ORIGINAL ARTICLE

Qiangzhi Decoction (羌跖汤) Protects Mice from Influenza A Pneumonia through Inhibition of Inflammatory Cytokine Storm*

ZHU Hai-yan (朱海燕)*, HUANG Hai (黄 海)*, SHI Xun-long (史训龙)*, ZHOU Wei (周 伟)*, ZHOU Pei (周 磊)*, YAN Qian-lin (颜乾麟)*, ZHU Hong-guang (朱宏光)*, and JU Dian-wen (鞠佃文)*

ABSTRACT  Objective: To investigate the preventive effects of Qiangzhi Decoction (羌跖汤, QZD) on influenza A pneumonia through inhibition of inflammatory cytokine storm in vivo and in vitro. Methods: One hundred ICR mice were randomly divided into the virus control, the Tamiflu control and the QZD high-, medium-, and low-dose groups. Mice were infected intranasally with influenza virus (H1N1) at 10 median lethal dose (LD50). QZD and Tamiflu were administered intragastrically twice daily from day 0 to day 7 after infection. The virus control group was treated with distilled water alone under the same condition. The number of surviving mice was recorded daily for 14 days after viral infection. The histological damage and viral replication and the expression of inflammatory cytokines were monitored. Additionally, the suppression capacity on the secretion of regulated on activation normal T cells expressed and secreted (RANTES) and tumor necrosis factor-α (TNF-α) in epithelial and macrophage cell-lines were evaluated. Results: Compared with the virus control group, the survival rate of the QZD groups significantly improved in a dose-dependent manner (P<0.05), the viral titers in lung tissue was inhibited (P<0.05), and the production of inflammatory cytokines interferon-γ (IFN-γ), interleukin-6 (IL-6), TNF-α, and intercellular adhesion molecule-1 (ICAM-1) were suppressed (P<0.05). Meanwhile, the secretion of RANTES and TNF-α by epithelial and macrophage cell-lines was inhibited with the treatment of QZD respectively in vitro (P<0.05). Conclusions: The preventive effects of QZD on influenza virus infection might be due to its unique cytokine inhibition mechanism. QZD may have significant therapeutic potential in combination with antiviral drugs.

KEYWORDS  herbal medicine, influenza A virus, antiviral activity, pneumonia, inflammatory cytokine, Chinese medicine

Infections caused by influenza viruses are a considerable threat to human health around the world. Influenza virus pandemics have occurred in 1918, 1957, and 1968.[1] Further, the outbreak of a novel influenza A (H1N1) virus has been identified in 2009.[2] Recognition and rapid clearance of pathogens by the innate immune system provide the first line of defense. High morbidity and mortality from influenza virus infection can be exacerbated by robust cytokine production (cytokine storm), excessive inflammatory infiltrates from the innate immunity, and virus-induced tissue destruction.[3] Influenza viruses replicate in the epithelial cells of the upper respiratory tract, monocytes/macrophages, and leukocytes.[4] Productive influenza A virus infection in epithelial cells destroys host cell pre-mRNAs, and kills the host cells either by cytolytic or apoptotic mechanisms.[5,6]

The means to prevent and control influenza include vaccines and antiviral substances. Many classes of anti-influenza drugs have been approved for clinical use, including M2 blockers, neuraminidase inhibitors and inosine monophosphate dehydrogenase inhibitors. However, their application is limited by side effects and the emergence of resistant viral strains. Hence, it is necessary to develop drugs for the treatment of influenza infection that are less susceptible to virus selection, mutation and resistance. Additionally, modulation of the inflammatory response may provide protection from the cytokine storm induced by influenza virus infection.

Qiangzhi Decoction (羌跖汤, QZD), prepared
from aqueous extract of four medicinal plants including *Notopterygium incisum* Ting ex H. T. Chang, *Isatisindigotica* Fort. (*IsatidisFolium*), *Commelinacommunis* L. and *Isatisindigotica* Fort. (*Isatidis Radix*), was developed by Dr. Yan De-xin, a famous Chinese medicine (CM) expert. The prescription has been employed in clinical use since 1960s for the treatment of exogenous fever and is well-received by patients in China and other Asian countries. Based on the rich practical experience in the treatment of epidemic diseases, the prescription had exerted significant adjunctive effect for pneumonia induced by causing severe acute respiratory syndrome (SARS) in China in 2004. These results provided indirect evidence for QZD's application to anti-influenza infection. However, treatment and prevention of H1N1 infection with QZD has not been investigated. The major components of QZD have been studied for their biological effects as following. *N. incisum* is reported to exert inhibitory effects on coronavirus, herpetic and hepatitis B virus;\(^7\)\(^-\)^\(^9\) *Isatidis Folium* has been shown to have activity against herpes simplex virus type I (HSV-1);\(^1\)^\(^0\) and *Commelinacommunis* L. and *Isatidis Radix* have activity against influenza-virus in vitro.\(^1\)^\(^1\),\(^1\)^\(^2\) However, these four Chinese herbs in combination has not been studied for anti-H1N1 virus activity.

The innate immune system within the airway and lungs can respond with rapid identification and elimination of invading virus by local and systemic inflammation.\(^1\)^\(^3\) Therefore, modulation of the host immune response has a potential advantage of exerting less-selective pressure on viral populations. In order to understand the protective mechanism of QZD, we hypothesized that QZD would induce distinct innate immune responses, and modulate the balance between suppression and activation of inflammatory cytokines to alleviate the lung injury. Therefore, we investigated antiviral effect of QDZ in lethal influenza A infection model and its immunodulatory potential in vivo and in vitro.

**METHODS**

**Virus Strains**

A mice-adapted strain A/FM/1/47 (H1N1) of influenza A virus, was obtained from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (Beijing, China). Influenza virus A3/Beijing/30/95 (H3N2) was kindly provided by Shanghai Municipal Center for Disease Control and Prevention (Shanghai, China). The virus strains were stored at −70 °C. Viruses were thawed and freshly prepared for each experiment.\(^1\)^\(^4\)

**Cell Cultures**

Madin Darby Canine Kidney (MDCK) cells, Ana-1 cells (a murine macrophage cell line) and airway epithelial carcinoma A549 cells were provided by the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, USA), 100 U/mL of penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and non-essential amino acids. The cells were maintained in a humidified atmosphere containing 5% CO2 at 37 °C.

**Laboratory Animals**

Inbred ICR mice, 100 male and female, weighing 16–18 g were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China with Certificate No. SCXX(SH)2003-0003, and maintained at the Animal Center of Fudan University. The animals had free access to food pellets and tap water. All animals were used in accordance with the policies and guidelines of Ethic Committee for Animal Use in Fudan University, China.

**Protective Effect of QZD in H1N1 Pneumonia Mice**

Mice were randomly divided into the control and QZD treatment groups (12 mice per group, 6 males and 6 females). After the mice were anesthetized with ether, all mice were infected with H1N1 virus intranasally at 10 median lethal dose (LD50) dosage in a volume of 30 μL per mouse. The initial drug treatment was at 2 h post virus challenge; QZD at dosages of 960, 320 or 107 mg/(kg•d) and Tamiflu at dose of 24 mg/(kg•d) were gavaged twice daily for 7 days. Mice in the normal control group were given saline alone under the same conditions. The survival of mice was monitored until day 14 post virus infection.

**Pathological Damages of Lung Tissue in H1N1 Infected Mice**

For the acute tissue damage, mice were randomly divided into the control and the QZD
treatment groups (8 mice per group, 4 males and 4 females). Mice, anesthetized with ether, were infected with H1N1 virus intranasally at 10 LD₅₀ dosage (30 μL per mouse). QZD and Tamiflu were intragastrically administered to the mice twice daily for 4 days. The mice from each group were euthanized at day 4, and lungs were collected. Lung tissues were fixed in formaldehyde for 24 h, embedded in paraffin, sliced into 5-μm sections, and stained with haematoxylin and eosin (HE). Histopathologic changes of the lungs from each mouse were examined by two blinded observers to determine the number of focal lesions and lung consolidation microscopically. Consolidation was scored using the following assigned values: 0 = no consolidation; 1+ = mild consolidation encompassing <25% of the lung; 2+ = moderate consolidation (25%–50% of lung); 3+ = extensive and dense consolidation (>50% of lung) as previous report.(10)

**Assay for Pulmonary Viral Titer**

Viral load was presented as hemagglutination (HA) titer, similarly to a previous report.(7) Briefly, each lung was homogenized to a 10% (w/v) suspension with phosphate buffered saline (PBS), and the homogenates were serially diluted 2-fold. Fifty microliters of two-fold serial dilutions of the homogenate was added into each well of V-bottom microplate, and 50 μL of 1% chicken red blood cells suspension in PBS was added to each well. The suspensions were mixed and incubated at room temperature for 30 min. The final dilution of homogenates that agglutinates red blood cells was considered the end point of the titration and the HA titer was calculated.

**Measurement of Inflammatory Cytokines and Chemokine**

Anti-mouse interferon-γ (IFN-γ), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), and chemokine regulated on activation normal T cells expressed and secreted (RANTES) antibodies were used to coat 96-well filtration plates (R&D Systems, Minneapolis, USA). Serum and lung homogenates were prepared with the concentration of 100 mg tissue/μL PBS, and then centrifuged for assay. Concentrations of IFN-γ, IL-6, TNF-α, ICAM-1, and RANTES in freshly prepared lung homogenates and serum were determined using specific sandwich enzyme-linked immunosorbert assay (ELISA) kits (Abcam, England) according to the manufacturer’s instructions.

**Cytotoxicity**

MDCK, A549, and Ana-1 cells were seeded into 96-well culture plates at a density of 1 × 10⁵ cells per well. Cells were incubated for 24 h until 90% confluence. QZD was dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mg/mL, then was diluted in RPMI-1640 medium containing 10% FCS, penicillin G (100 U/mL) and streptomycin (100 μg/mL) to 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 μg/mL. These dilutions were incubated with monolayer MDCK, A549, and Ana-1 cells at 37 °C under 5% CO₂ in humidified air for 72 h. Then cell growth was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT assay; Sinopharm Chemical Reagent Co., Ltd. China) and cell viability was expressed as optical density.(8)

**Antiviral Effect of QZD In Vitro**

The antiviral effect of QZD on influenza virus was measured by cytopathic effect (CPE) assay. MDCK cells were seeded into 96-well culture plates at a density of 1 × 10⁴ cells per well and incubated for 24 h for until 90% confluence. Monolayer MDCK cells were washed twice with serum-free 1640 culture medium, and then infected with influenza virus H3N2 at 100 tissue culture infectious dose 50% (TCID₅₀), and dissolved in cooled serum-free 1640 culture medium containing 0.025% trypsin. After incubation at 37 °C in 5% CO₂ for 1.5 h, uninfected virus was removed by pipetting. Samples of QZD (below the limit of toxic concentration) were added into the 96-well plates for further 72 h incubation. Cell viability was observed by a modified MTT assay.

**Effect of QZD on Chemokine**

A549 cells were seeded into 6-well culture plates at a density of 1 × 10⁵ cells per well and incubated for 24 h for until 90% confluence. Then the A549 cells were infected with influenza virus H3N2 at TCID₅₀. After incubation for 1.5 h with the virus, QZD dilutions were added into the 6-well plates for 24 h. Culture supernatants were collected for the RANTES ELISA assay.

**Effect on Cell Proliferation and Cytokine Production**

Ana-1 cells were seeded into 6-well culture plates at a density of 1 × 10⁶ cells per well and
incubated for 24 h until 90% confluence. Then the cells were infected with influenza virus H3N2 at 10 TCID\textsubscript{50}. After 24 h of infection, cell viability was assayed and the inhibitory effect of QZD on cell proliferation after infection was evaluated. On the other plate, the Ana-1 cells were infected with influenza virus H3N2 at TCID\textsubscript{50}. After incubation for 1.5 h, the uninfected virus was removed and QZD dilutions were added for further incubation. Culture supernatants were collected at 6 and 12 h after the infection. The levels of TNF-\alpha were determined by an ELISA method.

Statistics
Data was analyzed with Stat View ver. 5.01 SAS Institute Inc and SPSS 11.5 software package (SPSS Inc, Chicago, IL). All experimental values obtained from above assays were expressed as mean ± standard deviation, and examined by the two-sample test or one-way analysis of variance (Dunnett's t test). A probability of less than 0.05 was considered to be statistically significant.

RESULTS
Protective Effects of QZD in vivo
Mice infected intranasally with influenza virus showed signs of piloerection, lethargy, and reduced food intake 3 days after infection. The infection was lethal in several animals by day 4. After day 11, no additional animals died, and the survival rate (survival/total) remained stable in each group. No mice survived in the control group, or in the QZD 107 mg/(kg•d) group. In contrast, after treatment with QZD 320 and 960 mg/(kg•d), the survival rate of mice on day 14 was 41.7% (5/12, \textit{P}<0.05) and 66.7% (8/12, \textit{P}<0.01), respectively (Figure 1). The mean survival days (MSD) of the control mice was 5.39 ± 0.98 days. Administration of QZD at 107, 320, and 960 mg/(kg•d) increased the MSD of mice to 6.50 ± 1.93 days, 9.91 ± 3.96 days (\textit{P}<0.01) and 12.4 ± 2.57 days (\textit{P}<0.01), respectively. The MSD increased by 20.6%, 83.8% and 130%, respectively, compared with the control group. Additionally, there was a clear correlation between treatment with QZD and subsequent survival in mice (\textit{r}=0.93, \textit{P}<0.01).

QZD Protected the Pulmonary Damages
Viral pneumonia was the main pathological damage in the mice infected with influenza virus H1N1, which was characterized by interstitial pneumonia. The pathological damages were observed on the day 4 after virus inoculation. Most infected mice showed severe infiltration of monocytes and lymphocytes, thickened alveolar walls, and exudation of inflammatory cells into the alveolar space, whereas treatment with QZD ameliorated the pathological injury in a dose-dependent manner (Figure 2). QZD at the dose of 960 mg/(kg-d) significantly decreased the number of influenza-related focal lesions and lung consolidation (\textit{P}<0.05, Figure 3). In addition, the infiltration of monocytes and lymphocytes were significantly reduced.

QZD Inhibited HA Titers
The pulmonary viral HA titer in mice treated with QZD of 960, 320, 107 mg/(kg•d) was reduced to 68.75%, 33.3% and 25%, respectively, as compared with the infected control (Figure 4). The HA titer in the lungs in QZD treated mice [960 mg/(kg•d)] were significantly lower than that in the control mice (\textit{P}<0.05).

QZD Suppressed Cytokine/Chemokine Production
Influenza virus infection induces a robust inflammatory reaction, hallmarkmed by the production of the antiviral cytokines type I and type II IFN.\textsuperscript{(15-17)} To determine the immune regulatory effect of QZD, serum and lungs were collected at day 4 post-
infection and cytokines were measured. Treatment with QZD [960mg/(kg•d)] showed a significant inhibition of IFN-γ, TNF-α and IL-6, as well as ICAM-1 and RANTETS production compared with saline controls from influenza virus-infected mice (Figure 5). Importantly, QZD-dependent down-regulation of the production of cytokine/chemokine/ICAM-1 by innate immune cells, including epithelial cells and macrophages, did not increase viral burden. This demonstrates that QZD enhanced protection without altering the host’s ability to control infection. Quantitative analysis showed that QZD treatment reduced cytokines in a concentration-dependent manner.

CPE Induced by H3N2 Influenza Virus Was Not Inhibited by QZD

Microscopic examination showed that the virus-infected control MDCK cells were nearly destroyed by influenza virus 3 days after incubation. Treatment with QZD did not exert significant antiviral activity in infected MDCK cells (data not shown).

QZD Increased the Cell Viability under Virus Infection

Microscopic examination showed that the proliferation of macrophage Ana-1 cells was significantly
inhibited after inoculation of H3N2 virus. Non-cytotoxic doses of QZD (30, 15 and 7.5 $\mu$g/mL) were added to the infected Ana-1 cells monolayer. After incubation for 24 h, cell survival was measured by MTT assay. The results revealed that the forty-five percent of cells were killed after H3N2 infection with a 10TCID$_{50}$ virus titer. However, QZD at 30 and 15 $\mu$g/mL significantly increased cell viability to 95% and 82% of the control cells, respectively (Figure 6).

**QZD Inhibited TNF- $\alpha$ from Infected Ana-1 Cells**

TNF- $\alpha$ was found at 104.9±11.6 and 184.8±37.8 pg/mL in the medium at 6 h and 12 h after infection, respectively. At non-toxic doses (30 and 15 $\mu$g/mL), QZD exerted a meaningful inhibitory responsiveness on TNF- $\alpha$ secretion (113.7±38.7 pg/mL, 132.7±23.1 pg/mL) at 12 h (Figure 7). These results suggested that QZD exerted the inhibitory activity on TNF- $\alpha$ induced by H3N2 virus in Ana-1 cells.

**QZD Inhibited RANTES from Infected A549 Cells**

RANTES was detected in the culture medium of bronchial A549 epithelial cells at 48 h after influenza viral infection (with a low CPE). QZD at non-cytotoxic doses (30, and 15 $\mu$g/mL), exerted an inhibitory response on virus-stimulated RANTES secretion (956.2±29.5 and 1082.3±155.8 pg/mL vs. 1599.5±101.8 pg/mL of the control, Figure 8).

**DISCUSSION**

In our preliminary study, the toxicity of QZD was evaluated in ICR mice. According to the commendatory dose [120 mg/(kg•d)] of QZD for adults, three-fold and eight-fold doses were tested. The results showed that administration of 960 mg/(kg•d) had no toxic effects on mice (data not shown). Therefore, we chose three lower doses for the current experiments.

H1N1-infected mice are a common experimental model for acute viral pneumonia. In such model, mice with viral infection showed various symptoms, from morbidity to death at different times. Our results showed that mice treated with QZD 960 mg/(kg•d) had a significant improvement in the overall survival rate, reduction of adverse pathological changes and inhibition of virus titer in pulmonary homogenates. Tamiflu administration was found to exert a much better inhibitory effect on the virus titer in the lungs than QZD. However, QZD could significantly reduce the cytokine/chemokine/ICAM-1 production in the infected mice.

In order to provide further evidence for the suppression of inflammatory cytokines, we used various cell models for evaluation the immune modulation with QZD treatment. While QZD had no antiviral activity in MDCK cells, it reduced mouse macrophage apoptosis, and suppressed the release of TNF- $\alpha$ and RANTES from macrophage and endothelial cells after influenza virus infection. Combined with the data in vitro, our
results suggested that QZD did not elicit protection from the influenza virus through direct inhibition of influenza virus replication. The suppression of cytokine/chemokine/ICAM-1 release could assist recovery from the lethal pulmonary injury.

In the early lethal influenza virus infection, the prognosis of viral pneumonia depends on the innate immune response, in which cytokines and other inflammatory mediators are released from immune cells. Mice infected with influenza virus produce cytokine and chemokine responses, including IL-1, IL-1α, IL-6, IFN-γ, TNF-α, IL-8, macrophage inflammatory protein (MIP)-1α, RANTES, and ICAM-1 at days 3-5 post-infection.(19) The interplay of IL-1β, TNF-α, IFN-α/β, IL-18, IFN-γ, and chemokines forms a complex positive feedback network leading to inflammatory response and the development of influenza-specific Th1 response.(20) TNF-α and IL-6 possess multifunctional activities, and are associated with morbidity during influenza virus infection. Chemokines, such as RANTES, induce the recruitment of innate immune cells into the lung, which can release more cytokines exacerbating cytokine storm and further damage the lung.(21) This study showed that the influenza virus transiently altered endothelial function expressed as ICAM-1. Endothelial dysfunction may be explained by the expression on the endothelial surface of adhesion molecules, including ICAM-1, and promote the adherence of leukocytes with the consequent initiation of vascular damage.(19) RANTES, a member of the C-C chemokine family, is a chemoattractant for monocytes, T lymphocytes, basophils, and eosinophils, and expressed by endothelial cells and T lymphocytes. It has been found in nasal secretions of patients suffering from upper respiratory tract infection with influenza virus, parainfluenza virus and adenovirus.(22) RANTES may contribute to the accumulation of these inflammatory cells into the epithelium during viral infection.

We found that QZD exerted no antiviral activity in vitro, but robustly inhibited the expression of viral protein HA in the lung of mice. The significant antiviral efficiency in vivo may due to suppression of cytokine/chemokine/ICAM. Immune response is a double-edged sword. The immune response presents a powerful barrier against virus and can target virus-infected cells. However, the uncontrolled immune response may cause more damage to host cells than could be attributed to the replication of the virus alone.(23,24) Excessive inflammatory cytokine released from immune cells leads to lung injury and dysfunction of respiratory system. Administration of QZD significantly dampened interferon, cytokine, chemokine and ICAM-1 release into the bronchoalveolar lavage, and resulted in a significant decrease in infiltration of monocytes, macrophages, neutrophils in the lung. In addition, we found that QZD had the ability to inhibit RANTES release by H3N2 infected A549 cells. As a result of no obvious cytopathic change in A549 cells 48 h after virus inoculation, the suppression of RANTES by QZD might not contribute to inhibition of virus replication. Influenza virus-infected monocytes and macrophages responded with a cell-specific response involving cytokines (TNF-α, IL-1, IL-6, and IFN-α/β).(25) We found that with the higher virus titer, QZD at the concentration of 30 μg/mL inhibited cell apoptosis, whereas with the lower virus titer, QZD reduced TNF-α in Ana-1 cells.

As a result, pathological changes in the lung were reduced through inhibition of this cytokine storm. It has been confirmed that N. forbesii and I. indigota have anti-inflammatory activity to reduce cell damage caused by virus, lipopolysaccharide, or radiation.(25-27) QZD, which contains these herb components, may exert multiple anti-inflammation activities for indirect modulation of virus replication.

In conclusion, the results presented here clearly demonstrate that QZD has a promising anti-viral effect against influenza virus infection, leading to improved survival rate, attenuation of lung lesions, reduction of virus HA titers in the lung of infected mice, and attenuation of the cytokine network after viral infection. However, further understanding to cellular signaling pathways and cytokine storm would provide insight into influenza pathogenesis, and provide the potential application for CM in infectious disease.

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