

Original Article

San-Ao Decoction (三拗汤) Regulates Urine Volume on Bronchial Asthma Model Mice*

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ABSTRACT **Objective:** To observe the effect of San-Ao Decoction (三拗汤, SAD) on water metabolism of bronchial asthma model mice. **Methods:** Forty-five female BALB/c mice were randomly divided into control, model and SAD groups by a random number table, 15 mice in each group. A composite method with ovalbumin (OVA) sensitization and challenge was developed to establish bronchial asthma model. Mice in the control group were intraperitoneally injected with distilled water without aerosol inhalation challenge. On day 15–22, 0.3 mL SAD was administered via gastric route in SAD group, one time per day, while an equivalent volume of normal saline was used for gastric administration in the control and model groups. Changes in airway resistance in the inspiratory phase (RI-R-Area) were detected using an AniRes2005 system, and 5-h urine output was collected by metabolic cages. Histopathological changes in lung and kidney were observed by hematoxylin-eosin staining. mRNA expressions of aquaporin (AQP) 1 and AQP2 in kidney were detected by reverse transcription-polymerase chain reaction, and the protein expressions of AQP1 and AQP2 in kidney were detected by immunohistochemistry. Enzyme-linked immune sorbent assay was used to detect the OVA-specific endothelium-1 (ET-1), antidiuretic hormone (ADH), atrial natriuretic peptide (ANP), prostaglandin E₂ (PGE₂), and angiotensin II (Ang II) levels in serum, lung and kidney tissues, respectively. The nitric oxide (NO) contents in serum, lung, and kidney tissues were tested by chemical method, respectively. **Results:** Compared with the control group, the serum IgE level in model group increased ($P < 0.01$). Following the pathologic changes in lung tissue, no significant change in kidney tissue was observed among 3 groups. Compared with the control group, the mice in the model group showed elevated airway resistance during inhalation phase, higher mRNA and protein expression levels on AQP1 and AQP2 in kidney tissue and higher ET-1 levels in serum, lung and kidney tissues, ADH and ANP in lung and serum, PGE₂ in kidney, Ang II in lung and kidney tissues ($P < 0.05$ or $P < 0.01$), but decreased in 5-h urinary output as well as NO and PGE₂ contents in serum and lung tissues ($P < 0.05$ or $P < 0.01$). Compared with the model group, the mice in the SAD group showed a weakened airway resistance in inspiratory phase, lower mRNA and protein expressions of AQP1 and AQP2 in kidney tissues, lower levels of ET-1, ADH, ANP in serum as well as ET-1, ANP, Ang II levels in kidney tissues ($P < 0.05$ or $P < 0.01$), whereas 5-h urinary output, NO content in kidney, ADH, ANP and PGE₂ levels in lung and Ang II in serum increased ($P < 0.05$ or $P < 0.01$). **Conclusion:** San-Ao Decoction can regulate the urine volume through regulating AQP1 and AQP2 expression, and the expression of these in the kidneys might be regulated by ET-1, NO and Ang II.

KEYWORDS regulation of fluid passage, urine volume, aquaporin, San-Ao Decoction, bronchial asthma

Bronchial asthma (BA) is a heterogeneous disease characterized by chronic airway inflammation with respiratory symptoms of wheezing, shortness of breath, chest tightness, and coughing, with variable airflow limitation and intensity that vary with time. Patients with severe asthma may also show a decrease in the urine volume.⁽¹⁾ Clinical studies have found that altered respiratory function might lead to changes in the urine volume.^(2,3) Some studies have

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demonstrated that the abovementioned phenomenon might be induced by unilateral variation in the systemic hormones such as antidiuretic hormone (ADH) and atrial natriuretic peptide (ANP),^(4,5) while others speculated it as the result of a joint action from multiple factors including the stimulation of sympathetic angiotensin system or re-adjustment of the angiotensin aldosterone system.^(6,7) More than 100 bioactive substances have been found successively, including matrix molecules, neuropeptides, growth factors, and surfactants, which are excreted, synthesized, and metabolized in the lungs.⁽⁸⁾ We carried out a comprehensive analysis of these bioactive substances and found that ANP, endothelium-1 (ET-1), prostaglandin E₂ (PGE₂), angiotensin II (Ang II), nitric oxide (NO) and ADH play an important role in renal urine metabolism and is closely related to the lung. All these 6 substances help in the functioning of the lung by regulating renal humoral metabolism, which subsequently coincides with the theories of Chinese medicine: "Fei (Lung) governing the regulation of fluid passage" and "Fei is the upper source of water."

San-Ao Decoction (三拗汤, SAD) is one of the representative prescriptions for "dispersing Fei qi," and is capable of ameliorating pulmonary function in asthma patients and alleviating airway obstruction.⁽⁹⁾ Previous study have found that SAD might ameliorate the volume of urine in chronic obstructive pulmonary disease (COPD) model rats, while NO, PGE₂, Ang II, ANP and ADH exerted regulatory effects on COPD rats.⁽¹⁰⁾ In clinical practice, Chinese herbal medicine for "Fei-dispersing" are frequently used to treat multiple humoral metabolic diseases such as cirrhosis ascites,⁽¹¹⁾ retention of urine,⁽¹²⁾ and prostate hyperplasia.⁽¹³⁾ The approach is known as "Lifting the lid from the teapot".⁽¹⁴⁾

In this study, we explored the relationship between regulation of breathing and water pathways of the Fei by detecting the changes in airway resistance and urine output. Meanwhile, by detecting the expressions of mRNA and protein of aquaporin 1 (AQP1) and AQP2 in the kidney, and the contents of substances such as ET-1, NO, PGE₂, Ang II, ANP, ADH in the serum, lung, and kidney tissues to explore the mechanism of action of SAD on water metabolism.

METHODS

Experimental Animals

Forty-five healthy female BALB/c mice (specific

pathogen-free grade), aged 6–8 weeks and body weight 18–22 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. [certificate No. SCXK (Jing) 2012-0001]. Seven-day adaptive feeding was provided for the animals with food and water *ad libitum*. All mice were maintained in animal center under standard laboratory conditions for 1 week prior to the experiments (a 12-h light/dark cycle at 25 ± 1 °C, with relative humidity of 40%–60% and automatic day-night rhythm). The mice had free access to lab chow and tap water. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the animals were handled in accordance with the dictates of the National Animal Welfare Law of China.

Herb Preparation

SAD, consist of *Ephedrae Herba* (batch No. 03100800), *Armeniacae Semen Amarum* (batch No. 200023594) and *Glycyrrhizae Radix et Rhizoma* (batch No. 201003599) were purchased from the Beijing Tongren Drug Store and identified as authentic products by Basic Theory and Key Technology Research Center of Beijing University of Chinese Medicine.

The mass of the drugs comprising the SAD were calculated based on the maximum human dosage (10 g raw *Herba Ephedrae*, 10 g *Semen Armeniacae Amarum*, and 10 g raw *Radix Glycyrrhizae*) prescribed by Pharmacopoeia of the People's Republic of China (2010 version).⁽¹⁵⁾ Dosages per unit weight of mice were determined based on human dosage, 10-fold, yielding 1.67 g/kg. The 3 herbs were macerated together with the following procedures. Firstly, the components were immersed in distilled water for 1 h, boiled with high heat, and then maintained in the boiling state with gentle fire for 15 min. After the first 2 times of boiling, the decoctions were mixed, filtered through 2-layer gauze, heated within the water bath for evaporation and condensation, and then preserved at 4 °C in a refrigerator for use.

Main Reagents

Ethanol and xylene were purchased from Beijing Chemical Plant (batch No. 20140323 and 1330-20-7, China), Na₂HPO₄, KH₂PO₄, NaCl, phosphate buffer saline (PBS) solution, and methanol

were purchased from Sinopharm Chemical Reagent (batch No. 8012-95-1, China). Ovalbumin (OVA) model-prepared drugs were purchased from Sigma (batch No. A5503-5G, China). Aluminum hydroxide gel was purchased from Pierce Company (batch No. 77161, Massachusetts, USA). Rabbit anti-AQP1 antibody (batch No. GR20414-1), and rabbit anti-AQP2 antibody (batch No. GR27165-1) were purchased from Abcam Company (Cambridge, UK). Immunohistochemistry hypersensitivity kit (batch No. 1305209706), 3,3'-N-diaminobenzidine tetrahydrochloride (DAB) kit (batch No. 1403030031), IgE kit (batch No. G13032045), ADH kit (batch No. E11036562), ET-1 kit (batch No. E11036144), PGE₂ kit (batch No. C0916430356), Ang II kit (batch No. B05030782) and ANP kit (batch No. B10035329) were all purchased from Wuhan Huamei Bioengineering Co., Ltd, China. TRIzol (batch No. 66218) and moloney murine leukemia virus (M-MLV) reverse transcriptase (batch No. 1530776) were purchased from Invitrogen Company (Carlsbad, Canada). dNTP mix (batch No. CW0940A) and Oligo(dT, batch No. CWO731) were purchased from Kangwei Company (Beijing, China). ABI power SYBR Green was purchased from Applied Biosystems (batch No. 272005354; Foster, CA). The design and synthesis of the primers were carried out by Primer 5 software. GAPDH, the internal marker gene, AQP1 and AQP2, and other primers were synthesized by Invitrogen Company (CA). Primers for fluorescent quantitative PCR are listed in Table 1.

Table 1. Primers for Fluorescent Quantitative PCR

Name of primer	Primer sequence from 5'-3'	Annealing temperature	Size of the fragment (bp)
GAPDH			
Forward	AAATGGTGAAGGTCGGTGTGAAC	60 °C	23
Reverse	CAACAATCTCCACTTTGCCACTG	60 °C	23
AQP1			
Forward	TTCACACACACAGCCTTCCT	60 °C	20
Reverse	TACACGGGCAGACCCACTA	60 °C	19
AQP2			
Forward	GTCATCGGTTCCCTCCTCTA	60 °C	20
Reverse	CGTTCCTCCAGTCAGTGT	60 °C	19

Development of Bronchial Asthma Mice Model

Forty-five BALB/c mice were randomized into the control, model and SAD groups by a random number table, with 15 mice in each group. Bronchial asthma model was established by a composite method

with OVA sensitization and challenge.⁽¹⁶⁻¹⁸⁾ On day 1 and 13, mice in the model and SAD groups were intraperitoneally injected with 0.2 mL mixture containing 100 µg OVA and 1.5 mg aluminum hydroxide. On day 15, 5 mice from each group were placed in an organic glass box, sized 20 cm × 30 cm × 40 cm, and challenged with 1% (w/v PBS) OVA aerosol inhalation using a medical ultrasonic atomizer (402B, batch No. 16042704; Shanghai Yuyue Medical Equipment Co., Ltd, China). During the challenge, the mice were exposed to OVA aerosol for 30 min, 2 times per day (at an interval of 4 h) for 7 consecutive days. The mice in the control group were intraperitoneally injected with distilled water, and without aerosol inhalation challenge. The success of the bronchial asthma model can be determined when the mice show signs of wheezing, shortness of breath, frequent noises and sneezing, as well as pathological changes in lung tissues, elevated serum immunoglobulin E (IgE) level and high airway hyper-responsiveness.⁽¹⁹⁾

Administration

Totally 0.3 mL SAD was administered via intragastric administration in the SAD group, one time per day from day 15–22 successively (there was an interval of half a day between aerosol inhalation and gastric administration), while an equivalent volume of normal saline was used for gastric administration in the control and model groups. The mice were fasted for 12 h after the last medication, and the samples were collected and analyzed for the corresponding parameters.

General Observation

During the experiment, the general condition of mice was observed closely.

Assessment of Airway Resistance in Mice

The bronchial challenge test was performed after 24-h OVA inhalation. The animals were anesthetized by intraperitoneally injecting 2% pentobarbital, at a dosage of 90 mg/kg body weight. After the pain reflex had disappeared, the mice were laid on its back in the volume recording box for the lung function detection and analysis system for laboratory animal (AniRes2005; Beijing Bestlab High-Tech, China). The subcutaneous tissue was cut and separated to expose the trachea, which was then connected to an animal respirator, and tracheal intubation was performed. The

external jugular vein was dissociated and punctured using the fastened needle handle and enclosed in a scanning box. Acetylcholine was intravenously injected based on the concentration multiplication method (0.025, 0.05, 0.1, and 0.2 mg/kg, respectively), and for each time, a dosage of 0.04 mL was injected via external jugular vein. The subsequent dosage was administered when the airway resistance was dropped to the normal level, and the wave patterns and data were recorded as reported previously.⁽²⁰⁾

Determination of Urine Volume

Mice were fasted for 12 h after the last aerosol inhalation. On the following day, 1 mL normal saline was injected intraperitoneally as the water was loaded. The lower abdomen of the mouse was pressed gently to discharge the residual urine. About 2–3 mice were placed immediately in the metabolic box fixed with a weighed filter paper at the bottom of the box. The weight of filter paper was recorded every hour for 5 h, and the feces was removed after weighing the filter paper. The urine volume discharged was detected simultaneously and the mean urine volume of each cage was estimated.

Sample Collection and Preparation

Blood was withdrawn through eyeball enucleation, maintained at 4 °C for 2 h, followed by centrifugation (3,000 r/min for 10 min) to separate the serum, and preserved at –80 °C for use. The dead mice were laid on ice, chest and abdominal cavities were cut open to separate the upper left lung and right kidney and afterward fixed with 4% paraformaldehyde for pathological morphology and immunohistochemistry (IHC) analysis. The lower left kidney was preserved at –80 °C for reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Histopathological Analysis

Paraformaldehyde-fixed lung and kidney tissues were washed overnight in water, dehydrated in gradient alcohol, hyalinized with xylene, embedded in paraffin, and sectioned into 5–6 µm-thick slices. The slices were HE stained, dehydrated, hyalinized again, mounted with neutral gum, and imaged by microscopy.

Estimation of Mice-Specific Serum IgE, Contents of NO, ET-1, ADH, ANP, PGE₂ and Ang II in Serum, Lung and Kidney Tissues

The OVA-specific IgE in serum and the contents

of ET-1, ADH, ANP, PGE₂ and Ang II in serum, lung and kidney tissues were estimated by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. The levels of NO in serum, lung and kidney tissues were detected by chemical method according to the kit instructions.

Fluorescent Quantitative PCR Analysis

TRIzol was used to extract the RNA from kidney tissues and reverse transcribed into cDNA for quantitative PCR analysis. The reaction system of real-time quantitative PCR consisted of 0.4 µL upstream and downstream primers. The cDNA was diluted to 10-fold with 10 µL of SYBRGreen, and 7.6 µL ddH₂O, in a total volume of 20 µL. The reaction conditions included pre-denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation for 5 s at 95 °C and 30 s at 60 °C. Subsequently, the melting curve was analyzed to determine the specificity of the reaction. Real-time PCR was performed using a fluorescence quantitative PCR machine. The dissolution and amplification curves were analyzed, and then converted it to Ct value using following formula: $\Delta Ct = Ct(AQP1/AQP2) - Ct(GAPDH)$, $\Delta\Delta Ct = \Delta Ct(model) - \Delta Ct(control)$, AQP1/AQP2 mRNA relative content = $2^{-\Delta\Delta Ct}$.

IHC for Kidney

The expression levels of AQP1 and AQP2 in renal tissue was detected by IHC using standard procedures. The samples were analyzed based on the randomly chosen fields (40×10 times) for each slice using the cellSens image collecting software and Image Pro-plus (IPP) image analysis software, yielding the mean optical density of the positive area. The expression levels of AQP1 and AQP2 proteins were computed as follows: mean optical density = cumulative optical density (IOD sum)/total area.

Statistical Analysis

All data were analyzed using SPSS17.0 software. Values are presented as means ± standard deviation ($\bar{x} \pm s$). Statistical analyses were performed using analysis of variance (ANOVA) followed by least significant difference (LSD). $P < 0.05$ was considered statistically significant.

RESULTS

General Conditions

The control group mice were in good shape, and

no obvious abnormality. During the process of OVA aerosol inhalation, the mice in model group manifested asthma symptoms, such as wheeze, polypnea, scratching nose frequently, cough, sneezing, red nose, dysphoria even jumping up. Compared with the model group, the SAD group were obviously relieved, only a few of the above symptoms occurred.

Airway Resistance during Inhalation Phase

Compared with the control group, a significant higher IgE content and elevated airway resistance during inhalation phase in the model group was observed ($P<0.01$, Figure 1A). Compared with the model group, mice in SAD group showed a lowered airway resistance during the inhalation phase ($P<0.01$, Figure 1B).

Urine Volume

Compared with the control group, mice in the model group showed a decrease in 5-h total urine volume ($P<0.05$). SAD group showed an increase in 5-h total urine volume as compared with the model group ($P<0.01$, Figure 1C).

Morphology of Lung and Kidney in Mice

The lung tissue in the control group appeared clear with complete structure (Figure 2A). The model group mice showed pulmonary alveoli with fuzzy structures, apparent thickening of the alveoli wall, increased secretion of mucus, and aggravated infiltration of lymphocytes compared to the control group (Figure 2B). However, in comparison with the model group, mice in the SAD group showed a relatively clear structure in pulmonary alveoli, slight thickening in some of the alveoli walls, and a marked decrease in the infiltration of inflammatory cells (Figure 2C). No distinct pathological changes were observed in the structure of renal tissues in all groups. Also, the cellular morphology and tissue structure of glomerulus and tubules remained normal (Figures 2D–2F).

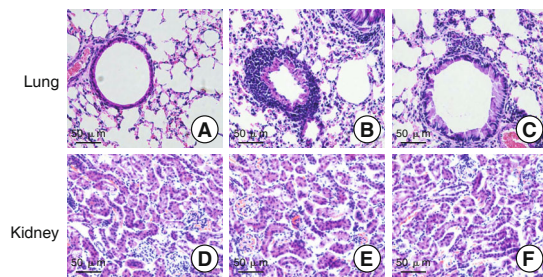


Figure 2. Morphology of Lung and Kidney Tissues in Mice of Each Group (HE staining, 400 ×)

Notes: A, D: control group; B, E: model group; C, F: San-Ao Decoction group

Determination of the Contents of NO, ET-1, ADH, ANP, PGE₂, and Ang II in Serum, Lung and Kidney Tissues

In comparison with the control group, mice in the model group displayed significantly lower levels of NO and PGE₂ in serum and lung tissue ($P<0.05$ or $P<0.01$), but higher levels of ET-1 in serum, lung and kidney tissues, ADH and ANP in serum and lung tissue, PGE₂ in kidney tissue, Ang II in lung and kidney tissues ($P<0.05$ or $P<0.01$; Table 1 and Figure 3).

Table 1. NO Levels in Serum, Lung and Kidney Tissues of Mice ($\mu\text{mol/L}$, $\bar{x} \pm s$)

Group	n	NO		
		Serum	Lung	Kidney
Control	5	55.51 $\pm$ 19.22	0.62 $\pm$ 0.29	0.11 $\pm$ 0.03
Model	5	33.74 $\pm$ 13.77*	0.32 $\pm$ 0.11*	0.08 $\pm$ 0.02
SAD	6	40.58 $\pm$ 11.81	0.46 $\pm$ 0.15	0.17 $\pm$ 0.08 [△]

Notes: SAD: San-Ao Decoction; * $P<0.05$, vs. control group; [△] $P<0.01$, vs. model group

Compared with the model group, mice in the SAD group showed lower levels of ET-1, ADH, ANP in serum and ET-1, ANP, Ang II in kidney tissue ($P<0.05$ or $P<0.01$), whereas higher levels of NO in kidney, ADH, ANP and PGE₂ in lung tissue, Ang II levels in serum were observed ($P<0.05$ or $P<0.01$; Table 1 and Figure 3).

mRNA and Protein Expression Levels of AQP1 and AQP2 in Kidney Tissues of Mice

In comparison with the control group, the mRNA

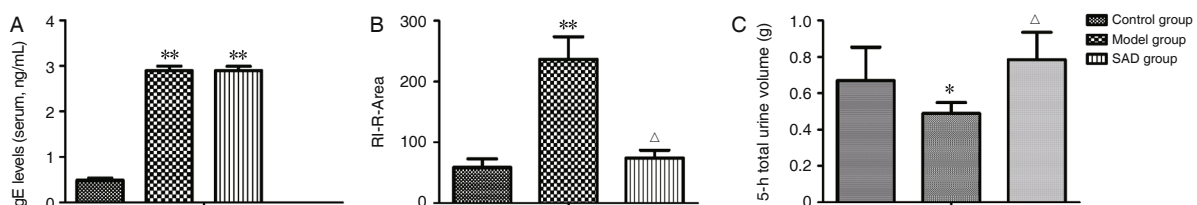


Figure 1. IgE Levels in Serum, Airway Resistance During Inhalation Phase and 5-h Total Urine in Each Group ($\bar{x} \pm s$)

Notes: SAD: San-Ao Decoction; * $P<0.05$, ** $P<0.01$, vs. control group; [△] $P<0.01$, vs. model group

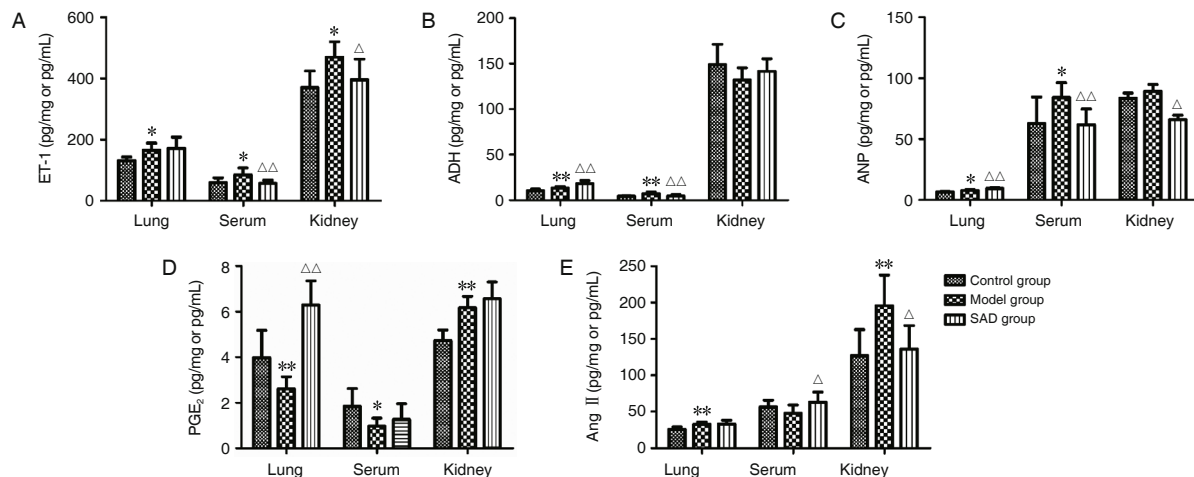


Figure 3. ET-1, ADH, ANP, PGE₂ and Ang II Levels in Serum, Lung and Kidney Tissues in Each Group ($\bar{x} \pm s$)

Notes: ET-1: endothelium-1; ADH: antidiuretic hormone; ANP: atrial natriuretic peptide; PGE₂: prostaglandin E₂; Ang II: angiotensin II. * $P < 0.05$, ** $P < 0.01$, vs. control group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, vs. model group

and protein expression levels of AQP1 and AQP2 in the kidney tissues were higher in the model group (both $P < 0.01$). Compared with the model group, the expression levels in the SAD group were lower ($P < 0.01$, Figure 4).

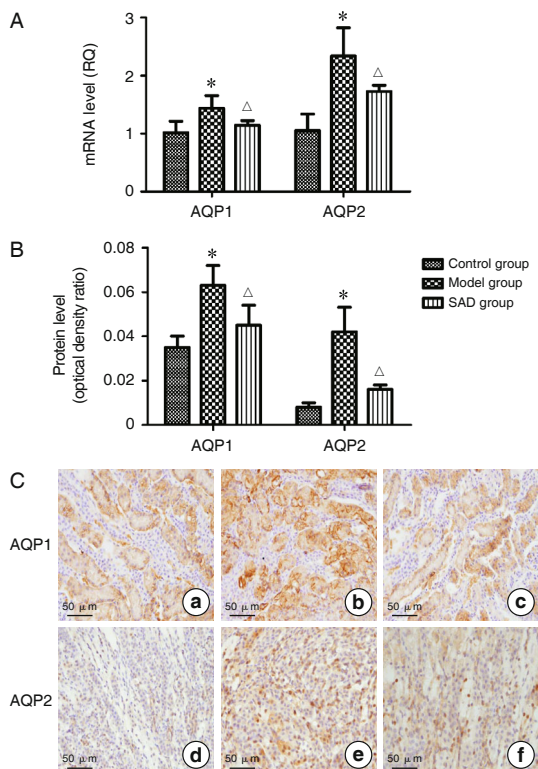


Figure 4. AQP1 and AQP2 mRNA and Protein Expressions in Kidney Tissues

Notes: A: mRNA expressions; B: protein expressions; C: IHC (40×); a, d: control group; b, e: model group; c, f: San-Ao Decoction group; * $P < 0.01$, vs. control group; $\Delta P < 0.01$, vs. model group

DISCUSSION

Bronchial asthma is a chronic airway

inflammatory disease characterized by wheezing, shortness of breath, and involves a complex gene-environment interactions.⁽²¹⁾ AQPs belong to the membrane protein family, which improve the rapid passage of water and other small molecular substances through biological membranes with high selectivity.^(22,23) AQP1 is widely distributed in the human body and expressed in lung and kidney, and is involved in the regulation of systemic humoral metabolism. The regulation of AQP2 on humoral metabolism primarily involves its influence on urine volume. A study found that 24-h urine volume was decreased significantly in rats with nephrotic syndrome and a significant increase in the AQP2 content was observed.⁽²⁴⁾ In case of bilateral urethral obstruction and after its relief, the AQP2 content showed a distinct decrease, which resulted in persistent polyuria. These results suggested a correlation between altered AQP2 expression and impaired urine concentration/delayed recovery.⁽²⁵⁾ The synthesis and excretion of ET-1, NO, ANP, PGE₂, ADH, and Ang II are regulated by lungs and act on kidneys which participate in urine production.

In this study, the mice in the model group showed wheezing, shortness of breath, frequent noises and sneezing, and a higher levels of IgE in serum compared with the control group, indicating that the BA model was successfully established. Airway resistance in the inspiratory phase was increased in the model group and the urine output was decreased significantly in 5 h, suggesting a relationship between the change in respiratory function and reduction in

urine output. In Chinese medicine theory, urine is the main form of water discharge, and the effect of water pathways regulation by the Fei is reflected mainly by urine. Changes in the respiratory function of BA mice lead to failure of diffusion and a decrease in the lung function of water metabolism as well as 5-h urine volume. Damage to the structure and impaired function of the Fei governing qi also affected the regulation of water pathways.

We measured the expression of genes and proteins of AQP1 and AQP2 in the renal tissues and detected the contents of ET-1, NO, ANP, PGE₂, ADH, and Ang II in serum, lung and kidney tissues, respectively. The results showed that the mRNA and protein expression of AQP1 and AQP2 in renal tissues in the model group mice was increased compared with the control group. The contents of NO in lung and serum were decreased, as well as the contents of ET-1 in serum, lung, and kidney and Ang II in lung and kidney were increased significantly in the model group. As reported by several studies,⁽²⁶⁻²⁹⁾ ET-1 can affect the expression of AQP1 and AQP2 in renal tissue, NO and Ang II also affect the expression of AQP1 in kidney or lung tissue. Therefore, we speculated that the alteration of ET-1, NO and Ang II might regulate the expression of AQP1 and AQP2 in renal tissue and then regulate the urine volume in the BA mice.

After intervention using the Fei-diffusing decoction, the pathologic changes in lung tissue were reduced, the airway resistance in the inspiratory phase and the content of IgE in serum, the expression of AQP1 and AQP2 genes and proteins in kidney tissue, the contents of ET-1 in serum, lung, and kidney as well as Ang II in lung and kidney tissues were all decreased compared with the model group. At the same time, 5-h total urine output and the content of NO in lung and serum were increased. These observations suggested that SAD improved the pulmonary function of BA model mice. By lifting the lid from the teapot, SAD can increase the urine volume in BA mice and this might be related to the expression of AQP1 and AQP2, which in turn regulated by ET-1, NO and Ang II.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this study.

Author Contributions

The paper was written by Wang M. Li YH contributed to the conception and design of the study. Wang M, Sun Y and Zhang SJ analyzed the data and drafted the paper. All by-line authors read and approved the final paper.

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