Research Note: Anti-inflammatory effects and antiviral activities of baicalein and chlorogenic acid against infectious bursal disease virus in embryonic eggs

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ABSTRACT The purpose of this study was to investigate if baicalein and chlorogenic acid could inhibit the inflammatory responses induced by and protect against infectious bursal disease virus (**IBDV**) in chicken embryonic eggs. Nine-day-old embryonated chicken eggs were randomly divided into 3 groups of 50 eggs per group: 1) treatment with varying concentrations of baicalein, 2) treatment with varying concentrations of chlorogenic acid, or 3) left untreated as a control. Forty-eight hours after hatching, each group was inoculated with a very virulent IBDV isolate, and the survival of the embryo was monitored daily until the embryonic livers were collected 72 h after inoculation. After IBDV infection, the viral loads in the embryonic livers were evaluated using qRT-PCR, and the hepatic content of inflammatory mediators, such as histamine, interleukin 1β (**IL-1β**), tumor necrosis factor alpha (**TNF-α**), and nuclear factor-kappa B (**NF-κB**), were examined. Significant antiviral potential was demonstrated at concentrations of 108 and 215 μg/egg of baicalein and chlorogenic acid, respectively. We observed a concentration-dependent response in the antiviral properties of these chemicals. Treating the embryos with baicalein and chlorogenic acid significantly reduced histamine production. Moreover, pretreatment with baicalein and chlorogenic acid significantly inhibited NF-κB activation, and this inhibited the subsequent production of the proinflammatory cytokines TNF-α and IL-1β in the context of IBDV infection. These findings suggest that baicalein and chlorogenic acid have anti-IBDV properties, and they may be useful in the prevention of inflammation-related diseases.

Key words: infectious bursal disease virus (IBDV), baicalein, chlorogenic acid, inflammatory mediator, embryonated chicken egg

2021 Poultry Science 100:100987
https://doi.org/10.1016/j.psj.2021.01.010

INTRODUCTION

Infectious bursal disease (**IBD**) is an immunosuppressive and highly contagious disease that affects young chickens worldwide. Infectious bursal disease virus (**IBDV**) is an etiological agent that belongs to the *Birnaviridae* family which destroys the premature B-lymphocyte precursors in the bursa of Fabricius (Muller et al., 1979). IBDV-infected chickens experience severe immunosuppression that leads to an increased susceptibility to other diseases and poor immune responses to vaccines (Aricibasi et al., 2010). Currently, there is a limited understanding of the molecular mechanisms responsible for IBDV infection and pathogenesis. A better understanding of how IBDV pathogenesis leads to immunosuppression and death in chickens is needed to develop efficient control measures. IBDV-infected bursa of Fabricius undergo a depletion of lymphoid B cells due to both necrosis and apoptosis (Muller et al., 1979). When an animal is infected with a pathogen, a complex network of cytokines controls both the inflammatory and specific immune responses. Therefore, cytokines regulate both inflammation and immunity, and there is growing evidence that the pathogenesis of IBD is controlled by innate immunity, particularly proinflammatory mediators (Rauf et al., 2011).

Traditionally, broilers were protected from IBDV infection through passive immunization. However, inactivated or attenuated vaccines do not provide sufficient

Received July 29, 2020.
Accepted January 2, 2021.

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protection against IBDV. Hence, there is a dire need to investigate alternative strategies to control for IBDV. One such alternative strategy is to use different indigenous medicinal plants with antiviral properties. Natural products from medicinal plants are being developed to prevent a variety of animal diseases (Tan and Vanitha, 2004), and these often have fewer side effects than pharmacological medicines. In addition, there is a lower chance of viral strains developing a resistance to natural medicinal plants with antiviral properties because they often function through a multistage mode of action (Ahmad et al., 2014).

Generating treatments against viruses using medicinal ingredients from plants combines the experience and creativity of folk medicine with the techniques of modern pharmacology and phytochemistry (Ahmad et al., 2014). The active ingredients isolated from medicinal plants have been widely used as traditional herbal medicines. These have been reported to have antiviral and anti-inflammatory activity in addition to antioxidant and immunomodulatory effects (Sithisarn et al., 2013). Baicalein (5,6,7-trihydroxyflavone) is a flavonoid extracted from the root of *Scutellaria baicalensis* that has numerous pharmacological properties, including antibacterial, antioxidant, antiviral, and anti-inflammatory activities (Lin et al., 1996). Chlorogenic acid is a common polyphenol that is found in various foods and beverages. Chlorogenic acid is known to possess various pharmacologic effects, such as anti-inflammatory and multiantiviral activities (Shin et al., 2015). However, the effects of these compounds on IBDV activity remain elusive. Here, we investigated and compared the variety of beneficial effects of baicalein and chlorogenic acid on IBDV replication in chicken embryos. We further characterized the effects of baicalein and chlorogenic acid on IBDV-induced nuclear factor-kappa B (**NF-κB**) expression and related proinflammatory cytokines, including IL-1β, tumor necrosis factor alpha (**TNF-α**), and histamine levels, in embryonic chicken livers. The overall aim of this work was to evaluate the anti-inflammatory effects and antiviral activities of baicalein and chlorogenic acid against IBDV in embryonic eggs, to understand the molecular mechanism underlying these effects.

MATERIALS AND METHODS

Chemical and Biological Agents

Baicalein (98% pure) and chlorogenic acid (98% pure) were purchased from Henan Lyle Wormwood Biological Technology Co., Ltd. (Henan, PR China). Methyl thiazolyl tetrazolium (**MTT**) and dimethyl sulfoxide were obtained from Beyotime Biotechnology Company (Jiangsu, PR China). L-Histamine was purchased from TakaRa Biotechnology Company (Dalian, PR China). All reagents were of analytical grade. The IBDV strain XA2004 was provided by the Animal Science and Technology College, Henan University of Science and Technology, PR China. The published ELD_{50} of the particular virus stock is $2.5 \times 10^{7.40}$/mL (Li et al., 2015). Specific pathogen-free eggs were purchased from the Poultry Science and Technology Limited Company (Jinan Spirax Ferrer, PR China).

Cell Culture Conditions

Chick embryo fibroblasts (**CEF**) were propagated in DMEM media with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO_2. Cells were seeded on 96-well plates and maintained at a density of 1×10^6 per mL.

Measuring the Safe Concentration of Baicalein and Chlorogenic Acid in CEF Cells

Cytotoxicity was assessed by the MTT assay. Briefly, baicalein (20, 10, 5, 2.5, 1.25, and 0.625 μg/mL) and chlorogenic acid (200, 100, 50, 25, 12.5, and 6.25 μg/mL) were diluted into 6 gradient concentrations in Dulbecco's modified Eagle's medium (**DMEM**) medium. CEF cells were cultured in triplicate for each of the compounds tested. Uninoculated cells served as a negative control, and all cells were incubated for 72 h at 37°C. The cell supernatants were removed, and then, 90 mL of DMEM and 10 mL of MTT (5 mg/mL) reagents were added to the wells. The supernatant was carefully removed after 4 h, and 100 μL of dimethyl sulfoxide was added to each well. The plate was shaken for 10 min to completely dissolve any crystals. The OD values at 490 nm were measured using a microplate reader, and the viability of the cells calculated determined by calculating the 50% inhibiting concentration (**IC50**). The maximum concentration of baicalein and chlorogenic acid was determined by IC50.

Grouping and Incubation of Specific Pathogen-Free Chicken Embryos

Nine-day-old embryonated chicken eggs were randomly divided into 3 groups with 50 eggs per group. Two groups were treated with different concentrations of baicalein and chlorogenic acid; each group had 10 eggs per concentration of each compound. The uninoculated group was used as the control. The experiments were performed in duplicate.

Baicalein, Chlorogenic Acid, and Virus Inoculation in Embryonated Eggs

A serial dilution method was used to prepare baicalein and chlorogenic acid at a safe concentration and at 1:2, 1:4, 1:8, and 1:16 concentrations. Two groups were inoculated with 0.2 mL of baicalein and chlorogenic acid at different concentrations through the allantoic cavity. Forty-eight hours after hatching, embryonated eggs were inoculated with 0.2 mL of the IBDV isolate. Untreated embryonated eggs were inoculated with equal amounts of either normal saline or virus. The embryonated eggs were incubated at 37°C for 72 h. Embryo

survival was assessed daily. At 72 h after inoculation, livers were collected from 6 embryos of each group.

Sampling and Processing of Embryonic Chicken Livers

Embryonic livers were prepared using a previously published method (Li et al., 2015), with some modifications. Briefly, the embryonated eggs were sacrificed 72 h after inoculation, and the embryos were culled by decapitation. Livers were promptly dissected out and were then washed twice with ice cold isolation buffer (300 mmol sucrose, 1 mmol ethylenediaminetetraacitic acid, and 10 mmol Tris base, pH7.5) to remove excess blood, after which they were weighed and homogenized. Livers were then placed into fresh isolation buffer (1:10 w/v) in a chilled glass tissue homogenizer. Liver homogenates were then collected. All procedures were performed at 0°C to 4°C.

Assay of Viral RNA Loads in Chicken Embryonic Livers

Viral RNA loads in embryonic livers were measured by the qRT-PCR method, as previously described (Li et al., 2015). Briefly, each sample (20 mg) was homogenized in a sample tube with 0.5 mL of DEPC-dH_2O and then centrifuged at 5,000 × *g* for 5 min at 4°C. Total RNA was extracted from the supernatants (0.2 mL) using the Trizol reagent (Invitrogen, Waltham, MA), according to the manufacturer's instructions. The qRT-PCR reactions were performed using the one-step SYBR ExScript RT-PCR Kit (Takara Bio, Dalian, PR China), according to the manufacturer's instructions. A pair of primers, 5′-GCCGATGATTACCAATTCTCATC-3′ (located at 633–656 bp) and 5′-CATAGTCTGCGGCCACAGCTC-3′ (located at 822–843 bp), were designed based on the sequences of the *VP2* gene of the XA2004 IBDV isolate. PCR was performed in an iQ5 Real-Time PCR Detection System (Bio-Rad, Irvine, CA). Concentrations of viral RNA were determined using the threshold cycle method. To quantitate viral RNA in livers, the concentration of the plasmid pMD19-VP2 was determined using a spectrophotometer. Serial dilutions ranging from 10^9 copies/μL to 10^1 copies/μL were used as standard controls. To generate a standard curve, the Ct of the standard dilutions was plotted against the number of plasmid copies. The viral RNA loads in chicken embryonic livers are presented as log copies/mg wet weight of the liver.

Assay of Histamine Content in the Livers of Chicken Embryos

The histamine content was assayed as previously described (Li et al., 2015). Briefly, each of the embryonic liver samples (50 mg) were homogenized with 1.5 mL of 25% (w/v) trichloroacetic acid and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant (1.0 mL) was mixed with 1.0 mL of double-distilled water, 0.5 mL of 0.4 N NaOH, and 0.1% o-Phthalaldehyde methanol. This was incubated at 22°C in the dark for 10 min. This reaction was stopped by the addition of 0.5 mL of 0.5-N HCl. A blank was created by combining, in order, 0.5 mL each of 0.5-N HCl, 0.1% o-Phthalaldehyde methanol, and 0.4-N NaOH. The fluorescence intensities were determined by fluorescence spectrophotometry (Shanghai Precision & Scientific Instrument Co., Ltd., PR China) using an activation wavelength of 360 nm and fluorescence monitoring at 450 nm. The histamine concentration is presented as μg/g wet weight of the liver.

Assay of TNF-α and IL-1β Content in Embryonic Livers

The embryonic livers were collected from the surviving embryos at 72 h after inoculation. Each of the embryonic liver samples (50 mg) was homogenized in 1.5 mL of phosphate buffer (pH 6.8). The concentrations of TNF-α and IL-1β were assayed by using an ELISA Kit (Shanghai HuaLing Biotechnology Co., Ltd., PR China) according to the manufacturer's instructions. The TNF-α and IL-1β concentrations are presented as μg/g wet weight of liver.

Assay of NF-κB and NF-κB p65 Content in Embryonic Livers

The same liver homogenates described previously were used to extract nucleoprotein and plasmosin using the nucleoprotein extraction Kit (Shanghai BangYi Biol. Tech. Co. Ltd., PR China), according to the manufacturer's instructions. The NF-κB content in plasmosin and the NF-κB p65 content in nucleoprotein were assayed by an ELISA Kit (Shanghai QiMing Biotechnology Co., Ltd., PR China), according to the manufacturer's instructions. The NF-κB and NF-κB p65 concentrations are presented as pg/g wet weight of the liver.

Statistical Analysis

All assays were performed in duplicate. Results are expressed as mean ± SD. All data were analyzed using a one-way ANOVA using GaphPad Prism 5.01 software. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The IC50 of baicalein and chlorogenic acid to CEFs was 7.2 μg/mL and 28.6 μg/mL, respectively. Based on the IC50 and an average of 30 mL of allantoic fluid per egg, 2 groups were inoculated with 0.2 mL of different concentrations of baicalein (216, 108, 54, 27, and 13.5 μg/egg) and chlorogenic acid (858, 429, 214.5, 107.25, and 53.125 μg/egg) through the allantoic cavity. Medicinal plants can have broad-spectrum antiviral activities against RNA and DNA viruses (Ahmad et al., 2014). Based on these criteria, we used ingredients isolated from Chinese herbal medicinal plants that are commonly

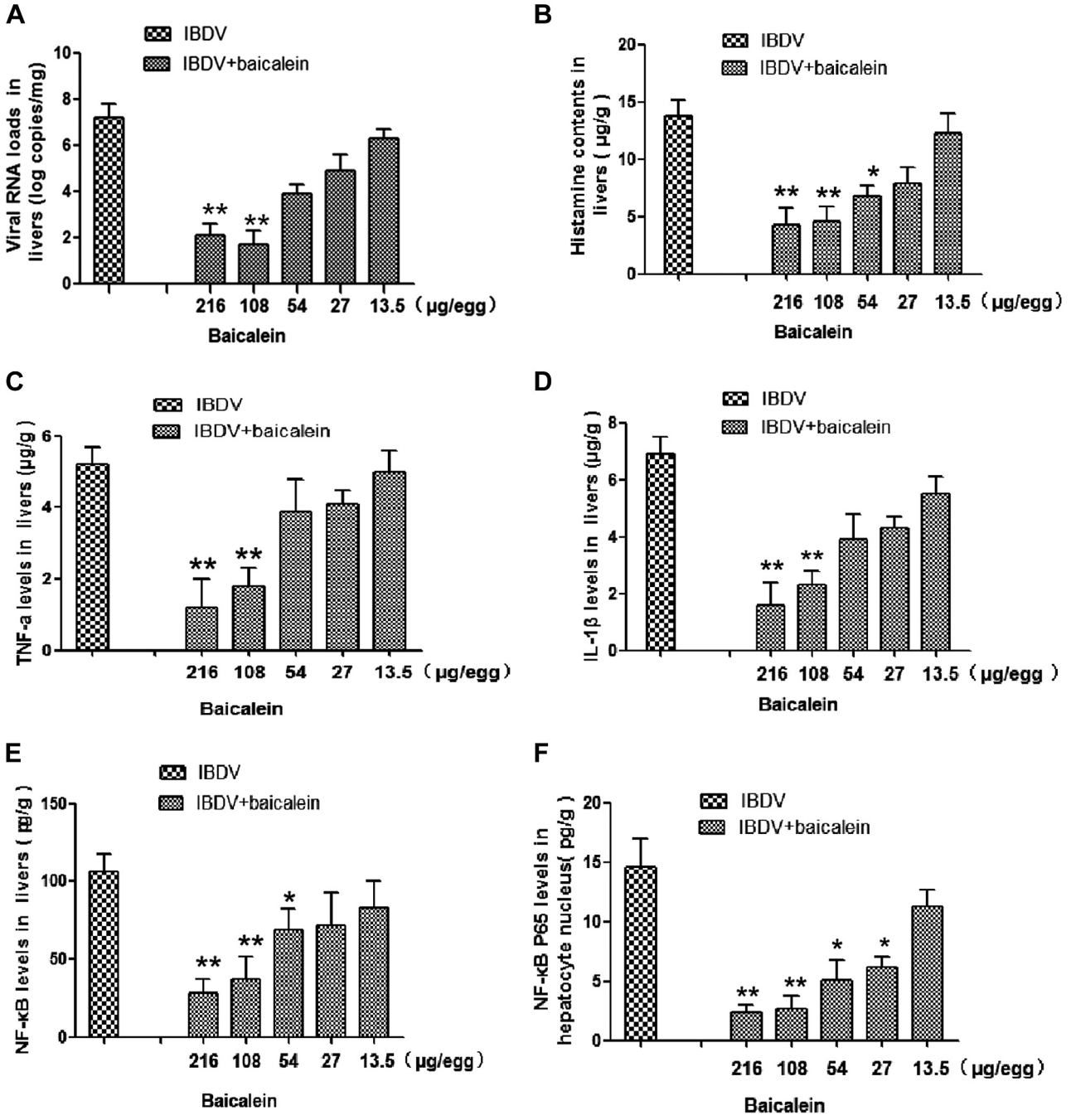

Figure 1. Baicalein inhibited IBDV replication and attenuated IBDV-induced inflammatory mediator expression in embryonic livers. Viral RNA loads (A) as measured by real-time qRT-PCR. Levels of inflammatory mediator histamine (B), TNF-α (C), IL-1β (D), NF-κB (E), and activated NF-κB p65 (F) in embryonic livers treated with different concentrations of baicalein compared with the control group. Data are expressed as mean ± SD of 6 embryonic livers treated with different concentrations at 72 h after inoculation. $^{*}P < 0.05$ and $^{**}P < 0.01$ different from the control group. Abbreviations: IBDV, infectious bursal disease virus; IL-1β, interleukin 1β; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor alpha.

known as baicalein and chlorogenic acid to investigate their antiviral potential against IBDV. For baicalein and chlorogenic acid, the cytotoxic activity was determined at concentrations of 216 and 858 μg/egg, and the antiviral potentials were found at the concentrations of 108 μg/egg ($P < 0.01$; Figure 1A) and 107 μg/egg ($P < 0.05$; Figure 2A), respectively. Our results demonstrated that both baicalein and chlorogenic acid have therapeutic benefits against IBD by inhibiting IBDV propagation, with the antiviral responses being concentration-dependent, especially for chlorogenic acid.

Histamine is an important inflammatory mediator. Previous studies from our laboratory have indicated that the histamine content correlates with the inflammatory responses of chicken embryos infected with IBDV (Li et al., 2015). IBDV-infected embryos treated with baicalein (108 μg/egg) and chlorogenic acid (429 μg/egg) had a significantly lower histamine content in the embryonic livers than untreated IBDV-infected embryos ($P < 0.01$; Figures 1B and 2B). Baicalein and chlorogenic acid suppressed histamine production mediated by IBDV. Therefore, the antiviral effects of these

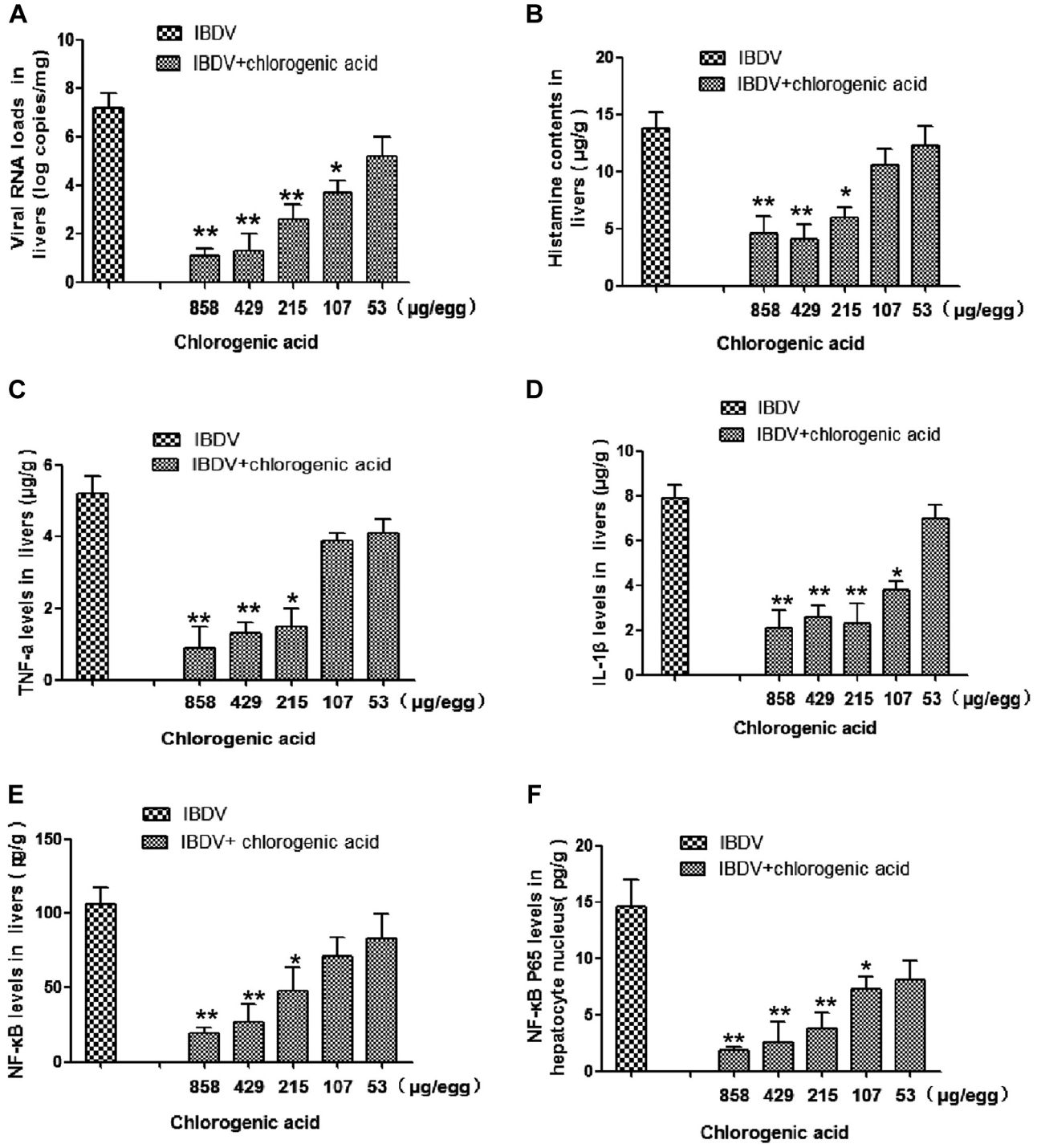

Figure 2. Chlorogenic acid inhibited IBDV replication and attenuated IBDV-induced inflammatory mediator expression in embryonic livers. Viral RNA loads (A) as measured by real-time qRT-PCR. Relative levels of inflammatory mediator histamine (B), TNF-α (C), IL-1β (D), NF-κB (E), and activated NF-κB p65 (F) in embryonic livers treated with different concentrations of chlorogenic acid treated compared with the control group. Data are expressed as the mean ± SD of 6 embryonic livers treated with different concentrations at 72 h after inoculation. $^{*}P < 0.05$ and $^{**}P < 0.01$ different from the control group. Abbreviations: IBDV, infectious bursal disease virus; IL-1β, interleukin 1β; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor alpha.

ingredients may also act in part by changing the histamine content of the embryo.

Previous studies have demonstrated that baicalein can inhibit apoptosis by altering the activity of the NF-κB pathway (Sithisarn et al., 2013). NF-κB is an important transcription factor that controls the expression of inflammatory mediators, such as TNF-α and IL-1β (Munhoz et al., 2008). NF-κB p65 proteins are the active subunits of the NF-κB complex, which is transported into the nucleus and binds to NF-κB binding sites in the promoter regions of inflammatory mediators, inhibiting their expression. IBDV infection leads to a robust upregulation of proinflammatory mediators and cytokines in the acute phase, and it can even cause death

during this period (Rauf et al., 2011). The results showed that IBDV increased NF-κB expression and NF-κB p65 content in the nucleus. Chicken embryos treated with baicalein (108 μg/egg) and chlorogenic acid (429 μg/egg) had a significantly lower NF-κB content in the cytoplasm of hepatocytes than the control embryos ($P < 0.01$; Figures 1E and 2E). In addition, NF-κB p65 levels were significantly lower in the nucleus of embryonic hepatocytes treated with 108 μg/egg baicalein ($P < 0.01$; Figure 1F) and 215 μg/egg chlorogenic acid ($P < 0.01$; Figure 2F). Baicalein and chlorogenic acid affected NF-κB production and blocked NF-κB p65 translocation. The TNF-α content in livers treated with baicalein (108 μg/egg) and chlorogenic acid (429 μg/egg) was significantly lower than that of the control embryos ($P < 0.01$; Figures 1C and 2C). The IL-1β levels were significantly lower at concentrations of 108 μg/egg ($P < 0.01$; Figure 1D) and 215 μg/egg ($P < 0.01$; Figure 2D) for baicalein and chlorogenic acid, respectively. These results imply that the IBDV-mediated NF-κB activation was inhibited by baicalein and chlorogenic acid, which may also contribute to NF-κB p65 nuclear translocation. These data indicate that these ingredients have inhibitory effects on the NF-κB pathways specific to IBDV-induced expression of the inflammatory mediators TNF-α and IL-1β in embryonated eggs. Therefore, the anti-inflammatory effects of baicalein and chlorogenic acid can be explained by their suppression of these inflammatory mediators that occurred in a dose-dependent manner. Comparison of the doses for baicalein and chlorogenic acid reveals that the anti-inflammatory effects and antiviral activities of baicalein were likely more potent than those of chicoric acid.

The present study has demonstrated the antiviral activities of baicalein and chlorogenic acid against IBDV and their ability to attenuate the hepatic inflammatory response in chicken embryos. Therefore, we recommend the use of baicalein and chlorogenic acid as potential agents to control IBDV infections in chickens. The pharmacological mechanisms of Chinese herbal medicines are often complex because of the various chemical ingredients present in the medicine. Thus, studies to design an optimized mixture of herbal medicinal ingredients for the treatment of IBD are currently being conducted.

DISCLOSURES

The authors declare no conflict of interest.

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