation, we hypothesized that AQP3 in human fetal membranes may be a target for the treatment of oligohydramnios.

Several studies showed that Danshen extract may play a crucial role in regulating the AQPs expression in various tissues. Li *et al.* (15) demonstrated that the treatment with tanshinone IIA, one of the main active components from Danshen extract, could significantly alleviate seawater exposure-induced acute lung injury in rats through the inhibition of AQP1 and AQP5 over-expression in injured lungs probably. However, Lithospermic acid B, another active component from Danshen extract had an ameliorative effect on renal functional parameters associated with AQP2 over-expression in the ischemia/reperfusion-induced acute renal failure in rats (16). Thus, the efficacy of Danshen extract for oligohydramnios may be related to the expression of AQPs in fetal membranes.

The present study aims to investigate the effect of Danshen extract on AQP3 expression in human amniotic epithelial cells from term pregnancies with oligohydramnios or normal amniotic fluid volume. The time and dose response curves of Danshen extract mediated AQP3 regulation are also constructed.

3. MATERIALS AND METHODS

3.1. Tissue specimens

The study has received ethical approval from our institutional Research Ethical Committee (No: 2010035) with written informed consent acquired from

patients prior to this research. Between June 2010 and December 2012, twenty-three women having elective cesarean deliveries due to social factors within our division of obstetrics, were consented for the study, in which ten pregnant women suffered from oligohydramnios and others had normal amniotic fluid volume. All patients aged between 22 and 36 years old and had a pregnancy from thirty-seven to forty weeks of gestational age. Those pregnancies with premature rupture of the membrane, echographically confirmed fetal abnormalities, fetal growth restriction or small-for-gestational-age infant were excluded. We also not included the patients having other disorders that may influence amniotic fluid volume, eg., hypertensive diseases, pre-gestational or gestational diabetes, autoimmune disease or cardiovascular disorders.

The amniotic fluid index (AFI) was measured with ultrasound before delivery. AFI was determined by the sum of maximum vertical pocket diameter of amniotic fluid, in each of the four quadrants of the uterus free from cord or fetal extremities. Amniotic fluid volume measurements were performed in triplicates and averaged in order to minimize errors. Oligohydramnios was defined as an AFI no more than 5 cm (1), while an AFI of 8 cm to 18.0 cm was characterized as normal AFI.

At time of cesarean delivery, the amniotic fluid volume was reevaluated with the technique reported in our previous study (14). To facilitate the amniotic fluid collection, large plastic pockets with plastic drapes were used at abdominal delivery. The amniotic fluid was inhaled into a suction collection device, which was in addition to that used to suck irrigating fluid or blood during the operation. The amniotic fluid was continued to be suctioned until it stopped flowing. Following the delivery of fetus, the remaining amniotic fluid in the operative field, drape pockets, and uterus was collected before the placenta was delivered. A tube containing ethylene diamine tetraacetic acid was used to collect 3ml fluid specimen. To ascertain the blood contamination volume, the collected specimen was used to measure the hemoglobin concentration. The blood volume in the amniotic fluid was calculated by using the patient's preoperative hemoglobin, which was deducted from the total fluid in order to get the calibrated amniotic fluid volume. Normal amniotic fluid volume was described as an amniotic fluid volume 300 ml to 2000 ml, while oligohydramnios was considered as an amniotic fluid volume less than 300 ml (17). The patients whose AFI measured before operation were discordant with the amniotic fluid volume evaluated at delivery were excluded.

3.2. Culture of human amniotic epithelial cells

After delivery, amnion were carefully isolated from chorion and cut broken with scissors. Small

pieces of tissue were trypsinized by using 0.2.5% trypsin (Sigma, St. Louis, MO) for 1 h. The cell suspension was subjected to centrifugation at 500 g (10 min) and then the resultant supernatant was centrifuged at 2,500 g for 20 min. The cells were plated on 100-mm plastic culture dishes and maintained in Dulbecco's Modified Eagle Medium (Gibco BRL Life Technologies, USA) containing 1% L-glutamine, 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) at 37°C in 95% air/5% CO₂ incubator. The cells were digested by using 0.2.5% trypsin and split into new dishes.

The treatment with Danshen extract (Shanghai New Asia Pharmaceutical Gaoyou Co. Ltd., China, batch number: 101227-3, specifications: 2 ml) was carried out according to the following steps. After the cell confluence reached 80%, cells were treated with various concentrations of Danshen extract (0 mg/ml, 0.5. mg/ml, 5 mg/ml, 10 mg/ml, 30 mg/ml or 50 mg/ml) for 24 h. In addition, cells were incubated with 5 mg/ml of Danshen extract for 0, 6, 12, 24, 48 h, respectively.

3.3. Immunocytochemical analysis of cultured human amniotic epithelial cells

Purity of the cell preparation was assessed before treatment by immunocytochemistry. Briefly, the human amniotic epithelial cells were fixed with 4% paraformaldehyde for 20 min, and then permeabilized for 20 min in 0.3.% Triton-X100. The human amniotic epithelial cells were then incubated in 0.3.% hydrogen peroxide in methanol for 15 min to terminate endogenous peroxidase activity, and blocked non-specific binding with 1.5.% non-immune goat serum for 15 min. Then the cells were incubated with an anti-cytokeratin 18 antibody (Sigma St. Louis, MO), at a dilution of 1:200 at 4 °C overnight. After incubation with biotinylated goat anti-rabbit secondary antibodies at room temperature for 30 min, streptavidin peroxidase was added for an additional 10 min and diaminobenzidine as chromogen for 1 to 2 min. The cells were washed with phosphate buffered saline after every step of the experiment, and counterstained with Mayer hematoxylin, dehydrated and mounted. For negative controls, the cells were incubated with phosphate buffered saline without the primary antibody.

3.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from human amniotic epithelial cells using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Using the M-MLV (200 U/μl, Fermentas, Hanover, MD), 2 μg total RNA was reversely transcribed, according to the manufacturer's instructions. The sequences of primers used were as follows: AQP3 primer

(forwards: AGGATGGCGGTTGGTCTATG, reverse: TGGCTTTGCTGTCACTCTGG), β-actin primer (forward: GCTGGGGTGTGAAGGTCTC, reverse: ACAGACTCCCCATCCCAAGA). PCR was performed for 35 cycles of 94 °C denaturing for 30 seconds, 65 °C annealing for 30 seconds, and extension at 72 °C for 30 seconds, the final extension was performed at 72 °C for 10 min. The RT-PCR product was electrophoresed in 3% agarose gel, and subsequently by Quantity-One densitometry software package (BioRad, Hercules, CA). All experiments were performed in triplicates. AQP3/β-actin pixel signal ratio was used to represent the level of AQP3 gene expression.

3.5. Western blotting

The protein samples were extracted from the whole cells of human amniotic epithelium with the procedures as followed. The cells were lysed with lysis buffer (PH 7.5., 20 mM Tris, 1 mM ethylene-diaminetetra-acetate, 2.5. mM sodium pyrophosphate, 150 mM NaCl, 1% Na₃VO₄, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 0.5. μg/ml leupeptin), and further broken by ultrasound. For maximal protein extraction, the lysed cell solution was kept on the ice for 30 min. It was centrifuged and the supernatant was collected. The concentration of protein was measured in the supernatant using the bicinchoninic acid procedure (Pierce Biotechnology, Rockford, USA) as recommended by the manufacturer. After boiling the samples for 5 min (stored at -20 °C), the protein samples (8 μg) were separated by 10% polyacrylamide gel (Bio-Rad Laboratories, Hercules, USA) electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane were then blocked for the nonspecific binding sites by 5% non-fat milk in tris-buffered saline containing 0.1.% Tween for 2 h at room temperature and equilibrated in tris-buffered saline with 0.1.% Tween three times for 10 min. The membranes were then incubated overnight at 4 °C with anti-AQP3 polyclonal rabbit antibody (Santa Cruz Biotechnology Inc, CA, USA) at a dilution of 1:300. Membranes were washed and incubated with a secondary rabbit polyclonal antibody conjugated to horseradish peroxidase at room temperature for 1 h. After washing, blots were treated with enhanced chemiluminescence reagent (Amersham Life Technologies, Aylesbury, UK) and exposed. Using Quantity-One densitometry software package, western blot bands were quantified by measuring the band intensity (area × OD) for each group and normalizing to tubulin (diluted at 1:1000) as an internal control. The western blot analysis was performed in triplicates.

3.6. Statistical Analysis

The analysis was performed by using SPSS 13.0. statistical software package (Chicago, IL, USA). The Kolmogorov-Smirnov test of normality was

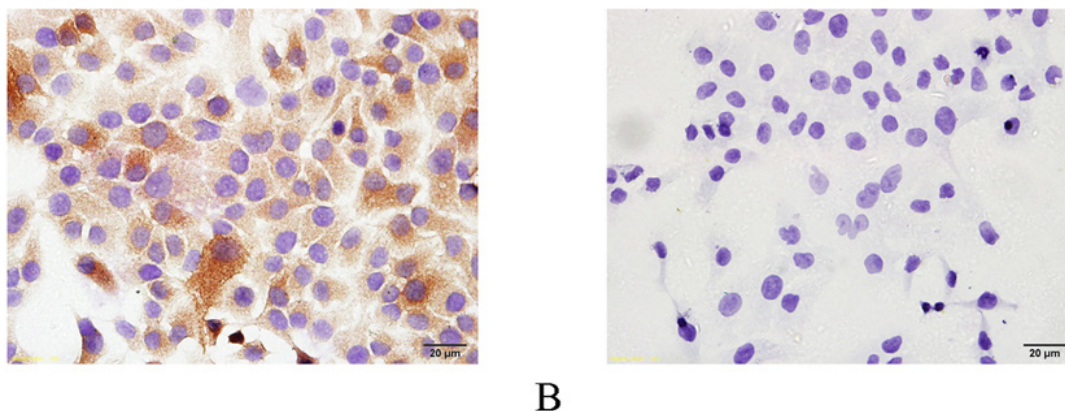


Figure 1. Immunocytochemical staining of human amniotic epithelial cells for the expression of cytokeratin 18. (A) Positive staining of cytokeratin 18 in human amniotic epithelial cells (SP staining, $\times 400$). The majority of the cells ($> 90\%$) stained positive in cytoplasm for cytokeratin 18 confirming that they are human amniotic epithelial cells. (B) Negative control. (SP staining, $\times 400$).

Table 1. Maternal characteristics of oligohydramnios and normal amniotic fluid volume groups

Variable	Normal amniotic fluid volume (n=13)	Oligohydramnios (n=10)
Maternal age (years)	27.9 \pm 1.6.	28.4 \pm 2.2. ¹
Gestational age (weeks)	39.1 \pm 1.2.	38.9 \pm 0.7. ¹
Parity (percentage of nulliparous)	90	93.3. ¹
Weight (kg)	70.8 \pm 3.9.	71.0 \pm 4.5. ¹

¹: no significant difference when compared with normal amniotic fluid volume group ($P > 0.0.5$).

applied. Continuous variables normal distribution is shown as means \pm standard deviation.

The Student's unpaired t-test was performed to compare the variables (such as gestational age, maternal age and weight) between two groups, while the non-parametric tests either Kruskal-Wallis test or Mann-Whitney U test was used for evaluating non-normal distribution variables. A 2-sided P value of less than 0.0.5 was considered statistically significant.

4. RESULTS

4.1. Immunocytochemical identification

Expression of cytokeratin 18 was used to check the purity of the isolated human amniotic epithelial cells. The majority of the cells ($> 90\%$) stained positive for cytokeratin 18 as brown color in cytoplasm confirming that they were human amniotic epithelial cells (Figure 1).

4.2. Maternal characteristic of two groups

All participants in the study met the inclusion criteria. The maternal demographics, eg., age, gestational age at the time of delivery and weight, are listed in Table 1. There was no significantly different in these variables between the two groups.

4.3. AQP3 mRNA and protein expression in two groups

In comparison to pregnancies with normal amniotic fluid volume group, there were significant decreases of AQP3 mRNA and protein in oligohydramnios group ($P < 0.0.5$), which are shown in Figure 2 and 3.

4.4. Alteration of AQP3 expression after treatment with various concentrations of Danshen extract

To elucidate the effects of Danshen extract on AQP3 mRNA expression in human amniotic epithelial cells, the AQP3 mRNA levels after the treatment with various concentrations of Danshen extract (0, 0.5, 5, 10, 30 or 50 mg/ml) for 24 h were measured by RT-PCR. AQP3 mRNA levels in human amniotic epithelial cells of both groups reached the peak when the concentration of Danshen extract was 5 mg/ml, and then decreased with its concentrations higher than 5 mg/ml, as shown in Figure 4.

Similarly, treatment with Danshen extract at 5 mg/ml for 24 h significantly increased AQP3 protein in the two groups and reached the peak, then decreased with the concentrations higher than 5 mg/ml ($P < 0.0.5$), which are presented in Figure 5.

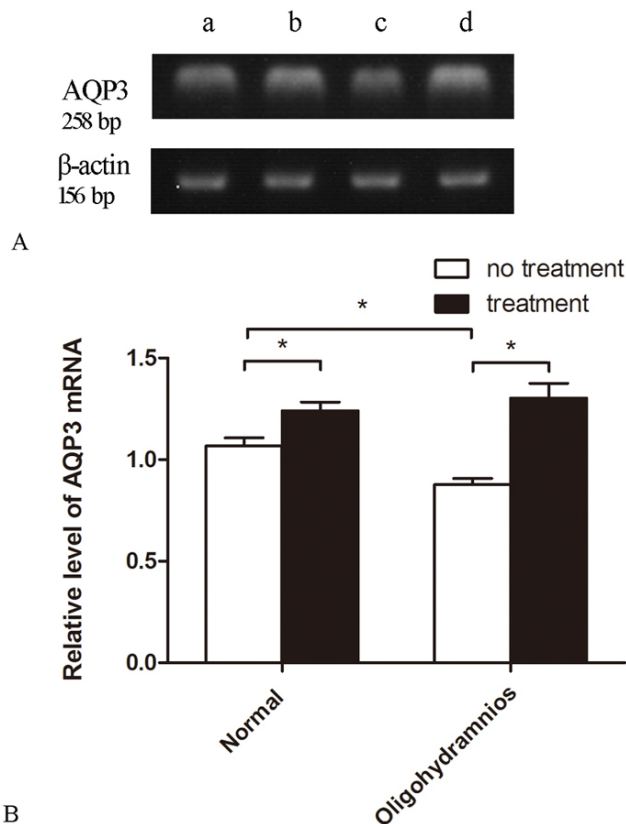


Figure 2. Effect of Danshen extract on AQP3 mRNA expression in primary culture of the human amniotic epithelial cells from donors with normal amniotic fluid volume or with oligohydramnios. (A) The expression of AQP3 mRNA in the human amniotic epithelial cells after treatment with Danshen extract at 5 mg/ml for 12 h. AQP3 mRNA expression was detected by RT-PCR. β-actin level was used as internal controls. a: Normal amniotic fluid volume group, without treatment; b: Normal amniotic fluid volume group, with Danshen extract; c: Oligohydramnios group, without treatment; d: Oligohydramnios group, with Danshen extract. (B) Densitometer analysis results. The data are presented as relative expression level of AQP3 mRNA. Normal: Normal amniotic fluid volume group. Each bar represents mean ± standard deviation of 6 independent experiments (* indicates $P < 0.05$).

4.5. Alteration of AQP3 expression after treatment with Danshen extract for various times

The expression of AQP3 mRNA showed a biphasic change when human amniotic epithelial cells from the two groups were treated for different times (0, 6, 12, 24 or 48 h) with Danshen extract at 5 mg/ml. It was increased in a time-dependent manner up to 12 h of treatment and then steadily decreased afterwards (Figure 6). Similarly, Danshen extract at 5 mg/ml caused AQP3 protein expression in human amniotic epithelial cells from the two groups to be at the peak after being treated for 12 h, and then decreased between 12 h and 48 h as shown in Figure 7.

4.6. Alteration of AQP3 expression after treatment with optimal concentration and time of Danshen extract

After treated with optimal concentration and time of Danshen extract, which was 5 mg/ml concentration for 12 h, both AQP3 mRNA and AQP3 protein expression increased obviously in oligohydramnios group than that in normal amniotic

fluid volume group ($P < 0.05$), as shown in Figure 2 and 3.

5. DISCUSSION

Since AQP3 exists in fetal membranes and is characterized as a water channel, it is reasonable to speculate that AQP3 can mediate fluid resorption. Prat *et al.* (18) investigated the expression of AQP3 in amnion and chorion throughout the first, second, and third trimesters of pregnancy. They found a sharp increase in AQP3 expression from 12 to 26 weeks of gestation. However, weak expression of AQP3 was observed during the third trimester of pregnancy. The average amniotic fluid volume increases from 20 ml to 630 ml in the course of pregnancy from 10 weeks to 22 weeks of gestation progressively and reaches 770 ml at 28 weeks of gestation, but there is little subsequent change between 29 and 37 weeks of gestation (19). Thus, the expression of AQP3 in fetal membrane appears to be positively correlated with amniotic fluid volume. In our previous study (14), AQP3 expression in amnion with oligohydramnios was significantly downregulated in comparison with

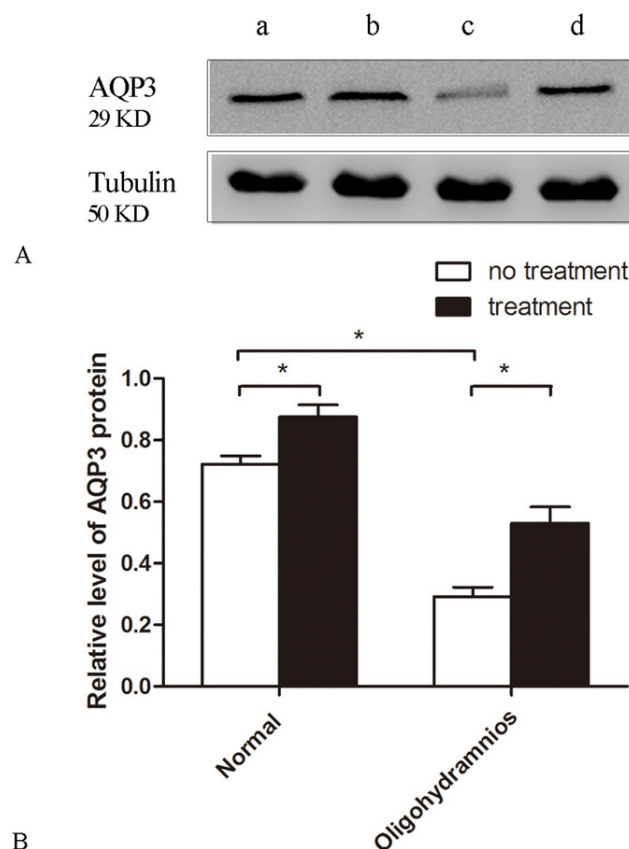


Figure 3. Effect of Danshen extract on AQP3 protein expression in primary culture of human amniotic epithelial cells from donors with normal amniotic fluid volume or with oligohydramnios. (A) The expression of AQP3 protein in the human amniotic epithelial cells after treated with Danshen extract at 5 mg/ml for 12 h. AQP3 protein expression was detected by Western blotting. Tubulin was used as internal controls. a: Normal amniotic fluid volume group, without treatment; b: Normal amniotic fluid volume group, with Danshen extract; c: Oligohydramnios group, without treatment; d: Oligohydramnios group, with Danshen extract. (B) Densitometer analysis results. The relative expression level of AQP3 protein. Normal: Normal amniotic fluid volume group. Each bar represents mean \pm standard deviation of 6 independent experiments (* indicates $P < 0.05$).

that in normal amniotic fluid volume group. In the present study, using human amniotic epithelial cells primary culture, we investigated AQP3 expression and found that compared with normal amniotic fluid volume group, there was also a modest decrease of AQP3 expression in human amniotic epithelial cells with oligohydramnios consistent with our previous findings (14).

Anti-symptomatic treatment, such as trans-abdominal amniocentesis or maternal hydration, has been applied for treating polyhydramnios or oligohydramnios, respectively. There is no target therapy against the cause of abnormal amniotic fluid volume. Danshen extract, has been used to improve blood circulation and treating vascular diseases (4-5). It was reported that Danshen extract could protect hepatocytes and myocardial cells against oxidative damage (20-21). As a traditional Chinese medicine, it has been to treat oligohydramnios for decades. Chu *et al.* (9) identified that Danshen extract was an effective medicine to increase the amniotic fluid volume in pre-term patients suffering oligohydramnios, and the

absolute (increased fluid) effective rate of Danshen extract was approximately 70%. But the molecular pathway responsible for the beneficial effects of Danshen extract on oligohydramnios has not been elucidated. Our study showed that AQP3 expression in human amniotic epithelial cells from pregnancies with normal amniotic fluid volume or oligohydramnios was increased after being treated with Danshen extract, providing molecular mechanisms through which Danshen extract exert its regulatory function in human amniotic epithelial cells.

In the present study, we observed biphasic reaction of human amniotic epithelial cells to Danshen extract. The maximal stimulation of AQP3 expression in human amniotic epithelial cells by the treatment with Danshen extract was observed at 5 mg/ml for 12 h. The effect was then decreased when the concentration was higher than 5 mg/ml or the treatment time was longer than 12 h. It is suggest that Danshen extract regulate the AQP3 expression in a short time. Further increasing of Danshen extract concentration beyond 5 mg/ml might be harmful to the cells.

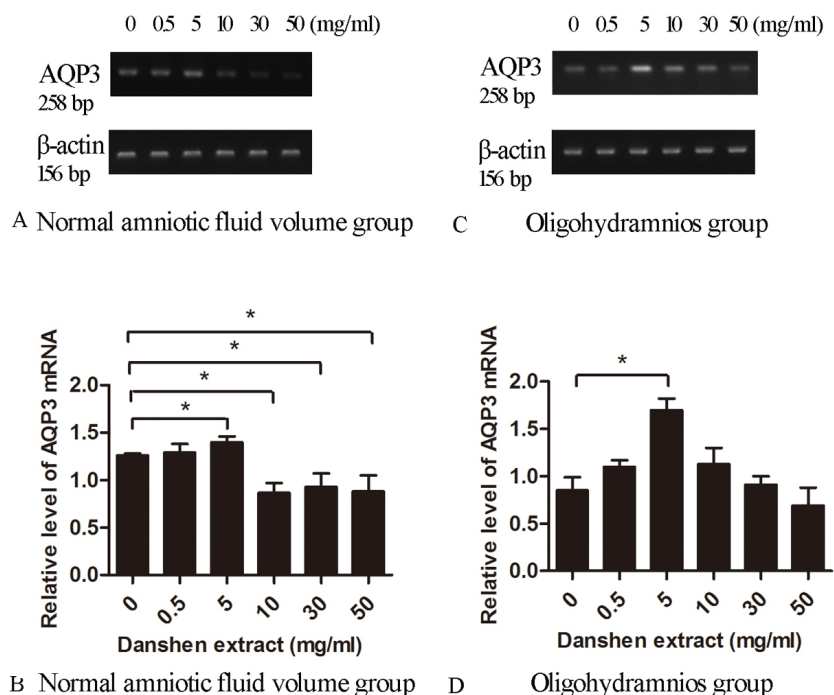


Figure 4. AQP3 mRNA expression in human amniotic epithelial cells from donors with normal amniotic fluid volume or with oligohydramnios before and after treatment with various concentrations of Danshen extract. (A-B) The expression of AQP3 mRNA in the human amniotic epithelial cells from normal amniotic fluid volume after treatment with Danshen extract at 0, 0.5, 5, 10, 30, or 50 mg/ml for 24 h. AQP3 mRNA expression was detected by RT-PCR. β -actin level was used as internal controls. The data are presented as relative expression level of AQP3 mRNA. (C-D) The expression of AQP3 mRNA in the human amniotic epithelial cells from oligohydramnios after treatment with Danshen extract at 0, 0.5, 5, 10, 30, or 50 mg/ml for 24 h. The data are presented as relative expression level of AQP3 mRNA. Each bar represents mean \pm standard deviation of 6 independent experiments (* indicates $P < 0.05$).

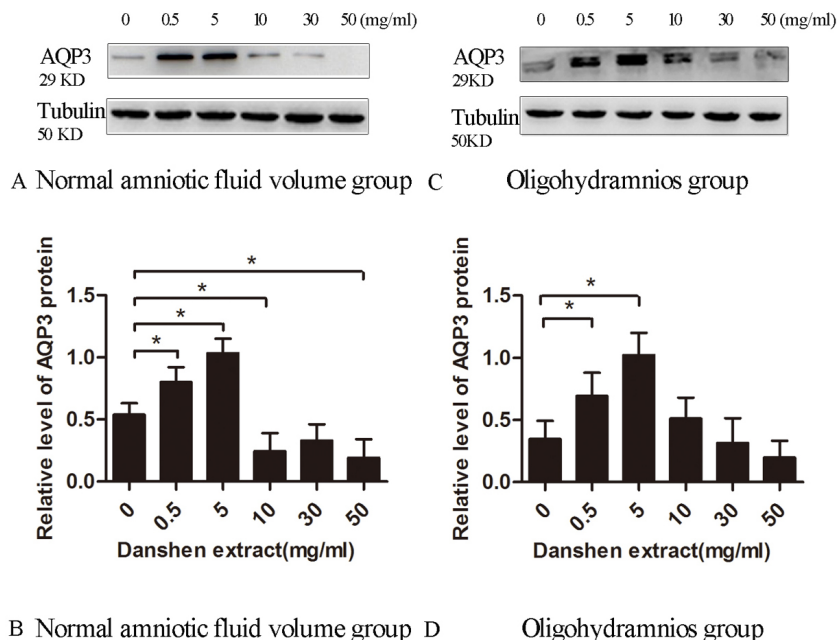


Figure 5. AQP3 protein expression in human amniotic epithelial cells from donors with normal amniotic fluid volume or with oligohydramnios before and after treatment with various concentrations of Danshen extract. (A-B) The expression of AQP3 protein in the human amniotic epithelial cells from normal amniotic fluid volume after treatment with Danshen extract at 0, 0.5, 5, 10, 30, or 50 mg/ml for 24 h. AQP3 protein expression was analyzed by Western blotting. Tubulin was used as internal controls. The data are presented as relative expression level of AQP3 protein. (C-D) The expression of AQP3 protein in the human amniotic epithelial cells from oligohydramnios after treatment with Danshen extract at 0, 0.5, 5, 10, 30, or 50 mg/ml for 24 h. The data are presented as relative expression level of AQP3 protein. A P value less than 0.05 was considered as statistically significant. Each bar represents mean \pm standard deviation of 6 independent experiments (* indicates $P < 0.05$).

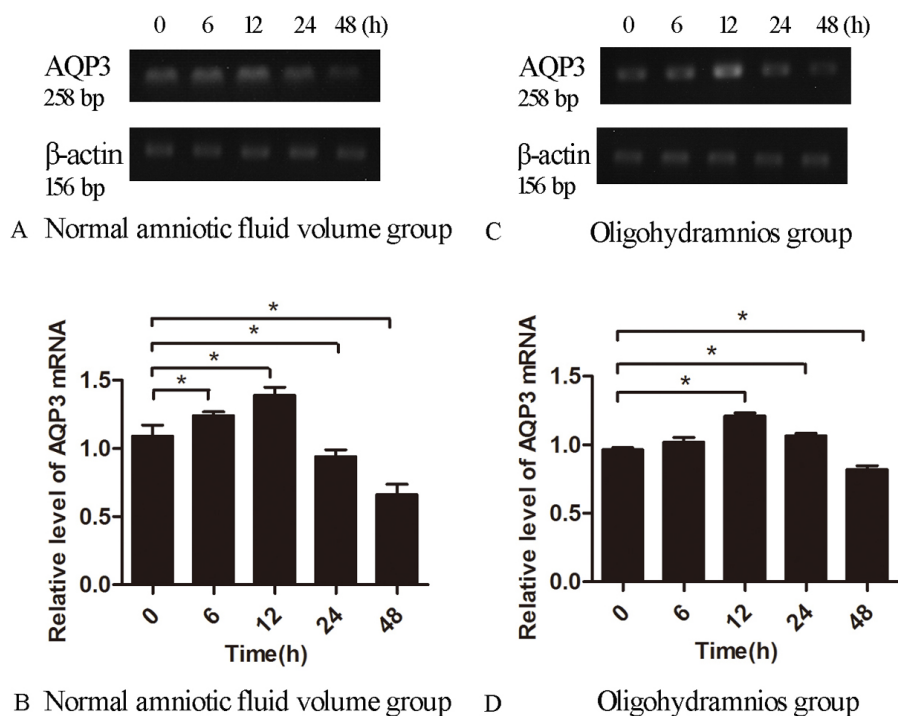


Figure 6. AQP3 mRNA expression in human amniotic epithelial cells from donors with normal amniotic fluid volume or with oligohydramnios before and after treatment with Danshen extract for various times. (A-B) The expression of AQP3 mRNA in human amniotic epithelial cells from pregnancies with normal amniotic fluid volume after treatment with Danshen extract for 0, 6, 12, 24, or 48 h at 5 mg/ml. The relative expression level of AQP3 mRNA is presented. (C-D) The expression of AQP3 mRNA in human amniotic epithelial cells from pregnancies with oligohydramnios after treatment with Danshen extract for 0, 6, 12, 24, or 48 h at 5 mg/ml. The data are presented as relative expression level of AQP3 mRNA. Each bar represents mean \pm standard deviation of 6 independent experiments. (* indicates $P < 0.05$)

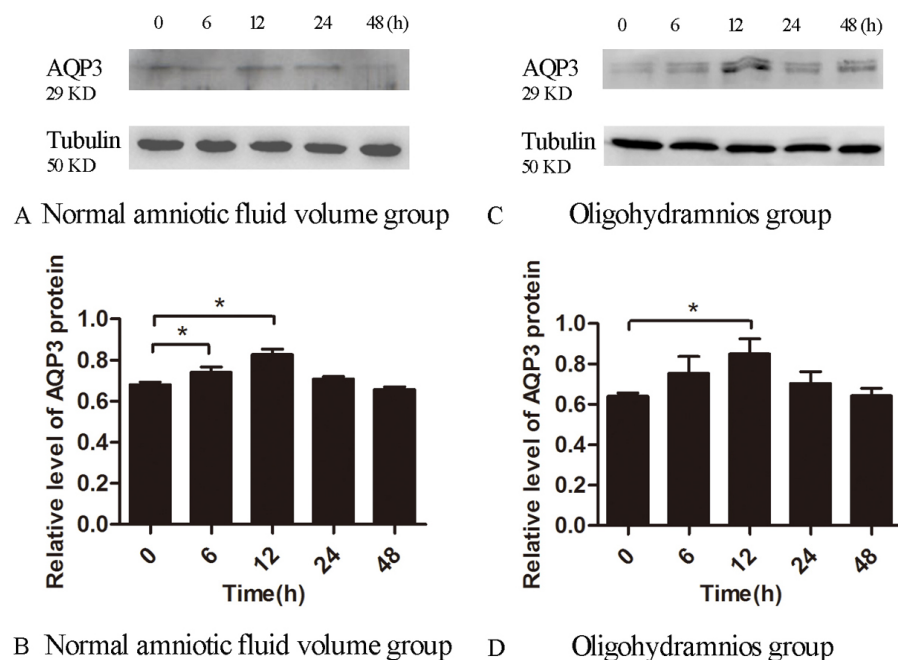


Figure 7. AQP3 protein expression in human amniotic epithelial cells from donors with normal amniotic fluid volume or with oligohydramnios before and after treatment with Danshen extract for various times. (A-B) The expression of AQP3 protein in human amniotic epithelial cells from donors with normal amniotic fluid volume after treatment with Danshen extract for 0, 6, 12, 24, or 48 h at 5 mg/ml. The relative expression level of AQP3 protein is presented. (C-D) The expression of AQP3 protein in human amniotic epithelial cells from donors with oligohydramnios after treatment with Danshen extract for 0, 6, 12, 24, or 48 h at 5 mg/ml. The data are presented as relative expression level of AQP3 protein. Each bar represents mean \pm standard deviation of 6 independent experiments. (* indicates $P < 0.05$)

Compared with normal amniotic fluid volume group, the human amniotic epithelial cells from oligohydramnios patients appeared a bit more sensitive to Danshen extract. This provides the reason for clinical application of Danshen extract in treating patients with oligohydramnios. Further investigations are necessary to explore the signal transduction pathway(s) regulating the expression of AQP3 in human amniotic epithelial cells by Danshen extract.

In summary, we found that compared with human amniotic epithelial cells from donors with normal amniotic fluid volume; there was a modest decrease of AQP3 expression in human amniotic epithelial cells from oligohydramnios patients. In addition, we demonstrated that Danshen extract could regulate the AQP3 expression in human amniotic epithelial cells with normal amniotic fluid volume or with oligohydramnios. The optimal concentration and time for Danshen extract to stimulate AQP3 expression in human amniotic epithelial cells was 5 mg/ml for 12 h. These findings shed new insights on the bioactivity of Danshen extract in human amniotic epithelial cells, which explains the beneficial effects of Danshen extract for oligohydramnios.

6. ACKNOWLEDGMENTS

This work was supported by grants from Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents. The study sponsors had no involvement in the collection, analysis and interpretation of data and in the writing of the manuscript.

7. REFERENCES

- Phelan JP, Smith CV, Broussard P, Small M: Amniotic fluid volume assessment with the four-quadrant technique at 36-42 weeks' gestation. *J Reprod Med* 32, 540-542. (1987)
- Moore TR: Clinical Assessment of Amniotic Fluid. *Clin Obstet Gynecol* 40, 303-313. (1997)
DOI: 10.1097/00003081-199706000-00007
- Manzanares S, Carrillo MP, Gonza' lez-Pera'n E, Puertas A, Montoya F: Isolated oligohydramnios in term pregnancy as an indication for induction of labor. *J Matern Fetal Neonatal Med* 20, 221-224. (2007)
DOI: 10.1080/14767050601127391
- Adams JD., Wang R, Yang J, Lien EJ: Preclinical and clinical examinations of Salvia miltiorrhiza and its tanshinones in ischemic conditions. *Chin Med* 1, 663-694. (2006)
DOI: 10.1186/1749-8546-1-3
- Sun J, Huang SH, Tan BK, Whiteman M, Zhu YC, Wu YJ, Ng Y, Duan W, Zhu YZ: Effects of purified herbal extract of salvia miltiorrhiza on ischemic rat myocardium after acute myocardial infarction. *Life Sci* 76, 2849-2860. (2005)
DOI: 10.1016/j.lfs.2004.11.016
- Ahn YM, Kim SK, Lee SH, Ahn SY, Kang SW, Chung JH, Kim SD, Lee BC: Renoprotective effect of Tanshinone IIA, an active component of Salvia miltiorrhiza, on rats with chronic kidney disease. *Phytother Res* 24, 1886-1892. (2010)
DOI: 10.1002/ptr.3347
- Oh SH, Nan JX, Sohn D-, Kim YC, Lee BH: Salvia miltiorrhiza inhibits biliary obstruction-induced hepatocyte apoptosis by cytoplasmic sequestration of p53. *Toxicol Appl Pharmacol* 182, 27-33. (2002)
DOI: 10.1006/taap.2002.9367
- Wang Y, Wang XX, Zhang LN, Jin SM, Zhang J: Effects of traditional Chinese medicine on bone remodeling during orthodontic tooth movement. *J Ethnopharmacol* 141, 642-646. (2012)
DOI: 10.1016/j.jep.2011.09.003
- Chu HN, Shen MJ: Treating oligohydramnios with extract of salvia miltiorrhiza: a randomized control trial. *Ther Clin Risk Manag* 4, 287-290. (2008)
- Wang F, Feng XC, Li YM, Yang H, Ma TH: Aquaporins as potential drug targets. *Acta Pharmacol Sin* 27, 395-401. (2006)
DOI: 10.1111/j.1745-7254.2006.00318.x
- Damiano AE: Review: Water channel proteins in the human placenta and fetal membranes. *Placenta* 32 Suppl 2, S207-211. (2011)
DOI: 10.1016/j.placenta.2010.12.012
- Takata K, Matsuzaki T, Tajika Y: Aquaporins: water channel proteins of the cell membrane. *Prog Histochem Cytochem* 39, 1-83. (2004)
DOI: 10.1016/j.proghi.2004.03.001
- Wang S, Amidi F, Beall M, Gui L, Ross MG: Aquaporin 3 expression in human fetal membranes and its up-regulation by cyclic adenosine monophosphate in amnion epithelial cell culture. *J Soc Gynecol Investig* 13, 181-185. (2006)
DOI: 10.1016/j.jsjg.2006.02.002

14. Zhu XQ, Jiang SS, Zhu XJ, Zou SW, Wang YH, Hu YC: Expression of aquaporin 1 and aquaporin 3 in fetal membranes and placenta in human term pregnancies with oligohydramnios. *Placenta* 30, 670–676. (2009)
DOI: 10.1016/j.placenta.2009.05.010
15. Li J, Xu M, Fan Q, Xie X, Zhang Y, Mu D, Zhao P, Zhang B, Cao F, Wang Y, Jin F, Li Z: Tanshinone IIA ameliorates seawater exposure-induced lung injury by inhibiting aquaporins (AQP) 1 and AQP5 expression in lung. *Respir Physiol Neurobiol* 176, 39–49. (2011)
DOI: 10.1016/j.resp.2011.01.005
16. Kang DG, Oh H, Sohn EJ, Hur TY, Lee KC, Kim KJ, Kim TY, Lee HS: Lithospermic acid B isolated from *Salvia miltiorrhiza* ameliorates ischemia/reperfusion-induced renal injury in rats. *Life Sci* 75, 1801–1816. (2004)
DOI: 10.1016/j.lfs.2004.02.034
17. Croom CS, Banias BB, Ramos-Santos E, Devoe LD, Bezhadian A, Hiatt AK: Do semiquantitative amniotic fluid indexes reflect actual volume? *Am J Obstet Gynecol* 167, 995–999. (1992)
DOI: 10.1016/S0002-9378(12)80026-3
18. Prat C, Blanchon L, Borel V, Gallot D, Herbet A, Bouvier D, Marceau G, Sapin V: Ontogeny of aquaporins in human fetal membranes. *Biol Reprod* 86, 48. (2012)
DOI: 10.1095/biolreprod.111.095448
19. Wang S, Chen J, Beall M, Zhou W, Ross MG: Expression of aquaporin 9 in human chorioamniotic membranes and placenta. *Am J Obstet Gynecol* 191, 2160–2167. (2004)
DOI: 10.1016/j.ajog.2004.05.089
20. Li HY, Li Y, Yan CH, Li LN, Chen XG: Inhibition of tumor growth by S-3-1, a synthetic intermediate of salvianolic acid A. *J Asian Nat Prod Res* 4, 271–280. (2002)
DOI: 10.1080/1028602021000049069
21. Liu J, Shen HM, Ong CN: Role of intracellular thiol depletion, mitochondrial function and reactive oxygen species in *Salvia miltiorrhiza*-induced apoptosis in human hepatoma HepG2 cells. *Life Sci* 69, 1833–1850. (2001)
DOI: 10.1016/S0024-3205(01)01267-X

Key Words: Aquaporin 3, oligohydramnios, amniotic epithelial cells, Danshen

Send correspondence to: Xueqiong Zhu, No.109 Xueyuan Xi Road, Department of Obstetrics and Gynecology, the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325027, China, Tel: 86-577-88002796, Fax: 86-577-88832693, E-mail: zjwzzxq@yeah.net

Abbreviations: AQP3, aquaporin 3; AFI, amniotic fluid index