

## Original Article

# Catalpol inhibits inflammatory response in rat alveolar macrophages infected with influenza virus by downregulating TLR7, MyD88, NF- $\kappa$ B, and PLA<sub>2</sub>

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**Abstract:** Objective: We aim to evaluate the effects of catalpol, extracted from a traditional Chinese herb *Rehmannia glutinosa*, on inflammatory response in the rat alveolar macrophage cell line NR8383 infected with the influenza A virus adapted to rat A/FM/1/47 (H1N1). Methods: After 1 hour of influenza virus absorption, 500  $\mu$ M catalpol were added to NR8383 cells. At 12, 24, and 36 hours after catalpol treatment, cells supernatants were analyzed for concentrations of tumor necrosis factor (TNF)- $\alpha$  and monocyte chemoattractant protein MCP-1 by ELISA. Expression of these cytokines was also quantified at the mRNA level. In addition, secreted prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and leukotriene B<sub>4</sub>, as well as the levels of the enzyme phospholipase A<sub>2</sub> (PLA<sub>2</sub>), were quantified by RIA. Furthermore, at 36 hours after catalpol treatment, mRNA and protein expression of the Toll-like Receptor (TLR) 7, Myeloid Differentiation Factor (MyD) 88, and Nuclear Factor (NF) - $\kappa$ B were quantified by, respectively, PCR and Western blot analyses. Results: Influenza virus infection upregulated secretion of TNF- $\alpha$ , MCP-1, PGE<sub>2</sub>, and LTB<sub>4</sub>. This inflammatory response was attenuated by catalpol treatment. Furthermore, catalpol decreased the levels of PLA<sub>2</sub>, and mRNA and protein levels of TLR7, MyD88 and NF- $\kappa$ B. Conclusion: Influenza virus upregulates transcription and secretion of inflammatory factors in rat alveolar macrophages, and the traditional Chinese herbal medicine catalpol was found to attenuate this inflammatory response. Thereby, catalpol can relieve the excessive immune response associated with influenza.

**Keywords:** Catalpol, *Rehmannia glutinosa*, influenza virus, rat, alveolar macrophage, TNF- $\alpha$ , MCP-1, TLR7, MyD88, NF- $\kappa$ B, PGE<sub>2</sub>, LTB<sub>4</sub>, PLA<sub>2</sub>

## Introduction

Influenza is a respiratory viral infection that leads to high morbidity and significant mortality [1]. There have been 10 influenza A pandemics in the past 300 years. Not surprisingly, there are concerns about new pandemics that could kill millions of people [2, 3]. Development of effective preventive vaccines against influenza virus has been hampered by its high genetic variability [4]. Chemical drugs which are used to treat the influenza fall into two types. The first type is the M2 proton channel blockers amantadine and rimantadine, whereas the other types are the neuraminidase inhibitors zanamivir and oseltamivir. The effectiveness of phar-

macological therapy of influenza is limited by evolving of drug-resistant viruses. In addition, these drugs may cause serious side effects. Therefore, alternatives to these drugs for the treatment of influenza infection are needed.

Traditional Chinese medicine has attracted attention as alternative and supplemental medicine. Many traditional Chinese medicine drugs have direct antiviral effects and regulate excessive immune response caused by viral infection [5, 6]. *Rehmannia glutinosa* is an important traditional Chinese herbal medicine which is widely used to replenish vitality and strengthen the liver, kidney, heart, and for other diseases, such as diabetes, anemia, or urinary tract problems.

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The recommendations on the use of this herbal medicine are presented in the Chinese Pharmacopoeia [7].

Catalpol is an iridoid glycoside isolated from the fresh root of *Rehmannia glutinosa*, which has a molecular formula of  $C_{15}H_{22}O_{10}$ . Previous studies demonstrated that catalpol can protect the lipopolysaccharide-induced acute lung injury by inhibiting the inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, IL-4 and IL-1 $\beta$ , and up-regulating the anti-inflammatory cytokine IL-10 [8]. In animal models, catalpol was found to protect against the cerebral ischaemia/reperfusion injury by scavenging free radicals and suppressing lipid peroxidation [9]. In addition, several recent studies demonstrated a broad range of biological and pharmacological effects of catalpol, such as neuroprotection and anti-cancer effects [10-12].

Influenza virus infection is associated with increased presence of inflammatory cells (neutrophils, monocytes, macrophages) and overproduction of pro-inflammatory cytokines and chemokines in the airways. This indicates that excessive host immune response is one of the main factors responsible for the damage caused by the influenza virus [13]. After infection with influenza virus, the key cells to initiate the inflammatory response in the airways are alveolar macrophages [14]. These cells secreted pro-inflammatory cytokines TNF- $\alpha$ , IL-1, and IL-8, produce oxygen free radicals, nitric oxides and arachidonic acid metabolites [13, 15, 16]. Potential anti-inflammatory effects of catalpol on alveolar macrophages infected with influenza virus have not been studied so far. Here we report the results of our study on the effects of catalpol on inflammatory responses of rat alveolar macrophages infected with the influenza A virus. These results provide the scientific evidence and experimental basis for pharmacological treatment of influenza infection with catalpol.

### Materials and methods

#### Reagents

Catalpol, RIPA lysis buffer, methyl thiazolyl tetrazolium (MTT), dimethylsulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Boster Biology Co. (Wuhan, China). The TNF- $\alpha$  and monocyte chemoattractant protein (MCP)-

1 ELISA kits were from Aviva Systems Biology Co. (Beijing, China). The prostaglandin  $E_2$  (PGE<sub>2</sub>), phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) radioimmunoassay kits were purchased from the Cellular Institute of the Shanghai Institutes for Biological Sciences (Shanghai, China). The protease inhibitor cocktail, phenylmethylsulfonyl fluoride and Pierce chemiluminescence kit were from Sigma Chemical (Louis, USA). Biotinylated mouse polyclonal antibodies to NF- $\kappa$ B, Toll-like receptor (TLR) 7, myeloid differentiation factor (MyD) 88, GAPDH, and HRP-conjugated streptavidin were purchased from Zhongshan Co. (Beijing, China). The RNeasy Mini kit and the First Strand cDNA Synthesis kit were from Sinopharm Chemical Reagent Co. (Shanghai, China).

#### Cell infection

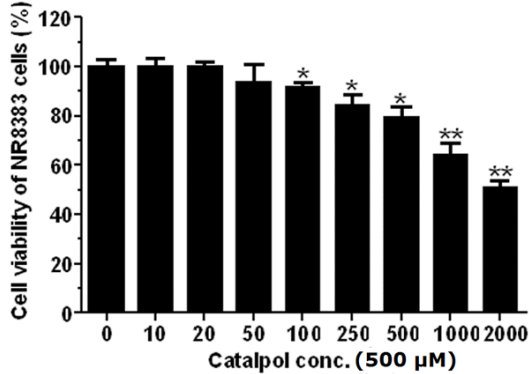
The influenza A/FM1/1/47 (H1N1) virus used in this study was obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China). The virus was grown for 2 days in chorio-allantoic fluid of 10-day-old embryonic hen eggs at 37°C. After harvesting, the allantoic fluid was filtered through a 0.22- $\mu$ m cellulose acetate membrane. The filtered liquid was stored aliquoted at -70°C until further use.

The rat alveolar macrophage cell line NR8383 was obtained from the Shanghai Institutes for Biological Sciences. This cell line was propagated in RPMI1640 (Zhongshan Co., Beijing, China) supplemented with 10% FBS at 37°C/5% CO<sub>2</sub>. The cells were divided into three groups treated with the following conditions: physiological saline, infected with H1N1 virus, and virus-infected and treated with catalpol. Throughout the manuscript, these experimental conditions are referred to as "Normal", "Virus", and "Virus/Catalpol" cells. Catalpol was added to the cells after 1 hour of adsorption of the influenza virus.

#### MTT cytotoxicity test

Potential cytotoxic effects of catalpol on NR8383 cells were assessed using the MTT assay [17]. The cells were seeded at a density of 5000/well in 96-well plates and incubated overnight at 37°C. Then the cells were treated with catalpol at concentrations of 10, 20, 50, 100, 250, 500, 1000, and 2000  $\mu$ M for 48 hours. Cell viability was quantified by adding a 5 mg/ml solution of MTT to the wells, followed by cell incubation at 37°C for 4 hours. The

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**Figure 1.** Effects of catalpol on viability of NR8383 cells. Viability of NR8383 cells 48 hours after treatment with different concentration of catalpol was quantified by MTT assay. The values represent mean  $\pm$  SEM of five experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control cells ("0  $\mu\text{M}$ ").

absorbance was measured at 570 nm. Cell viability was quantified as absorbance of treated cells/absorbance of control cells  $\times$  100%. Each experiment was repeated five times.

### *TNF- $\alpha$ and MCP-1 ELISA*

Twelve, 24, and 36 hours after catalpol treatment, cell culture supernatants were collected and centrifuged (12,000 rpm) for 20 min to remove cell debris. The supernatants were collected for TNF- $\alpha$  and MCP-1 ELISAs. The standard curves were constructed using the non-linear regression models to calculate the concentration of cytokines of interest in the supernatants. Each experiment was repeated five times.

### *PGE<sub>2</sub>, PLA<sub>2</sub> and LTB<sub>4</sub> RIA*

Cell culture supernatants were collected as above and subjected to RIA to quantify PGE<sub>2</sub>, PLA<sub>2</sub> and LTB<sub>4</sub> levels. Each experiment was repeated five times.

### *RNA isolation and PCR*

After 36 hours of catalpol treatment, cells were collected to quantify mRNA levels of TNF- $\alpha$ , MCP-1, NF- $\kappa$ B, TLR7, and MyD88. Total RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions. Five  $\mu\text{g}$  of total RNA were reverse transcribed using the First Strand cDNA Synthesis kit. The following primer sequences were used: TNF- $\alpha$  forward 5'-CCAGCTCTTCTGTCTACTG-3' and

reverse 5'-GCTACGGGCTTGCTACTC-3', MCP-1 forward 5'-GGGTCCAGAAGTACATTAGA-3' reverse 5'-GCTGAAGTCCTTAGGGTTGA-3', NF- $\kappa$ B forward 5'-ACCTGCAGTTCGATGCTGAT-3' and reverse 5'-CCTGTCACCAGGCGGATTTAT-3', TLR7 forward 5'-TCTCCAGACTCCTCCATAG-3' and reverse 5'-GGAAGATCCTGTGGTATCTC-3', and MyD88 forward 5'-CGACGCCTTCATCTGCTACT-3' and reverse 5'-ATGAGCTCGCTGGCGATGGA-3. Expression of  $\beta$ -actin was used as an endogenous control (primer sequences: forward 5'-CCACTGCCGCATCCTCTT-3' and reverse 5'-GCATCGGAACCGCTCATT-3'). Primers were synthesized at the Cellular Institute of the Shanghai Institutes for Biological Sciences (Shanghai, China). Forty cycles of PCR comprised 30 sec at 95°C, 30 sec at 55°C, and 60 sec at 72°C. Each experiment was repeated five times.

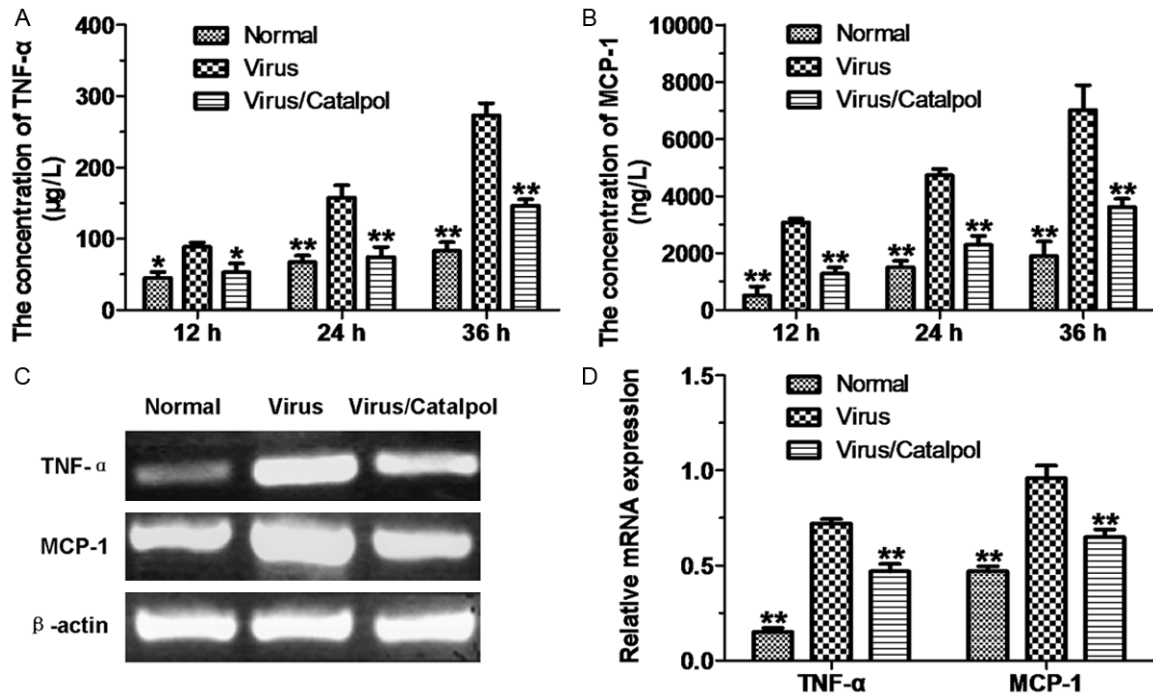
### *Western blot analysis*

After 36 hours of catalpol treatment, cell proteins were isolated as previously described [18]. Briefly, cells were lysed on ice in 1 $\times$  PBS supplemented with 1% Nonidet P-40, 0.1% SDS, and freshly added 20  $\mu\text{l}/\text{mL}$  of protease inhibitor cocktail and 2 mM of phenylmethylsulfonyl fluoride. Protein concentration was measured by Bradford method [19]. The lysates were stored at -40°C until Western blot analysis.

For Western blot, equal amounts of the lysates were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in TBS-T buffer (20 mM Tri-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) and incubated overnight at 4°C with antibodies against NF- $\kappa$ B (1:500 dilution), or TLR7 (1:500 dilution), or MyD88 (1:500 dilution), or GAPDH (1:1000 dilution). The membrane was washed with TBS-T buffer and incubated with HRP-streptavidin. Protein bands were visualized using the enhanced Pierce chemiluminescence kit. The optical density of the bands were normalized to those of GAPDH using the Image Pro Plus image analysis system. Each experiment was repeated five times.

### *Statistical analysis*

The SPSS statistical package, version 17.0 for Windows (SPSS Inc., Chicago, USA), was used



**Figure 2.** Effects of catalpol on TNF- $\alpha$  and MCP-1 production in NR8383 cells infected with influenza virus. A. Concentrations of secreted TNF- $\alpha$  in control cells (“Normal”), cells infected with influenza virus (“Virus”), and virus-infected cells treated with catalpol (“Virus/Catalpol”) were quantified by ELISA. The values represent mean  $\pm$  SEM of five experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. “Virus” cells at the same time point. B. Concentrations of secreted MCP-1 in “Normal”, “Virus” and “Virus/Catalpol” cells were quantified by ELISA. The values represent mean  $\pm$  SEM of five experiments. \*\* $P < 0.01$  vs. “Virus” cells at the same time point. C. mRNA expression of TNF- $\alpha$  and MCP-1 in “Normal”, “Virus” and “Virus/Catalpol” cells was quantified by PCR. A representative gel is shown. D. Relative quantification of mRNA expression in “Normal”, “Virus” and “Virus/Catalpol” cells. The values represent mean  $\pm$  SEM of five experiments. \*\* $P < 0.01$  vs. “Virus” cells at the same time point.

for statistical treatment. Data are presented as the mean  $\pm$  SEM. Statistical significance was determined using the one-way ANOVA analysis with the Bonferonni correction. The  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### Viability NR8383 cells treated with catalpol

As shown in **Figure 1**, cell viability was reduced by 1000  $\mu$ M of catalpol to less than 70%. Therefore, to avoid confounding effects on cell viability, the concentration of catalpol used in subsequent experiments was 500  $\mu$ M.

### Catalpol downregulates transcription and secretion of TNF- $\alpha$ and MCP-1 in NR8383 cells infected with influenza virus

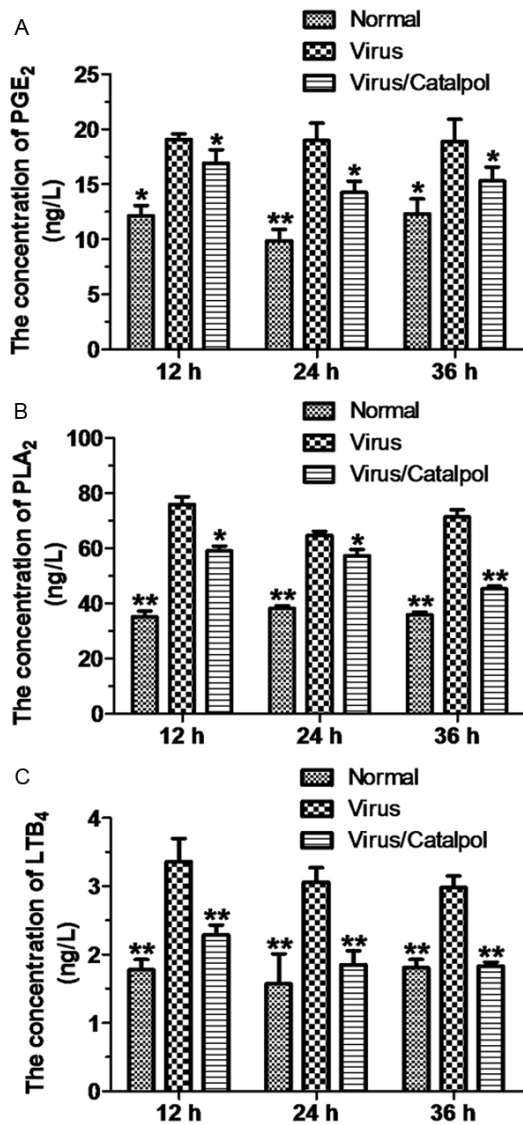
We next quantified production of inflammatory cytokines in cells infected with influenza virus

and treated with catalpol. We observed that concentration of TNF- $\alpha$  in the supernatant of infected cells (“Virus” cells) was significantly up-regulated at each studied time point ( $P < 0.05$  for all studied time points; **Figure 2A**). Treatment with catalpol decreased upregulation of TNF- $\alpha$  production (**Figure 2A**). Similar trend was observed with regard to MCP-1 (**Figure 2B**).

We further documented that influenza virus infection markedly upregulated mRNA levels of both TNF- $\alpha$  and MCP-1 (respectively, **Figure 2C** and **2D**), and that catalpol decreased this mRNA upregulation (**Figure 2C** and **2D**).

### Catalpol downregulates the levels of PGE<sub>2</sub>, PLA<sub>2</sub> and LTB<sub>4</sub> in NR8383 cells infected with influenza virus

Subsequently, we tested the effects of catalpol on secretion of PGE<sub>2</sub> and LTB<sub>4</sub>, and the levels of PLA<sub>2</sub>, in virus-infected NR8383 cells. As shown



**Figure 3.** Effects of catalpol on secretion of PGE<sub>2</sub>, PLA<sub>2</sub>, and LTB<sub>4</sub> in NR8383 cells infected with influenza virus. A. Concentrations of secreted PGE<sub>2</sub> in control cells (“Normal”), cells infected with influenza virus (“Virus”), and virus-infected cells treated with catalpol (“Virus/Catalpol”) were quantified by RIA. The values represent mean ± SEM of five experiments. \**P* < 0.05, \*\**P* < 0.01 vs. “Virus” at the same time point. B. Concentrations of secreted PLA<sub>2</sub> in “Normal”, “Virus” and “Virus/Catalpol” cells were quantified by RIA. The values represent mean ± SEM of five experiments. \**P* < 0.05, \*\**P* < 0.01 vs. “Virus” cells at the same time point. C. Concentrations of secreted LTB<sub>4</sub> in “Normal”, “Virus” and “Virus/Catalpol” cells were quantified by RIA. The values represent mean ± SEM of five experiments. \*\**P* < 0.01 vs. “Virus” at the same time point.

in **Figure 3A**, influenza virus markedly upregulated the levels of all three studied factors, whereas catalpol attenuated this upregulation.

*Catalpol downregulates mRNA and protein levels of NF-κB, TLR7 and MyD88 in NR8383 cells infected with influenza virus*

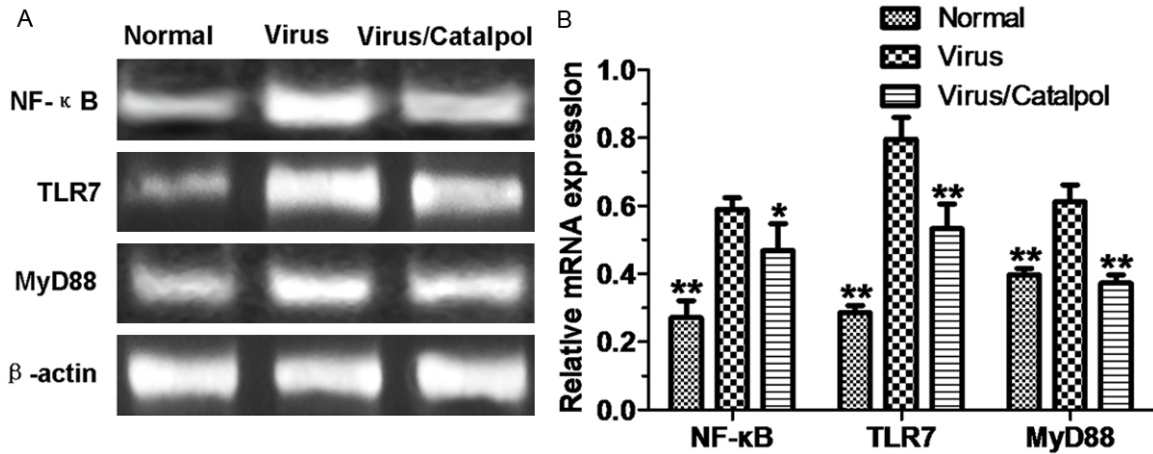
To study the mechanisms of catalpol-induced downregulation of inflammatory response in virus-infected cells, we examined expression of NF-κB, TLR7, and MyD88. As expected, mRNA levels of NF-κB, TLR7 and MyD88 were up-regulated by virus infection, and this was attenuated by catalpol treatment (**Figure 4A** and **4B**). A similar trend was observed at the protein level (**Figure 5A** and **5B**).

**Discussion**

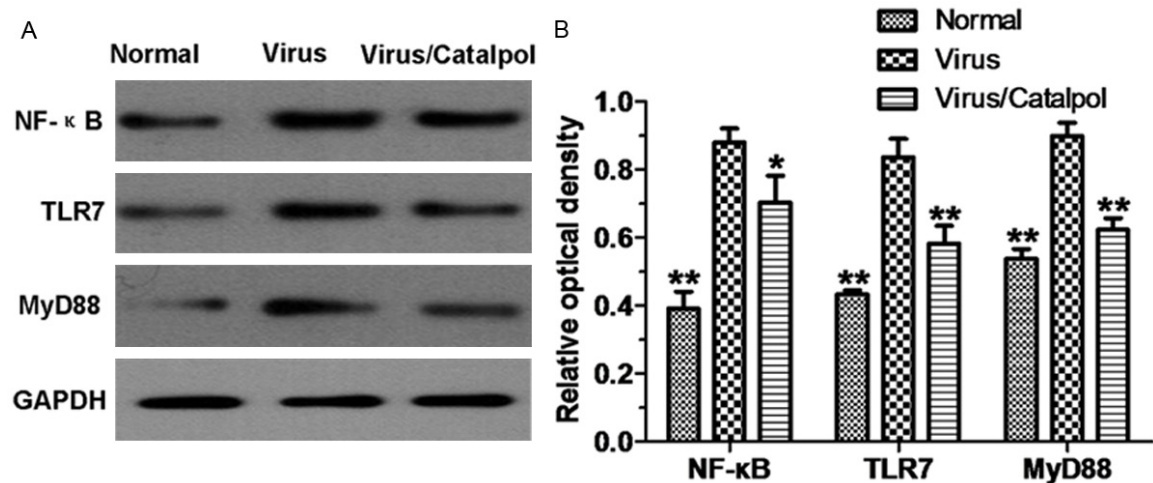
Influenza virus infection is a highly contagious respiratory disease which spreads rapidly and leads to a high mortality rate [20]. It has led to severe global pandemics and is thus considered a serious threat to public health [21]. Consistent with previous literature reports, we here demonstrate that influenza virus infection upregulates secretion of inflammatory cytokines TNF-α and MCP-1, and low-molecular weight inflammatory factors PGE<sub>2</sub> and LTB<sub>4</sub> in rat alveolar macrophages.

Influenza virus is a coated RNA virus which consists of seven or eight segmented single strands [22]. Previous studies demonstrated that single-stranded RNA of influenza virus is recognized by TLR7, which subsequently activates the cells through the MyD88-dependent pathway [23]. This involves activation of the transcription factor NF-κB [24]. In our study, influenza infection upregulated transcription of TNF-α, MCP-1, NF-κB, TLR7 and MyD88 in rat alveolar macrophages.

Traditional Chinese herbal medicine has been used for treatment of human infectious diseases for centuries. One of traditional herbal medicines, catalpol, was found in our study to attenuate virus-induced upregulation of the aforementioned inflammatory markers. We demonstrate here that catalpol decreases the levels (both mRNA and protein) of TLR7, MyD88, and NF-κB. This is a likely mechanism of attenuation of transcription and secretion of TNF-α and MCP-1 in virus-infected cells treated with catalpol. Furthermore, catalpol diminished secretion of LTB<sub>4</sub> and PGE<sub>2</sub> in virus-infected cells by decreasing the levels of the enzyme PLA<sub>2</sub>. Therefore, beneficial effects of catalpol in cells infected with influenza virus comprise attenua-



**Figure 4.** Effects of catalpol on NF-κB, TLR7, and MyD88 mRNA expression in NR8383 cells infected with influenza virus. A. mRNA levels of NF-κB, TLR7 and MyD88 control cells (“Normal”), cells infected with influenza virus (“Virus”), and virus-infected cells treated with catalpol (“Virus/Catalpol”) were quantified by PCR. A representative gel is shown. B. Relative quantification of mRNA expression in “Normal”, “Virus” and “Virus/Catalpol” cells. The values represent mean ± SEM of five experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. “Virus” cells at the same time point.



**Figure 5.** Effects of catalpol on protein expression of NF-κB, TLR7 and MyD88 in NR8383 cells infected with influenza virus. A. Protein levels of NF-κB, TLR7 and MyD88 control cells (“Normal”), cells infected with influenza virus (“Virus”), and virus-infected cells treated with catalpol (“Virus/Catalpol”) were quantified by Western blot analysis. A representative Western blot is shown. B. Relative quantification of mRNA expression in “Normal”, “Virus” and “Virus/Catalpol” cells. The values represent mean ± SEM of five experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. “Virus” cells at the same time point.

tion of both protein and low-molecular weight inflammatory markers.

In conclusion, influenza virus upregulates transcription and secretion of inflammatory factors in rat alveolar macrophages, and the traditional Chinese herbal medicine catalpol was found to attenuate this inflammatory response. Thereby, catalpol can relieve the excessive immune response associated with influenza. The results of our study provide an experimental basis for

further clinical studies of usability of catalpol for future treatment of influenza.

**Disclosure of conflict of interest**

None.

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