Epigallocatechin-3-gallate exhibits antiviral effects against the duck Tembusu virus via blocking virus entry and upregulating type I interferons

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ABSTRACT The duck Tembusu virus (**DTMUV**) is a novel mosquito-borne *Flavivirus* which caused huge economic losses for poultry industries in Southeast Asia and China. Currently, no effective antiviral drugs against this virus have been reported. (-)-Epigallocatechin-3-gallate (**EGCG**), a polyphenol present in abundance in green tea, has recently been demonstrated to have an antiviral activity for many viruses; however, whether EGCG can inhibit DTMUV infection remains unknown. Here, we tried to explore the anti-DTMUV effects and mechanisms of EGCG both in vitro and in vivo. Several EGCG treatment regimens were used to study the comprehensive antiviral activity of EGCG in DTMUV-infected baby hamster kidney cell line (**BHK-21**). The DTMUV titers of mock- and EGCG-treated infected cell cultures were determined using the tissue culture infective dose assay and the DTMUV mRNA copy number as determined using quantitative Real Time PCR. Moreover, the therapeutic efficacy of EGCG against DTMUV was assessed in DTMUV-infected ducklings. Our results suggested that EGCG significantly reduced the viral infection in BHK-21 cells in a dose-dependent manner, as reflected by the reduction of virus titers, virus copy number, and the expressions of viral E protein. We also observed that EGCG exhibited direct virucidal abilities against DTMUV. Notably, a significant reduction in virus binding ability was also observed, indicating that EGCG possesses excellent inhibitory effects on the viral adsorption step. In addition, DTMUV replication was also suppressed in BHK-21 cells treated with EGCG after viral entry, likely because of upregulation of the levels of interferon alfa and interferon beta. Finally, we also proved that EGCG exhibited anti-DTMUV efficacy in a duckling infection model because the survival rate was significantly improved. This is the first study to demonstrate the protective effect of EGCG against DTMUV, suggesting its potential use as an antiviral drug for DTMUV infection.

Key words: duck Tembusu virus, (-)-epigallocatechin-3-gallate, antiviral activity, virus entry, type I interferon

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INTRODUCTION

In 2010, a newly emerging duck Tembusu virus (**DTMUV**) disease outbroke in south eastern China and caused serious economic losses (Cao et al., 2011; Li et al., 2013). The duck Tembusu virus is a novel member of *Flavivirus*, which is a positive-sense single-stranded RNA enveloped virus. Until recently, DTMUV has been found to be able to infect many species besides duck, including egg-laying chickens (Chen et al., 2014), geese (Ti et al., 2015), pigeons (Dai et al., 2015), and sparrows (Tang et al., 2013). To date, although some commercial vaccines have been developed, the new outbreaks of DTMUV infection are still being reported in China and recent outbreak of DTMUV infection have resulted in large economic losses for poultry industries in China (Homonnay et al., 2014; Thontiravong et al., 2015; Lu et al., 2016). Current researches mainly focused on the development of vaccines to prevent this disease, but there is little report on the antiviral drugs against this virus. Thus, there is an urgent need to develop effective antiviral drugs for DTMUV infection.

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(-)-Epigallocatechin-3-gallate (**EGCG**), the most abundant and bioactive catechin in green tea, has antiviral activities on various types of viruses, including the human immunodeficiency virus (Nance et al., 2009), herpes simplex virus (Isaacs et al., 2008), and influenza virus (Song et al., 2005). Calland et al. have shown that EGCG could block an early step of the hepatitis C virus (**HCV**) entry process and inhibit HCV cell-to-cell transmission (Calland et al., 2012). Muhareva et al. reported that EGCG exerted its antiviral effect mainly at the early stage of dengue virus infection by interacting directly with virions (Raekiansyah et al., 2018). Ge et al. have found that EGCG suppressed porcine reproductive and respiratory syndrome virus replication in Marc-145 cells because of upregulation of proinflammatory cytokines, such as TNF-α, IL-6 and IL-8 (Ge et al., 2018). These findings suggest that EGCG play protective effects against many viruses infection via multiple antiviral approaches. However, the anti-DTMUV activity of EGCG has not been evaluated, and the underlying mechanisms remain to be investigated.

In this study, we first explored the antiviral activity of EGCG against DTMUV and determined the antiviral mechanisms in baby hamster kidney cell line (**BHK-21**) cells. Furthermore, we investigated the therapeutic effects of this drug in ducklings. All these findings suggest that EGCG might be a candidate drug for effective control of DTMUV infection.

MATERIALS AND METHODS

Virus, Cell, and Drug

The duck Tembusu virus AH-F10 strain isolated in 2010 in our laboratory was used in the present study (GenBank accession No. KM102539.1). And, this virus stock was propagated in BHK-21 cells. Viral titers were determined as median tissue culture infective dose (**$TCID_{50}$**)/mL by infection of BHK-21 cells and calculation by Reed and Muench method.

BHK-21 cells were purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle medium (**DMEM**) (Life Technologies, Waltham, MA) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) at 37°C in an atmosphere with 5% (v/v) CO_2.

Epigallocatechin-3-gallate (purity≥98%) was purchased from Shanghai PureOne Biotechnology (Cat No.: P0033, Shanghai, China). (-)-Epigallocatechin-3-gallate solution was prepared in DMEM and used it immediately.

Cytotoxicity of EGCG on BHK-21 Cells

To confirm that the inhibitory effect observed was not a result of a change in cellular conditions, we assessed the cytotoxicity of EGCG in BHK-21 cells by Cell Counting Kit–8 assay (Beyotime Biotechnology, Shanghai, China). Briefly, BHK-21 cells were added at a density of 1×10^4 cells per well to a 96-well plate and incubated for 24 h at 37°C. Media were removed and replaced with DMEM containing different concentrations of EGCG (0–200 μM). At 24 h, 10 μL Cell Counting Kit–8 solution was added to each well and incubated at 37°C in a CO_2 cell incubator for 90 min, then the absorbance rates were measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, Massachusetts). All experiments were performed in triplicate.

Virus Infection and EGCG Treatment

Dose-Dependent Effects of EGCG Against DTMUV To determine the antivirus effect of EGCG on DTMUV, BHK-21 cells (2.0×10^5/well) were seeded in a 6-well plate and incubated at 37°C with 5% CO_2 for 24 h and then treated for 4 h with different concentrations of EGCG (0 μM, 10 μM, 20 μM, 50 μM, 100 μM, and 200 μM) and incubated at 37°C in a 5% CO_2 environment. After the treatment, the cells were washed twice using serum-free DMEM and then infected with 0.1 multiplicity of infection (**MOI**) of DTMUV. After incubation for 48 h with fresh DMEM consisting of 2% fetal bovine serum and the different EGCG concentrations, the cells were collected for interferon alfa and interferon beta mRNA, E protein expression, and virus copy number analysis. The supernatant was also collected, and the virus titers weres determined by the $TCID_{50}$ assay.

The Effects of Different EGCG Treatment Conditions To investigate whether EGCG inhibits DTMUV infection through direct interaction with DTMUV particles or interfere with the cellular receptors used by DTMUV, the timing of EGCG treatment after infection was planned into 2 treatment regimens. The first regimen (**pre + virus**) was preincubation of DTMUV strain AH-F10 at an MOI of 0.1 with 100 μM of EGCG for 1 h before infection of cells. The second regimen (**pre + cells**) was a preinfection EGCG treatment, where the BHK-21 cells were pretreated for 1 h with 100 μM of EGCG before virus infection. After 48 h, the cells were collected for E protein expression by Western blot assay and indirect immunofluorescence assay. The supernatant was collected, and the virus titers were determined by the $TCID_{50}$ assay.

Antiadsorption Assay To investigate if EGCG would inhibit DTMUV binding to the host cells, the experiments were designed as follows. The first regimen (adsorption) was preincubation of DTMUV strain AH-F10 at an MOI of 0.1 with 100 μM of EGCG or 50 μg of heparin (used as positive control) per mL. The cells were then incubated for 1 h at 4°C. Subsequently, the cells were washed with ice-cold PBS 3 times and cultured with 2% DMEM–fetal bovine serum for 48 h at 37°C in a 5% CO_2 environment. The negative control was 0.1% dimethyl sulfoxide (**DMSO**). The second regimen (Postadsorption) was preincubation of DTMUV at an MOI of 0.1 at 4°C for 1 h. Subsequently, the cells were washed with ice-cold PBS 3 times and cultured with

fresh medium with EGCG (100 μM) at 37°C for 48 h. The third regimen (simultaneously) was preincubation of DTMUV at an MOI of 0.1 with 100 μM of EGCG at 37°C for 48 h. Then, the cells were collected for E protein expression and virus copy number analysis. The supernatant was collected, and the virus titers were determined by the $TCID_{50}$ assay.

Real-time RT-PCR Total RNA was extracted from BHK-21 cells using RNAsimple Total RNA Kit (Cat No.: DP419; TIANGEN, Beijing, China) as per the manufacturer's instructions. cDNA was synthesized with the FastKing RT Kit (Cat No.: KR116, TIANGEN, Beijing, China), as per the manufacturer's protocol. To quantify the viral copies, absolute quantitative real-time PCR was performed using a ChamQ Universal SYBR qPCR Master Mix (Cat no.: Q711-02; Vazyme Biotech Co., Ltd., Nanjing, China) on a Thermo Scientific PikoReal PCR Instrument (Thermo Scientific, Waltham, Massachusetts). For the standard curve, serial dilutions of a plasmid pcDNA3.1-E, constructed as described previously, were used to quantify the virus genomic copy number.

Relative quantitative RT-PCR was performed to evaluate the RNA transcription of inflammatory cytokines. β-actin was used as an internal reference gene. Each sample was amplified in triplicate. Analyses of gene expression were performed by the $2^{-\Delta\Delta Ct}$ method. The following primers were used: interferon alfa, forward 5′-GCTGGCAAGATTGAGTGAAGAG-3' and reverse 5′-ATAACAAATAGGTGCGGATACA-3'; interferon beta, forward 5′-TGCGTTCCTGCTGTGCTTCT-3' and reverse, 5′-CGCCCTGTAGGTGAGGTTGA-3'; β-actin, forward 5′-CTGTCCCTGTATGCCTCTGGTC-3′ and reverse 5′-TCTTTGATGTCACGCACGATTT-3'.

Immunofluorescence At 48 h after infection, the cells were fixed in absolute ethyl alcohol for 30 min at room temperature followed by blocking with 1% BSA in PBS for 30 min. After washing twice with PBS, the fixed cells were incubated with primary antibodies (mouse anti-E serum prepared by our laboratory, 1:1,000) for 2 h at room temperature. Then, the cells were washed with PBS, followed by incubation with the secondary antibody, fluorescein isothiocyanate-Labeled Goat Anti-Mouse IgG (H + L) (A0568; Beyotime Biotechnology, Shanghai, China; 1:500) for 30 min. Nuclei were stained with 1 μg/mL 4′, 6-diamidino-2-phenylindole (Beyotime Biotechnology). Samples were observed under an inverted fluorescence microscope.

Western Blot Cells were lysed with radio-immunoprecipitation assay buffer (Cell Signaling Technology), and the protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). The protein lysates (50 μg) were electrophoretically transferred onto polyvinylidene fluoride membranes (Beyotime Biotechnology, Shanghai, China), followed blocking with 5% skim milk at 4°C overnight. The membranes were incubated with primary antibodies at 37°C for 1 h. Mouse anti-E protein antibody was prepared by our laboratory. β-actin (Cat no. AF5001; Beyotime Biotechnology, 1:1,000) was used as an internal control for protein loading. The rabbit anti-mouse IgG-HRP (Cat no. A0216; Beyotime Biotechnology, 1:1,000) was used as the secondary antibody. The relative intensity of each band was detected using an electrochemiluminescence detection system (GE Healthcare Life Sciences) and blot bands were semiquantified using ImageJ software, version 1.46 (National Institutes of Health, MD).

Infection of Ducklings We confirmed that all animal experiments were carried out in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals set by Anhui Agricultural University. The ethical approval was approved by the Animal Care and Use Committee of Anhui Agricultural University.

Forty healthy 7-day-old ducklings, negative of any DTMUV and their antibody in vivo using RT-PCR and ELISA, were used in this experiment. The ducklings were randomly divided into 4 groups (10 per group). In the DTMUV group, ducklings were intramuscularly injected with DTMUV (0.4 mL per duckling). In the control group, all ducklings were intramuscularly injected with 0.4 mL sterile PBS as a negative control group. In the DTMUV + EGCG (300 mg/kg) and DTMUV + EGCG (600 mg/kg) groups, ducklings in each group were orally administered EGCG, freshly dissolved in distilled water (1 mL) daily, to the concentration described. Treatment began 4 h after virus infection. The different tissues (brain, lung, spleen, and blood) from the dead ducklings throughout the experiment were collected and subjected to pathologic examinations and RT-PCR. At 15 d after infection, all surviving ducklings were euthanized, different tissues collected, and dealt as previously mentioned.

Hematoxylin and Eosin Staining The liver and brain tissues from ducklings in different groups were taken and fixed in 4% paraformaldehyde (4°C) in PBS (pH 7.4) for 24 h and then embedded in paraffin. Hematoxylin and eosin staining was performed by Servicebio Co. Ltd. (Wuhan, China). The morphological changes were observed by light microscopy (BX-FM; Olympus Corp, Tokyo, Japan) (400 × magnification).

Statistical Analysis Statistical analysis was performed using GraphPad Prism for Windows, version 7 (Graph Pad Software Inc., San Diego, CA). The results are presented as the means ± SD. Student t test was performed for statistical comparisons between 2 groups. One-way ANOVA was used to compare 3 or more groups. A P value of < 0.05 was considered significant.

RESULTS

EGCG Inhibited DTMUV Infection in a Dose-Dependent Manner

(-)-Epigallocatechin-3-gallate, a polyphenol present in large quantities in green tea, has been shown to have an intense antiviral activity for many viruses (Calland et al., 2012). In this study, we evaluated its activity against DTMUV. Initially, Cell Counting Kit–8 assay

was used to determine the potential toxicity of EGCG against BHK-21 cells. BHK-21 cells were treated with increasing concentrations of EGCG: 10, 20, 50, 100, and 200 μM and incubated for 48 h before cytotoxicity evaluation. It was observed that the BHK-21 cells treated with 10, 20, 50, and 100 μM EGCG did not cause significant cytotoxicity to BHK-21 cells, while 200 μM EGCG treatment showed a significant antiproliferative effect, compared with the untreated group (Figure 1A). Therefore, the optimal concentration of EGCG suggested was at 100 μM for subsequent experiments.

To investigate the antiviral effects of EGCG against DTMUV, we determined the virus replication inhibition in cells treated with different concentrations of EGCG. As shown in Figures 1B and 1C, EGCG inhibited the virus titers and DTMUV RNA copy numbers in BHK-21 cells in a dose-dependent manner. In addition, immunofluorescence staining was used to detect the effects of EGCG on the expression of the viral E protein in BHK-21 cells. It was shown that EGCG dose-dependently inhibited the expression of the E protein in BHK-21 cells (Figure 1D). A similar result in the expression of E protein was obtained via Western blot

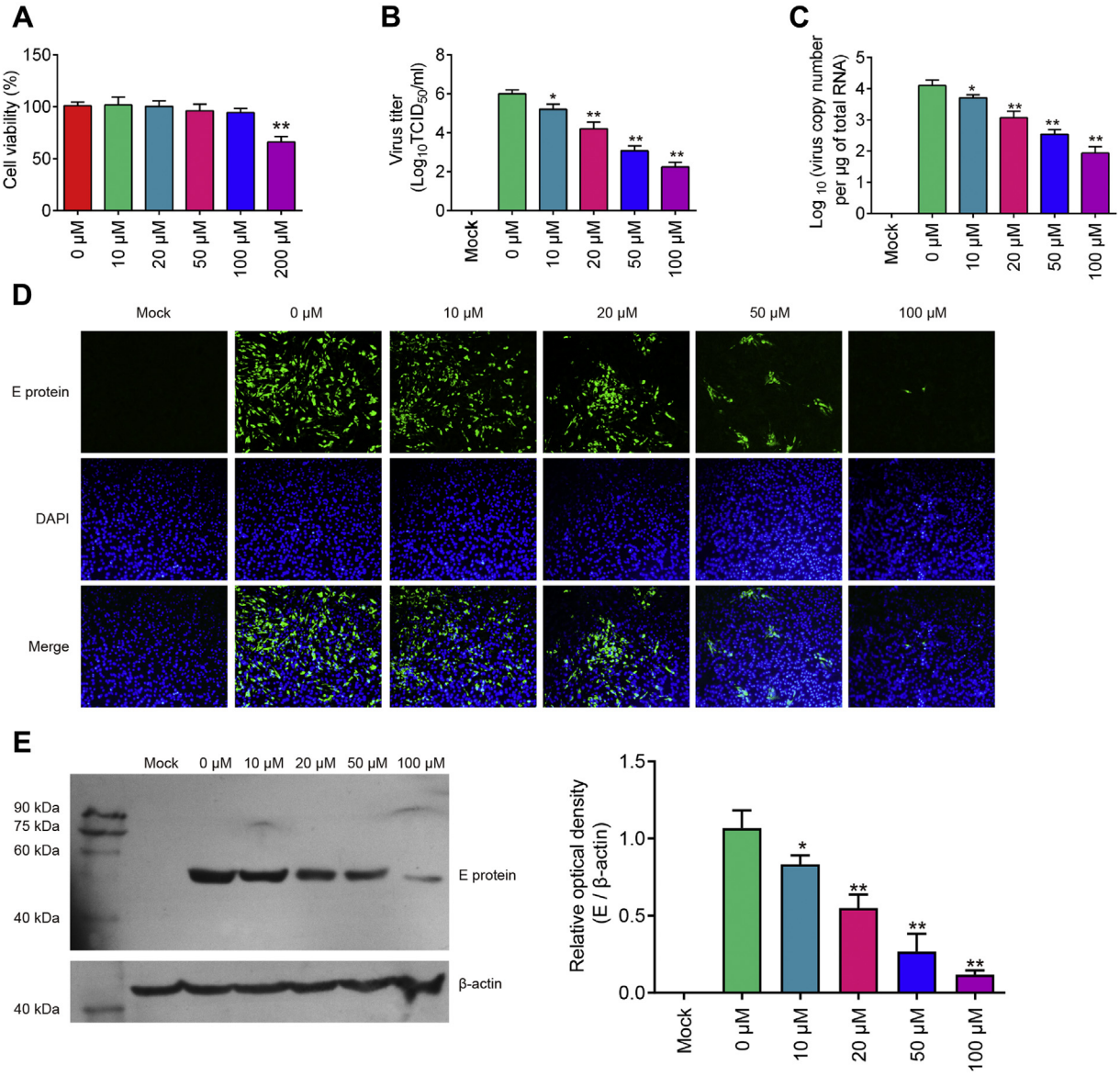

Figure 1. Dose-dependent inhibitory effect of EGCG on DTMUV. (A) BHK-21 cells were treated with EGCG at the concentrations of 0 μM, 10 μM, 20 μM, 50 μM, 100 μM, and 200 μM for 48 h, and then, the cytotoxicity of EGCG was determined using the CCK-8 assay. Data represent the mean ± SD of 3 independent experiments. $^{**}P < 0.01$ vs. untreated cells. BHK-21 cells were treated with different concentrations of EGCG at 37 °C and subsequently infected with DTMUV strain AH-F10 at an MOI=0.1 for 48 h. The virus titers were determined by measuring the $TCID_{50}$ assay (B). The virus copy numbers were quantified by qRT-PCR (C). DTMUV E protein expression in cells was detected using indirect immunofluorescence staining and Western blot (D, E). Data represent the mean ± SD of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock-treated cells. Abbreviations: CCK-8, Cell Counting Kit–8; DTMUV, duck Tembusu virus; EGCG, (-)-Epigallocatechin-3-gallate; $TCID_{50}$, tissue culture infective dose.

assay (Figure 1E). All these data suggest the inhibitory effects of EGCG against DTMUV replication.

EGCG Inhibited DTMUV Infection Through Direct Interaction With DTMUV Particles

Previous studies have shown that EGCG may also directly affect the infectivity of the virus (Carneiro et al., 2016; Raekiansyah et al., 2018). To obtain insights into the potential antiviral mechanisms of EGCG, an extracellular virucidal assay was performed. The timing of EGCG treatment after infection was planned into 2 treatment regimens: pre + virus and pre + cells. After 48 h, the supernatants were harvested, and $TCID_{50}$ assay and qRT-PCR were performed. As shown in Figures 2A and 2B, compared with the mock-treated

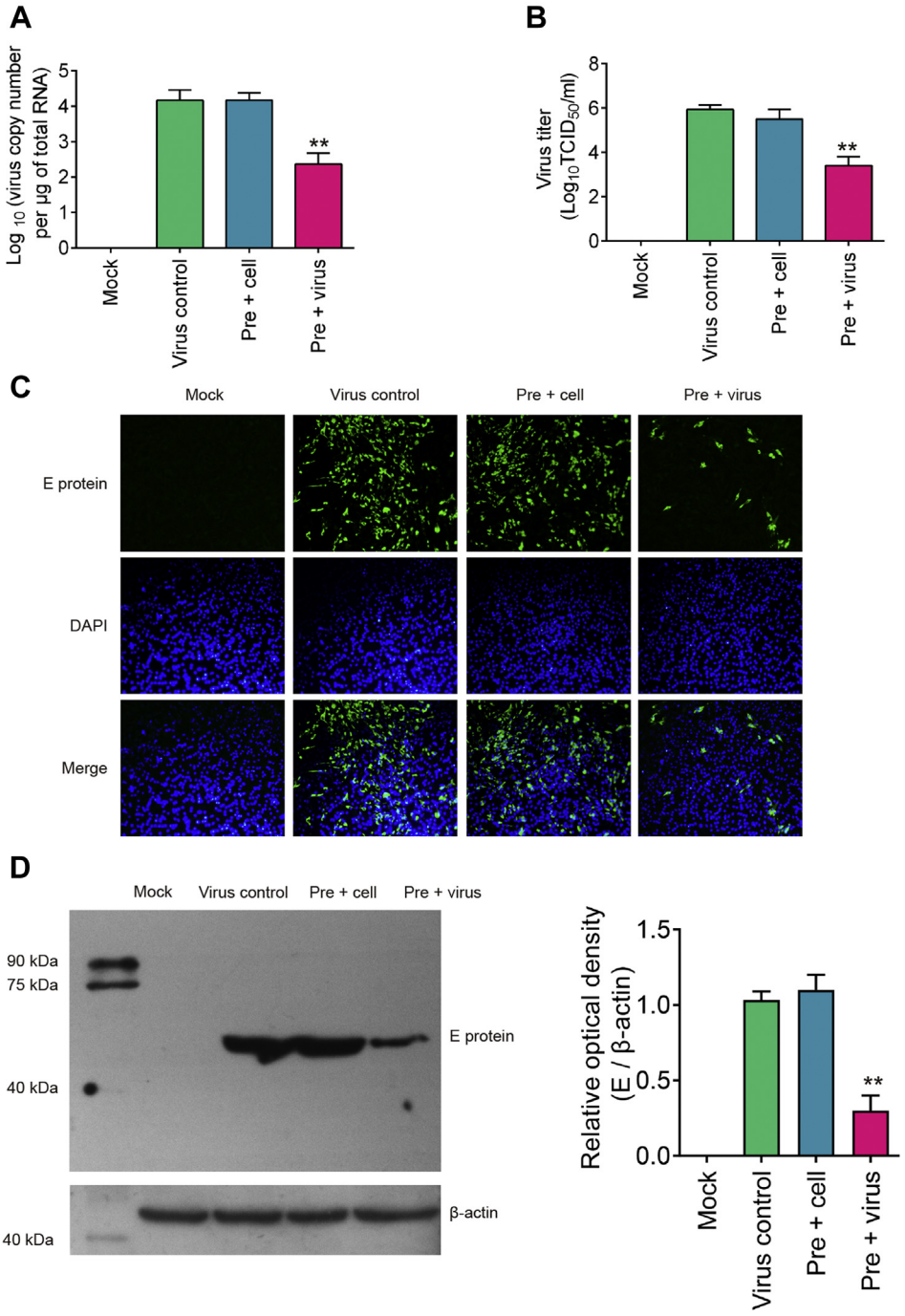

Figure 2. EGCG inhibited DTMUV infection through direct interaction with DTMUV particles. The timing of EGCG treatment after infection was planned into 2 treatment regimens: pre + virus and pre + cells. The cells and cell supernatants were collected after 48 h infection for subsequent experiments. (A) The virus titers were determined by $TCID_{50}$ assay. (B) The virus copy numbers were measured by qRT-PCR. (C, D) DTMUV E protein expression in cells was detected using indirect immunofluorescence staining and Western blot, respectively. Data represent the mean ± SD of 3 independent experiments. $^{**}P < 0.01$ vs. mock treated cells. Abbreviations: DTMUV, duck Tembusu virus; EGCG, (-)-Epigallocatechin-3-gallate; $TCID_{50}$, tissue culture infective dose.

cells, the virus copy numbers and virus titers were decreased significantly in the pre + virus group, whereas no significant reduction of the virus copy numbers and virus titer was observed in the pre + cell group. Meanwhile, the expression of DTMUV E protein in the cell lysates was also determined by the immunofluorescence assay and Western blot. As expected, the expression of E protein decreased in the pre + virus group compared with the mock-treated cells, while no obvious change was observed in the pre + cell group (Figures 2C and 2D). These results clearly suggest that EGCG could act directly against the extracellular DTMUV particles.

EGCG Inhibited DTMUV Infection Through Blocking the Adsorption of DTMUV in BHK-21 Cells

Because EGCG could directly act on DTMUV particles, we examined further if EGCG would inhibit DTMUV binding to the host cells. Antiadsorption assay, where EGCG was present during virus adsorption at 4°C for 1 h, was performed. As shown in Figure 3A, EGCG treatment resulted in a significant reduction in bound virus compared with the DMSO group, which is similar with the result of heparin treatment. This result suggests that EGCG treatment interfered with DTMUV binding.

Subsequently, the impact of EGCG on postbinding events of the virus was further investigated. As shown in Figures 3B and 3C, the virus titers and virus copy numbers were significantly decreased in simultaneously, adsorption and postadsorption groups compared with the virus control group. Notably, the "simultaneously" group showed greater activity of the drug than the adsorption or postadsorption group. A similar result was obtained in the expression of E protein (Figure 3D). All these data suggest that, in addition to viral binding, EGCG might also inhibit postbinding events such as viral entry or postentry steps.

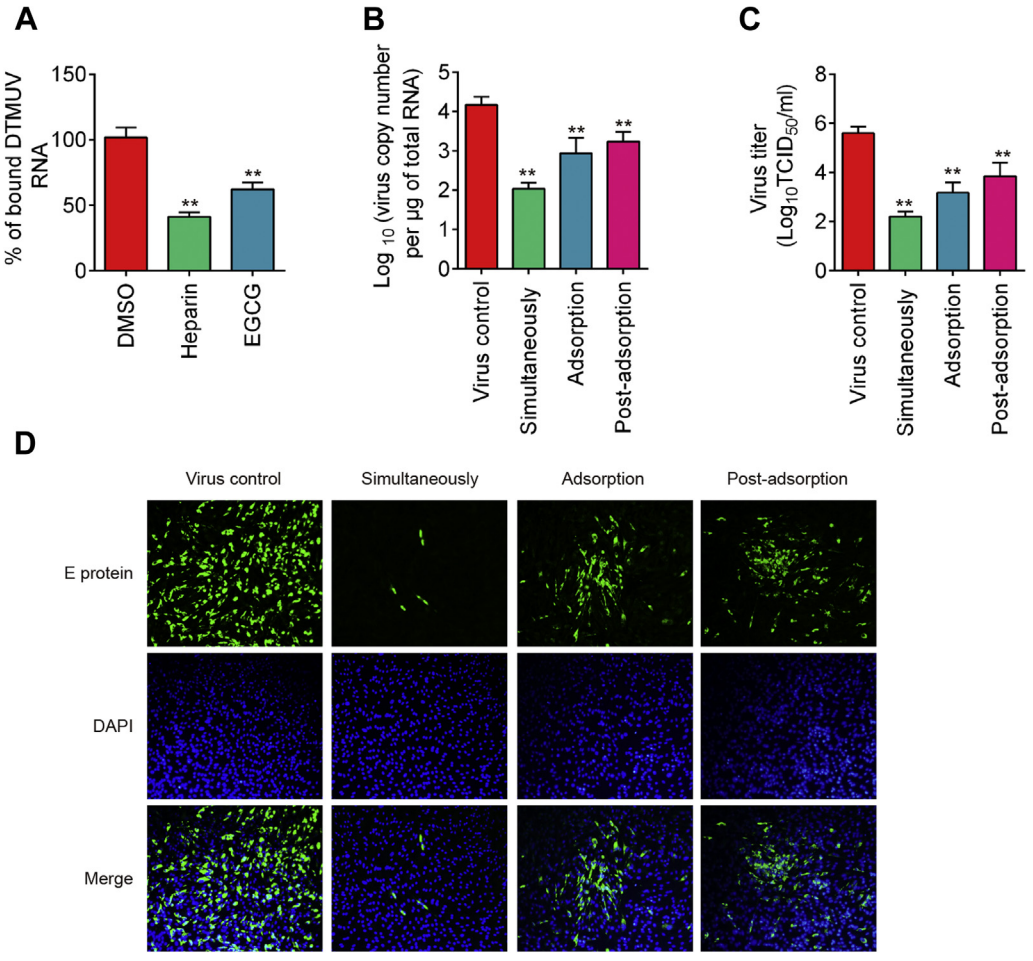

Figure 3. EGCG inhibited DTMUV infection through blocking the adsorption of DTMUV in BHK-21 cells. BHK-21 cells underwent 3 types of treatment as follows: 1) Simultaneous treatment: BHK-21 cells were treated with 100 μM EGCG at 37°C and subsequently infected with DTMUV strain AH-F10 at an MOI=0.1. 2) Adsorption: BHK-21 cells in 6-well plates were infected with DTMUV at an MOI of 0.1 for 1 h at 4°C in the presence of 100 μM EGCG, or 50 μg of heparin per mL. After removal of the pretreatment medium, the cells were incubated with compound-free media for 48 h. 3) Postadsorption: DTMUV at an MOI of 0.1 was preincubated with BHK-21 cells in 6-well plates for 1 h at 4°C. After removal of the pretreatment medium, the cells were incubated with 100 μM EGCG at 37°C for 48 h. (A) Viral RNA was quantified using qRT-PCR assay. Data represent the mean ± SD of 3 independent experiments. $^{**}P < 0.01$ vs. DMSO group. (B) The virus copy numbers were measured by qRT-PCR. (C) The virus titers were determined by $TCID_{50}$ assay. (D) DTMUV E protein expression in cells was detected using indirect immunofluorescence staining. Data represent the mean ± SD of 3 independent experiments. $^{**}P < 0.01$ vs. virus control group. Abbreviations: DTMUV, duck Tembusu virus; EGCG, (-)-Epigallocatechin-3-gallate; $TCID_{50}$, tissue culture infective dose.

EGCG Inhibited DTMUV Infection Partially Through Promoting Antiviral Interferon Alfa and Beta Gene Expression

Previous reporters have demonstrated that EGCG exerts its antiviral activities as an immunomodulatory regulator against the various types of viruses, such as porcine reproductive and respiratory syndrome virus and HCV (Wang et al., 2016; Ge et al., 2018). To determine whether EGCG could induce interferon alfa and beta expressions via this effect, we measured the mRNA expression levels of interferon alfa and interferon beta by qRT-PCR. As shown in Figures 4A and 4B, EGCG treatment can significantly increase the interferon alfa and interferon beta mRNA levels in a dose-dependent manner. Taken together, these results demonstrated that EGCG inhibited DTMUV infection partially through modulating the expression of interferon alfa and interferon beta.

EGCG Inhibited DTMUV Infection In Vivo

To evaluate the inhibitory abilities of EGCG in DTMUV infection in vivo, the ducklings were infected with DTMUV at 4 h before the beginning of the EGCG treatment. The animal survival rate was measured for 15 d to evaluate the therapeutic efficacy of EGCG. As shown in Figure 5A, compared with the control group, the survival rate of infected ducklings treated with 300 mg/kg and 600 mg/kg of EGCG was noticeably increased. In parallel, DTMUV infection resulted in a significant increase of virus copy numbers in the spleen, liver, blood, lung, and brain compared with the control group, whereas EGCG treatment significantly reduced the virus copy numbers in different tissues of ducklings compared with the DTMUV group (Figures 5B-5F). We further analyzed the histopathologic lesions of the brain and liver in all group ducklings. As for the brain, DTMUV infection caused lymphoid perivascular cuffing, swelling of the neuron and fatty degeneration. On the contrary, there were just slight pathologic changes in the DTMUV-infected brains with EGCG treatment, which indicating that treatment with EGCG protected the brains of ducklings from damage caused by DTMUV infection (Figure 6). As for the liver, hematoxylin and eosin staining exhibited degeneration and necrosis of hepatocytes in liver of ducklings after DTMUV infection, while the lesions were dramatically alleviated in the high and low dosages of the EGCG-treated group (Figure 6), further supporting the protective effect of EGCG against DTMUV infection in ducklings.

DISCUSSION

In the present study, we demonstrate that EGCG showed significant inhibitory effect on DTMUV infection in vitro and in vivo. (-)-Epigallocatechin-3-gallate directly suppress DTMUV infection through its virucidal effects, as well as interfering with the virus binding to the host cells. Notably, EGCG treatment could promote innate immune responses to inhibit DTMUV infection. Our findings indicated that EGCG may be used as a potent antiviral drug against DTMUV infection.

Growing evidence has shown that EGCG displays strong antiviral effects against viral infection, including human immunodeficiency virus (Nance et al., 2009), herpes simplex virus (Isaacs et al., 2008), influenza virus (Song et al., 2005), and so on. However, the antiviral activity and mechanism of EGCG against DTMUV has never been studied. In the present study, our results suggested a dose-dependent antiviral effect of EGCG on DTMUV, as evidenced by the reduction of virus titers, virus copy number, and the expressions of E proteins. Moreover, EGCG showed no cytotoxicity effects against BHK-21 cells, even when used at 100 μM concentration, which implied that the antiviral activity of EGCG against DTMUV is not caused by cell death. In addition, EGCG inhibited viral copies in different tissues from DTMUV-infected ducklings and prolonged the survival rates of DTMUV-infected ducklings. Therefore, our data suggest that EGCG treatment is remarkably effective in preventing DTMUV infection in vitro and in vivo.

It is well-known that EGCG exhibits its antiviral effects through multiple potential mechanisms of viral inhibition. For example, EGCG directly interacted with

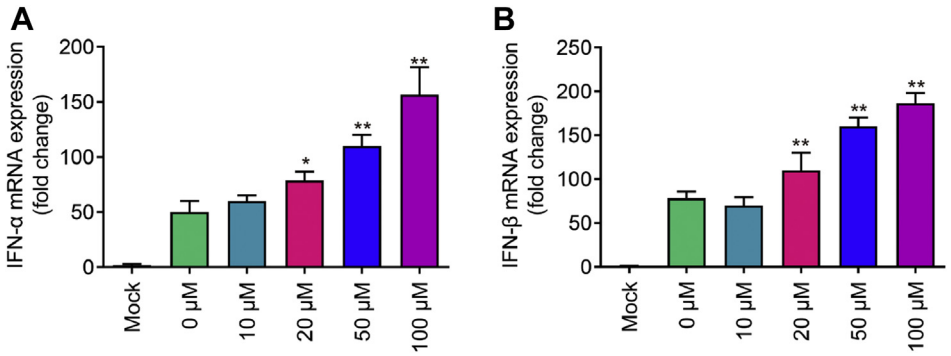

Figure 4. EGCG inhibited DTMUV infection through promoting antiviral interferon alfa and beta gene expression. BHK-21 cells were treated with different concentrations of EGCG at 37°C, and subsequently infected with DTMUV strain AH-F10 at an MOI=0.1. (A, B) The mRNA expression levels of interferon alfa and interferon beta were detected by qRT-PCR. Data represent the mean ± SD of 3 independent experiments. $^{**}P < 0.01$ vs. untreated cells. Abbreviations: DTMUV, duck Tembusu virus; EGCG, (-)-Epigallocatechin-3-gallate.

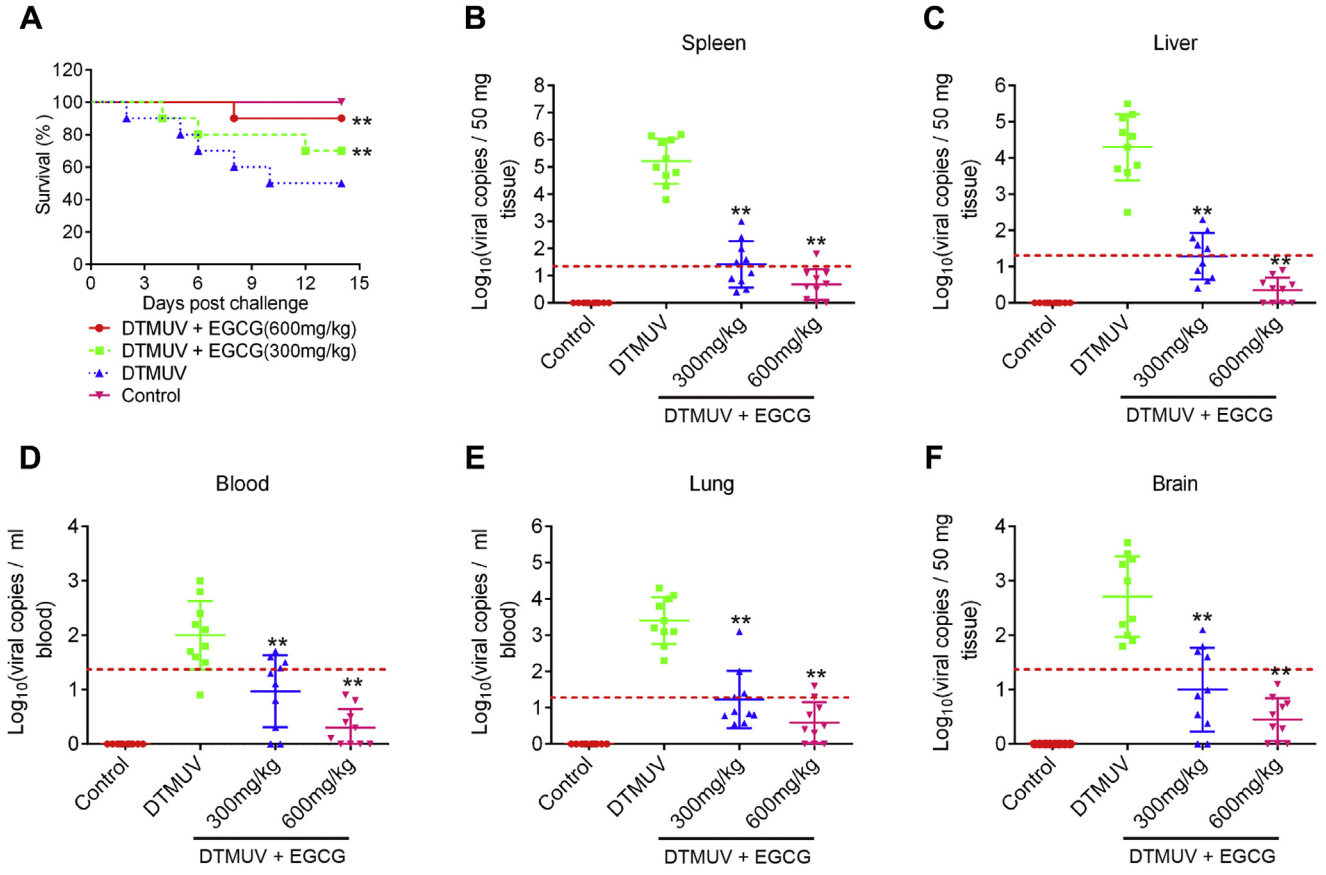

Figure 5. EGCG inhibited DTMUV infection in vivo. The ducklings were infected with DTMUV at 4 h before the beginning of the EGCG treatment. (A) The survival rate was measured for 15 d. (B–F) The virus copy numbers in the spleen, liver, blood, lung, and brain were detected using qRT-PCR in all group ducklings. Data represent the mean ± SD of 3 independent experiments. $^{**}P < 0.01$ vs. virus group. Abbreviations: DTMUV, duck Tembusu virus; EGCG, (-)-Epigallocatechin-3-gallate.

lipid envelope, lead to a subsequent destruction of the virus particles, thus inhibiting the entry of the Zika virus into the host cell (Carneiro et al., 2016). Kim et al. demonstrated that EGCG inhibited influenza virus infection by the direct damage on influenza virus membrane with the loss of viral penetration activity (Kim et al., 2013). Wang et al. reported that EGCG exerted antiviral activity with highly inhibitory effects on Japanese encephalitis virus attachment and entry (Wang et al., 2018). In addition, EGCG could effectively block the binding of herpes simplex virus-1 and HCV particles to the heparan sulfate on the cell surface (Colpitts and Schang, 2014). Heparan sulfate have the binding affinity to several flaviviruses E protein, suggested as the virus receptors (Chen et al., 1997, 2010; Kroschewski et al., 2003). In this study, we demonstrated that the antiviral activity of EGCG against DTMUV in different approaches in the following order: virucidal ability, virus attachment, and virus entry. Thus, the data revealed that the antiviral mechanism of EGCG against DTMUV was associated with blocking the early steps (including attachment and entry) of DTMUV infection.

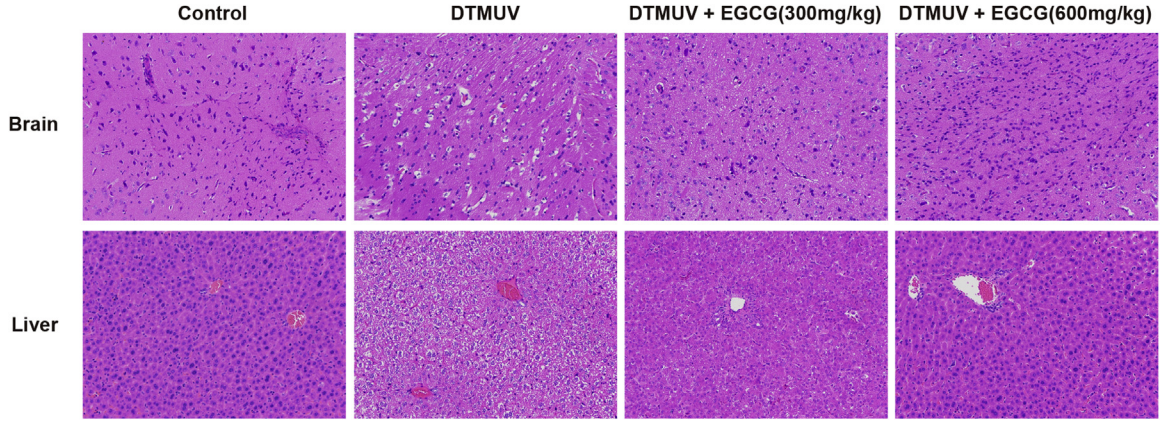

Figure 6. The histopathologic lesions of the brain and liver after EGCG treatment in DTMUV-infected ducklings. Histopathologic changes (200 ×) of the brain and liver tissues obtained from ducklings. Abbreviations: DTMUV, duck Tembusu virus; EGCG, (-)-Epigallocatechin-3-gallate.

During the virus infection, type I interferon (interferon alfa/beta)–mediated immune response effectively prevents the replication of DTMUV (Li et al., 2015; Chen et al., 2016, 2017; Yu et al., 2018). Interferons bind their cognate receptors and subsequently initiate a signaling cascade through the Janus kinase–signal transducer and activator of transcription pathway, triggering the expression of hundreds of interferon-stimulated genes, which play a crucial role in host resistance to DTMUV infection (Bi et al., 2017; Chen et al., 2017). Notably, EGCG exhibits the potential of antiviral activity through enhancing antiviral innate immune responses. For example, Wang et al. showed that EGCG has the ability to enhance HCV dsRNA–induced intracellular antiviral innate immunity against HCV (Wang et al., 2016). However, whether EGCG exerts its anti-DTMUV effect through enhancing innate immune responses remains unknown. In this study, our results showed that EGCG could promote the expressions of interferon alfa and beta at mRNA levels in DTMUV-infected BHK-21 cells. These data suggest that EGCG inhibits DTMUV infection partially by its immunomodulatory activity.

Increasing evidence indicates that utilization of EGCG in animals can be effective in various doses. For example, Lai et al. showed the inhibitory effects of EGCG on hepatitis B virus infection by monitoring hepatitis B virus DNA and HBsAg in mouse models for hepatitis B virus (Lai et al., 2018). Xiao et al. found that after oral administration of EGCG, the death rate and pulmonary pathologic lesions were decreased, and the mean survival d were prolonged in the mice infected by influenza A virus (Xiao et al., 2008). However, whether EGCG displays its inhibitory abilities in vivo remains unknown. In our study, we found that the pathologic lesions of brain and liver tissues were alleviated, and the survival rate was raised by EGCG treatment in the ducklings infected with DTMUV. These data suggest that EGCG also exhibits its antiviral activity in vivo.

In conclusion, EGCG exhibits the potential of antiviral activity against DTMUV via significantly inhibiting virus attachment, receptor binding, and entry, as well as inducing the expression of interferon alfa and beta. Therefore, EGCG might be used as a potential drug in therapy and prevention of infections caused by DTMUV.

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DISCLOSURES

The authors declare no conflicts of interest.

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