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ORIGINAL ARTICLE

In Vivo and *in Vitro* Antiviral Effects of Berberine on Influenza Virus*

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ABSTRACT **Objective**: To explore the potential effects of berberine on influenza virus infection both *in vitro* and *in vivo*. **Methods**: *In vitro* anti-influenza virus assays were performed by cytopathogenic effect and neuraminidase assays in Madin Darby canine kidney cells. *In vivo* anti-influenza virus assays were performed on the viral pneumonia model of mice. The numbers of mice that died within day 2 to day 14 postinfection were recorded to calculate the mortality. On days 2, 4, and 6, the viral titers in the lungs were determined by hemagglutination assay; hematoxylin/eosin staining was used to assess the pathogenic changes of lung tissues; the concentrations of tumor necrosis factor-alpha (TNF-α) and monocyte specific chemoattractant molecule (MCP-1) were measured by radio immunoassay or enzyme-linked immunosorbent assay; the concentrations of nitric oxide (NO) and inducible nitric oxide synthetase (iNOS) were detected by colorimetric method; reverse transcription polymerase chain reaction was used to detect the mRNA level of TNF-α and MCP-1. **Results**: Berberine showed inhibitory effects on cytopathogenic effects and neuraminidase activity of virus, with the therapeutic index 9.69. *In vivo*, berberine decreased mice mortality from 90% to 55%, reduced virus titers in the lungs on day 2 postinfection ($P<0.05$). The lung histology scores were 1.50±0.67, 4.50±1.00, and 5.50±1.00 in the berberine group on days 2, 4, and 6, respectively, which were significantly reduced compared to 2.17±0.22, 6.83±0.44, and 8.50±0.33 in the infected group ($P<0.05$). The productions of NO and iNOS were repressed by berberine compared with those in the infected group ($P<0.01$). The transcription and expression of TNF-α were inhibited by berberine on day 4 ($P<0.01$) and day 6 ($P<0.05$), and those of MCP-1 were inhibited on day 6 ($P<0.01$) compared with the infected group. **Conclusions**: Berberine exhibited antiviral effects on the influenza virus both *in vitro* and *in vivo*. The possible therapeutic mechanism of berberine on influenza-induced viral pneumonia might be inhibiting the virus infection, as well as improving the pathogenic changes by repressing inflammatory substances release.

KEYWORDS *in vivo, in vitro*, antiviral effects, berberine, influenza virus

Influenza is a crucial annual respiratory infection that remains one of the leading causes of illness and death throughout the world. Influenza viruses may cause primary viral pneumonia in humans with fatal outcome as soon as the pathogen spreads from the upper respiratory tract to the alveolar air space[1]. Although inactivated vaccine has achievely a certain amount of protection in healthy subjects, it is less effective in elderly patients and cannot prevent new emerged mutated viruses from infection[2]. Amantadine and rimantadine or new neuraminidase inhibitors have been available for the therapy of prevention; however, a few adverse reactions and the emergence of resistant viral strains have been reported previously[3-5].

The application of Chinese medicines on clinical therapy of influenza virus infection has achieved great therapeutic efficiency. A lot of Chinese medicines have been proved to have antiviral effects on influenza virus infection with less adverse reactions, for example, scutellaria, licorice root, and arctium[6,7]. Berberine is an isoquinoline derivative alkaloid isolated from many medicinal herbs, such as *Rhizoma coptidis* and *Cortex phellodendri*. It is widely used in Chinese medicine for antimicrobial and anti-inflammatory activities. In recent years, berberine has been reported to have a wide range of pharmacological effects, including immunological regulation, myocardial protection,

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inhibition of tumor cell proliferation, and invasion[8]. Recently, it was reported that HB-13, an active compound from berberine derivatives, had a strong anti-HSV-1 and anti-HSV-2 activity *in vitro* and *in vivo*[9,10]. This suggested that berberine might exert some unknown functions on virus infection and virus-related diseases. Based on that, our study was to explore the potential effects of berberine on the influenza virus infection and flu-induced pneumonia.

METHODS

Virus

Influenza virus A/FM/1/47 (H1N1), adapted to the mice, was kindly provided by the Institute for Virology, Chinese Academy of Preventive Medicine. Viruses were passaged in embryonated chicken eggs (1 to 3 passages), and samples of allantoic fluid were stored at −70 ℃ until needed. The hemagglutination (HA) titer of virus was determined as 1:512 by titration of virus samples in phosphated buffered saline with thoroughly washed Leghorn RBC (Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China).

Cell Line

Madin Darby canine kidney (MDCK) cells (American Type Culture Collection, America) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and gentamicin. The 50% tissue culture infective dose ($TCID_{50}$) titer of the virus on MDCK cells was determined by the cytopathogenic effect (CPE) methods[11] and calculated as $10^{-4.6}$/0.1 L by the method of Reed and Muench[12].

Drugs and Drug Cytotoxicity

Berberine (batch code: 110713-200609, purity of ＞99%) was provided by the National Institute for the Control of Pharmaceutical and Biological Products, China. Ribavirin (batch code: 06101623), which served as a positive control, was provided by Tianjin Pharmaceutical Group Jiaozuo Co., LTD., China. The noncytotoxic dose (TD_0) and the 50% cytotoxic dose (TD_{50}) of single drug on MDCK cells were detected with methyl thiazolyl tetrazolium (MTT) method.

Animals

Eighty SPF ICR mice, outbred, male/female, weighing 15 ± 1 g, and 180 SPF BALb/c mice, inbred, female, weighing 18 ± 1 g, were provided by Vital River Laboratory Animal Technology Co., Ltd., Beijing, China[12].

In Vitro Anti-Influenza Activity Detected by CPE and Neuraminidase Assay

The 96-well microtiter plates were seeded with 3×10^4 MDCK cells per well. When the monolayer was developed to 90% or greater confluency 24 h later, the culture medium was removed from replicates. The wells were washed with phosphated buffered saline and inoculated with 0.1 mL of 100 $TCID_{50}$ virus diluted in the growth medium without serum. After 1 h adsorption, the medium containing virus was removed, and 0.1 mL berberine diluted in DMEM with the final concentrations as 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.001563 g/L, respectively, was added to each of the six wells in serial half dilutions. The infected cell controls and normal uninfected cell controls were included in each test, and ribavirin, which was concomitantly evaluated for antiviral activity in a similar manner, served as the positive control drug. Then, the plates were sealed and incubated at 37 ℃. Every day the cell monolayers were examined microscopically, and the virus-induced CPE was scored on a scale as 0 (no CPE), 1 (< 25% CPE), 2 (25%–50% CPE), 3 (50%–75% CPE), and 4 (100% CPE).

At 48 h postinfection, when the cytopathogenic effect on the infected cells mounted to 100%, the observation ceased and the CPE scores of every monolayer were recorded. The half inhibitory concentration (IC_{50}) was determined for each drug, and the therapeutic index (TI) was calculated by the formula $TI=TD_{50}/IC_{50}$. TI had a weighted measurement of antiviral activity that took into account the degree of inhibition of virus-specific CPE and the degree of cytotoxicity produced by the test drug, determined by a modification of the method of Ehrlich, et al[11]. A TI >1.0 indicated definite antiviral activity; otherwise, there was no significant antiviral activity. Data were compiled from results from three independent experiments.

The activity of viral neuraminidase (NA) was determined with neuraminidase assay kit (Sun Biomedical Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Mice Mortality

A total number of 80 ICR mice were randomly

divided into four groups, 20 in each group: (1) Infection group. The mice were anaesthetized with ethylether and intranasally infected with 50% lethal dose (LD_{50}) influenza virus in a 25 μL volume to induce viral pneumonia[7]. (2) Berberine group. At 1 h after infection, the mice were treated by intraperitoneal injection with berberine at a dose of 0.005 g/(kg·d) for 7 days. (3) Ribavirin group. At 1 h after infection, the mice were treated by intraperitoneal injection with ribavirin at a dose of 0.1 g/(kg·d) for 7 days. (4) Normal control group. The mice were intranasally given 25 μL phosphate-buffered saline instead. Water was given to mice in the infected group and control group, twice a day, by using stomach tube for 7 days. The numbers of mice that died within days 2 to 14 postinfection were recorded.

Virus Titration and Histology

The 180 BALb/c mice were randomly divided into four groups, 54 for the infection group, 54 for the berberine group, 54 for the normal control group, and 18 for the ribavirin group. On days 2, 4, and 6 after infection, six mice of each group were sacrificed. The left lung was homogenized by grinding in 1 mL of sterile 0.9% sodium chloride, and the supernatants were extracted by centrifugation at 4 ℃, 2 500r/min for 20 min. The viral titers were determined by hemagglutination (HA) assay[13].

For histology, the lower lobe of the right lung was fixed in 4% formalin. After fixation, the samples were dehydrated and embedded in paraffin. The 6-μm-thick sections were cut and stained with hematoxylin/eosin (HE). The samples were analyzed and scored microscopically in a blind study on coded slides as described[14]. Three types of pathological changes were scored separately. The mononuclear cells and lymphocytes infiltration were termed as inflammatory cell on a scale as 0 (normal) to 3 (a large number of inflammatory cells infiltration in endobronchial); the histopathologic changes around vessels were termed lung micrangium on a scale as 0 (normal) to 3 (severe damaged vessel wall); histopathologic changes within the alveolar parenchyma were scored as alveolar structure also on a scale as 0 (normal) to 3 (consolidation in most of lung tissues); and a final score of pneumonitis from 0 to 9 was assigned for each pathologic process as the product of severity grade by the extent. The final score that was presented reflects the sum of all scores.

Oxygen Radical Detection

On days 2, 4, and 6 after infection, eight mice in each group of the infection, berberine, and normal control were sacrificed, respectively. The entire lung was homogenized by grinding in 1 mL of sterile 0.9% sodium chloride and then centrifugated at 4 ℃ 2 500 r/min for 20 min. The supernatants were collected and frozen at –20 ℃ for use. The concentrations of nitric oxide (NO) and inducible nitric oxide synthetase (iNOS) were detected with nitric oxide detection kit and nitric oxide synthetase detection kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Cytokine Assay

On days 2, 4, and 6 after infection, eight mice in each group of the infection, berberine, and normal control were sacrificed, respectively. The eyes of the mice were extracted to collect the blood. The sera were separated by centrifugation at 3 500 r/min for 15 min. The concentrations of tumor necrosis factor-alpha (TNF-α) and monocyte-specific chemoattractant molecules (MCP)-1 in the mice sera were determined with radio immunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) kits (Beijing Sinouk Institute of Biological Technology, China).

RT-PCR Assay

On days 2, 4, and 6 after infection, four mice in each group of the infection, berberine, and normal control were sacrificed, respectively. The entire lung RNA was extracted with Trizol agent (Invitrogen, American) according to the manufacturer's instructions. The primers (forward: 5'-CATGCGTCC AGCTGACTA-3', reverse: 5'-GGCTACAGGCTTGTC ACT-3') were designed to amplify TNF-α gene, and the length of RT-PCR product would be 646 bp. The primers for MCP-1 gene were forward: 5'-TAGGCTG GAGAGCTACAAGA-3' and reverse: 5'-TCACAGTC CGAGTCACACTA-3'), which resulted in a product at the length of 329 bp. The primers for the endogenous gene β-actin were forward: 5'-TGACGGCCAGGTC ATCACTA-3', reverse: 5'-TCCTGCTTGCTGATCCA CAT-3', with a RT-PCR product of 354 bp in length. RT-PCR reaction was performed by using RT-PCR kit (Fermentas, America) according to the manufacturer's instructions. PCR products were separated on 1% agarose gels, and the signal intensity of each band was quantified by using GeneTools from SynGene (Synoptics Ltd. England).

Statistical Analysis

All statistical computations were performed with SPSS 12.0. All data were expressed as the mean ± standard deviation. The Kaplan-Meier method was used to calculate the mice mean survival time, and the log-rank method was used to perform nonparametric test on the survival rate between different groups[15]. One-way ANOVA was used to compare the difference between two or more groups in other assays and the LSD test was used for individual comparisons. The *P* values less than 0.05 were considered statistically significant.

RESULTS

In vitro Anti-Influenza Virus Effects of Berberine

The antiviral efficacies of the berberine were evaluated in MDCK cells by two methods: CPE assay, which allowed detecting the cytotoxicity of infected-cells under the protection of drugs, and NA assay, which reflected the effects of drugs on virus activity. CPE method showed that berberine exhibited obvious antiviral activity against influenza virus in MDCK cells. The extent of cytotoxicity of berberine-treated MDCK cells was decreased, compared with that in the infection group. The TI of berberine was 9.69, which suggested that berberine had definite antiviral activity (Table 1). The NA assay further demonstrated the anti-influenza activity of berberine, which showed that when the concentration of berberine was between 0.00625–0.05 g/L, the viral NA activity was dramatically reduced, compared with that in the infected group ($P<0.05$, Table 2). As the data showed, ribavirin showed stronger anti-influenza activity than berberine *in vitro*, both in CPE and NA assay.

Table 1. *In Vitro* Antiviral Effect of Berberine on Influenza Virus in MDCK Cells

Drug	TC_0 (g/L)	TC_{50} (g/L)	IC_{50} (g/L)	TI
Berberine	0.05	0.242	0.025	9.69
Ribavirin	1.25	2.220	0.051	43.45

Berberine Decreased Mice Mortality

The mice mortality rate was calculated by recording the number of mice that died within day 2 to day 14 postinfection. The data showed that none of the uninfected mice died. The mortality rate in influenza-infected mice was 90% (18/20), while that in the berberine-treated group was reduced to 55% (11/22) and that in the ribavirin-treated group was reduced to 5% (1/20). The mean survival duration in the berberine group was 9.7 days, which was significantly longer than 6.3 days in the infection group ($P<0.05$). The mean survival duration in the ribavirin group was 13.55 days ($P<0.01$).

Table 2. Effect of Berberine on Viral NA Activity (n=6 in Each Group, $\bar{x} \pm s$)

Drug	Concentration (g/L)	NA (U)
Berberine	0.05	1.09 ± 0.04*
	0.025	1.16 ± 0.01*
	0.0125	1.25 ± 0.03*
	0.00625	1.28 ± 0.02*
	0.003125	1.36 ± 0.02
	0.001563	1.34 ± 0.02
Ribavirin	1.25	1.03 ± 0.01*
	0.625	1.07 ± 0.01*
	0.310	1.04 ± 0.02*
	0.155	1.05 ± 0.02*
	0.078	1.13 ± 0.02*
	0.039	1.25 ± 0.02*
Normal control		1.00 ± 0.00*
Infection control		1.36 ± 0.05
F value		44.819
P value		0.000

Note: *$P<0.05$, compared with the virus control group

Berberine Reduced Virus Titers in the Lungs

To explore whether berberine repressed influenza virus proliferation in mice, the virus titers in the lung were assessed dynamically. The hemagglutination titers were 2.03 ± 0.35, 1.34 ± 0.41, and 1.24 ± 0.25 in the infection group on days 2, 4, and 6, respectively, and 1.15 ± 0.40, 1.35 ± 0.41, and 1.28 ± 0.27 in the berberine group at the corresponding days. The data showed that berberine treatment obviously reduced the viral titers on day 2 postinfection compared with virus-infected mice ($P<0.05$), while almost similar viral titers were observed on days 4 and 6 between the two groups. The positive control drug, ribavirin, showed significantly strong inhibition on influenza replication, and the virus titers were reduced to nearly undetectable ($P<0.01$, Figure 1).

Berberine Improved the Lung Pathological Changes

The effects of berberine on the pathogenesis of influenza virus-induced pneumonia in mice were observed by HE staining, and a final score of pneumonitis from 0 to 9 was assigned for each pathologic process (inflammatory cell, lung

Chin J Integr Med 2011 Jun;17(6):444-452

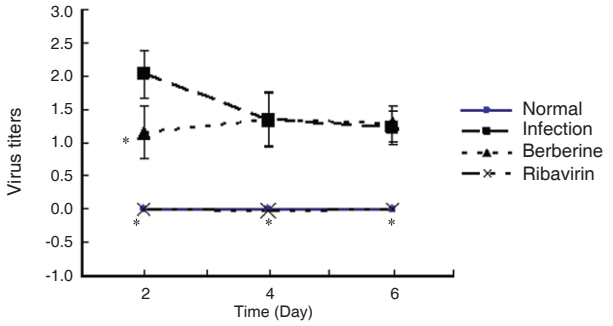

Figure 1. Effects of Berberine on Virus Titers in the Lungs on Days 2, 4, and 6 after Infection

Note: $^{*}P<0.01$, compared with the infection group on the same day

micrangium, and alveolar structure). The noninfected mice had normal lungs in terms of color, texture, and size, at various time points (Figure 2A). By contrast, on day 2 postinfection, the lung interstitials of infected-mice were infiltrated with a small amount of mononuclear cells and lymphocytes, and the lungs had minimal bronchitis; on day 4, the pneumonia developed with more inflammatory cells infiltration, some alveolar were filled with hemorrhage exudates, alveolar walls widened, and part of the bronchial mucosa had shedding or erosion. Moreover, other lung tissues

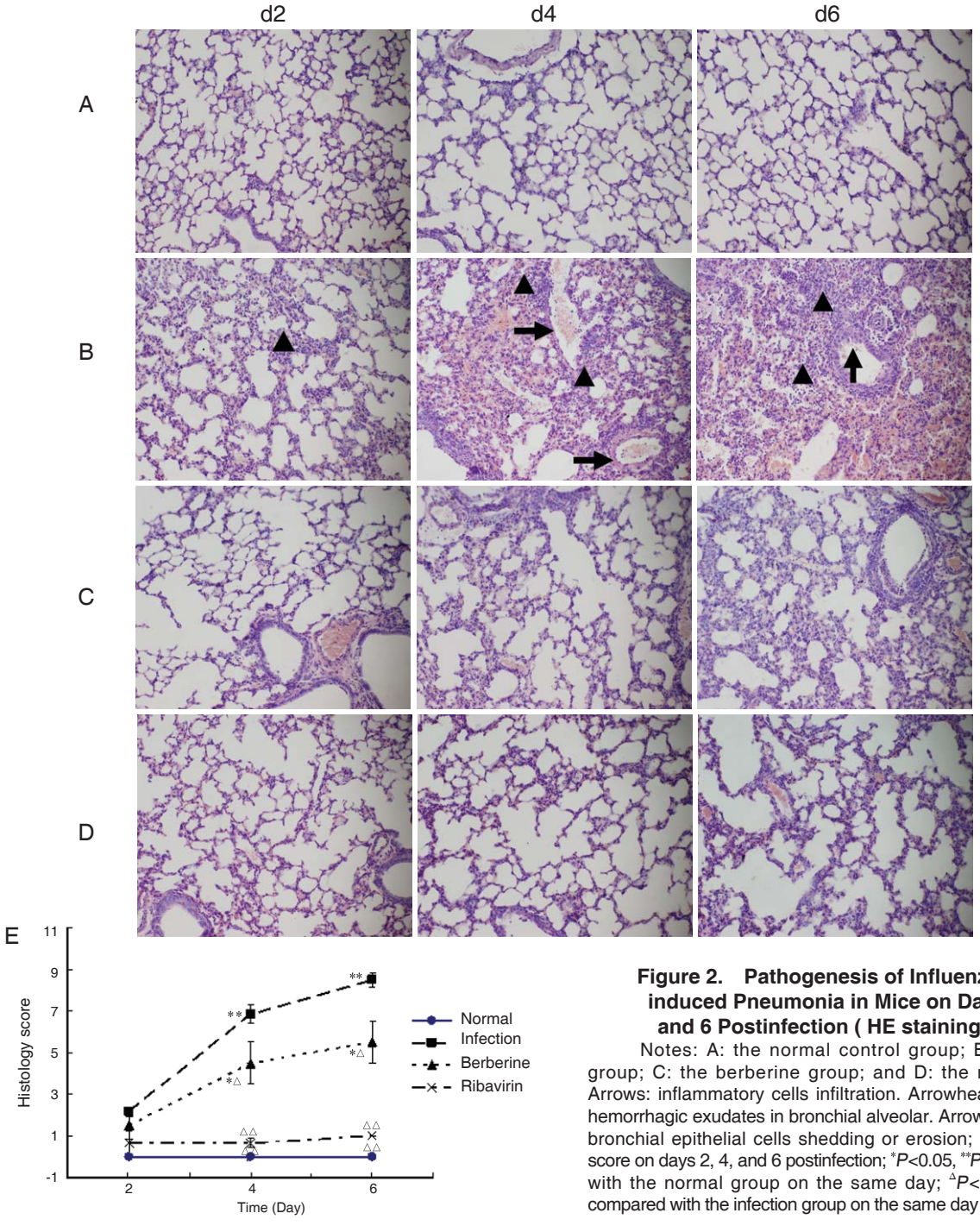

Figure 2. Pathogenesis of Influenza Virus-induced Pneumonia in Mice on Days 2, 4, and 6 Postinfection (HE staining, × 20)

Notes: A: the normal control group; B: the infection group; C: the berberine group; and D: the ribavirin group. Arrows: inflammatory cells infiltration. Arrowheads (horizontal): hemorrhagic exudates in bronchial alveolar. Arrowheads (vertical): bronchial epithelial cells shedding or erosion; E: the histology score on days 2, 4, and 6 postinfection; $^{*}P<0.05$, $^{**}P<0.01$, compared with the normal group on the same day; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, compared with the infection group on the same day

around the lesions showed significant compensatory emphysema; on day 6, the pneumonias were more severe, the pathological changes involved in the entire lung, with inflammatory cells exudation, a large number of epithelial shedding, and the bronchial epithelial hyperplasia significantly (Figure 2B). However, the pathogeneses in both berberine and ribavirin groups were significantly reduced, compared with the infection group on the same day (Figures 2C, 2D).

The histology score showed the development of pathogenesis in viral pneumonia. The histology scores were 2.17±0.22, 6.83±0.44, and 8.50±0.33 in the infection group, 1.50±0.67, 4.50±1.00, and 5.50±1.00 in berberine group, and 0.67±0.56, 0.67±0.22, and 1.00±0.00 in the ribavirin group on days 2, 4, and 6, respectively. As the histology score showed, the pathological extents in the berberine group were significantly reduced (P<0.05, Figure 2E).

Inhibitory Effects of Berberine on NO and iNOS Production in the Lungs

To observe the effects of berberine on the oxygen radical release in pathologic process of viral pneumonia in mice, the concentrations of NO and iNOS were assayed. The data showed that the NO secretions increased in the lung tissues of infected group from the beginning and peaked on day 6. Berberine significantly reduced the concentrations of NO compared with those in the infected group on days 2, 4, and 6 (P<0.01). Interestingly, the NO concentrations in the berberine group were even lower than those in the normal group on days 2 (P<0.01) and 4 (P<0.05, Figure 3A).

The iNOS concentrations in the lung tissues of the infected group were significantly higher on days 2 and 4 and reduced to the normal level on day 6. Berberine repressed the iNOS expressions on days 2 and 4, with the lower iNOS concentrations compared with the infected group (P<0.01). Similarly, the iNOS concentrations in the berberine group were lower than those in the normal group on days 2 and 4 (P<0.05, Figure 3B).

Berberine Repressed TNF-α and MCP-1 Transcription and Expression

To explore the effects of berberine on the inflammatory cytokines in viral pneumonia of mice, the mRNA and protein concentrations of TNF-α and

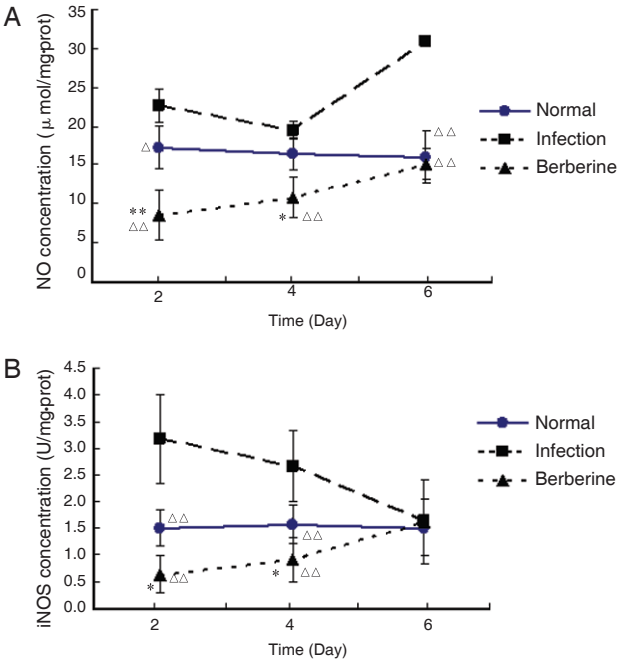

Figure 3. Effects of Berberine on Oxygen Radical Release in Pathologic Process of Viral Pneumonia in Mice

Notes: A: the NO concentration in the lungs; B: the iNOS concentration in the lungs; $^{*}P$<0.05, $^{**}P$<0.01, compared with the normal group on the same day; $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, compared with the infection group on the same day

MCP-1 were detected. The data showed that TNF-α concentrations in the infected group increased on days 4 and 6, and those in the berberine group were significantly lower than those in the infected group on days 4 (P<0.01) and 6 (P<0.05, Figure 4A). The effects of berberine on TNF/α transcription were coordinated with the effects on TNF-α expression (Figure 4B). The MCP-1 concentrations in the infected group increased significantly on days 4 and 6, and those in the berberine group were significantly low compared with the infected group on day 6 (P<0.01, Figure 4C). The effects of berberine on MCP-1 mRNA levels were coordinated with the effects on its protein concentration (Figure 4D).

DISCUSSION

Berberine (natural yellow 18, 5,6-dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo (5,6-α) quinolizinium), a benzyltetra isoquinoline plant alkaloid derived from the berberidaceae family, has been extensively used for many centuries in Chinese and native American medicines[16]. Previously, we reported berberine exerted antitumoral effects by suppressing the activity of the AP-1 signaling pathway and inhibiting the binding of transcription factors to the

Chin J Integr Med 2011 Jun;17(6):444-452

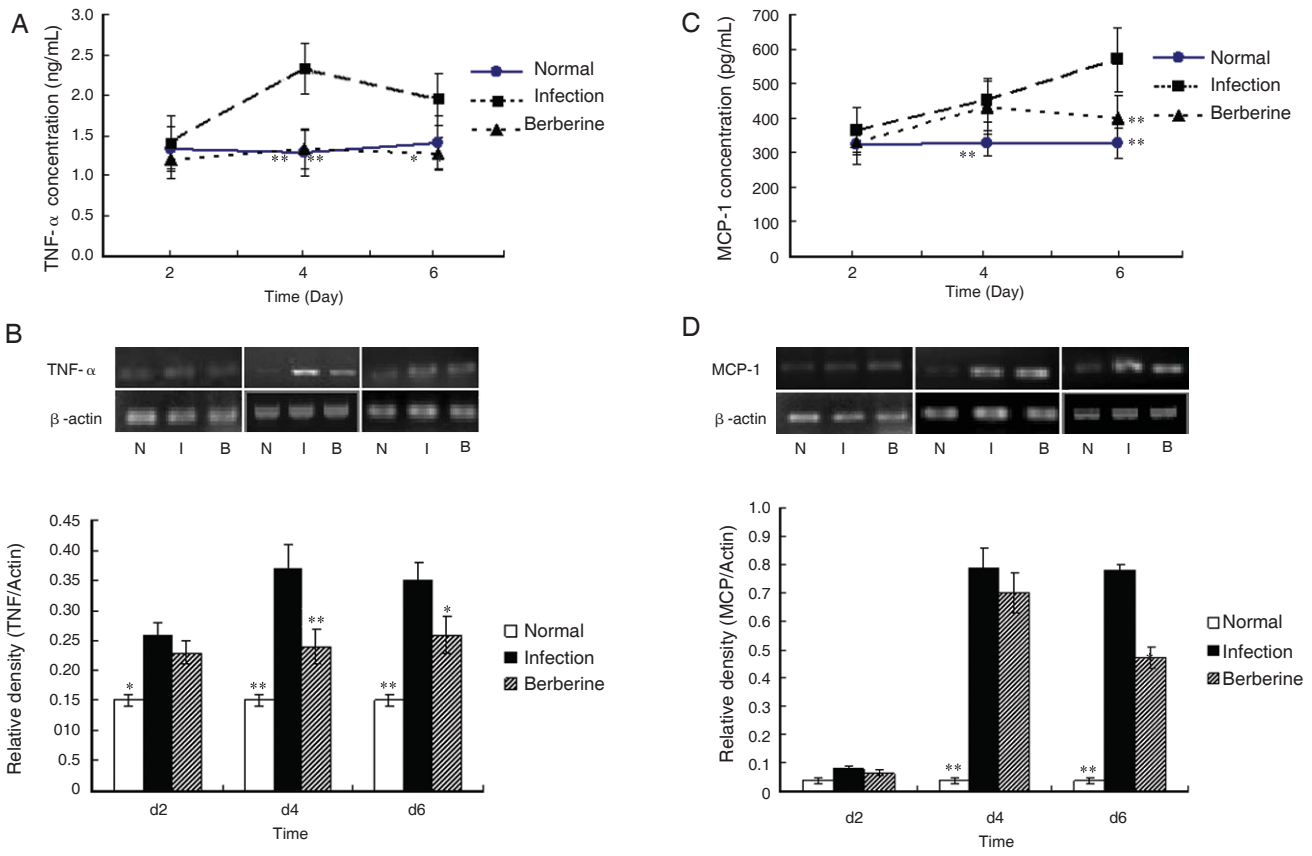

Figure 4. Effects of Berberine on the Inflammatory Cytokines Transcription and Expression in Viral Pneumonia of Mice

Notes: A: the TNF-α concentrations in mice sera. B: RT-PCR analysis revealing the mRNA level of TNF-α in mice lungs with β-actin gene as an internal control for RNA loading. The quantitative analysis of three RT-PCR analyses and the bar graphs represent relative density of TNF-α/β-actin. N represents the normal group; I represents the infection group; and B represents the berberine group. C: the MCP-1 concentrations in mice sera. D: RT-PCR analysis revealing the mRNA level of MCP-1 in mice lungs and the quantitative analysis of three RT-PCR analyses. $^{*}P<0.05$, $^{**}P<0.01$, compared with the infection group on the same day

CCND1 AP-1 motif[8]. Here, we assessed the potential effects of berberine on the influenza virus both *in vitro* and *in vivo*.

Our results showed that berberine repressed the CPE of MDCK cells induced by influenza virus and reduced viral neuraminidase activity *in vitro*. In mice models of influenza virus infection, berberine dramatically decreased mice mortality, prolonged the mice mean survival duration, and reduced virus titers in the lungs. The pathological changes in mice lungs were greatly relieved by berberine. These results demonstrated that berberine had direct antiviral effects on the influenza infection *in vitro* and *in vivo*.

To explore the effects of berberine on inflammatory reaction in flu-induced pneumonia, we detected the concentrations of oxygen radical (NO and iNOS) and the transcription and expression levels of inflammatory cytokines (TNF-α and MCP-1) after berberine treatment. The results showed that berberine dramatically reduced NO, and iNOS released and repressed TNF-α and MCP-1 transcription and expression.

Interestingly, we found that the inhibitory effects of berberine on the inflammatory substances were inconsistent with the changes in virus titers. The data showed that berberine treatment obviously reduced the virus titers on day 2 postinfection, while no inhibition was observed on days 4 and 6. However, berberine showed no inhibitory effects on TNF-α and MCP-1 on day 2, while significant inhibition was observed on day 4 for TNF-α and on day 6 for MCP-1. Moreover, for NO and iNOS, their concentrations in the berberine treatment group were dramatically reduced even compared with the normal group. These results demonstrated that the reduction of the inflammatory substances was not only due to the less stimulation of virus but also, more

importantly, due to the inhibitory effects of berberine on inflammatory response.

A robust cytokine response is the first line of lung defense against external challenges. However, excessive innate immune responses may also cause additional tissue damage and inflammation. This scenario may be especially true with pandemic and avian flu infections in which it is proposed that a cytokine storm is a major reason for the increased levels of mortality[17]. TNF-α, an important proinflammatory cytokine produced by macrophages, is pivotal in the establishment of an acute inflammatory response through its actions of activating endothelium and leukocytes and induction of increased vascular permeability, which in the lung may lead to increased pulmonary recruitment of inflammatory cells as well as the development of pulmonary edema and hemorrhage.

MCP-1, secreted by TNF-α-activated endothelial cells to recruit monocytes at the sites of vascular injury and inflammation, plays a primary role in inflammatory states of vessels[18]. It has been suggested that inhibition of tumor necrosis factor could reduce the severity of virus-specific lung immunopathology[19].

Our present study clearly showed that berberine significantly inhibited TNF-α transcription and expression along with the reduced expression of MCP-1, without exerting an effect on the pulmonary virus titers (the data on days 4 and 6 after infection). This suggested that berberine could be an effective treatment for viral pneumonia as a TNF-α antagonist. Our results were consistent with the data reported by Lee, et al[20], which suggested that berberine could inhibit TNF-α-induced inflammatory response in human umbilical vein endothelial cells.

NO is a simple inorganic radical and is produced by different isoforms of NO synthase[21]. It is well known that iNOS can be induced in various cells after stimulation with proinflammatory cytokines, such as TNF-α. The overproduction of NO is the primary pathogenic molecules in influenza virus-induced pneumonia in mice[22]. Lin, et al[23] suggested an increased production of iNOS and TNF-α significantly contributed to immunopathology and lung injury in flu-induced pneumonia. Our present study suggested that berberine could inhibit the production of iNOS and NO, which might account for the attenuation of pathologic changes in flu-induced pneumonia.

In conclusion, our present study indicated that berberine inhibited influenza virus infection both *in vitro* and *in vivo*. The results suggested another novel function of berberine as an anti-influenza formula, which had not been reported before. Meanwhile, berberine exerted strong inhibition on the inflammatory substances production, which greatly improved the pathologic changes in the influenza-induced viral pneumonia in mice model. Thus, the present study suggested two potential mechanisms of berberine treatment in the influenza-induced viral pneumonia. Our further studies will be required to determine the elaborated antiviral mechanism of berberine on influenza virus infection.

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