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Apigenin inhibits infectious bronchitis virus replication *in ovo*

M.J. SAADH[1], S.A. JABER[1], M. ALARAJ[1], A. ALAFNAN[2]

[1]Faculty of Pharmacy, Middle East University, Amman, Jordan
[2]Department of Pharmacology and Toxicology, College of Pharmacy, University of Hail, Hail P.O. Box 2440, Saudi Arabia

Abstract. – OBJECTIVE: Infectious bronchitis virus (IBV), for which no effective drugs are available, is among the most important causes of economic loss within the poultry industry. Apigenin is a flavonoid that can be isolated from plants. Apigenin has low toxicity with anti-viral activity. However, the effects of apigenin against IBV remain unclear.

MATERIALS AND METHODS: Thus, here we investigate the anti-viral effect of apigenin on IBV using 10 day-old embryonated eggs by determining the virus titer by embryo infective doses50 (EID50/mL) and determining IBV genomes copy number (per μL) of allantoic fluid.

RESULTS: We found that apigenin protected embryonated eggs from IBV. Additionally, apigenin reduced the log titer of the IBV with a significant correlation of up to 9.4 times at 2 μg/egg. Also, apigenin appears to significantly reduce IBV genomes copy number (per μL) in the allantoic fluid.

CONCLUSIONS: Apigenin may be a promising approach for the treatment of IBV, since it protects embryonated eggs from IBV *in ovo* and suppresses viral replication.

Key Words:
Apigenin, Antiviral activity, Infectious bronchitis virus, IBV genomes, Coronaviruses.

Introduction

Coronaviruses (*Coronaviridae* family, genus Gammacoronavirus) are enveloped, non-segmented, single-stranded, positive-sense ribonucleic acid (+ssRNA) viruses and can be classified into four genera (alpha-, beta-, gamma-, and delta coronaviruses)[1-3]. IBV is a highly infectious avian coronavirus that affects birds and is responsible for great losses to the poultry industry worldwide[4]. In hens, IBV can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IBV infection is also the major cause of animal death[4,5].

Chickens with IBV experience diffuse alveolar harm and discharge in the lungs, brought about by overactive inflammatory responses[5]. Overproduction of inflammatory cytokines, such as interleukin 6 (IL-6) in chicken, is similar to mammalian IL-6 in humans infected with coronavirus, referred to as cytokine storm and recognized as the fundamental driver of death related to this virus[5].

Vaccines remain the best approach to preventing coronavirus infections. However, vaccine value is diminished because IBV infections have a high transformation rate, creating different serotypes of the virus for which the vaccine may not cross protect, requiring booster doses and adjustments in the immunization composition. Also, vaccine creation is always time-consuming and costly[6-8]. In addition, numerous drugs are used to treat IBV infection, but there is no significantly effective drug for IBV infection, and these anti-viral medications have adverse side effects[9,10].

Apigenin is a nontoxic and non-mutagenic flavonoid of the flavone family, found in various plants such as parsley, artichoke, basil, celery, celeriac, and chamomile[11]. Apigenin has antioxidant[12], antihyperglycemic[13], anti-inflammatory[14], and anti-apoptotic, anti-cancer, and anti-viral activities[12-15]. In this study, we investigate the anti-viral efficacy of apigenin against IBV *in ovo*, and the activity against viral IBV genomes synthesis.

Materials and Methods

Materials

Apigenin (purity ≥ 95%) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Anti-Viral Activity Assay in Ovo

The propagation of infectious bronchitis virus-H120 (IBV-H120) virus was performed using

Corresponding Author: M.J. Saadh, MD; e-mail: msaadeh@meu.edu.jo

an embryonated chicken egg (ECE) system. The master seed of IBV-H120 vaccine strain was obtained (Jordan Bio-Industries Center – JOVAC, Amman, Jordan), containing 10^8 EID_{50}/mL, and was diluted in 1 mL of sterile phosphate buffer saline (PBS; pH 7.2), then subsequently diluted serially using sterile PBS (pH 7.2) until reaching the desired dilution (10^5 EID_{50}/mL). About 0.1 mL of 10^5 EID_{50}/mL in the presence of different concentrations of apigenin (0, 1, 1.5, 2, 2.5, and 3.5 µg/egg) was mixed and incubated at 37°C for 1 hour with different concentrations, then inoculated into the allantoic cavity of 10 day-olds embryonated Specific-Pathogen-Free (SPF) hen's eggs.

Eggs were then incubated at 37°C and 60% humidity. Then, they were candled daily for 7 days. Mortality within the first 24 h was considered non-specific. At 7 days after infection, the tops of the eggs were removed, and the allantoic fluids of all eggs harvested and pooled. Allantoic fluids were harvested by suction, excluding yolk material and albumin.

All fluids were immediately stored at 4°C[16-18]. Following titration using the ECE system, as previously described, the EID_{50}/mL values of the control and IBV-infected samples were calculated (0.1 mL $10^5$$EID_{50}$/mL of IBV-H120 virus without any treatments)[18].

IBV-H120 Viral Titration

To determine viral EID_{50} titers, we injected 100 µL of 10-fold dilutions of the virus into the allantoic cavities of 10-day-old SPF eggs. Five eggs/each dilution were used. According to Reed and Muench[19], 50% of endpoints were calculated for EID_{50}/mL and were expressed as $\log_{10}$ EID_{50}/mL.

Viral RNA Extraction and qRT-PCR

The RNA was extracted from the crude harvest of allotonic fluid using the NZY Viral RNA Isolation kit (NZYTech, Lisbon, Portugal) according to the manufacturer's protocol. The reaction mixture was prepared for each RNA sample according to the manufacturer's instructions.

The ORF1a gene was amplified using IBV One-Step RT-qPCR Kit, RUO (NZYTech, Portugal). Following the manufacturer's protocol, a standard curve was included for quantitative analysis.

Statistical Analysis

All analyses were done using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and SPSS (IBM Corp., Armonk, NY, USA). Differences among the studied groups were determined based on one-way ANOVA followed by Tukey's multiple comparisons as a post-hoc test. $p < 0.05$ was considered significant.

Results

Anti-Viral Activity of Apigenin Against IBV-H120 in Ovo

When injecting 10-day-old SPF embryonated chicken eggs with high concentrations of apigenin (1,500 µg/ml) to determine the lethal dose of 50 (LD_{50}), we detected no toxicity. Beyond this concentration, it became difficult to inject eggs with high concentrations due to the solvents' toxicity. Therefore, injecting eggs with a concentration of more than 1,500 µg/ml is not practical. Thus, the LD_{50} of apigenin was not calculated. At first, embryonated SPF eggs were infected with 0.1 mL of 10^5 EID_{50}/mL IBV-H120 and various concentrations of apigenin (1, 1.5, 2, 2.5, and 3.5 µg/egg) to determine the anti-viral effect.

Seven days after infection, the allantoic fluids of all eggs were pooled after harvesting, and the $\log_{10}EID_{50}$ was calculated. The reduction $\log_{10}EID_{50}$ displays the effect of apigenin on IBV-H 120 replication as dose-dependent. At 2, 2.5, and 3.5 µg/egg, apigenin had excellent inhibitory activity against infectious IBV-H120 ($p < 0.001$). At $\geq$ 2 µg/egg, the apigenin level has a highly potent inhibitory effect against IBV *in ovo*. The $\log_{10}EID_{50}$/ml for IBV-H120 virus value at 2 µg/egg was reduced by approximately 9.4-times (Figure 1A).

The morphological changes in the chicken embryos at different drug regimens following IBV challenges are shown in Figure 1B.

Apigenin Inhibits IBV Genomes Synthesis

The quantification of IBV copy number (per µl)/quantitation Cycle (Cq) value in the allantoic fluids confirms the potent inhibitory effect of apigenin on IBV production, with a significant reduction in IBV genomes synthesis *in ovo* (Figure 2).

Discussion

Coronaviruses have become an important pathogen for humans and animals because of their pandemic potential and high zoonotic infec-

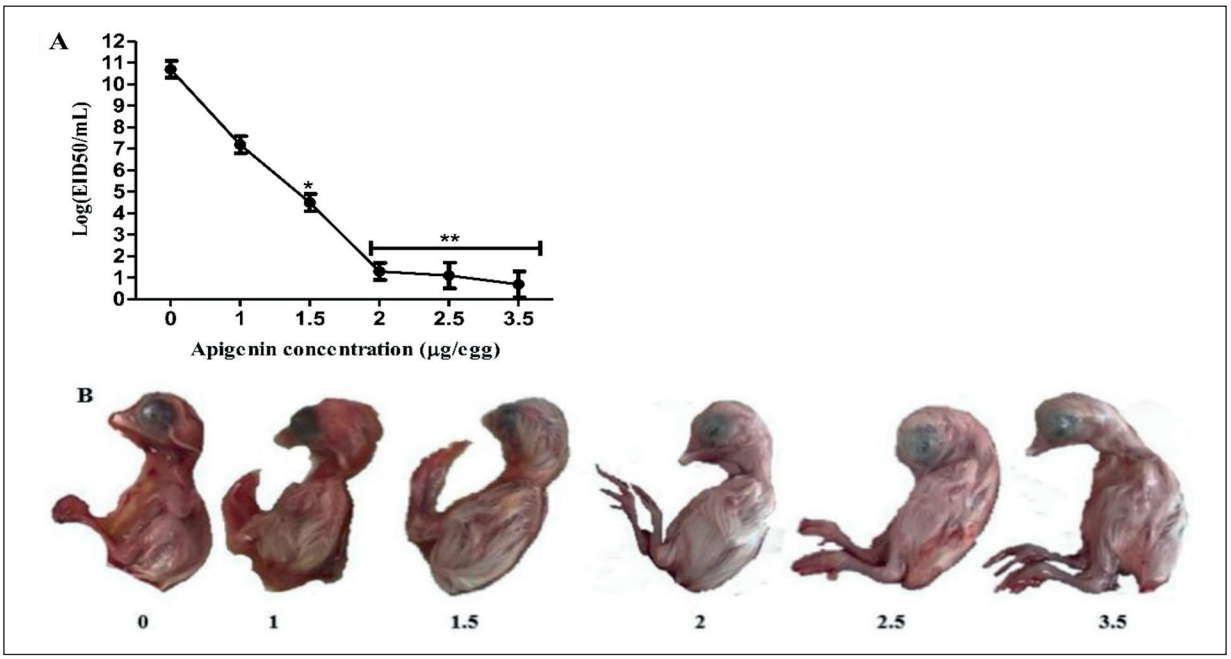

Figure 1. *In ovo* anti-viral efficacy of Apigenin against IBV. **A**, *In ovo* anti-IBV efficacy of the Apigenin: 10-day-old SPF embryonated chicken eggs were infected with IBV at 0.1 mL of 10^5 EID_{50}/mL in the presence of different concentrations of apigenin and observed daily for mortality of the embryos for 7 days after infection. After harvest, the allantoic fluid from all eggs was pooled. The EID_{50}/mL was calculated by the Reed-Muench method. **B**, Morphological changes in the chicken embryos at different drug regimens following IBV challenges are shown. *$p < 0.01$, **$p < 0.001$.

tion rates. Coronaviruses cause mild to severe respiratory infections[1]. However, no effective drugs are available to control IBV[9].

Apigenin is a natural product belonging to the flavone class, and it has multiple biological and pharmacological activities, such as anti-cancer[16], anti-inflammatory[14], and anti-viral[17]. Our study shows that apigenin can significantly reduce the titers of IBV-H120 ($log_{10}EID_{50}$/mL) at the post-entry stages *in ovo*. Apigenin can prevent the development of pock lesions on the CAM of embryonated eggs, giving protection against IBV-H120, and it can be used and developed as an anti- IBV-H120 agent. Its anti-viral activity was dose-dependent. In similar results, apigenin exhibits various anti-viral activities against numerous viruses *in vitro* and *in vivo*, such as enterovirus 71 (EV71)[18], hepatitis C virus (HCV)[19], human immunodeficiency virus (HIV)[20], adenoviruses[21], and buffalopox virus (BPXV)[22].

In addition, apigenin has excellent inhibitory activity of IBV genomes synthesis. Therefore, the best activity of apigenin is shown at the early stage; this result supports this hypothesis. Several mechanisms of apigenin action have been proposed. Apigenin is mediated via inhibiting viral DNA, RNA, and protein synthesis in infected

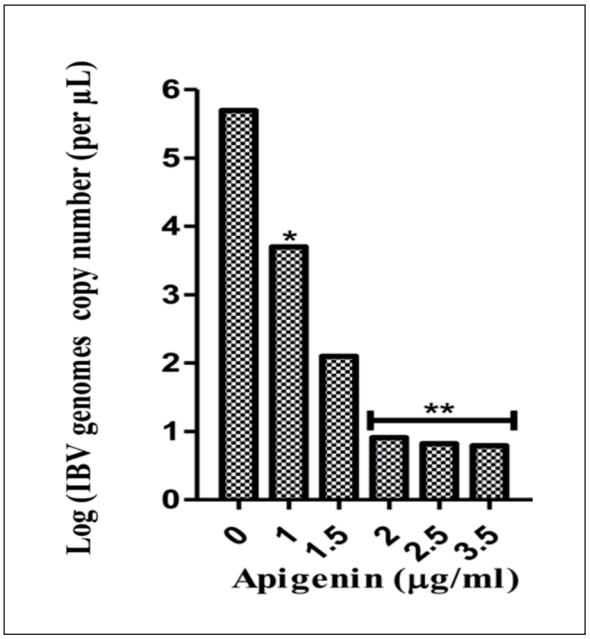

Figure 2. The effect of apigenin on quantitation of IBV genomes synthesis on the allantoic fluids. Ten-days-old SPF embryonated chicken eggs were infected with IBV at 0.1 mL of 10^5 EID_{50}/mL in the presence of different concentrations of apigenin and observed daily for mortality of the embryos for 7 days after infection. The allantoic fluids of all eggs were collected. The IBV genomes was quantitated in the allantoic fluids by qRT-PCR. *$p < 0.01$; **$p < 0.001$.

cells, rather than directly inactivating virion particles[22]. Also, apigenin could directly inhibit viral polymerase activity[23]. For example, apigenin disrupts the association of viral RNA and internal ribosomal entry site (IRES) to heterogeneous nuclear ribonucleoproteins (hnRNPs) in inhibiting picornavirus and FMDV infection[18,23,24].

Apigenin treatment is an inhibitor of IL-6 transcription, one of the mechanisms by which apigenin exerts its anti-cancer and anti-viral effects[25,26]. Therefore, the use and development of apigenin may be promising in treating SARS-CoV-2 infections.

Conclusions

Apigenin is a promising therapeutic agent in the early stage against IBV and can protect embryonated eggs against IBV, targeting IBV replication. Apigenin seems to inhibit IBV *via* multiple mechanisms. Thus, apigenin's anti-IBV activity is unaffected by virus mutations. More *in vivo* studies on the use of apigenin against IBV and other viruses are now needed.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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ORCID ID

M.J. Saadh: 0000-0002-5701-4900.

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