The Impact of Baicalin on Influenza Virus Gene Replication and Oxidative-Stress Damage

Xiaodan Qi¹, Haitao Yu², Yan Sun³, Hao Cheng⁴ and Chunjing Zhang⁵*

¹Department of Clinical Biochemistry, ²Department of Biology Genetics, ³Department of Clinical Pathogenic Microorganism, ⁴The Clinical College Students, ⁵Department of Biochemistry and Molecular Biology, Qiqihar Medical University, Qiqihar, China.

(Received: 07 April 2013; accepted: 28 May 2013)

Influenza A virus targets the lung epithelial cells for infection and produces clinical outcomes ranging from a mild upper respiratory infection to severe pneumonia. Baicalin is Chinese herbal monomer of phenolic hydroxyl flavonoids extracted from scutellaria baicalensis of heat-clearing and detoxicating drug, has better anti-inflammatory function, antioxidant function and antiviral activity, but the mechanism of the anti-influenza viral activity of baicalin has not been revealed. The research object of the study is to discuss the significant activity and part mechanism of baicalin against influenza A viruses. Antiviral efficiency of baicalin in vitro was observed with trypan blue staining method. RT-PCR was used to study the impact of baicalin on influenza A virus structural protein HA, NA, M gene replication. Intracellular generation of reactive oxygen species (ROS) was examined by flow cytometry using probe DCFH-DA. Antioxidant reagent kits were applied to detect Superoxide dismutase (SOD) activity, malondialdehyde (MDA) content. The results showed that baicalin has a better protective effect on cell damage from influenza A virus. Compared to virus infected group, HA, NA, M gene replication in high concentration baicalin (50µg/ml) treatment group was in a decreasing trend respectively, but there has no significant difference. Baicalin can reduce influenza virus-induced ROS production in a dose-dependent manner, and decrease the production of malondialdehyde (MDA) the end product of lipid peroxidation, improve the activity of antioxidant enzyme SOD, and so reduce influenza virus infection-induced oxidative-stress damage.

Keywords: Baicalin, influenza virus, HA, NA, M gene replication, oxidative stress.

Influenza A virus infection is an ongoing clinical problem and thus, there is an urgent need to understand the mechanisms that regulate the lung inflammation in order to unravel novel generic pharmacological strategies. Baicalin is a kind of phenolic hydroxyl flavonoids derived from plants (Fig 1), the glucuronide kind. The chemical composition is flavonoid glycosides which is integrated with flavone and glucuronic acid, further hydrolysis eventually produces baicalein, is the main quality control index of scutellaria baicalensis and its preparation, pharmacologic action is mostly identical to scutellaria baicalensis. IN our knowledge, baicalin has the functions of antioxidant, anti-inflammatory, antivirus and bacteriostasis (Qiu et al., 2012; Liu et al., 2011; Wan et al., 2012; Chu et al., 2007).

In former studies we observed that baicalin exerted an inhibitive effect on cytopathic effect (CPE) in a dose-dependent manner after influenza A H1N1 virus infection, regulated cell cycle distribution, protected cells from apoptosis damages through suppressing the activation of Caspase8/Caspase3 (Zhang et al., 2011). Further, we
found that the mechanism of antiinfluenza virus infection of baicalin may be related with the following aspects: to decrease the transcriptional activity of the oxidative stress sensitive transcription factor NF-kappaB and AP-1 by moderately decrease the higher expression level of TLR3 mRNA and the higher expression level of protein; and to further inhibit the mRNA expression of the downstream target genes IL-1\(\beta\), IL-8, RANTES and IFN-\(\beta\) thereby alleviate the inflammatory injuries and restore the stability and balance of immune function in vitro (Zhang et al., 2012). This study was to investigate the impact of baicalin on influenza virus gene replication and the oxidative-stress damage in vitro.

**MATERIALS AND METHODS**

**Cell culture and reagents**

**Cell culture**

Human lung adenocarcinoma cell line A549 cells were obtained from Cell Culture Center for Peking Union Medical College (Beijing, China). The cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin at 37\(^\circ\)C in an atmosphere containing 5\% CO\(_2\). Cells at logarithmic growth phase were used for the experiments.

**Reagents**

Influenza A H3N2 virus (A3/Gui Fang/81/23) and influenza A H1N1 virus (A1/Qian Fang/166/85), the virus titers are 2.4 and 2.7 respectively, protected in -75\(^\circ\)C refrigerator. TCID\(_{50}\) of A549 cell infected by influenza A H3N2 virus (A3/Gui Fang/81/23) is 10\(^{1.5}\), 293T cell is 10\(^{4}\). TCID\(_{50}\) of A549 cell infected influenza A FM1 virus is 10\(^{3}\), 293T cell is 10\(^{5.5}\). Baicalin was obtained from National Institute for the Control of Pharmaceutical and Biological Products, 95.2% purity, \(C_{21}H_{18}O_{11}\), Mr 446.35. The positive reference drug: Shuanghuanglian injection (freeze-dried powder), batch number is 0501004, were obtained from Harbin Pharmaceutical Group the second Chinese medicine plant. Ribavirin, batch number is 0506251, were obtained from CSPC OUYI Pharmaceutical Co., LTD.

**Cytopathic effect (CPE) and trypan blue stain assay**

A549 cells were seeded in 6-well cell culture plate (1×10\(^{5}\) cells/mL), 2ml/well. A549 cells were cultured with different conditions: A549 cells (control group), adding final concentration of 100TCID\(_{50}\) H3N2 to infect cells for 2h (damage group), adding different levels of baicalin and others the same as control group (baicalin group), adding different levels of baicalin and others the same as damage group (baicalin treatment group), ribavirin (Rib) or Shuanghuanglian injection (SHL) (positive drug control group), n=3, repeat three times. The death rate was calculated by trypan blue stain assay: morphologic changes were observed using phase-contrast microscopy. Using Reed & muench method calculated TC\(_{50}\) (the maximum non-toxic concentration) and TC\(_{50}\) (Median toxic concentration) of baicalin, and IC\(_{50}\) (Median inhibition concentration) of baicalin under CPE induced by virus infection, TI (therapeutic index) or SI (selection index) (TI or SI = TC\(_{50}\)/IC\(_{50}\)). Median inhibition concentration (of baicalin under CPE induced by virus infection, TI (therapeutic index) or SI (selection index) (TI or SI = TC\(_{50}\)/IC\(_{50}\)).

**RT-PCR analysis**

A549 cells were seeded in 60mm cell culture plate (1.5-2×10\(^{6}\) cells/mL), 5ml/plate. Total RNA and influenza A virus RNA were extracted, Total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturers protocol. Influenza A virus RNA were extracted using ZR Viral RNA kit. RT-PCR were detected using PCR Kit(AMV)Ver. 3.0 (Takara Biotechnology) and Titan (tm) One Tube RT-PCR System (Roche) according to the manufacturer’s instructions. After Agarose gel electrophoresis, band intensities of M gene (506bp), HA gene...
Antioxidant index detection
A549 cells were cultured with different conditions
A549 cells (control group), adding final concentration of 100TCID₅₀ H3N2 to effect 2 hour (damage group), adding different levels of baicalin (25, 50, 100µg/mL) and others the same as damage group (baicalin treatment group), N-acetyl- L-cysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) (positive drug control group), n=3, repeat three times. Added 10µmol/L DCFH-DA (Sigma, USA) to treated cells. Flow cytometry (COULTER EPICS®XL, Becton,USA) detected fluorescence intensity (FI), the excited wavelength is 488nm and the emitted wavelength is 510nm. According to the operation manual to detect SOD activity (Jiancheng, China) and MDA content (Jiancheng, China) after adding baicalin.

Statistical analysis
The differences were tested using ANOVA. All values are expressed as x±S.D., and statistical significance was defined as p<0.05. Each data represents the average of three independent experiments.

RESULTS
Pharmacodynamic observation of baicalin's antiviral activity
Using Reed & muench method to calculate TC₅₀ and TC₀ after baicalin infected A549 cells for 48 h, the results showed that TC₅₀ is 850-1000µg/mL and TC₀ is 400-500µg/mL. Cytopathic effect (CPE), IC₅₀ and SI, SI=TC₅₀/IC₅₀ (Table 1, Pre-group: Pre-treatment with drug before infection with virus, Co-group: Co-treatment with drug and virus, Post-group: Post-treatment with drug after infection with virus). After treatment for 48 h, the result of trypan blue stain assay showed that damage group was compared with control group, the mortality increased significantly. However, 50µg/mL baicalin treatment group was compared with damage group, the mortality reduced significantly (Fig 2).

Table 1. IC₅₀ (µg/mL) and SI analyses

<table>
<thead>
<tr>
<th></th>
<th>TC₅₀ µg/mL</th>
<th>IC₅₀ µg/mL</th>
<th>SI</th>
<th>Inhibition effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemoprophylaxis group</td>
<td>850-1000</td>
<td>&gt;100</td>
<td>—</td>
<td>Bad preventive effect</td>
</tr>
<tr>
<td>antiviral adsorption group</td>
<td>850-1000</td>
<td>40-45</td>
<td>&gt;8</td>
<td>Inhibiting viruses' adsorption</td>
</tr>
<tr>
<td>antiviral biosynthesis group</td>
<td>850-1000</td>
<td>45-50</td>
<td>&gt;8</td>
<td>Suppressing replication after adsorption</td>
</tr>
</tbody>
</table>

Effect of baicalin on HA gene mRNA expression
HA gene amplification primer sequence of human influenza H3N2 virus (Lu et al., 2005), as follows:
upstream primer
52-GACCTTTTGTGGTGAACGCAG-32
downstream primer
52 -TCCATTTGGAGTGTGCATT-32
amplification product is 605bp (Fig 3). Results show that, HA gene of influenza A virus of mRNA expression is significantly higher starting 12 to 24 hours after infection than control group in A549 cells (***P<0.01). But HA gene mRNA expression of ribavirin positive control group is significantly lower than virus infection group (**P<0.05). And positive control group of Shuanghuangliang injection and high concentration baicalin (50µg/
mL) comparing with virus infection group, have the trend of lower, but there was no dramatically difference.

![Fig. 3.](image)

**Effect of baicalin on M gene mRNA expression**

M gene amplification primer sequence of human influenza H1N1 and H3N2 virus (Zhu et al., 2000), as follows: upstream primer: 5' -ATTTGTGTTCACGCTCACCG-32 downstream primer: 5' -ACCAGCACTGGAGCTAGGAT-32 amplification product is 506bp (Fig 4). Results show that, M gene of influenza A virus of mRNA expression is significantly higher starting 24 hours after infection than control group in A549 cells (**P<0.01). And high concentration baicalin (50µg/mL) comparing with virus infection group, have the trend of lower, but there was no dramatically difference (P>0.05).

**Effect of baicalin on NA gene mRNA expression**

NA gene amplification primer sequence of human influenza H3N2 virus (Lu et al., 2005), as follows:

**upstream primer**

52 -ATGTCCAGCTCAAGTTGTCA-32

![Fig. 4.](image)

**Downstream primer**

52 -TTCCAGTTGTCTCTGCAGA-32 amplification product is 456bp (Fig. 5). Results show that, NA gene of influenza A virus of mRNA expression is significantly higher starting 24 hours after infection than control group in A549 cells (**P<0.01). But NA gene mRNA expression of ribavirin positive control group is significantly lower than virus infection group (#P<0.05). And positive control group of Shuanghuanglian
injection (SHL) and high concentration baicalin (BG) (50µg/mL) comparing with virus infection group, have the trend of lower, but there was no dramatically difference, among them, antiviral adsorption group (Co-) and antiviral biosynthesis group (Post-) have similar action.

**Reactive oxygen species production**

Flow Cytometer test results, as the figure shows, after A549 cells (red) were infected by influenza virus for 48h, ROS dramatically increased, increase fluorescence intensity comparing with control group cells (blue) by 40-50%. After cells (orange) treated with baicalin (50µg/mL), fluorescence intensity reduced, which is fundamentally the same as control group.

![Flow Cytometry Image](image)

**Fig. 6.** Intracellular generation of ROS was examined by flow cytometry using probe DCFH-DA. DCF-sensitive ROS (up) and DCF Intensity (down). *p<0.05 vs cell control group, ^p<0.05 vs virus infection group

**Content of MDA and Activation of SOD**

After A549 cells were infected by influenza virus for 48h, SOD activity significantly decreased (**P<0.01), but treated with baicalin, 10mM NAC and 50µM PDTC, SOD activity significantly increased (^P<0.05). MDA content significantly increased after infected by influenza virus (**P<0.01), MDA content of treated with baicalin groups are significantly higher than control group, but lower than virus infection group (*P<0.05).

![SOD and MDA Image](image)

**Fig. 7.** The influence of baicalin on the content of MDA and activation of SOD. **p<0.01 vs cell control group, ^p<0.01 vs virus infection group, ^p<0.05 vs virus infection group

**DISCUSSION**

Influenza is an acute respiratory infectious disease caused by influenza virus, especially influenza A virus, but because of its high variability, which has been risk factor for the most common and highly contagious diseases. Influenza viruses around the world continue to inflict significant global morbidity and mortality. Seasonal and pandemic influenza infections over the last century have claimed over 50 million lives and impose a huge socio-economic burden.

In our country influenza is prevalent, often can form erupt popular in local areas nearly every year, and the incidence ranks first among other infectious diseases. Because influenza virus strains are so good at mutating to form new ones, which brings much difficulties for prevention with vaccine, and there are at present no effective treatments for influenza. In this situation, search for effective drugs in the treasure-house of Chinese traditional medicine and create new agent to prevent and treat influenza, which become meaningful research work. Although influenza virus has been widely researched in pathology and clinics, we still don't understand it completely about the pathogenesis of influenza. So the effect of traditional Chinese medicine(TCM) effective constituents against influenza viruses and its mechanism would be further investigated, to promote TCM applied better to prevent and treat influenza.
This study shows that baicalin has antiviral activity in vitro, the efficiency is high starting 24 to 48 hours after infection, which can basically inhibited damage induced by influenza virus, and has dosage dependent and antiviral effect at high concentrations (baicalin ≥50µg/mL) shows no substantial change with time. Antiviral efficiency of baicalin will change with different dosing mode. Chemoprophylaxis group, studying if baicalin has a blocking effect on virus invading cells, the result shows that the effect of preventing absorption of influenza virus is weak after baicalin treated cells. Antiviral adsorption group, researching if baicalin can block viruses’ adsorption to the host cells, the result indicates that baicalin can inhibit virus absorption. Antiviral biosynthesis group, discussing if baicalin can inhibit cytopathic effect induced by viruses, the result demonstrates that baicalin can suppress replication induced by viruses’ adsorption. The effect is similar to ribavirin, but compared with Shuanghuanglian injection baicalin predominate on antiviral activity. The above results show that antiviral activity of baicalin is stable and obvious, and can be carried out in any of several ways. So we need to do some in-depth research on antiviral mechanism of baicalin.

Influenza A virus has a broad host range, can infect humans and other species of animals, trigger an influenza pandemic across the world. It is further divided into 16 H subtypes (H1-H16) and 9 N subtypes (N1-N9) according to HA, NA antigenicity difference on the surface of viral envelopes (Keawcharoen et al., 2010). H3N2 and H1N1 are the most popular in human at present. Research shows HA, M, NA are key viral envelope glycoproteins and primary targets of anti-influenza drugs. The research on how baicalin affect influenza A virus gene duplication in vitro shows that HA, M, NA genes of influenza A virus of mRNA expression are significantly higher starting 12 to 24 hours after infection than control group in A549 cells (**P<0.01). But HA, M, NA genes mRNA expression of ribavirin positive control group are significantly lower than virus infection group (# P<0.05). And positive control group of Shuanghuanglian injection and high concentration baicalin (50µg/mL) comparing with virus infection group, have the decreasing trend, but there was no significant difference.

In summary, baicalin has obvious antiviral activity in vitro through pharmacodynamics observation, and inhibit virus reproduction starting 24 to 48 hours after infection with dose-dependence, antiviral activity is evident at higher concentrations (50µg/mL), suppress reproduction induced by viruses’ adsorption. The effect of baicalin is less than ribavirin, but it predominate when compared with Shuanghuanglian injection. These results indicate that antiviral activity of baicalin may be unrelated to inhibit influenza A virus structural gene replication, anti-proliferation of influenza virus maybe accomplish through other means in vitro.

Influenza viruses cause oxidative stress and acute respiratory inflammation (Fukuyama et al., 2011; Phillips et al., 2010). Accumulated animal and human studies provide compelling evidence for a new paradigm whereby excessive production of reactive oxygen species (ROS), such as superoxide anion, and its derivatives hydrogen peroxide and peroxynitrite, are crucial mediators of the acute lung injury to influenza A virus infection (Vlahos et al., Selendis et al., 2013). Recent studies have focused on the role of lung antioxidant defense systems against injury induced by this virus because they likely play a role in virus-associated inflammation, viral susceptibility and immune clearance (Kesic et al., 2011; Yageta et al., 2011; Kosmider et al., 2012). It has been shown that antioxidant compounds inhibit influenza virus replication and diminish the release of inflammatory and apoptotic mediators during virus infection (Mata et al., 2011). Moreover, the combination of antioxidants with antiviral drugs synergistically reduces the lethal effects of influenza virus infections. This suggests that agents with antiviral and antioxidant activities could be a strategy for the treatment of patients with severe influenza-associated complications (Uchide et al., 2011).

The results show SOD activity of baicalin treatment group is significantly higher than H3N2 infected group for 48h, ROS production and MDA content is significantly lower than H3N2 infected group, which prompt that baicalin as antioxidant can reduce increased production of ROS induced by influenza virus infection, while relieve lipid peroxidation, reduce formation of lipid peroxidation end products, improve the activity of antioxidant enzyme SOD, and so reduce influenza virus
infection-induced oxidative-stress damage. So we will further study pathogenesis on pathological lesion caused by influenza virus infection-induced oxidation/antioxidation imbalance, and intervention mechanism of baicalin.

ACKNOWLEDGEMENTS

This work was funded by grants from The Natural Science Foundation of Heilongjiang Province (D201027 and D201134) and Committee of Education Science Foundation of Heilongjiang Province (12521627) and The Foundation of Qiqihar Science and Technology Bureau (SF2010-06).

REFERENCES


J PURE APPL MICROBIO, 7(2), JUNE 2013.