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Anti-NDV activity of baicalin from a traditional Chinese medicine *in vitro*

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ABSTRACT. The purpose of this study was to investigate the anti-Newcastle disease virus (NDV) activities of baicalin from *Scutellaria baicalensis*, a Traditional Chinese Medicine *in vitro*. Chicken embryo fibroblasts (CEFs) were infected with NDV, and quantitative analysis of apoptotic cells was performed using flow cytometry. Cytotoxicity and anti-viral activities of baicalin were studied using the MTT method. The results showed that the maximal safe concentrations of baicalin to CEFs was 1×2^{-2} mg/m*l*. Baicalin could directly kill NDV, inhibit the infectivity of NDV to CEF and block intracellular NDV. It inhibited the apoptosis of NDV-infected CEFs and suppressed the spread of NDV. These results indicate that baicalin has strong anti-NDV activity and has the potential for use as components of an antiviral drug.
KEY WORDS: apoptosis, Baicalin, newcastle disease virus (NDV), Virus inhibitory rate

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Traditional Chinese Medicine (TCM) is a complete and independent medical system, which has been used to diagnose, treat and prevent illnesses for thousands of years. Since the World Health Organization began exploring TCM in the 1950s, TCM has garnered increasing interest beyond China because of its particular therapeutic methods and effects. Currently, in many countries, TCM provides patients with a new array of therapeutic options [1, 11]. In recent years, it has been proved that many TCMs and their ingredients possess antiviral effects [15]. Some TCMs and their extracts, such as shuanghuanglian injections, a common patented Chinese drug, have been developed into antiviral agents. TCM has many biological activities, such as adjusting immunologic function [18], inhibiting viral proliferation and contributing to infection processes (adsorption, transcription, replication and release) [5], and provides reliable biological functions with little side effects and low toxicity. However, TCM also has many antiviral active materials and is rarely used in animal medicine today.

Baicalin (5,6,7-trihydroxyflavone) is one of the main active ingredients derived from the dried root of *Scutellaria baicalensis*, a popular herb in TCM used as an anti-pyretic [16]. It is a small-molecule monomer and exhibits anti-inflammatory [13], anti-oxidant, anti-apoptotic [12], anti-viral [20], anti-asthmatic [17] and antibacterial properties [34]. Baicalin has demonstrated an ability to inhibit influenza A (H1N1) infection *in vitro* and protect cells from apoptosis damage through regulation of the cell cycle and suppression of the activation of caspase-8 and caspase-3. Downregulation of caspase-8 and caspase-3 is significant and shows a dose-dependent relationship [30].

Newcastle disease (ND) is a highly contagious disease in chickens. It affects more than 250 bird species and typically manifests with respiratory, gastrointestinal and/or nervous system symptoms. The most severe form of Newcastle disease can result in mortality rates exceeding 90% in susceptible chicken flocks. The Newcastle disease virus (NDV) causes significant economic losses worldwide every year [22]. Thus far, this disease was mainly controlled with ND vaccines. Antiviral treatments are not available in poultry owing to their cost and toxicity, and thus, a promising alternative is the use of compounds of natural origin, which have been underexplored for this purpose [28]. Some Chinese veterinary herbal medical preparations, such as Shuanghuanglian injection, and injection of *Astragalus* polysaccharides have become one of the principal weapons in veterinary clinical therapy and play an important role in clinical epidemic prevention [31]. However, these preparations have not displayed satisfactory clinical effects for ND. Research and development of effective clinical medicines are still one of the primary tasks of many veterinary researchers.

The purpose of the present study is to investigate the antiviral properties of baicalin and its mechanism of activity against NDV (La Sota strain IV).

MATERIALS AND METHODS

Equipment and reagents: Baicalin was purchased from Shanghai Jinsui Bio-Technology Co., Ltd., Shanghai, China), purity ≥98%. The carbon dioxide (CO_2) incubator

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was purchased from Thermo Fisher Scientific (Shanghai, China). The flow cytometry was purchased from BD Biosciences (Shanghai, China). Unless otherwise stated, all other reagents were from Sigma-Aldrich (Shanghai, China).

Cells and virus: The chicken embryo fibroblasts (CEFs) were prepared using 10-day-old specific pathogen free chicken embryos. The chick embryo was removed right after the eggshell was disinfected and opened. The head, extremities and viscera were removed, washed with phosphate buffered solution (PBS), cut into 1- to 2-mm^3 pieces and washed three times with PBS. A 0.25% trypsin solution was added to the samples for 30 min at 37°C, and then, the samples were centrifuged. The precipitate was washed three times with PBS and filtered through a 4-tier gauze. Cells were counted and diluted into 1×10^6 ml^{-1} with 10% Dulbecco's modified Eagle's medium (DMEM) and inoculated in 96-well culture plates at 37°C in a humid atmosphere of 5% CO_2 for 12 hr.

The strain of NDV (La Sota strain IV) propagated in the 10-day-old specific pathogen-free chicken embryos, was obtained from the Poultry Research Laboratory of Sichuan Agricultural University. The virus was stored at −80°C until use.

Virulence of NDV assay: The virulence of NDV was expressed as median tissue culture infective dose ($TCID_{50}$). NDV was prepared by 10-fold dilution (10^{-1}–10^{-8}). Then, 100 μl of viral solution was added to each well of 96-well plates containing CEF monolayers (four wells for each concentration). CEF, added to 100-μl DMEM basal medium with 2 μl/m*l* TPCK-trypsin, was used as the control. After 2 hr of incubation at 37°C, 100 μl of DMEM basal medium with 2 μl/m*l* TPCK-trypsin was added to each well. After 72 hr, the cytopathic effect was observed and recorded, and the $TCID_{50}$ value of NDV was calculated using the Reed-Muench formula.

Cytotoxicity analysis: Baicalin was diluted two-fold serially from 2 mg/m*l* to eight concentrations with DMEM. CEFs were prepared and cultivated in 96-well plates. CEF monolayers in the 96-well plates were exposed to baicalin at a series of concentrations (four wells each concentration). After culture for 72 hr in a 37°C humid atmosphere of 5% CO_2, 30 μl of MTT was added to each well and incubated for 4 hr. The supernatant was then removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for 10 min to dissolve the crystals completely. The absorbance at 570 nm (A570 value) was measured using a microliter enzyme-linked immunosorbent assay reader. Larger A570 values contribute to more live cells, since A570 values are correlated with the number of live cells. When the A570 values of the Chinese crude drug group were not significantly lower than that of the cells in the control group (indicating that the Chinese crude drugs had no cytotoxicity), the corresponding concentrations were considered a maximally safe concentration for CEFs. All experiments were carried out in triplicate [3].

Antiviral assay: According to the results of the cytotoxicity analysis, baicalin at eight different concentrations, from 250 to 1.953 μg/m*l,* was used for determination of anti-viral activity using the MTT method. When treating CEFs cultured in monolayers, the two-fold serial dilutions of Chinese crude drugs and 100 $TCID_{50}$ NDV were added to the cell plates, respectively, using three different methods:

Pre-addition of the drug: Baicalin solution was first added to CEFs at 100 μl per well (four wells per concentration). After incubation for 4 hr, the baicalin solution was removed, cells were washed twice with PBS, and 100 μl of the virus solution was added to each well.

Post-addition of the drug: The virus solution was added to the CEFs first. After incubation for 2 hr, baicalin solution was removed, cells were washed twice with PBS, and baicalin solution was added to the wells (four wells each concentration).

Simultaneous-addition of the drug and virus after mixing: Each concentration of baicalin solution was mixed with the virus solution, incubated for 2 hr at 37°C and then added to the CEFs (four wells for each concentration). At the same time, the NDV control group (virus only), cell control group (DMEM only) and the black group (no cells) were established [32].

All CEF plates were placed in a 5% CO_2 incubator at 37°C. After 72 hr when the NDV control group showed the appearance of cytopathic effect (CPE), the CEF viability was measured using the MTT assay [26]. The mean cellular A570 values were used as indices of anti-viral activity. Baicalin was considered to have significant anti-viral activity when the A570 value of the Chinese crude drug group was significantly higher than that of the virus control group was [14].

The virus inhibitory rate was calculated based on the formula [10]: Virus inhibitory rate=(the highest Ā570 value of baicalin group −Ā570 value of the virus control group)/(Ā570 value of the cell control group −Ā570 value of the virus control group)×100%.

Flow cytometry analysis of apoptosis (FACS): The CEFs were inoculated in 6-well culture plates at 37°C in a humid atmosphere of 5% CO_2 until they were cultured into a monolayer. A 100 $TCID_{50}$ virus solution was added to the CEFs. After incubation for 2 hr at 37°C, the virus solution was removed, the cells were washed twice with PBS, and the maximal safe concentration of the baicalin solution was added (three wells for each concentration). At the same time, the NDV control group (virus only) and the cell control group (DMEM only) were established. All CEF plates were placed in a 5% CO_2 incubator at 37°C for 72 hr until the NDV control group had the appearance of CPE.

Each group was collected by trypsinization, washed twice with PBS, resuspended in one time binding buffer at a concentration of 1×10^6 cells/m*l* and subjected to Annexin V-FITC/PI staining. The samples were analyzed by FACS and Cell Quest Research Software [4].

Statistical analysis: Data were expressed as mean± standard error (SE), and Duncan's multiple range test was used to analyze the difference among the Chinese crude drug and control groups. Differences between means were considered significant at $P<0.05$.

RESULTS

The cytotoxicity of baicalin to CEFs: The A570 values of each group are listed in Table 1. The A570 values of baicalin in the 1×2^{-2} mg/m*l* groups were not significantly lower than those of the corresponding cell control groups ($P > 0.05$). Therefore, these concentrations could be considered as the maximum safe concentrations.

Antiviral activity of baicalin in CEF:

(1) Antiviral activity of baicalin before addition of the drug

The A570 values and the virus inhibition rates of each group are listed in Table 2. The A570 values of baicalin in the 1×2^{-9}–1×2^{-2} mg/m*l* groups were significantly higher than the A570 values of the NDV control groups were ($P<0.05$). The highest virus inhibitory rates of the baicalin groups were 100%.

(2) Antiviral activity of Baicalin in post-addition of the drug

The A570 values and the virus inhibition rates of each group are listed in Table 2. The A570 values of baicalin in the 1×2^{-9}–1×2^{-2} mg/m*l* groups were significantly higher than the A570 values of the NDV control groups were ($P<0.05$). The highest virus inhibition rates of the baicalin groups were >100%.

(3) Antiviral activity of baicalin in simultaneous-addition of the drug and virus after mixing

The A570 values and the virus neutralization rates of each group are listed in Table 2. The A570 values of baicalin in the 1×2^{-9}–1×2^{-2} mg/m*l* groups were significantly higher than the A570 values of the NDV control groups were ($P<0.05$). The highest virus neutralization rates of the baicalin groups were higher than 100%.

Annexin V-FITC/PI staining: FACS was used in the quantitative analysis of apoptotic cells after NDV infection by staining cells with a combination of fluorescent Annexin V-FITC and PI (Table 3, Fig. 1). The early and late stage apoptosis rates of the cell control groups were 20.75% and 27.37%, respectively. The early and late apoptosis rates of NDV control groups were 24.52% and 36.20%, respectively. The early and late apoptosis rates of the maximum concentration for the baicalin groups were 18.17% and 27.33%, respectively.

Table 1. The cytotoxicity of Baicalin to CEF (A570 value)

Concentration (mg/m*l*)	Baicalin (A570)
1×2	$0.211 \pm 0.007^{d)}$
$1 \times 2^{\circ}$	$0.347 \pm 0.009^{c)}$
1×2^{-1}	$0.421 \pm 0.007^{b)}$
1×2^{-2}	$0.472 \pm 0.011^{a)}$
1×2^{-3}	$0.477 \pm 0.015^{a)}$
1×2^{-4}	$0.481 \pm 0.007^{a)}$
1×2^{-5}	$0.479 \pm 0.004^{a)}$
1×2^{-6}	$0.477 \pm 0.007^{a)}$
Cell control	$0.471 \pm 0.007^{a)}$

Data are shown as the mean ± SE. a–d) Data within a column without the same superscripts $P<0.05$.

DISCUSSION

The cell absorbance value (A570 value) is an index that reflects the number of living cells and is related to cell growth. A higher value of A570 indicates higher activity of CEF [19]. In this study, a high A570 value reflected good anti-viral activity of the Chinese crude drugs. The Chinese crude drugs were indicated to have no cytotoxicity when their A570 values were not significantly lower than those of control group were, and the corresponding concentration could be considered as the maximally safe concentration for CEFs. The results of the safe concentration indicated that at baicalin concentrations higher than 1×2^{-2} mg/m*l*, cell growth was inhibited. Baicalin, within a certain range of concentrations, demonstrated cytotoxicity.

The virus inhibitory rate directly reflects the antiviral potential of a drug [10]. The pre-addition of baicalin was thought to exert inhibitory action at a very early stage in the viral infection cycle as the virus adsorbs or penetrates into the host cell [25]. NDV infection is achieved via two envelope proteins, hemagglutinin-neuraminidase (HN) and the fusion protein (F) [7]. HN binds to sialic acid-containing

Table 2. Effect of Baicalin on blocking, neutralizing and inhibiting NDV

Concentration (mg/m*l*)	Block		Neutralize		Inhibit	
	Baicalin (A570)	Blocking rate	Baicalin (A570)	Neutralization rate	Baicalin (A570)	Inhibition rate
1×2^{-2}	$0.446 \pm 0.005^{a)}$	100.00%	$0.447 \pm 0.007^{a)}$	100.41%	$0.455 \pm 0.006^{a)}$	103.70%
1×2^{-3}	$0.443 \pm 0.010^{a)}$	98.77%	$0.445 \pm 0.006^{a)}$	99.59%	$0.450 \pm 0.008^{a, b)}$	101.65%
1×2^{-4}	$0.443 \pm 0.007^{a)}$	98.77%	$0.437 \pm 0.005^{a, b)}$	96.30%	$0.449 \pm 0.006^{a, b)}$	101.23%
1×2^{-5}	$0.430 \pm 0.004^{b)}$	93.42%	$0.425 \pm 0.007^{b)}$	91.36%	$0.447 \pm 0.009^{a, b)}$	100.41%
1×2^{-6}	$0.429 \pm 0.008^{b)}$	93.00%	$0.412 \pm 0.004^{c)}$	86.01%	$0.443 \pm 0.005^{a, b)}$	98.77%
1×2^{-7}	$0.406 \pm 0.008^{c)}$	83.54%	$0.397 \pm 0.005^{d)}$	79.84%	$0.444 \pm 0.008^{a, b)}$	99.18%
1×2^{-8}	$0.372 \pm 0.011^{d)}$	69.55%	$0.381 \pm 0.004^{e)}$	73.25%	$0.432 \pm 0.009^{b)}$	94.24%
1×2^{-9}	$0.345 \pm 0.006^{e)}$	58.44%	$0.365 \pm 0.007^{f)}$	66.67%	$0.422 \pm 0.008^{b)}$	90.12%
Cell control	$0.446 \pm 0.008^{a)}$	-	$0.446 \pm 0.008^{a)}$	-	$0.446 \pm 0.008^{a, b)}$	-
NDV control	$0.203 \pm 0.008^{f)}$	-	$0.203 \pm 0.008^{g)}$	-	$0.203 \pm 0.008^{c)}$	-

Data are shown as the mean ± SE. a–g) Data within a column without the same superscripts $P<0.05$.

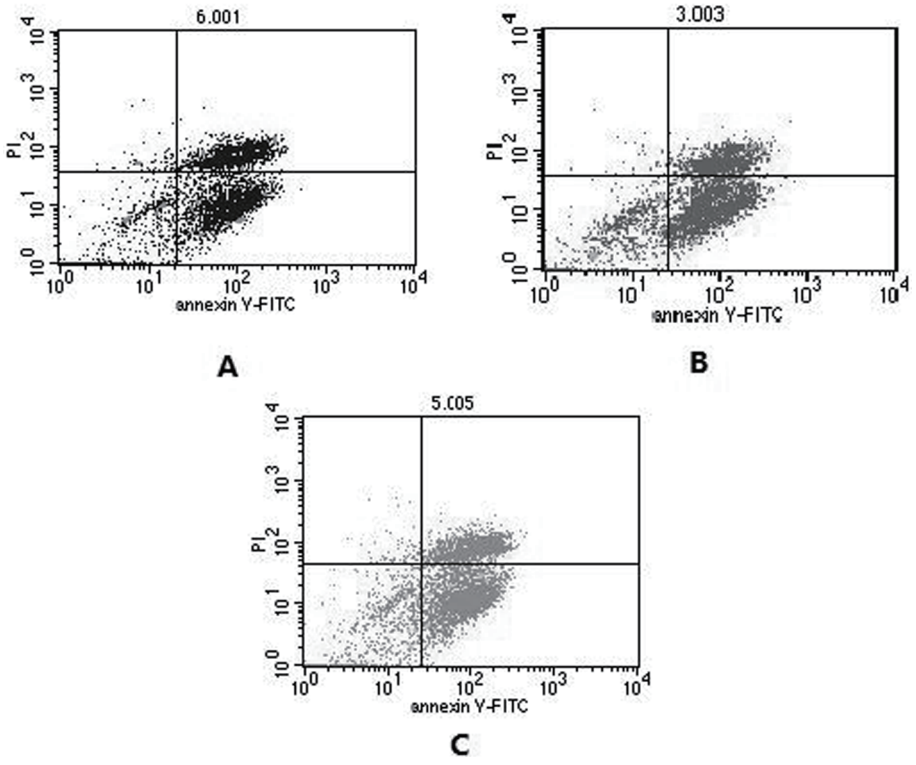

Fig. 1. Flow cytometry analysis of Annexin V/PI assay. In the four quadrants for each image, the right lower quadrant is for the early apoptosis rate, and the right upper quadrant is for the late apoptosis rate. A: Cell control; B: NDV control; C: CEFs treated with the maximum safe concentration of baicalin and 100 pfu/well of NDV.

Table 3. The Apoptosis rates of treatment groups

Classification	Apoptosis rates (%)	
	Early stage	Late stage
Baicalin	18.17 ± 0.61[c)]	27.33 ± 0.71[b)]
Cells control	20.75 ± 0.74[b)]	27.37 ± 0.59[b)]
NDV control	24.52 ± 0.72[a)]	36.2 ± 0.65[a)]

Data are shown as the mean ± SE. a–c) Data within a column without the same superscripts $P<0.01$.

receptors, leading to virus adsorption, and promotes membrane fusion causing virus penetration into host cells [8]. There are two sialic acid binding sites on the globular head region of HN [29]. Our results showed that the A570 values of all eight concentrations of the baicalin groups were significantly higher than the A570 values of the corresponding virus control groups were, which indicated that they could prevent NDV infection. Baicalin could block HN binding to sialic acid-containing receptors and virus adsorption.

The post-addition of Chinese crude drugs was thought to prevent viral protein synthesis. It may affect the transmembrane and intracellular signaling processes involved in regulating virus expression. These results were supported by previous findings that viral protein synthesis was prevented by carrageenan, as inhibition was observed only when the compound was present during virus entry [23]. Damonte *et al.* reported that the few attempts had been made to find anti-viral substances by mainly focusing on the screening of probable ribonucleic acid (RNA) synthesis inhibitors, such as ribavirin and other nucleoside analogs [9]. In agreement with this idea, our results showed that the A570 values of all eight concentrations of the baicalin groups were significantly higher than the A570 values of the corresponding virus control groups, which indicated that they could treat the NDV infection. Baicalin could inhibit viral protein or RNA synthesis.

The mixed-addition of Chinese crude drugs and viruses was thought to cause direct inactivation of the viruses. Wang *et al.* reported that sulfated *Lycium barbarum* polysaccharide at certain concentrations could directly kill NDV [27]. In agreement with this idea, our results showed that the A570 values of all eight concentrations of the baicalin groups were significantly higher than the A570 values of the corresponding virus control groups were, which indicated that they could directly kill NDV.

The NDV inhibitory rate of baicalin decreased in a dose-dependent manner. Both of the blocking rate and neutralization rate are significantly decreased as the decrease of the baicalin concentrations, but the inhibition rate is slightly decreased as the decrease of the baicalin concentrations. High concentrations of baicalin in the all three methods of addi-

tion, such as 1×2^{-2} mg/m*l*, 1×2^{-3} mg/m*l* and 1×2^{-4} mg/m*l*, had strong effects on NDV. However, low concentrations of baicalin in combination with the simultaneous-addition of the drug and virus after mixing, such as 1×2^{-7} mg/m*l*, 1×2^{-8} mg/m*l* and 1×2^{-9} mg/m*l*, had the strongest effect in directly killing NDV of the three methods of addition.

The highest virus inhibitory rate directly reflected the anti-viral potency. The highest virus inhibition rates and the highest virus neutralization rates of the baicalin groups were both higher than 100%. Our results showed that baicalin could suppress NDV as well as lead to CEF growth and proliferation. Zhao *et al*. reported that baicalin could promote CEF growth significantly [33]. This view is consistent with the results of our study.

NDV can induce apoptosis of infected CEF. To investigate the anti-viral mechanism of baicalin, we evaluated the apoptosis of CFE by flow cytometry (FCM) [6]. Our results showed that baicalin could inhibit NDV-infected CEF apoptosis in both early and late stages of the infection process. The stages of apoptosis can be categorized into early and late, and the sum of the stages represents the total apoptosis rate. NDV induces apoptosis in cells through caspase- and p38-MAPK-dependent pathways [2]. Baicalin may inhibit NDV-infected CEF apoptosis by blocking the signaling pathway and thus, reduce the release of NDV.

In recent years, the inhibitory effect of some TCM against NDV has been studied. Zhang *et al*. reported that some flavone ingredients from TCM could inhibit NDV-infected cells, such as baical skullcap root flavones, wild dendranthema flower flavones, epimedium flavones and sanchi flavones. However, their toxicity is higher than that of baicalin is, and only flavone prescriptions with suitable compatibility can show good anti-viral activity [31]. Some polysaccharides from TCM also could inhibit NDV infection of cells, such as *Chuanmingshen violaceum* polysaccharide, *Auricularia auricula* polysaccharide and *Tremella* polysaccharide. Anti-viral activity of these polysaccharides is insufficient, but sulfation could increase their anti-NDV activity [21, 24, 32].

In conclusion, baicalin can suppress, neutralize and block NDV *in vitro*, as well inhibit NDV-infected CEF apoptosis, leading to the subsequent suppression of the spreading of NDV infection. The compound has potential for use as components of future anti-viral regimens.

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