Effect of Maxing Shigan Tang on H1N1 Influenza A Virus-Associated Acute Lung Injury in Mice

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Keywords

Maxing Shigan Tang · H1N1 influenza A virus-associated acute lung injury · Mice

Abstract

Objective: This study is aimed at examining the effects of Maxing Shigan Tang (MST) treatment on H1N1-associated acute lung injury (ALI) and exploring the possible mechanism. **Material and Methods:** Mice were randomly divided into a control group, model group, peroxisomal proliferator activator receptor γ (PPAR γ) inhibition group (PPAR γ -), PPAR γ activation group (PPAR γ +), and MST group. Influenza A (H1N1) virus of the Fort Monmouth 1 (FM1) strain was used to induce an ALI mice model. Hematoxylin and eosin staining was performed to investigate the effect of MST treatment on H1N1-associated ALI. Cell apoptosis of lung tissues of each group were conducted through transferase-mediated dUTP nick end-labeling methods. Moreover, the expression level of caspase 3, activity of caspase 3, and serum level of tumor necrosis factor (TNF)- α of each group were also analyzed. Finally, quantitative real-time polymerase chain reaction and Western blotting analysis were carried out to detect angiopoietin-like 4 (ANGPTL4) expression level. **Results:** We

found that mice infected with the FM1 strain of H1N1 influenza A virus developed severe ALI, and MST could improve H1N1-induced ALI. Moreover, MST decreased lung cell apoptosis and reduced the serum content of TNF- α . In addition, MST significantly induced the ANGPTL4 expression in H1N1-induced ALI. **Conclusion:** MST improves H1N1-associated ALI maybe through targeting ANGPTL4 in mice.

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Introduction

Avian influenza, a highly contagious disease, causes acute respiratory distress syndrome and acute lung injury (ALI) [1]. The high morbidity and mortality associated with this pathogen have an impact on both economic and societal costs. During the spring of 2009, a novel swine-origin influenza A (H1N1) virus that caused human infections in Mexico, spread first to the United States and Canada, and then spread globally [2, 3]. As of August 31, 2010 the World Health Organization received reports of

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18,449 laboratory-confirmed deaths from H1N1pdm09 infection [4]. However, until now, the molecular mechanism of H1N1 influenza A virus-associated ALI remains unclear.

Maxing Shigan Tang (MST, also known as Maxing Ganshi Tang) is a classical complex prescription, derived from the well-known clinical textbook “Treatise on Cold-induced Febrile Diseases,” compiled by the most famous traditional Chinese physician Zhang Zhongjing [5]. MST is composed of Chinese Ephedra Herb (Ma Huang), Bitter Almond (Xing Ren), Gypsum (Shi Gao), and Liquorice Root (Gan Cao). MST has anti-virus effect and was widely used to relieve fever, it has anti-inflammatory properties and relieves cough. It has had an excellent safety record for thousands of years [6–9]. Evidences revealed that the inhibitory effects of MST on influenza virus were eliminated by directly killing the virus, interfering with the influenza virus absorption, inhibiting virus proliferation, and protecting the cells infected by the influenza virus [10]. However, few reports explored the effect of MST on influenza virus-associated ALI.

The peroxisomal proliferator activator receptors (PPARs) are ligand-activated transcription factors that play an important role in the regulation of lipid metabolism [11]. Three PPAR isoforms have been identified: PPAR α , PPAR β/δ , and PPAR γ . Each isoform has specific functions determined by their relative abundance in a cell and regulation of specific target genes [12, 13].

A highly sensitive PPAR target gene is represented by angiopoietin-like 4 (ANGPTL4), which was discovered by 3 independent groups in 2000. ANGPTL4 is produced in a number of organs including liver and adipose tissue, where its expression is governed by PPAR α and PPAR γ , respectively [14]. ANGPTL4 belongs to the family of angiopoietins and angiopoietin-like proteins, which are characterized by the presence of an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain; it is a remarkably multifaceted protein that is involved in many metabolic and non-metabolic conditions [15]. ANGPTL4 is widely expressed in vascular endothelial cells and protects endothelial cells from apoptosis through endocrine activity [16]. Furthermore, ANGPTL4 is also related to the progression of acute inflammatory response, cardiovascular events, and metastatic tumors [17, 18].

In this study, we reported that MST has an anti-virus effect and improved H1N1-associated ALI through targeting ANGPTL4 in mice.

Materials and Methods

Viruses and Animals

Influenza virus A H1N1, FM1 strain was supplied by the Guangdong Center for Disease Control and Prevention, China, and stored at -70°C . The FM1 strain was isolated in March 1947 from a patient in an Army Camp in Eastern United States [19]. Viruses were thawed and prepared freshly for each experiment. Experiments were performed in Bio-safety Level 3 facilities under governmental and institutional guidelines. Viruses were propagated by inoculation into 10–11-day-old SPF embryonated fowl eggs via the allantoic route.

Sixty of BALB/c mice (male and female), weighing 13–15 g, were obtained from the Experimental Animal Center, Traditional Chinese Medicine University of Guangzhou, China. Both animal care and the study protocol were conducted according to the Helsinki Declaration Accords. They also followed the guidelines of the Committee on Care and Use of Laboratory Animals in the animal center of Guangzhou University of Chinese medicine. Animals were housed under a 12/12 h light/dark cycle at a constant temperature of 23°C with Traditional Chinese Medicine University of Guangzhou, and laboratory animal guidelines were followed.

Reagents

PPAR γ inhibitors GW9662 and PPAR γ agonists rosiglitazone hydrochloride were purchased from Sigma-Aldrich (Sigma St. Louis, MO, USA). MST was obtained from Guangzhou Youdi Biocompany (Guangzhou, China), diluted in phosphate-buffered saline (PBS) for a concentration of 1 g/ml, and stored at -70°C . Other reagents were purchased from Sinopharm Chemical Reagent Co. Ltd., China, unless otherwise specified.

Virus Infection and MST Therapy

Sixty mice were randomly divided into 5 groups – control group, model group, PPAR γ inhibition group (PPAR γ -), PPAR γ activation group (PPAR γ +), and MST group. The mice in the model group, PPAR γ inhibition group, PPAR γ activation group, and MST group were lightly anesthetized with pentobarbital sodium and infected with fresh influenza virus A by intranasal application of the virus suspended in PBS containing 0.1% bovine serum albumin ($50 \times \text{LD}_{50}$) [20]. The control group was treated with saline solution in the same way, but not with influenza virus A.

For treatments, the animals in PPAR γ inhibition group, PPAR γ activation group, and MST group were anesthetized and administered $10 \mu\text{M}$ GW9662, $50 \mu\text{M}$ rosiglitazone hydrochloride, and $20 \text{ g}/(\text{kg} \times \text{day})$ of MST, respectively for 4 days [21, 22]. Mice in the model and control groups were anesthetized in the same way and given sterilized saline ($1.5 \text{ mL}/\text{kg}$) by inhalation, simultaneously.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA were extracted from the lung tissues of each group using TRIZOL reagent (Invitrogen). The amount and purity of the extracted RNA were determined by spectrophotometry. The cDNA Synthesis Kit (Takara) was used for the synthesis of cDNA according to the manufacturer’s instructions. Briefly, 800 ng of total RNA was added to a reaction containing $50 \mu\text{M}$ of oligo (dT)₁₈ primer, $1 \mu\text{L}$ of dNTP mixture (10 mM each), and RNase-free water to $10 \mu\text{L}$. Reactions were incubated at 65°C for 5 min,

Table 1. Primers used for q-PCR analysis

Gene	Name	Primers (5'–3')
M	M-F	AGTGAGCGAGGACTGCAGCGT
	M-R	TAGCTTTAGCTGTAGTGCTGGC
ANGPTL4	ANGPTL4-F	GCCTGCAGACACAACCTCAAG
	ANGPTL4-R	CCTCATGGTCTAGGTGCTTG
GAPDH	GAPDH-F	CCCATCACCATCTTCCAGGAGC
	GAPDH-R	CCAGTGAGCTTCCCTTCAGC
Actin	Actin-F	GTCCACACCCGCCACCAGTTC
	Actin-R	TCCCACCATCACACCCTGGTG

Primers were designed using the Primer Express version 2.0 software. Primer specificity was confirmed using Primer-BLAST web software (National Centre for Biotechnology Information).

followed by rapidly cooling on ice. Next, 4 μ L of reaction buffer (5 $\times$), 20 units of RNase inhibitor, and 200 units of PrimeScript RTase were added to each reaction. It was mixed gently and incubated at 42°C for 60 min followed by 95°C for 5 min, to inactivate the reverse transcriptase. The resulting cDNAs were diluted and used as templates for quantitative real-time polymerase chain reaction (q-PCR). Amplification of an actin cDNA was used to normalize results from different samples. q-PCR was accomplished using the iCycler iQ Detection System (Bio-Rad, Richmond, CA, USA) and interaction dye SYBR Green according to the manufacturer's instructions. The relative expression level was determined by comparing with the expression of actin ($2^{-\Delta\Delta C_t}$), where C_t represents the threshold cycle. The gene-specific primers are listed in Table 1.

Western Blotting

Total proteins were prepared from lung tissues of each group. Protein concentrations were determined using Bio-Rad protein assay system. Proteins were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, they were electro-transferred to the polyvinylidene difluoride membrane. The membrane containing the proteins was used for immunoblotting with appropriate antibodies (Abcam). Protein bands were scanned and quantified.

Measurement of TNF- α Level

Serum levels of tumor necrosis factor (TNF)- α of each group were detected using enzyme-linked immunosorbent assay kits (Sigma St. Louis, MO, USA) according to the manufacturer's instructions.

Hematoxylin and Eosin Staining

The lung tissues of each group were embedded in paraffin blocks, tissue sections (4–6 μ m) were mounted on slides, deparaffinized with xylene, rehydrated through graded alcohols, and stained with hematoxylin and eosin (H&E) [23]. Finally, they were observed with a Nikon Eclipse E600FN microscope (Nikon Instruments Inc., Melville, NY, USA) at a fixed magnification of 200 $\times$.

Fig. 1. MST reduces influenza RNA expression. Data are mean $\pm$ SEM, each bar represents the mean of 3 independent experiments carried out in triplicate. ## Compared with model group, $p < 0.01$.

Acute Pulmonary Edema (Wet/Dry Ratio)

To assess the extent of acute pulmonary edema, the lung wet-to-dry weight ratio was calculated. The wet weights of lungs were measured and heated to 68°C for 24 h, and then the dry weights of lungs were recorded, after which the wet-to-dry ratio was calculated.

Apoptosis Assay

Cell apoptosis of lung tissues of each group were conducted through terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). TUNEL was performed with TACS 2 TdT DAB kit (Roche, Mannheim, Germany), following the manufacturer's instructions.

Caspase-3 Activity Assay

The cytosolic extracts were incubated at 37°C with 200 mM Ac-DEVD-pNA in 96-well microtiter plates (Caspase-3 Cellular Activity Assay Kit Plus; Biomol Research Laboratory Inc., Plymouth Meeting, PA, USA). At different time points, hydrolytic activities were determined by measuring the absorbance of p-nitroaniline at 405 nm.

Statistical Analysis

All the original experimental data were analyzed by using SPSS 19.0. Comparison was carried out using Student t test. Results were expressed as mean $\pm$ SEM. Two-tailed values of $p < 0.05$ were considered as statistically significant differences.

Results

MST Improves H1N1-Associated ALI

To assess the effect of MST on viral mRNA synthesis, the expression of the M gene was used as an indicator of a successful replication of the influenza virus. No influenza was detected in the control group, and a significantly decreased expression of the M gene in MST group compared to the model group was observed (Fig. 1).

Fig. 2. MST improves H1N1-associated ALI. H&E staining was used to assess the effect of MST on H1N1-associated ALI. Original magnifications 200 $\times$. **a** Control group. **b** Model group. **c** PPAR γ - group. **d** PPAR γ + group. **e** MST group.

Fig. 3. MST decreases wet-to-dry ratio of lungs in H1N1-associated ALI. Data are mean $\pm$ SEM, each bar represents the mean of 3 independent experiments carried out in triplicate. * Compared with control group, $p < 0.05$; # compared with model group, $p < 0.05$.

Then, H&E staining was performed to further analyze the effect of MST on H1N1-associated ALI. As shown in Figure 2, there were no pathological changes in the lung tissues of the control group. In contrast, in the lung tissue

of the model group mice, significant infiltration of inflammatory cells, extensive thickening of alveolar wall, extensive alveoli, and demolished structure of pulmonary alveoli were observed. MST markedly alleviated H1N1-induced pathological changes of lung. These results indicated that BALB/c mice infected with the FM1 strain of H1N1 developed severe ALI (Fig. 2b), and MST could attenuate H1N1-induced ALI (Fig. 2c). Moreover, we also observed that wet-to-dry ratio of lungs, an important parameter for ALI, significantly increased in the model group and decreased in the MST group (Fig. 3).

MST Decreases Lung Cell Apoptosis in H1N1-Induced ALI

To assess the effect of MST on apoptosis, cell apoptosis of lung tissues of each group were conducted through TUNEL methods. As shown in Figure 4a, H1N1 influenza A virus significantly increased the number of apoptotic cells compared to the control group. However, the number of apoptotic cells decreased in the MST group.

To confirm the reliability of apoptosis result, we performed Western blotting analysis of caspase-3 expression level. We found that a strong increase in the expression

Fig. 4. MST decreases lung cell apoptosis in H1N1-induced ALI. **a** Cell apoptosis of lung tissues of each group were conducted through TUNEL methods. **b, c** Western blotting analysis of caspase-3 expression level. **d** Enzymatic assay of caspase-3 activity. Activity of caspase-3 was measured by using a caspase-3 color-

metricprotease assay kit. Data are mean $\pm$ SEM, each bar represents the mean of 3 independent experiments carried out in triplicate. * Compared with control group, $p < 0.05$; # compared with model group, $p < 0.05$.

of caspase-3 in the model group, in contrast, expression level of caspase-3 was markedly decreased in the MST group (Fig. 4b, c). In addition, we found that the caspase-3 activity was significantly higher in the model group, and decreased with MST treatment (Fig. 4d).

MST Reduces Serum Content of TNF- α in H1N1-Induced ALI

It was known that TNF- α activates several intracellular pathways to regulate inflammation, cell death, and proliferation. The content of TNF- α was detected and results revealed that TNF- α increased significantly in the model group compared to the control group, and decreased markedly in the MST group compared to the model group (Fig. 5). These data suggest that MST could reduce the content of TNF- α in H1N1-induced ALI.

MST Induce ANGPTL4 Expression in H1N1-Induced ALI

It was reported that ANGPTL4 participates in many inflammation responses, especially in lung inflammation and alveolar epithelial cell death, which could lead to ALI

Fig. 5. MST reduces the serum content of TNF- α in H1N1-induced ALI. Data are mean $\pm$ SEM, each bar represents the mean of 3 independent experiments carried out in triplicate. * Compared with control group, $p < 0.05$; # compared with model group, $p < 0.05$.

[24, 25]. Whether it also plays an important role in H1N1-induced ALI remains unclear. We, therefore, performed q-PCR and Western blotting analysis to investigate the ANGPTL4 expression of each group. The q-PCR data re-

Fig. 6. MST induces the ANGPTL4 expression in H1N1-induced ALI. **a** q-PCR results of the ANGPTL4 expression in different groups. **b, c** Western blotting results of the ANGPTL4 expression in different groups. Data are mean $\pm$ SEM, each bar represents the mean of 3 independent experiments carried out in triplicate. * Compared with control group, $p < 0.05$; # compared with model group, $p < 0.05$.

vealed that the ANGPTL4 mRNA expression level was reduced strongly in the model group and increased significantly in the MST group (Fig. 5a). Moreover, Western blotting result also clearly demonstrated that H1N1 influenza A virus could inhibit the ANGPTL4 expression, and MST treatment could enhance the ANGPTL4 expression (Fig. 6b, c).

Discussion

Traditional Chinese herbal medicine (TCHM) is the most important component of the traditional Chinese medicine system. TCHMs have been used to treat diseases for thousands of years in Chinese clinical practice [26–28]. For some places in poor financial condition, they have become popular complementary and alternative medicines in the treatment of chronic non-infectious diseases [29]. Today, TCHMs account for 10% of the prescription drugs in China.

TCHMs are widely used for the prevention and treatment of viral infectious diseases in China and many other Asian countries. A growing number of TCHMs with antiviral activity, such as *Radix isatidis*, *Radix bupleuri*, *Fructus forsythia*, has been approved by the China Food and Drug Administration [30]. TCHMs for respiratory

viral infections represent the majority of drugs in the market. In the present study, we reported that MST played an inhibited role in H1N1 by reducing influenza RNA expression. Further experiments revealed that MST could attenuate H1N1-induced ALI through decreasing lung cell apoptosis and reducing serum content of TNF- α . Therefore, MST may serve as a potential drug for influenza virus-associated ALI treatment.

To further probe into the molecular mechanism of MST on H1N1-associated ALI, ANGPTL4 expression was detected using q-PCR and Western blotting analysis, and we observed that H1N1 strongly down-regulated the ANGPTL4 expression both in mRNA and protein levels. In contrast, MST significantly enhanced the ANGPTL4 expression. We chose ANGPTL4 for further analysis, because its expression could be induced by acute phase response [31]. Liang et al. [32] showed that influenza infection could stimulate the expression of ANGPTL4, and treatment of infected mice with neutralizing anti-ANGPTL4 antibodies significantly accelerated lung recovery and improved lung tissue integrity. Moreover, ANGPTL4-deficient mice also showed reduced lung damage and recovered faster from influenza infection when compared to their wild-type counterparts. In addition, silencing ANGPTL4 protected against LPS-induced ALI via regulating SIRT1/NF-KB signaling pathway [33].

Taken together, these findings indicated that ANGPTL4 was involved in influenza-associated acute inflammatory response, and it may also be the target in H1N1-induced ALI development progress.

Unfortunately, the globalization and modernization of TCHMs remain very slow, because of the lack of explicit molecular mechanism and supporting clinical evidences collected under international standards [34]. In the present study, we investigated the role of MST in the H1N1-induced ALI, and our data revealed that MST improved H1N1-induced ALI via activation of the ANGPTL4 expression. However, there were some limitations in our study, for example, no clinical experiments were conducted to evaluate the effect of MST on H1N1-induced ALI. Moreover, the downstream targets of ANGPTL4 are still unclear. Therefore, more studies are needed for deeper understanding of the effect of MST on H1N1-induced ALI. In addition, more cautious and deeper researches were indispensable to assess the anti-virus effect of MST in clinical patients.

In summary, our data indicated that MST could effectively improve H1N1-associated ALI, decrease lung cell apoptosis, reduce serum content of TNF- α , and the molecular mechanism maybe through targeting ANGPTL4 in mice.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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