Diallyl Trisulfide Protects Against H9N2 Avian Influenza Virus Infection

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Research

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Abstract

Background

Diallyl trisulfide is a component of garlic. It has been shown to have anti-fungus, anti-cancer, and anti-virus activities. We hypothesized that diallyl trisulfide can enhance host defense mechanisms against the infection by influenza virus A subtype H9N2, which is the most prevalent subtype of influenza viruses in poultry in many countries in Asia.

Methods

In this study, the effects of diallyl trisulfide on influenza virus will be investigated in vivo and in vitro respectively. The expression levels of IL-6, TNF-α, RIG-I, IRF-3, IFN-β, and H9N2 avian influenza virus M gene were detected using Quantitative real-time RT-PCR.

Results

In vitro, we found that treatment of A549 cells before or after H9N2 virus infection resulted in reduced viral loads, increased expression of antiviral genes (RIG-I, IRF-3 and interferon-β), and decreased expression of inflammatory cytokines (TNF-α and IL-6). These effects were also observed in H9N2 virus infected mice.

Conclusions

These results suggest that diallyl trisulfide inhibits H9N2 virus replication and down regulate the expression of inflammatory cytokines. Also this research indicates that diallyl trisulfide might be a kind of effective drug against influenza virus infection.

Background

Influenza is a severe infectious disease of the respiratory tract and contributes to substantial morbidity and mortality. Previous estimates indicated that 290 000-650 000 influenza-associated respiratory deaths might occur annually (Iuliano et al, 2018), which provides a potential threat to global health problem. Currently two classes of anti-influenza virus drugs, M2 channel blockers and neuraminidase inhibitors, are available, but a drastic increase in viral resistance, cumulative neurotoxicity, and time-dependent effectiveness of these drugs limit their clinical use (Le et al, 2005; Bright et al, 2005). Therefore, novel and safe anti-influenza drugs are urgently needed.

The anti-viral activities of plant extracts and their derivatives are increasingly recognized in recent years (Sharma et al, 2015; Fang et al, 2019), with up to 40% of modern medicines are derived from plant materials (Rajasekaran et al, 2013). Generally, the plants can produce a vast array of metabolites that will provide an inhibitory effect on the protein's synthesis and the propagation of the virus. Several small molecules extracted from plants have been shown to possess obvious anti-influenza virus effects. For

instance, catechins in green tea have significant inhibitory effects on both A and B influenza viruses in vitro (Jae-min et al, 2005). The main active ingredients extracted from Dendrobium also have been shown to possess in vitro activity against H1N1 and H3N2 virus replication (Li et al, 2017). These plant extracts might provide alternative therapeutic measures for the treatment of influenza virus infection.

Garlic has been found to have a wide range of biological activities, including anti-fungus, anti-virus, anti-cancer, anti-oxidation, and anti-inflammation (Annamalai et al, 2017; Powolny et al, 2008). Fresh garlic extracts also has been observed to inhibit influenza virus infection (Mehrbod et al, 2013; Mehrbod et al, 2009). In addition to as an antiviral agent, garlic is also considered as a potential immune modulator, playing a very important role in immune regulation and anti-inflammatory response of the body (Arreola et al, 2015). In dengue virus infection, the active substance of garlic shown that it could significantly reduce the inflammatory and oxidative stress response (Hall et al, 2017). Organosulfur compounds are the main effective components of garlic, and among which diallyl trisulfide (DATS) is easily synthesized and more stable than the others (Corzo-Martínez M et al, 2007). It has been shown that DATS diminishes NF-κB and TNF-α activity in mice with acute LPS-induced lung injury (Yewdell et al, 1985). As we all known, except the direct damage caused by the virus itself, the severity of the influenza virus infection is usually closely related to the strong or uncontrolled immune response induced in the body (Brandes et al, 2013). However, the role of DATS and whether it can regulate the innate immune response during the influenza virus infection is not clear.

H9N2 subtype AIVs are highly prevalent in poultry in China, and can infect a wide variety of hosts, including bird, horse, pig, human, etc (Iqbal et al, 2013; Nili et al, 2003), which have caused great economic loss and health repercussions. In this study, we hypothesized that DATS can enhance innate immunity against infection by avian influenza virus (AIV) subtype H9N2 and its anti-viral activity was investigated in vitro and in vivo.

Materials And Methods

Virus

The H9N2 avian influenza virus (H9N2 AIV) stored in our laboratory was isolated from wild ducks in Jiangxi Province, China in 2013. H9N2 AIV has been shown able to infect and induce acute lung injury in mice (Zhu et al, 2013). The virus was propagated in 10 days old specific pathogen free chicken embryos (Ingelheim Vital Biotechnology Co, Ltd, Beijing, China) at 37^{o}C for 72h. The 50% tissue culture infection dose (TCID50) was determined in human alveolar epithelial cell line A549 cells, and the stocked virus titer was about $10^{6.9}$ TCID50/100 μl.

Cell culture and reagents

A549 cells were purchased from the Cell Bank Academy of Science (China) and grown in Dulbecco's Minimum Essential Medium (DMEM) (Gibco, USA) supplemented with 10% FBS at 37^{o}C with 5% CO_2.

MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (purity > 98%), amantadine hydrochloride (purity > 99%), and DATS (purity > 97%) were purchased from Sigma-Aldrich. DATS was dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 3mM as stock solution and stored at -20^0C before use. Amantadine hydrochloride (AMT) was dissolved in 0.1% DMSO at a concentration of 4 mM and used as positive anti-virus control drug. MTT (5 mg/ml) was dissolved in phosphate buffered saline (PBS) and filtered through a 0.2-μm microporous membrane (Millipore).

Cytotoxicity assay

The cytotoxicity of DATS and AMT for A549 cells were determined using the MTT assay. A549 cells were seeded at a concentration of 1×10^5 cells/well in 96-well plate (Corning) and incubated at 37ºC in 5 % CO_2 incubator for 24 h. When the cells reached 75 % confluency, they were replenished with 0.1ml of the maintenance medium (2% FBS) containing a series of concentrations of DATS (75 μM to 3000 μM) or AMT (125 μM to 4000μM). Five wells of cell were used in each concentration and incubated at 37ºC in 5 % CO2 incubator for 48 h. The wells with 0.1 mL maintenance medium were used as controls. After removal of the culture medium in each well, MTT (5 mg/ml, 20 μl) was added and incubated for 4 h at 37^{o}C. The reaction was stopped by addition of 100 μl of DMSO, and the absorbance (Abs) of the purple formazan formed due to MTT reduction by NAD (P) H-dependent cellular oxidoreductase enzymes in live cells was read at 570 nm using a microplate reader (Thermo Fisher Scientific,USA). The percentage of cell viability after drug treatment was calculated as follows: % cell viability = [Abs treatment group/Abs cell control group] x 100%.

DATS treatment in A549 cell line

The effects of DATS on H9N2 AIV infection were investigated under two different conditions, including pretreatment and post-infection. The A549 cells were seeded in 96-well plates and T-25 culture flasks, respectively, and after the confluent growth of A549 cells reached 75–90%, they were treated as the follows: For the pretreatment experiment, A549 cells were treated with three different concentrations (375μM, 187.5μM and 93.75μM) of DATS for 24 h, then the cells were washed twice with PBS, and infected with H9N2 virus. For the post-infection experiment, cells were infected with H9N2 virus for 1h, then the infected cells were washed twice with PBS and added maintenance medium containing three different concentrations (375μM, 187.5μM and 93.75μM) of DATS. 500 μM AMT was used as a positive drug control. For virus adsorption, the cells were inoculated with H9N2 AIV at a multiplicity of infection (MOI) of 0.1 except for control cells. After 1h of incubation at 37^{o}C, the cells in 96-well plates were washed twice with PBS and then overlaid with maintenance medium and cultured for 48h. The cell supernatants in 96 well plates were collected to perform the hemagglutination assay (HA). The inhibition effect of DATS on the CPE induced by H9N2 virus was determined by MTT assay as described above. The cells in T-25 culture flasks were incubated at 37 ◦C under 5 % CO2 with a small amount of maintenance medium, and then the cells and the supernatants were harvested together at 24 h and 48h,

respectively, and then they were tested for the expression levels of inflammatory cytokines by real-time PCR assay. All the experiments were performed in triplicates.

Hemagglutination (HA) assay

To evaluate the virus titer in cell culture, the U-bottom microtiter plates were used for the HA assay. Briefly, 50 µl of PBS were added to each wells of the plate, and then the 50 µl culture media was added in the first row of the plate, and serially diluted by transferring 50 µl from the first well to the successive well and so on. Finally, chicken red blood cells (cRBCs) (0.5%) were added to each well. Following incubation at least for 30 min at room temperature, the virus titer was read. The AMT positive control, the virus control and the blank control were kept in the same plate.

Quantitative RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed to measure the expression levels of IL-6, TNF-α, RIG-I, IRF-3, IFN-β, and H9N2 AIV M gene. All qRT-PCR primers were designed using the software Primer Premier 5.0 (Premier, Canada) and available if requested. Total RNAs were extracted from culture supernatants and cells using the RNeasy Mini Kit (QIAGEN). Synthesis of the first-strand complementary DNA was conducted using the Invitrogen Transcription SuperScript^{TM} RT Kit (Invitrogen, US). Each qRT-PCR reaction contained 10 µL SYBR Premix Ex Taq (2×), 0.4 µL forward primer, 0.4 µL reverse primer, 0.4 µL ROX reference dye (50×), 2 µL cDNA, 6.8 µL H_2O (total volume 20 µL), and SYBR Premix Ex Taq RR420A-Tli RNase H Plus (Takara Clontech, Dalian). PCR was performed as follows: 95°C for 30s followed by 40 cycles of 95°C for 5s and 60°C for 31s. Expression levels of various genes were normalized to that of the housekeeping gene GAPDH as its expression level was stable in A549 cells. Four independent PCRs were performed for each sample. All data were analyzed using the Sequence Detector Systems software (Applied Biosystems, USA). Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Li et al, 2013), where ΔCt values were calculated with the CT values of experiments minus CT values of the housekeeping gene. The ΔΔCt values were then calculated by subtracting ΔCt of the no-treated cell control groups from ΔCt of experiment groups.

In vivo experiments

Pathogen free BALB/c female mice (aged 6–8 weeks) were purchased from Shanghai Slake Co, Ltd. (China). To evaluate the effect of DATS on H9N2 AIV-induced lung injury, the mice were randomly divided into infected group, DATS-treated infected group, DATS control group, and uninfected control group (n = 26 mice/group). Mice in infected group and DATS-treated infected group were lightly anaesthetized with diethyl ether and inoculated intranasally with 80 µL of H9N2 AIV in allantois fluid (1×10^6 50% egg infection dose). Mice in uninfected control group were inoculated with the same dilution and volume of sterile allantois fluid intranasally. One day after virus inoculation, mice were injected with 30 mg/kg DATS

intraperitoneally or 0.9% saline every day. Five mice from each group were euthanized at days 2, 4, and 6 post-infection, and their whole lungs were harvested and weighed. The pulmonary index was calculated according to the following formula: pulmonary index = [lung weight (g)/body weight (g)] × 100. A portion of each lung was fixed in formalin and stained for histological examinations as described in our previous manuscript (Gui et al., 2017), and the other part of each lung was used for viral titration on MDCK cells and measure the expression of inflammatory cytokine genes and H9N2 AIV M gene by qRT-PCR. The remaining mice were monitored daily for clinical signs, body weight for 14 days after infection.

Statistical analyses

All data are expressed as mean ± standard deviation(SD). Statistical analyses were performed using SPSS for Windows, version 19.0 (SPSS Inc, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey test or unpaired two-tailed t-test was used to determine the significance of difference between two groups. In all statistical analyses, significant difference was accepted at $P < 0.05$ or $P < 0.01$.

Results

Cytotoxicity of DATS

The cytotoxicity of DATS and AMT on A549 cells was examined using the MTT based cell viability assay. Results showed that 375 μM of DATS and 500 μM of AMT had little or no cytotoxicity to A549 cells (Figure 1). Therefore, 93.75 μM-375 μM of DATS, and 500 μM of AMT were used for subsequent experiments.

Antiviral activity of DATS

To characterize anti-H9N2 AIV activity of DATS, its effects on various stages of infection were examined. DATS was added to A549 cell cultures to a final concentration of 93.75, 187.5, or 375 μM before (pretreatment), or after (post-infection) H9N2 AIV infection. H9N2 AIV-infected A549 cells were treated with 500 μM AMT as positive anti-viral control in the same manner. As AMT was dissolved in 0.1% DMSO, cells in the untreated control groups were treated with 0.1% DMSO before or after infection to serve as negative controls.

In the pretreatment experiment, the viability of untreated H9N2 AIV infected- cells was 43%, and that of AMT-treated infected group was 61%. The viability of infected cells treated with 93.75, 187.5, or 375 μM of DATS was 47%, 57%, and 57%, respectively (Figure 2A), suggesting that pretreatment of A549 cells with DATS rendered them less susceptible to H9N2 AIV infection. In the post-infection experiment, the viability of untreated H9N2 AIV infected-cells was 53%, and that of AMT-treated infected cells was 82%. The viability of infected cells treated with 93.75, 187.5, or 375 μM of DATS was 68%, 73%, and 79%,

respectively. These results suggested that DATS inhibited both infection and replication of H9N2 AIV. The effect of DATS was dose dependent, and the protection was more profound when DATS was added 1 hour post-infection.

The antiviral effect of DATS was also examined by the HA assay. As shown in Figure 2B, DATS exhibited remarkably inhibit the virus hemagglutination in pre- or post-treatment, but no dose dependent was observed in the assay. To further assess the effect of DATS on H9N2 AIV replication, the expression of the M gene of H9N2 AIV was examined by qRT-PCR. As seen in Figure 2C, M gene expression in AMT-treated infected cells was 0.71 and 0.54-fold of the level of untreated H9N2 AIV-infected cells in pretreatment and post-infection experiments, respectively. M gene expression in DATS pretreated A549 cells was about 0.6-fold (40% decrease) that of untreated H9N2-infected cells for all 3 DATS doses. The effect of DATS on H9N2 AIV replication was most profound in the post-infection experiment as M gene expression in A549 cells treated with 93.75, 187.5, or 375 μM of DATS after infection was 0.27, 0.18, and 0.16-fold that of untreated cells, indicating that DATS at 93.75, 187.5, and 375 μM inhibited H9N2 AIV replication by 73, 82, and 84% when it was added to the cells 1 hour after infection. Based on MTT and HA assay results, the protection was more effective when DATS was added 1-hour post-infection, so the 375μM of DATS post-infection were used in the following experiments.

DATS diminishes H9N2 AIV induced inflammation

To investigate the effect of DATS on H9N2 AIV-induced inflammation, the expression levels of inflammatory cytokines TNF-α and IL-6 in cells of post-infection were measured by qRT-PCR at two different time points.

In the experiment, A549 cells were infected with H9N2 virus for 1h, and then washed twice with PBS, followed by treatment with 375μM of DATS for 24 and 48h, respectively. For IL-6, H9N2 AIV infection increased its expression by 15.8-fold or 9.6-fold compared to uninfected control (set as 1.0) at the two indicated time points (Figure 3 A). Treatment of H9N2 infected cells with 500 μM AMT resulted in decreased expression (5.6-fold and 2.5-fold of control, respectively). Treatment of H9N2 infected cells with 375 μM of DATS reduced its expression to 9.0-fold and 2.3- fold of control, respectively. Treatment of H9N2 infected cells with 500 μMAMT also resulted in IL-6 decreased expression (5.6-fold and 2.5-fold, respectively of control). For TNF-α, H9N2 AIV infection increased its expression by 11.3-fold and 23.7-fold compared to uninfected control (set as 1.0), respectively (Figure 3B). Treatment of H9N2 infected cells with 500 μM AMT resulted in decreased its expression (2.9-fold and 2.3-fold of control, respectively). Treatment of H9N2 infected cells with DATS reduced its expression from 11.3-fold to 4.8-fold, 23.7-fold to 8.6-fold of control, respectively. These results showed that DATS could down-regulate proinflammatory response induced by H9N2 AIV infection.

DATS increases the antiviral immune response during H9N2 AIV infection

Type I IFN play an important role in the innate immune system, and IRF3 can regulate its production. In this study, the effects DATS treatment on the expression of RIG-I, IRF-3 and IFN-β in A549 cells were investigated by qRT-PCR. As shown in Fgure 3, H9N2 AIV infection increased RIG-1 expression 14.8-fold and 12.9-fold compared to uninfected control (set as 1.0) at two different time points. Treatment of A549 cells after H9N2 AIV infection with 500 μM AMT increased RIG-1 expression to 29.8 and 17.9-fold of control, respectively. Treatment of cells after infection with 375 μM of DATS increased RIG-1 expression to 26.9, and 15.3-fold of control, respectively. For IRF3, H9N2 AIV infection increased its expression 19.8-fold and 3.3-fold compared to uninfected control. Treatment of A549 cells after H9N2 AIV infection with 500 μM AMT increased its expression to 62.8 and 24.4-fold of control, respectively. Treatment of cells with 375 μM of DATS increased its expression to 51.8, and 34.2-fold of control, respectively. For IFN-β, H9N2 AIV infection increased its expression 6.1-fold and 4.7-foldd compared to uninfected control. Treatment of A549 cells after H9N2 AIV infection with 500 μM AMT increased its expression to 13.6 and 9.7-fold of control, respectively. Treatment of cells with DATS also increased its expression to 9.2, and 8.2-fold of control, respectively. These results suggested that DATS might exert its anti-H9N2 AIV by up-regulating the innate immune response.

DATS protects mice from H9N2 AIV infection

Based on results of in vitro experiments, we hypothesized that DATS can attenuate the symptoms of H9N2 AIV infection in mice. As reported previously, H9N2 AIV-infected mice exhibited marked inactivity, emaciation, ruffled fur, lack of appetite, labored breathing, respiratory distress, and decreased body weight (Gui et al, 2017). The decrease in body weight caused by H9N2 AIV infection was most profound 3 days after infection, from 100% to 87.2%. Treatment of infected mice with DATS (30 mg/kg) diminished such loss, from 100% to 91.8% on 3 days after infection. The body weight of DATS treated control group and uninfected control group was not significantly changed (Figure 4).

To investigate pulmonary edema caused by H9N2 virus infection, lung index (weight lung to body weight ratio) was determined at different time points during infection. As shown in Figure 5A, the lung index increased in the two infected groups on days 2-6 after H9N2 virus infection, but it was lower in the DATS treated group than in the infected group. At days 6, DATS effect was not significant. These results indicated that H9N2 infection caused edema, thus increasing the lung index, and DATS treatment could reduce the severity of edema. Similar patterns of DATS effect were observed at the titration of virus in lungs. A significant reduced lung titer on treatment with DATS on 2- and 4-days post infection (Figure 5B). Since influenza M protein can reflect the levels of virus replication, so we also checked the effect of DATS treatment on influenza M protein mRNA levels. The results showed that a significantly reduced the levels of M gene after treatment with DATS on 2- and 4-days post infection (Figure 5C). To further examine the lung lesion, HE staining of lung sections was carried out. As shown in figure 6, lungs of H9N2 AIV-infected mice showed collapse of alveolar spaces, infiltration of inflammatory cells, interstitial and alveolar edema, and hemorrhage. These pathological changes were relatively mild in DATS treatment.

These results all suggested that DATS treatment might reduce the severity of edema and the virus titers in lungs.

The expression levels of lung inflammatory cytokines (IL-6 and TNF-α) and antiviral cytokines (RIG-1 and IFN- β) induce by H9N2 AIV infection were then determined. As shown in Figure 7, the effects of H9N2 AIV infection and DATS treatment on the expression of TNF-α and IL-6 expression were most profound at 6 days post infection. At that time, H9N2 AIV infection increased TNF-α expression by 6.5-fold, and DATS treatment reduced it to 3.9-fold of uninfected control. For IL-6, H9N2 AIV infection increased its expression by 20-fold, and DATS treatment reduced it to 18-fold of uninfected control. The effect on RIG-I expression was most profound at day 6 post-infection. At which, H9N2 AIV infection increased its expression 16.9-fold, and DATS treatment further increased it to 29-fold of uninfected control. The effect on IFN-β expression was most profound at day 2 post-infection. H9N2 AIV infection increased its expression 10-fold, and DATS treatment further increased it to 12-fold of uninfected control at that time. These results showed that H9N2 AIV infection caused a profound increase in the expression of inflammatory and antiviral cytokines. DATS treatment of infected mice reduced the expression of TNF-α and IL-6, but increased the expression of RIG-1 and IFN-β.

Discussion

3-5 million severe respiratory cases every year worldwide was associated with Influenza (Iuliano et al, 2018). Treatment with conventional anti-influenza drugs usually results in resistance. In addition, influenza viruses undergo antigen drift and antigen shift, leading to difficulty in vaccine development (Tian et al, 2011). Therefore, exploring alternative antiviral agents are crucial prevent the spread of influenza. In this study, we assessed the antiviral effect of DATS on influenza infection in vitro and in vivo.

It has showed that the over expression of cytokines is a hallmark of severe influenza virus infection (Peeling et al, 2008). Several reports have demonstrated that the severity and higher mortality of influenza A viral infections were correlated with the excessive inflammation in the lungs attributed to IL-6 and TNF-a (McGill et al, 2009). Garlic has been shown to be effective to several diseases, and which largely due to the reduction of inflammation. DATS, one of the organosulfur compounds of garlic, has been shown to have immunomodulatory and anti-inflammatory effects in several types of cancer (Thomson et al, 2003). In this study, we also found that DATS pre- or post-treatment could alleviate the expression of the inflammatory cytokines (TNF-α and IL-6) induced by H9N2 AIV infection, but the mechanism of its reduces inflammation still is not clear.

It has been demonstrated that cytosolic RNAs derived from viral genome are mainly recognized by RNA helicases RIG-I and MDA5 encoded by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), respectively. RIG-I and MDA5 recruit virus-induced signaling adaptor (VISA) (also known as MAVS, IPS-1, and Cardif) (Pichlmair et al, 2006). VISA then forms a large prion-like complex and serves as a platform for the assembly of a signalosome, which contains multiple

components including TRAF proteins (TRAF2/3/5/6), TBK1, and IKKs kinases. TBK1 and IKKs phosphorylate IRF3 and NF-B, respectively, leading to the induction of type I interferons (IFNs) and pro-inflammatory cytokines (Hornung et al, 2006). RIG-I almost expressed in various cells, including immune cells and no-immune cells, such as lung epithelial cells, endothelial cells and fibroblasts), plays a vital role in the establishment of the innate immune after influenza virus infection. It can promote the expression of type I interferons to inhibit viral replication. In the present study, we showed that DATS up-regulated the expression of RIG-I in H9N2 AIV infected cells, and the elevated RIG-I might promote the expression of its downstream genes, such as IRF-3 and IFN-β (Figure 3). Therefore, RIG-I signaling pathways might play an important role in antiviral effect of DATS, but whether DATS inhibit H9N2 AIV replication through RIG-I signaling pathways remains to be further studied.

Conclusion

Results of our in vivo experiments showed that treatment of infected mice with DATS resulted in less weight loss, less lung damage, and reduced pulmonary inflammation and edema. Pathological examinations revealed that DATS decreased the infiltration of inflammatory cells. Previous studies have shown that both T cells and natural killer cells play an important role in anti-influenza virus infection (Vogt et al, 2008). We speculate that the DATS could mobilize immune cells such as natural killer cells, T cells, macrophages, and monocytes to clear the infection. DATS may also activate the complement system to form the membrane attack complex to destroy virus-infected cells (Hung et al, 2015). Moderate inflammatory response is protective, but severe inflammatory response is damaging (Matthay et al, 2012). Our results revealed that DATS significantly reduced the expression of IL-6 and TNF-α in H9N2 AIV infected cells, thus diminishing lung injury. DATS also enhanced the expression of RIG-I and IFN-β in the lungs to defend H9N2 AIV infection. Therefore, DATS, as an effective antiviral ingredient of garlic, might be considered a promising antiviral agent against influenza viruses.

Abbreviations

DATS : diallyl trisulfide

AIV : avian influenza virus

H9N2 AIV : H9N2 avian influenza virus

TCID50: tissue culture infection dose

DMEM: Dulbecco's Minimum Essential Medium

DMSO: dimethyl sulfoxide

AMT : Amantadine hydrochloride

PBS : phosphate buffered saline

Abs : absorbance

cRBCs : chicken red blood cells

SD : standard deviation

ANOVA: one-way analysis of variance

RIG-I : retinoic acid-inducible gene I

MDA5: melanoma differentiation-associated gene 5

VISA (also known as MAVS, IPS-1, and Cardif)**:** virus-induced signaling adaptor

Declarations

Ethical approval and conset to participate

All experiments were conducted under biosafety level (BSL)-2 conditions. Animals were maintained according to the National Institutes of Health (NIH) guidelines for the Care and Use of Experimental Animals. All animal experiments were performed according to established safety protocols and were approved by the Animal Ethics Committee of the East China Normal University.

Consent for publication

Not applicable.

Availability of data materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing Interest

The authors declare that they have no competing interests.

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Authors' contributions

GH conceived and designed the experiments. ML, ZL and XL performed the experiments. ML, CH, XL, WT, LT analysis the data. ZL and GH wrote the manuscript. All authors read and approved the final manuscript.

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Figures

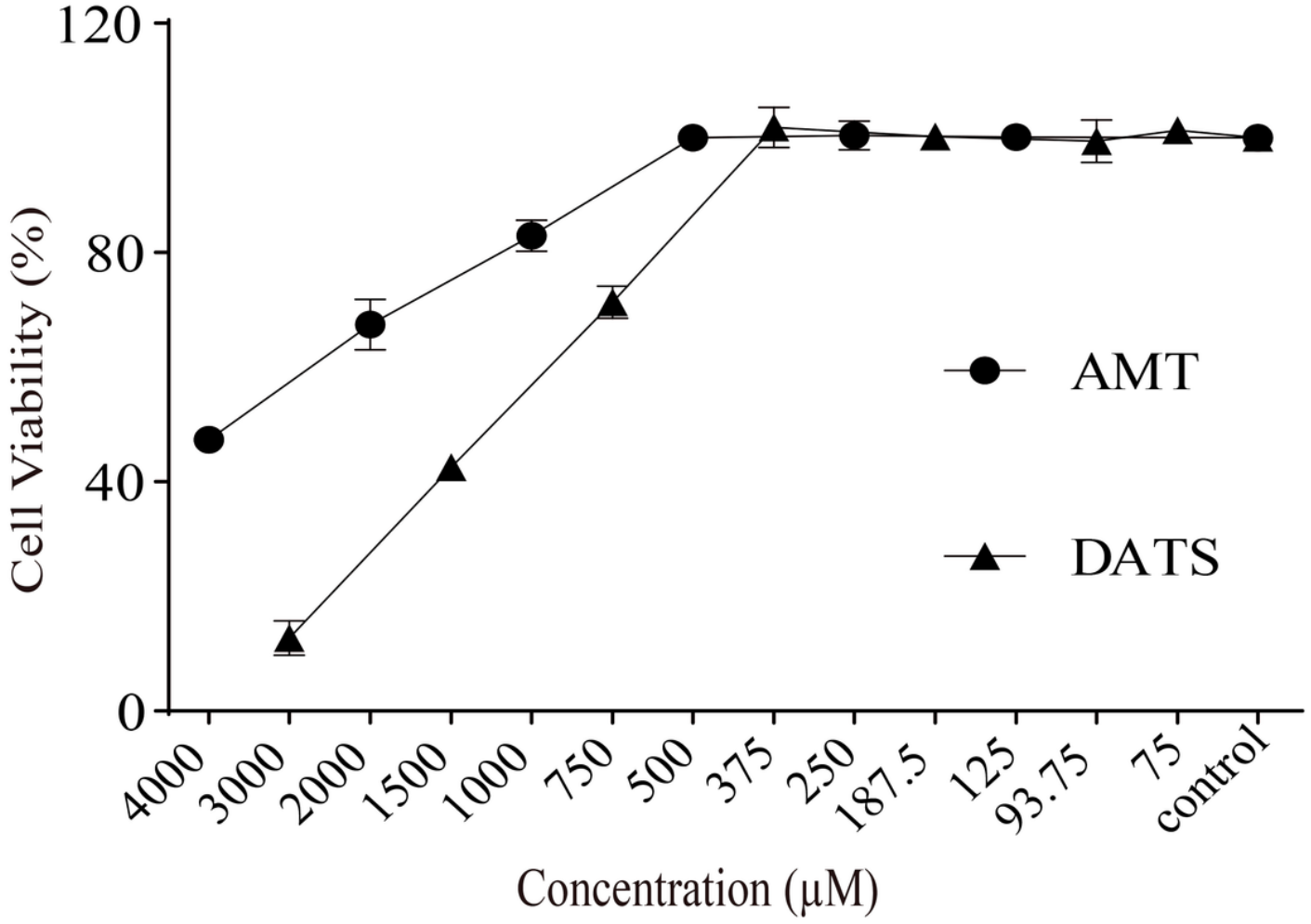

Figure 1

Cytotoxicity effects of diallyl trisulfide (DATS). The A549 cells were incubated with various concentrations of DATS or AMT for 48 h. The percentage of cell viability was calculated, the values are presented as means ± SD of each five detections.

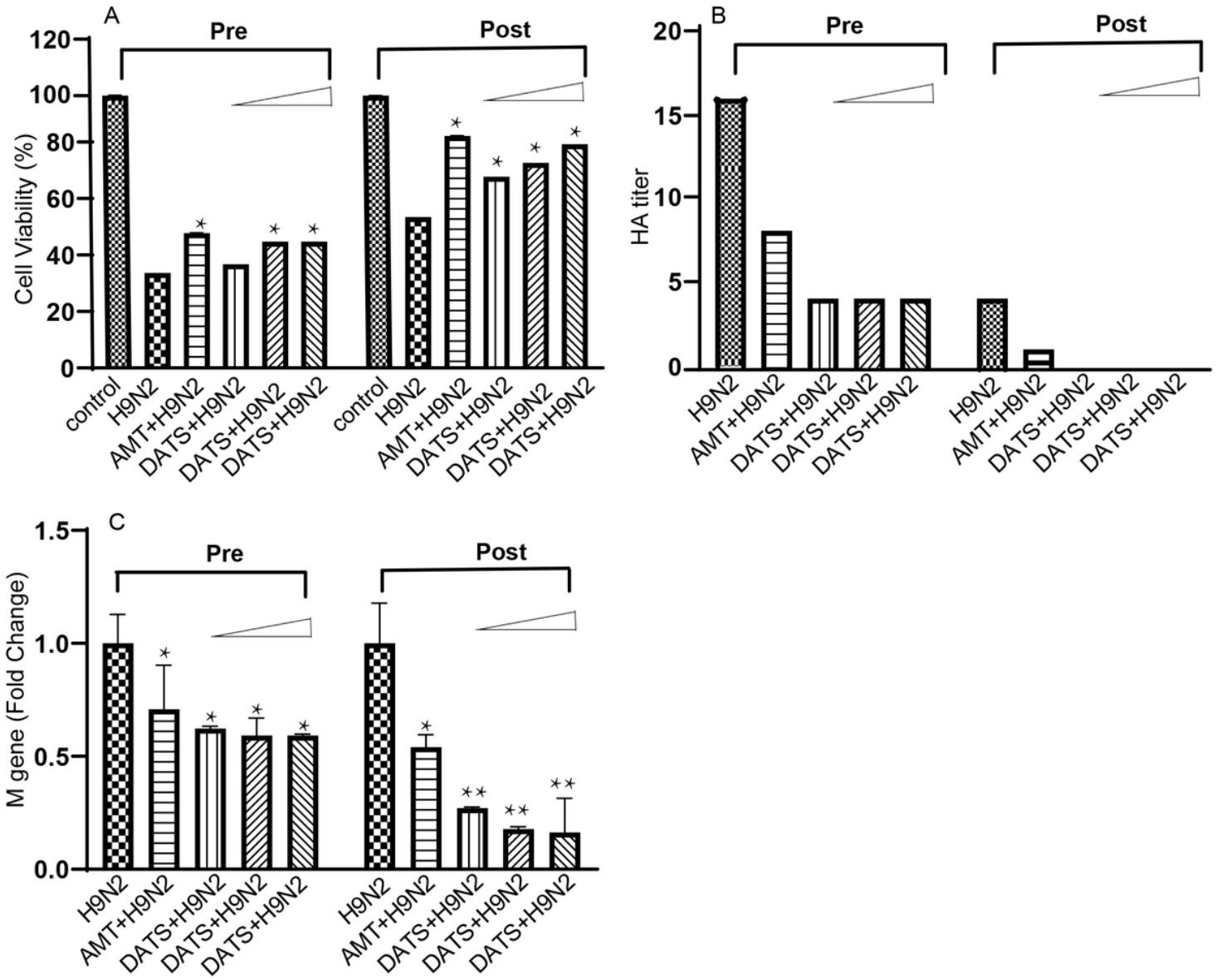

Figure 2

Effect of diallyl trisulfide on H9N2 AIV replication in A549 cells. A549 cells were treated with three different concentrations (375μM, 187.5μM and 93.75μM) of DATS before or after the H9N2 virus infection for 24h or 1h, respectively. then the cells were overlaid with maintenance medium and cultured for 48h. 500 μM AMT was used as a positive drug control. A. The cell viability of A549 cells were measured by MTT assay. B. The cell supernatants were collected, and the HA titer was measured by using chicken red blood cells (cRBCs). C. RNA was isolated from cell lysates and used in real-time RT-PCR to detect the expression levels of influenza M genes. Abbreviation: control, uninfected cells; H9N2, H9N2 AIV-infected; AMT+H9N2, cells treated with 500 □M AMT and infected with H9N2; DATS+H9N2, cells treated with DATS and infected with H9N2 AIV. Values are averages of three independent examinations. Significantly different compared to H9N2 infected group (*p<0.05, **p<0.01).

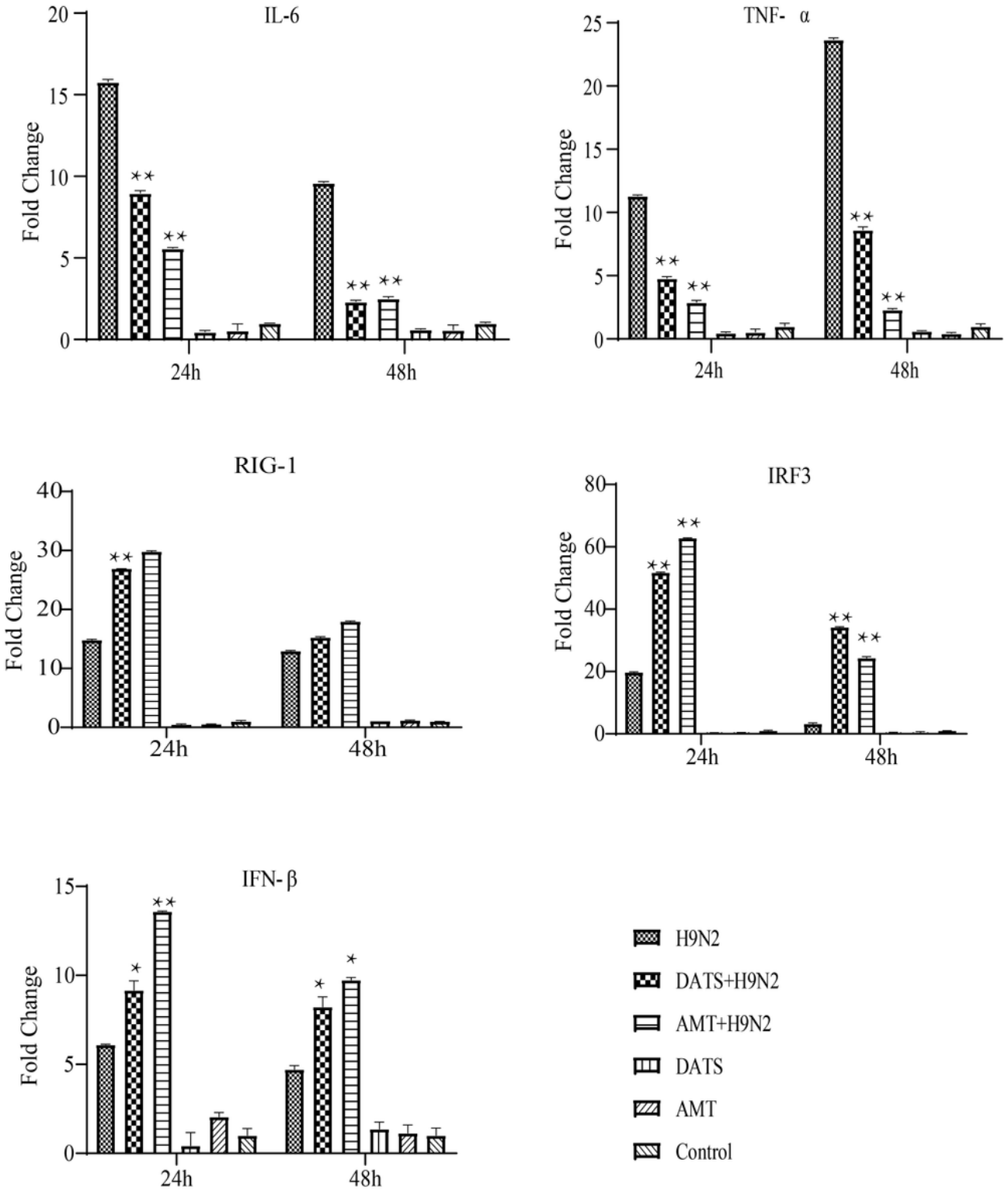

Figure 3

Effect of DATS on the expression of the inflammatory and antiviral cytokines. A549 cells were treated with DATS as described above, then the cells were overlaid with a small amount of maintenance medium and cultured for 24 and 48h, respectively. Then total RNAs were extracted, the expression levels of inflammatory and antiviral cytokines were measured by real-time PCR assay. GAPDH was used as an endogenous control. Fold changes in gene expression were calculated using the 2-ΔΔCt method. 500 μM

AMT was used as a positive drug control. The abbreviations were the same as the above figure. Values are averages of three independent examinations. Significantly different compared to H9N2 infected group (*p<0.05, **p<0.01).

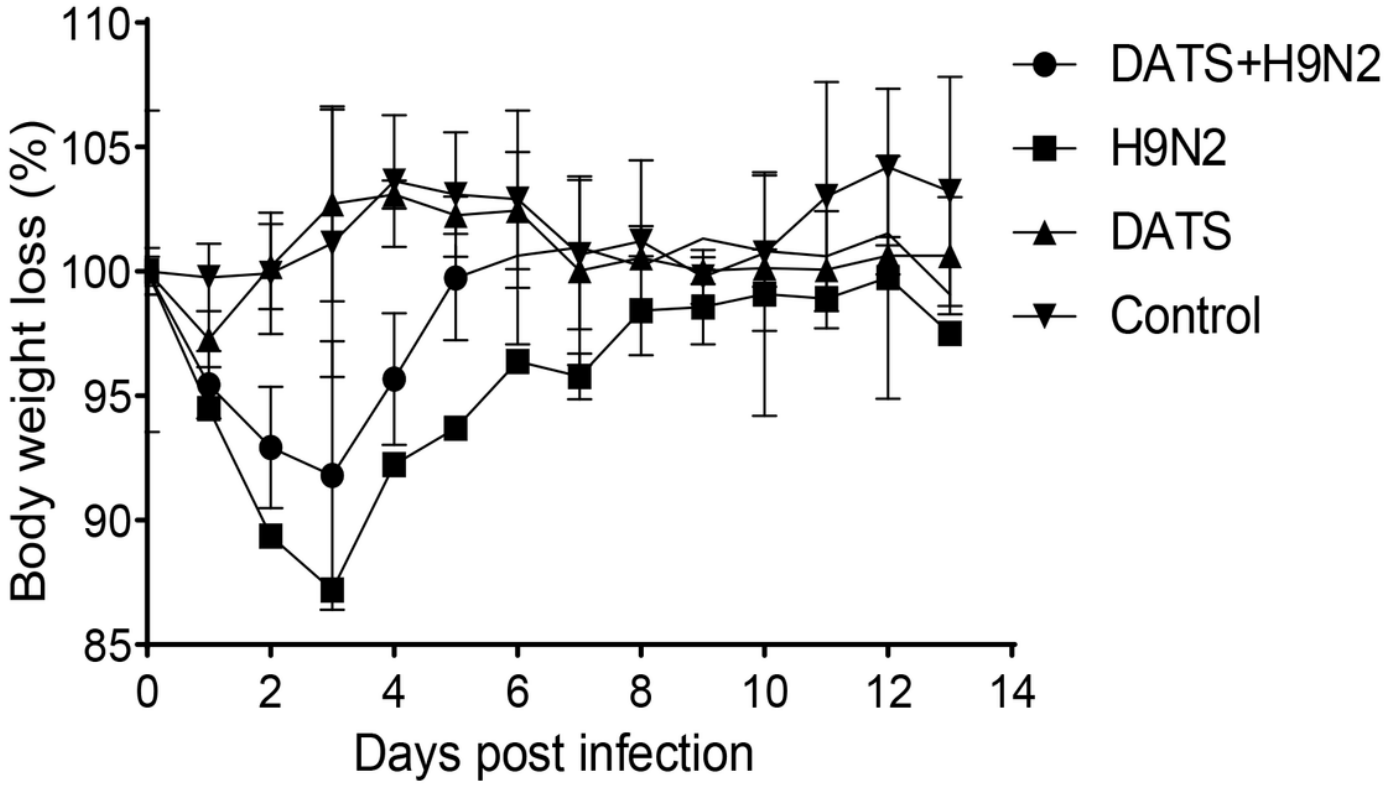

Figure 4

Effect of DATS on the body weight of H9N2 AIV infected mice. BALB/c female mice were inoculated intranasally with 80 μL of allantois fluid containing H9N2 virus (1 × 106 50% egg infection dose), One day after virus inoculation, mice were injected with 30 mg/kg DATS intraperitoneally or 0.9% saline every day. The mice body weight losses were calculated for 14 days. Abbreviations: Control, uninfected and untreated; DATS, DATS treated uninfected control; H9N2, H9N2 AIV-infected control; DATS+H9N2, mice infected H9N2 and then treated with DATS (H9N2). Data presented are the mean ± SD for more than 6 mice per group.

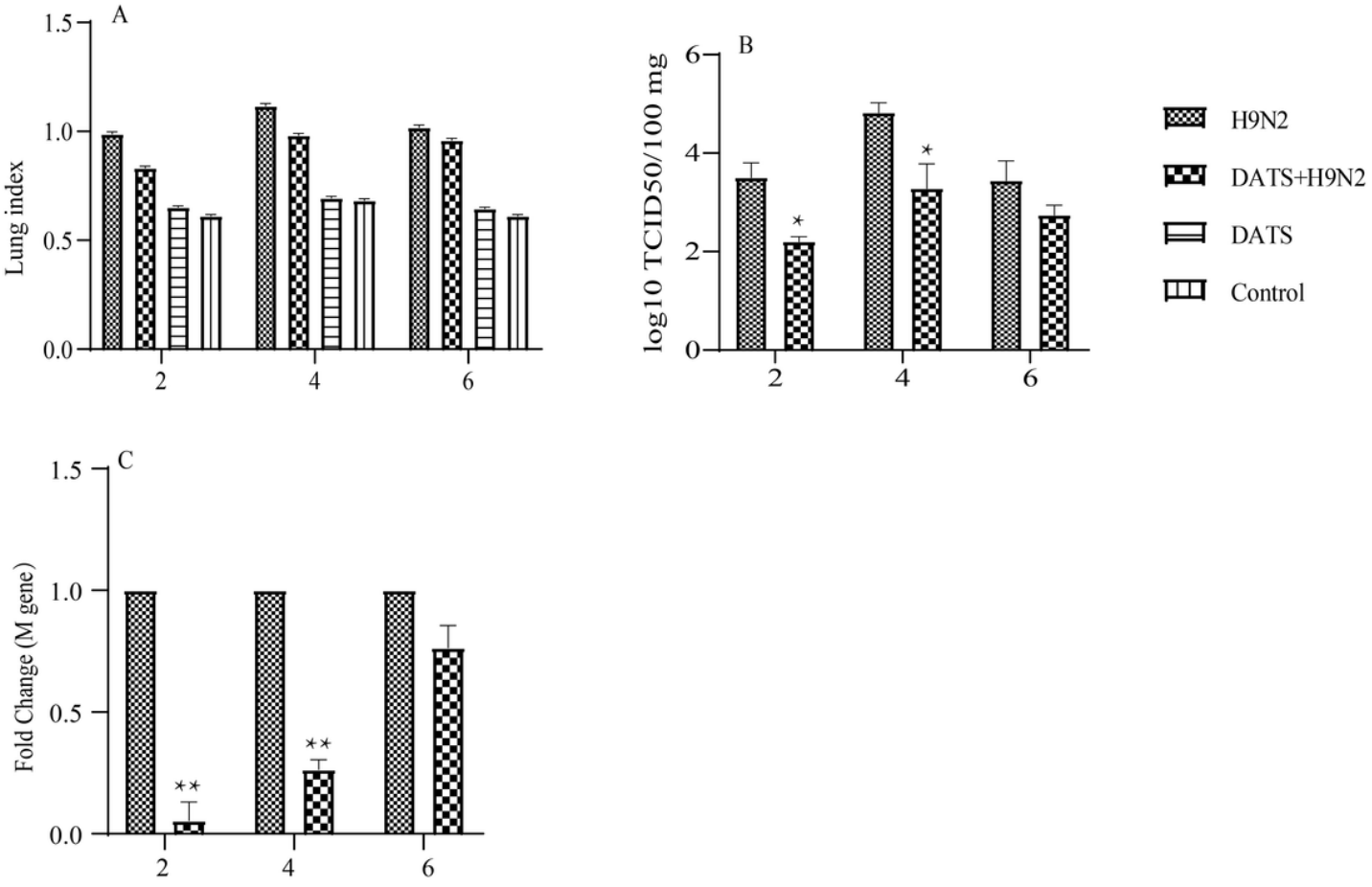

Figure 5

Effect of DATS on lung injury of mice. Five mice from each group were euthanized at days 2, 4, and 6 post-infection, and their whole lungs were harvested and weighed. The abbreviations were the same as the above figures. Significantly different compared to H9N2 infected group (*p<0.05, **p<0.01). A. The wet lung to body weight ratios were recorded. B. The virus titers in lungs were measured on MDCK cells by TCID50 assay. C. RNA was isolated from mice lungs and used in real-time RT-PCR to detect the expression levels of influenza M genes. Fold change were calculated using the 2-ΔΔCt method.

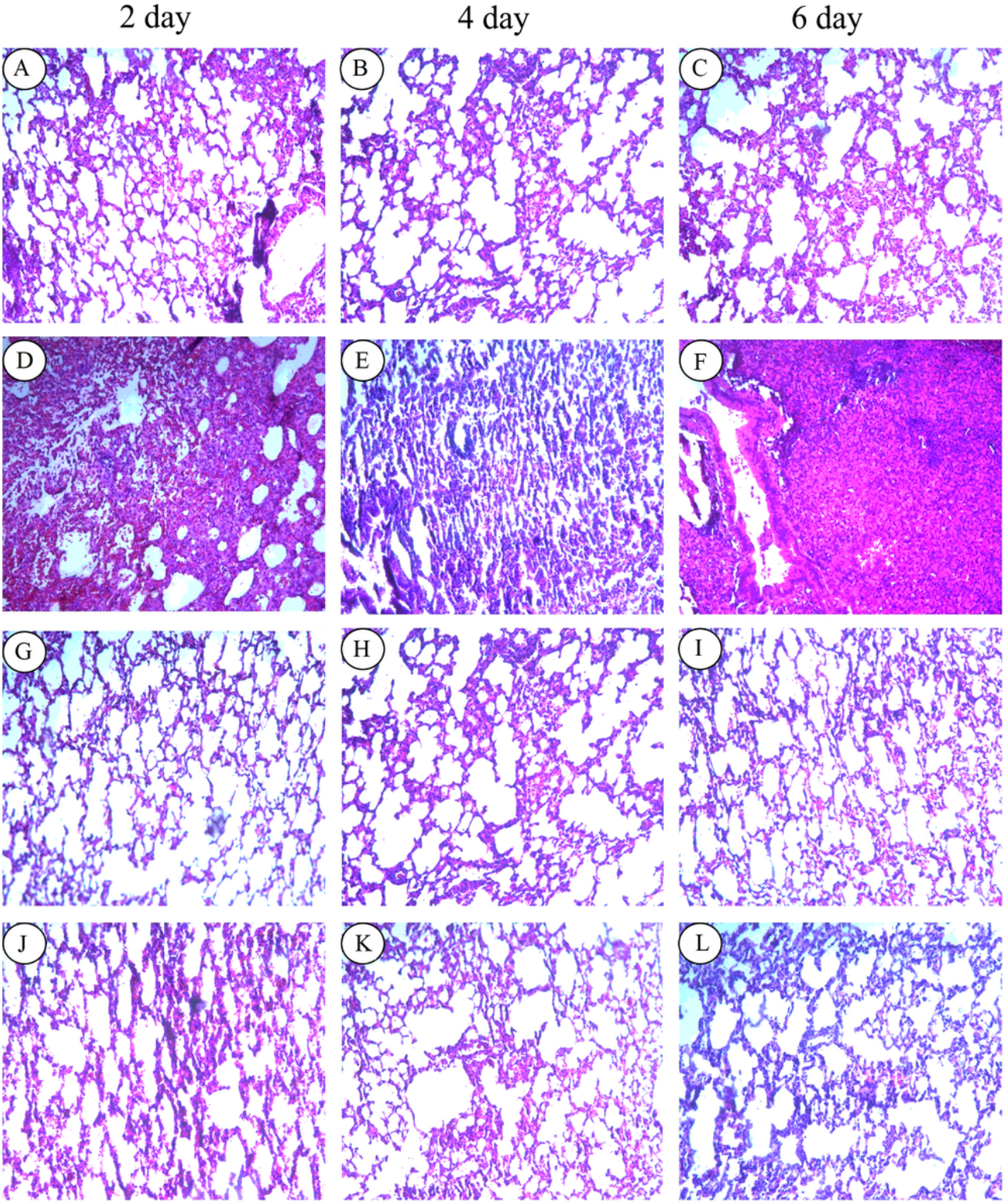

Figure 6

Effect of DATS on lung histology. Five mice from each group were euthanized at days 2, 4, and 6 post-infection, and part of their lungs were harvested and the histology of mouse lung sections were observed under the optical microscope. The abbreviations were the same as the above figures. DATS+H9N2 (A-C), H9N2 (D-F), DATS (G-I), and Control (J-L).

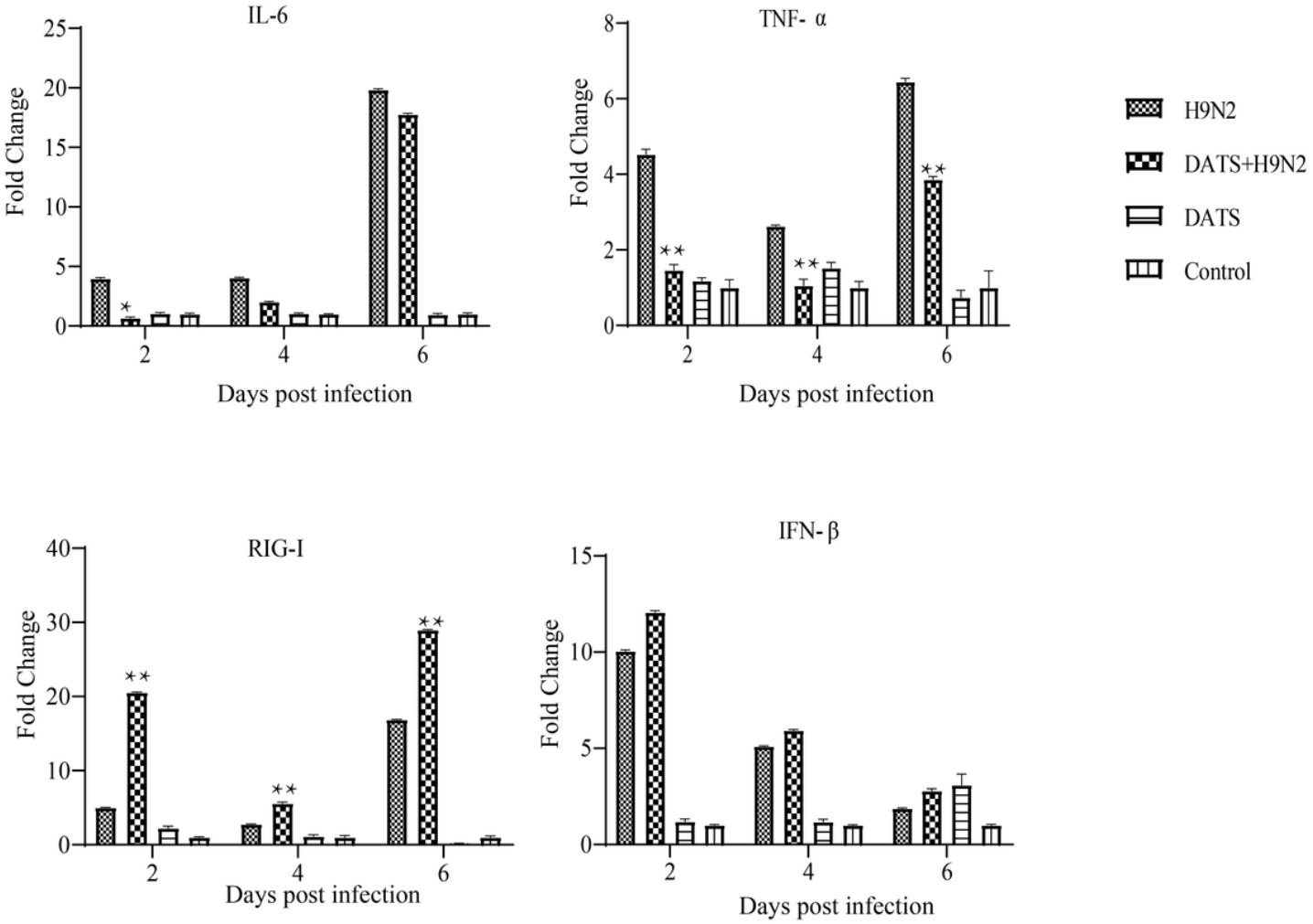

Figure 7

Effect of DATS treatment on the expression of the inflammatory and antiviral cytokines in mice. Five mice from each group were euthanized at days 2, 4, and 6 post-infection, and part of their lungs were harvested and total RNAs of them were extracted, the expression levels of inflammatory and antiviral cytokines were measured by real-time PCR assay. GAPDH was used as an endogenous control. Fold changes in gene expression were calculated using the 2-ΔΔCt method. The abbreviations were the same as the above figure. Values are averages of three independent examinations. Significantly different compared to H9N2 infected group (*p<0.05, **p<0.01).