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

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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)ÿthe end product of lipid peroxidationÿimprove 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 anti-oxidant, 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

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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-1β, IL-8, RANTES and IFN-β 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*.

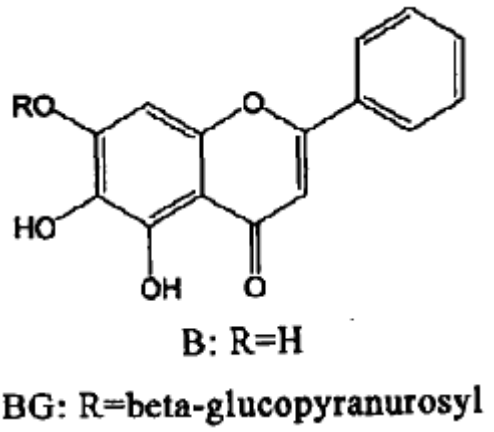

Fig. 1. Structures of baicalin (BG) and baicalein (B)

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 μg/ml streptomycin at 37°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^{-8} and 2^{-7} respectively, protected in -75°C refrigerator. $TCID_{50}$ of A549 cell infected by influenza A H3N2 virus (A3/Gui Fang/81/23) is $10^{-3.5}$, 293T cell is 10^{-5}. TCID50 of A549 cell infected influenza A H1N1 virus (A1/Qian Fang/166/85) is 10^{-3}, 293T cell is 10^{-4}. $TCID_{50}$ of A549 cell infected influenza A FM1 virus is 10^{-4}, 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 morphological changes of the cells were observed by using the phase contrast microscope . Using Reed & muench method calculated TC_0(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}). The death rate was calculated by trypan blue stain assay: morphologic changes were observed using phase-contrast microscopy, add 0.4% trypan blue stain to every well, calculate the mortality and survival rate.

RT-PCR analysis

A549 cells were seeded in 60mm cell culture plate ($1.5\text{-}2\times10^5$ 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

(605bp) and NA gene (456bp) of influenza A virus were recorded and analyzed by Bio-Rad Gel-Doc gel imaging system and Quantity One software respectively.

Antioxidant index detection

A549 cells were cultured with different conditions

A549 cells (control group), adding final concentration of 100$TCID_{50}$ 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 $\bar{x} \pm$ 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 TC_{50} and TC_0 after baicalin infected A549 cells for 48 h, the results showed that TC_{50} is 850-1000μg/mL and TC_0 is 400-500μg/mL. Cytopathic effect (CPE),IC_{50} and SI, $SI=TC_{50}/IC_{50}$ (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. IC_{50} (μg/mL) and SI analysises

	TC_{50} μg/mL	IC_{50} μg/mL	SI	Inhibition effect
Chemopro-phylaxis group(Pre-group)	850-1000	>100	—	Bad preventive effect
antiviral adsorption group(Co-group)	850-1000	40-45	>8	Inhibiting viruses' adsorption
antiviral biosynthesis group(Post-group)	850-1000	45-50	>8	Suppressing replication after adsorption

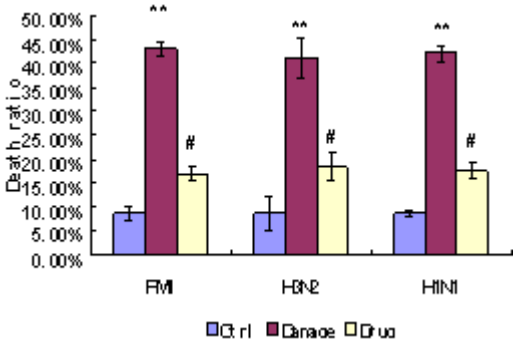

Fig. 2. The detected of cell death ratio using typan blue staining. Ctrl: control group, Cells under nomal condition; Damage: damage group, adding final concentration of 100$TCID_{50}$ virus to infect cells for 2h; Drug: baicalin treatment group, adding 50μg/mL baicalin and others the same as damage group. **p<0.01 vs Ctrl, #p<0.05 vs Damage

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 -GACCTTTTTGTTGAACGCAG-32

downstream primer

52 -TCCATTTGGAGTGATGCATT-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 Shuanghuanglian injection and high concentration baicalin (50μg/

mL) comparing with virus infection group, have the trend of lower, but there was no dramatically difference.

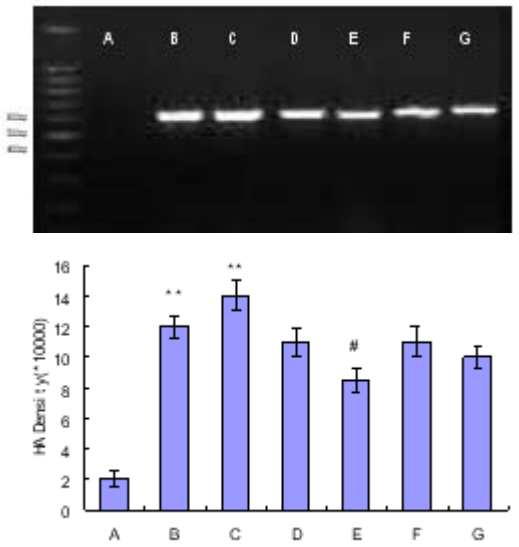

Fig. 3. The influence of baicalin on the mRNA expression level of HA gene. Agarose gel electrophoresistes (up) and Band Density (down). A: A549 cell control group; (Ctrl) B-C: H3N2 infected for 12 and 24h group (Virus group), D: Treated with SHL1.25mg/mL group; E: Treated with LBWL 0.5mg/mL group; F-G: Treated with Baicalin 25μg/mL and 50μg/mL group. **p<0.01 vs cell control group, #p<0.05 vs virus infection group

Effect of baicalin on M gene mRNA expression

M gene amplification primer sequence of human influenza H1N1and H3N2 virus(Zhu *et al*., 2000), as follows: upstream primer: 5-ATTTGTGTTCACGCTCACCG-32 downstream primer: 52 -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 -ATGGTCCAGCTCAAGTTGTCA-32

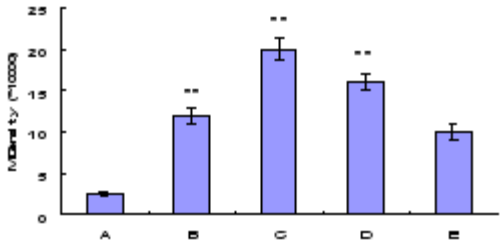

Fig. 4. The influence of baicalin on the mRNA expression level of M gene. A: A549 cell control group; B: H1N1 infected for 24h group; C: FM1 infected for 24h group; D: H3N2 infected for 24h group; E: Treated with Baicalin 50μg/mL infected by H1N1 group. **p<0.01 vs cell control group

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

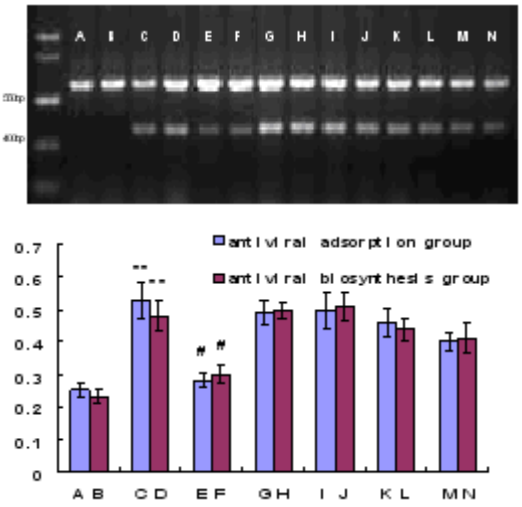

Fig. 5. The influence of baicalin to the mRNA expression level of NA gene . Agarose gel electrophoresis test (up) and Band Density (down). A-B: A549 cell control group; C-D: H3N2 infected for 24h group; E: Co-Ribavirin 0.5mg/mL; F: Post-Ribavirin 0.5mg/mL; G: Co-SHL1.25mg/mL; H: Post-SHL1.25mg/mL; I: Co-BG25μg/mL; J: Post-BG25μg/mL; K: Co-BG50μg/mL; L: Post-BG50μg/mL; M: Co-BG100μg/mL; N: Post-BG100μg/mL. **p<0.01 vs cell control group, #p<0.05 vs virus infection group

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.

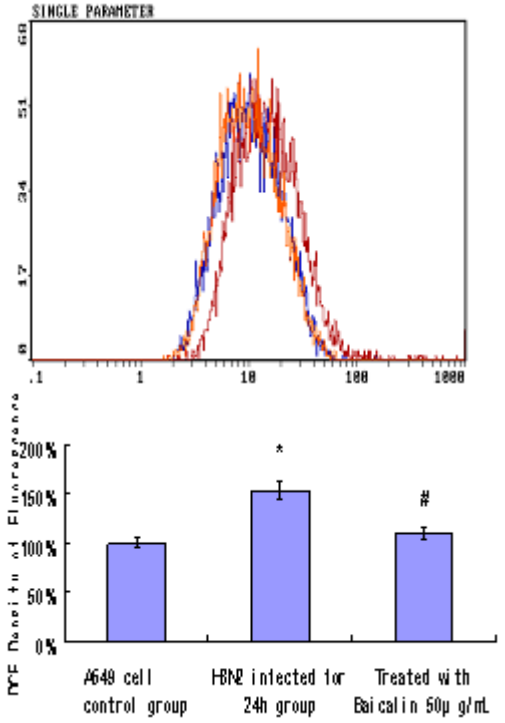

Fig. 6. Intracellular generation of ROS was examined by flow cytometry using probe DCFH-DA. DCF-sensitive ROS (up) and DCF Intensity (down). *p<0.05 vs cell control group, #p<0.05 vs virus infection group

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 increaseed (#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).

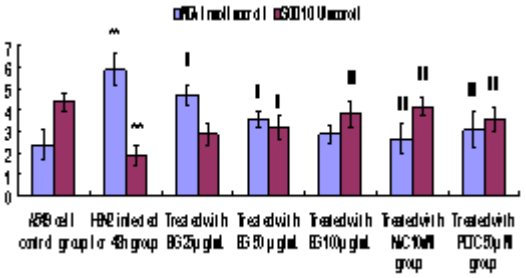

Fig. 7. The influence of baicalin on the content of MDA and activation of SOD. **p<0.01 vs cell control group, ##p<0.01 vs virus infection group, #p<0.05 vs virus infection group

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, studing 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 anti-virus 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 antivirus 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*., Selemidis *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.

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