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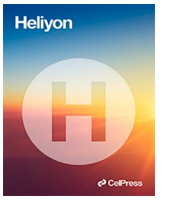

Research article

Baicalin reduced injury of and autophagy-related gene expression in RAW264.7 cells infected with H6N6 avian influenza virus

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ABSTRACT

In the present study, we investigated whether baicalin could reduce the damage caused to RAW264.7 cells following infection with H6N6 avian influenza virus. In addition, we studied the expression of autophagy-related genes. The morphological changes in cells were observed by hematoxylin and eosin (H&E) staining, and the inflammatory factors in the cell supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Transmission electron microscopy (TEM) was used to detect the levels of RAW264.7 autophagosomes, and western blotting and immunofluorescence were used to detect the protein expression of autophagy marker LC3. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to detect the mRNA transcription levels of autophagy key factors. The results showed that different doses of baicalin significantly reduced the H6N6 virus-induced damage of RAW264.7 cells. The contents of interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor (TNF)-α in the cell supernatant significantly decreased. In addition, the protein expression of LC3 and Beclin-1, ATG12, ATG5 the mRNA levels were significantly decreased. This study showed that baicalin can reduce cell damage and affect the H6N6-induced autophagy level of RAW264.7 cells.

1. Introduction

The avian influenza viruses of the Orthomyxoviridae family are known to cause a highly contagious disease, known as avian influenza (AI) in poultry and wild birds [1]. According to the pathogenicity of the avian influenza virus to chickens, the H6 subtype avian influenza virus belongs to the low pathogenic avian influenza virus (LPAIV) [2]. The H6N6 avian influenza virus does not demonstrate clinical symptoms in the host; however, the H6 subtype AIV often co-infects birds with the H5 and H9 subtypes of AIV, with the possibility of gene recombination of H5, H6, and H9 subtypes of AIV [3].

Research shows that the H6N6 virus binds to both avian-like and human-like receptors, confirming its ability to cross the species barrier to infect mice and human lungs without prior adaptation [4].

Autophagy specifically imparts innate immunity and serves as a major defense mechanism against pathogens entering the cells. Thus, it protects the host against microbial infections by promoting pathogen degradation and participating in acquired immunity [5]. Influenza A virus can induce autophagy, which is involved in virus replication and pathogenesis [6]. Recent studies have demonstrated

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that the active ingredients of traditional Chinese medicines can inhibit the proliferation of the influenza virus and reduce the expression of key genes involved in autophagy [7,8]. These data suggest that autophagy is involved in the pathogenesis of the influenza A virus. Baicalin is a flavonoid [9] with significant inhibitory activity against the influenza virus [10].

Baicalin has been reported to reduce the expression of inflammatory cytokines in the lung tissues of mice infected with the influenza virus and inhibit viral replication by downregulating the key factors of the RLR signaling pathway. In addition, it reduces the high expression of inflammatory cytokines in the lungs [11]. A recent study demonstrated the pharmacological action of baicalin against H3N2, where it inhibited virus-induced autophagy [12]. Thus, baicalin has emerged as a new candidate for targeted drug therapy for influenza virus infection. However, its action against the bird flu virus has not yet been reported. H6N6 avian influenza virus is known to be widespread in poultry, and the lack of effective vaccine prevention and control methods has been a major problem plaguing the poultry industry. No studies on the mechanism of baicalin against the injury and autophagy of H6N6 subtype avian influenza virus have been found, which is highly innovative. A study of the effect of baicalin on cells infected with the H6N6 avian influenza virus can deduce the relationship between anti-inflammatory and autophagy, which consequently can provide a new drug strategy for the treatment of influenza virus infection. Therefore, we investigated whether baicalin could reduced the H6N6 avian influenza virus-induced damage of RAW264.7 cells and its effect on autophagy-related genes. We believe this study will provide a novel theoretical basis for studying the mechanisms related to the prevention and treatment of avian influenza virus and autophagy.

2. Materials and methods

2.1. Materials and reagents

Mouse Mononuclear Macrophages Cells (RAW264.7 cells) were purchased from the Wuhan Procell Life Technology Co., Ltd. Details on the cell lines are as follows: source: *Mus musculus*. catalogue number: CL-0190. The cell lines were authenticated within 3 years of use in experiments and we have tested the cell lines for mycoplasma. The H6N6 subtype AIV strain (A/Duck/03/2016, GenBank accession no. MG434501) was procured from the Animal Disease Laboratory of Guizhou University. Baicalin (net content 90 %) was purchased from the Shanghai Aladdin Biochemical Technology Co., Ltd. Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco Co., Ltd. (Australia).

2.2. Cell culture

The H6N6 virus was amplified in the allantoic cavity of a 10-day-old chicken embryo. The RAW264.7 cells were cultured in 10 % fetal bovine serum and 1 % antibiotic–antimycotic solution at 37 °C with 5 % CO_2. The titer of the proliferating strain in RAW264.7 cells was measured by observing the cytopathic effects (CPEs), and the median tissue culture infective dose ($TCID_{50}$) was determined using the Reed–Muench method [13].

2.3. CCK-8 assay for cell viability

The RAW264.7 cells were inoculated into 96-well plates at a density of 1×10^4 cells per well and subsequently cultured for 12, 24, and 48 h. Afterward, a new culture medium with different concentrations of baicalin (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL) was added and cultured for 12, 24, and 48 h. These cells were subsequently treated with the CCK-8 reagent (GlpBio, USA), and the cells were incubated in the dark for 2 h, followed by measuring the absorbance at 450 nm using a microplate meter.

Next, the cells capable of growing into monolayers were inoculated with the H6N6 virus venom of 100 TCID50 for 2 h, following which the supernatants were discarded. The baicalin culture medium (0, 6.25, 12.5, 25, and 50 μg/mL) was added to the wells and cultured for 12, 24, and 48 h. The CCK-8 reagent was added to measure the rate of the vitality of cells.

2.4. Virus infection and grouping

RAW264.7 cells were seeded on cell culture plates of various specifications and grown overnight at 37 °C. The cells were infected with 100 TCID50 of the H6N6 virus for 2 h, washed twice or thrice with Hank's buffered, and subsequently treated with baicalin solution containing different concentrations (50, 25, and 12.5 μg/mL) for different time durations. The control group and H6N6 infection group were set up.

2.5. Determination of cytopathic effects

The RAW264.7 cells were cultured in 12-well plates (1×10^5 cells/well). After 24 h of incubation, the cells were infected with $100TCID_{50}$ H6N6 virus for 2 h and subsequently treated with different concentrations of baicalin (50, 25, and 12.5 μg/mL) for 24 h. The cells were made to grow on a carrier plate glass and fixed in a paraformaldehyde solution. These fixed cells were stained with hematoxylin–eosin (H&E) to observe the CPEs under a microscope (Olympus BX53 microscope, Japan).

2.6. Detection of inflammatory cytokine levels by enzyme-linked immunosorbent assay

According to Section 2.4, the supernatant from each group was collected at 12, 24, and 48 h, and the levels of IL-1β, IL-2, IL-6, and

TNF-α were measured using the mouse ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.) following the manufacturer's instructions.

2.7. Electron microscopy analysis

RAW264.7 cells were collected 24 h after infection with the H6N6 virus. The cells from each group were placed in a centrifuge tube and centrifuged at 1000 rpm for 5 min, The supernatant was discarded, and the cells were fixed in 2.5 % glutaraldehyde solution for 5 min, 1 % osmium tetroxide refixation. It was rinsed thrice with 0.1 M phosphoric acid buffer for 15 min, each time, and subsequently dehydrated by acetone for 15 min step by step (The concentration gradient of dehydrating agent is 30 %→50 %→70 %→80 %→90 %→95 %→100 %,100 % concentration three times), and finally buried overnight. The slices were sliced by an ultra-thin microtome into 60–90 nm thick sections, and double staining was performed with 3 % uranium acetate. It was stained with uranium acetate for 10–15 min, then with lead citrate for 1–2 min, and dyed at room temperature. Finally, the JEM-1400FLASH TEM (Japan Electronics) was used to observe and acquire images.

2.8. Western blotting

The total protein was extracted and quantified using a bicinchoninic acid (BCA) kit. The proteins of each group were boiled for 10 min and subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Next, the protein bands were transferred to a 0.22 μm nitrocellulose filter membrane, and 5 % skim milk powder was blocked and slowly shaken for 2 h. After continuous washing with TBST, primary antibodies against LC3 (Proteintech Co., Ltd.) and β-actin (Shanghai Beyotime Biotechnology Co., Ltd.) were added, and cells were incubated at 4 °C overnight. Next, corresponding goat anti-rabbit secondary antibodies (Shanghai Beyotime Biotechnology Co., Ltd.) were added and incubated for 2 h. The polyvinylidene difluoride (PVDF) film was imaged with enhanced chemiluminescence (ECL) solution.

2.9. Immunofluorescence

The RAW264.7 cells were cultured in 12-well plates (1×10^5 cells/well), mounted on a carrier plate glass, and fixed in the paraformaldehyde solution. The sections were incubated overnight with the rabbit anti-LC3 antibody (1:500, Proteintech Co., Ltd.) at 4 °C. Next, the cells were incubated with goat anti-rabbit IgG (1:400, Proteintech Co., Ltd.) at 37 °C for 1 h and the nuclei were restained with 4′,6-diamidino-2-phenylindole (DAPI) (Shanghai Beyotime Biotechnology Co., Ltd.). The images were captured using a fluorescence microscope (Olympus BX53 microscope).

2.10. Real-time quantitative PCR analysis

The total RNA was extracted from the cells using the TRIzol method. Reverse transcription was performed according to the instructions provided in the fluorescence quantitative reverse transcription kit. The PCR amplification assays were performed using the SYBR Premix Ex Taq II kit (TaKaRa Co., Ltd.) on an ABI 7300 Real-Time PCR System (Applied Biosystems). After adding all these components, the reaction tubes were placed in the real-time PCR (RT-PCR) instrument and the reaction protocol was set as follows: 95 °C for 30 s; 95 °C 5 s, Tm of 60 °C 20 s, 72 °C 30 s, 35 × cycle; melting curve 65.0–95.0 °C, with an increment by 0.5 °C for 5 s. After completion of the reaction, the relative expression of mRNA was compared using the relative quantitative $2^{-\triangle\triangle Ct}$ method. Primer sequences are shown in Table 1.

2.11. Statistical analysis

The data obtained from each experiment were statistically analyzed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) and are expressed as mean ± standard deviation (SD). The significance of the difference was compared using the one-way analysis of variance (ANOVA). Inconsistent capitalization indicates a significant difference ($P < 0.01$), and inconsistent lowercase

Table 1
Primers used for RT-qPCR.

Gene	Sequence (5′→3′)	Length/bp
β-actin	F:5′-CCTGAGGCTCTTTTCCAGCC-3′ R:5′-TAGAGGTCTTTACGGATGTCAACGT-3′	110
LC3	F:5′- AACGAAATTCCTGGTGCCTGA -3′ R:5′- AAGGCTTGGTTAGCATTGAGCTG -3′	90
Beclin-1	F: 5′-CCATAGGGAACAAGTCGGTAC-3′ R:5′-GAGTGGAATGAAATCAATGCTG-3′	107
ATG12	F: 5′-AAACAACTGTTCCGAGGC-3′ R: 5′-AAAGAAATGGGCTGTGGA-3′	94
ATG5	F: 5′-CACTGGGACTTCTGCTCCTG-3′ R:5′-TCCTTCAACCAAAGCCAAAC-3′	129

letters indicate significant differences ($P < 0.05$). There were no less than 6 samples in each group, and three biological replicates and three technical replicates were used for data analysis.

3. Results and discussion

3.1. Effect of baicalin on Raw264.7 cell viability

To determine the cytotoxicity of baicalin on RAW264.7 cells, the CCK-8 assay was performed. When Qingan was treated for 12 h, the cell activity was significantly decreased when the concentration was greater than 200 μg/mL, and the cell activity was significantly decreased when the concentration was greater than 100 μg/mL for 24 h and 48 h. In contrast, no cytotoxicity was observed under 50 μg/mL baicalin (Fig. 1B). Thus, 50, 25, and 12.5 μg/mL baicalin were found to be safe for cells.

3.2. Baicalin attenuated H6N6-induced RAW264.7 cell damage

The H&E staining was performed to investigate the effect of baicalin on the morphology of the cells infected with the H6N6 virus. The RAW264.7 cells were damaged in the group treated with H6N6 infection, showing gradual elliptic or round changes with irregular shapes, nuclear swelling, reticular and vacuolar cytoplasm, and severe partial nuclear fragmentation. At 24 h, reduced pathological damage to the cells in the three treatment groups was reported compared to that in IG (Fig. 2A).

The cell viability of RAW264.7 cells infected with the H6N6 virus (12, 24, and 48 h) significantly decreased. It substantially increased by adding different concentrations of baicalin drug solution medium (Fig. 2B). These data indicated that baicalin exerted a protective effect against the spread of the influenza A H6N6 virus.

3.3. Baicalin reduced H6N6-induced release of inflammatory factors in RAW264.7 cells

After the H6N6 infection, the number of adherent RAW264.7 cells gradually decreased, wrinkled, and underwent atrophy with the increase in infection time, which was the highest at 48 h of infection (Fig. 3A). The contents of interleukin (IL)-1β, IL-2, IL-6, and TNF-α in the cell supernatant were detected by ELISA (Fig. 3B). The levels of IL-1β, IL-2, IL-6, and TNF-α in RAW264.7 cells infected with the H6N6 virus (12, 24, and 48 h) significantly increased ($P < 0.01$). Different concentrations of baicalin (50, 25, and 12.5 μg/mL) reduced the release of IL-1β, IL-2, IL-6, and TNF-α to different degrees ($P < 0.01$) and showed dependence within a certain range.

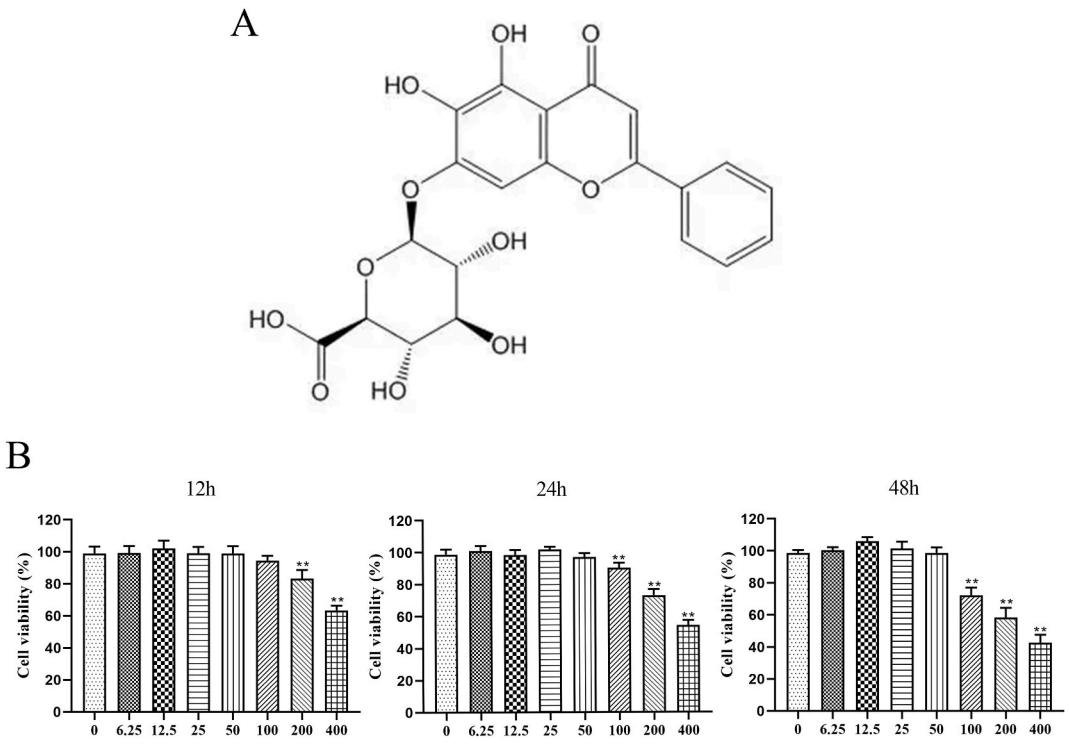

Fig. 1. Survival rates of RAW264.7 cells cultured in baicalin at various concentrations (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μg/mL). (A) The chemical structure of baicalin. (B) Toxicity of baicalin on RAW264.7 cells. Data are shown as means ± standard deviation (SD) of three independent experiments. The values $P < 0.05$ and $P < 0.01$ were considered significant and are indicated with * and **, respectively.

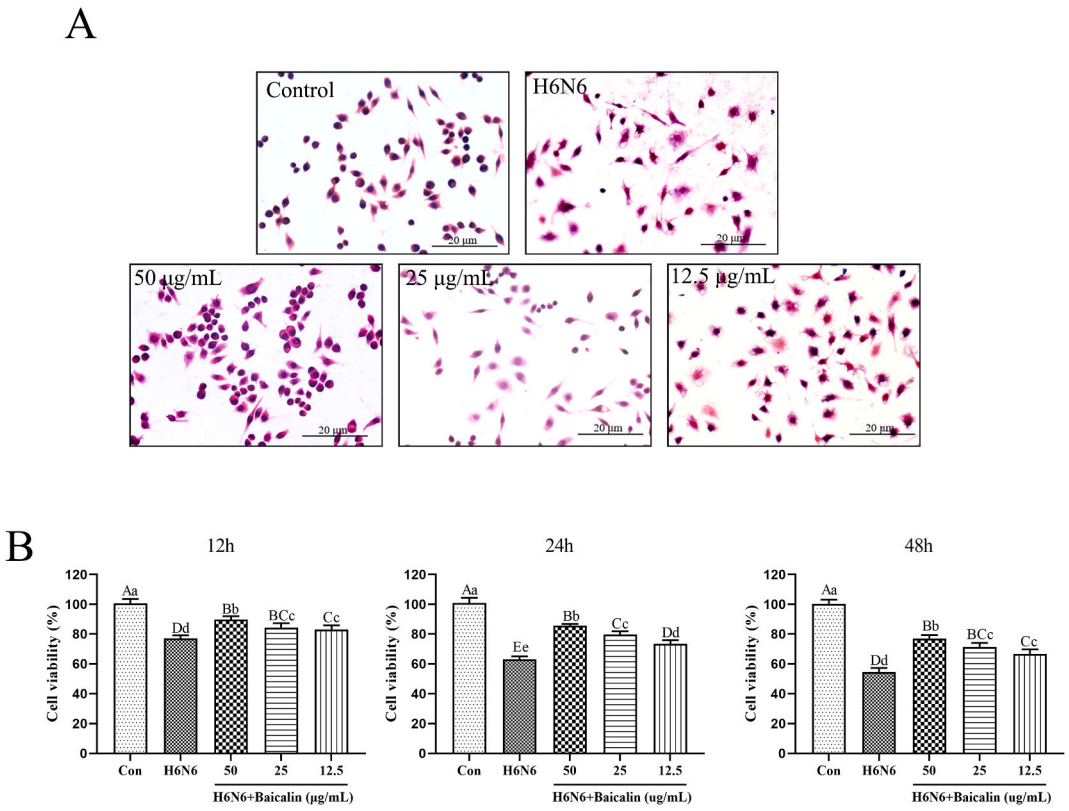

Fig. 2. Baicalin could reduced H6N6 virus-induced damage of RAW264.7 cells. (A) H&E staining was used to observe the changes in the cell morphology after 24 h of baicalin treatment (scale bar = 20 μm). (B) RAW264.7 cells infected with the H6N6 virus were treated with different concentrations of baicalin. Inconsistent capital letters indicate significant differences ($P < 0.01$), consistent uppercase letters indicated no significant difference ($P > 0.01$), and inconsistent lowercase letters indicate significant differences ($P < 0.05$), and consistent letters indicate no significant difference ($P > 0.05$).

3.4. Baicalin can regulate H6N6-induced autophagy in RAW264.7 cells

Transmission electron microscopy (TEM) was used to observe the ultrastructure of RAW264.7 cells infected with H6N6 for 24 h. As shown in (Fig. 4A), the cells in the control group exhibited normal morphological structure, oval nuclei, uniform chromatin distribution, majorly euchromatin, and a continuous and intact nuclear envelope. Intracytoplasmic mitochondria, rough endoplasmic reticulum, and ribosome organelles were clear with a complete structure, and an occasional small amount of autophagy was observed. The cells in the H6N6 infection group had markedly swollen mitochondria (crest dissolved fracture), rough endoplasmic reticulum in the cytoplasm is also more autophagy, H6N6 infection RAW264.7 cells can induce autophagy occurs. Compared with the model group, mitochondrial swelling and autophagy of RAW264.7 cells were reduced in the baicalin administration group.

After RAW264.7 cells infected with H6N6 for 24 h were treated with the baicalin solution (50, 25, and 12.5 μg/mL), the expression of autophagy proteins was detected by western blotting, and the control group and the H6N6 infection group were set up. The results showed low expression of LC3II/I in the control group (Fig. 4B). The expression of LC3II/I protein was significantly increased in the H6N6 infection group compared with the control group ($P < 0.01$). Compared with the H6N6 infection group, different concentrations of baicalin significantly decreased the expression of LC3II/I ($P < 0.01$). Significant differences were present among the three baicalin treatment groups ($P < 0.05$), with a certain dependence. The fluorescence intensity of autophagy LC3 protein was detected by immunofluorescence method, and the results showed that the fluorescence intensity of the control group was low (Fig. 4C). Compared with the control group, the fluorescence intensity of H6N6 infection group was significantly increased ($P < 0.01$). Compared with the H6N6 infection group, different concentrations of baicalin significantly reduced the expression of LC3 fluorescence intensity ($P < 0.01$). This result was consistent with the western blotting result. We detected the expression level of LC3 protein by observing the morphology of autophagy and immunoimprints by electron microscopy. The combination of these two methods can better reflect the level of autophagy, but there are still some limitations. In the subsequent studies, the inhibitor group may be added to better reflect the effect of *Baicalin* on the autophagy induced by H6N6 avian influenza virus.

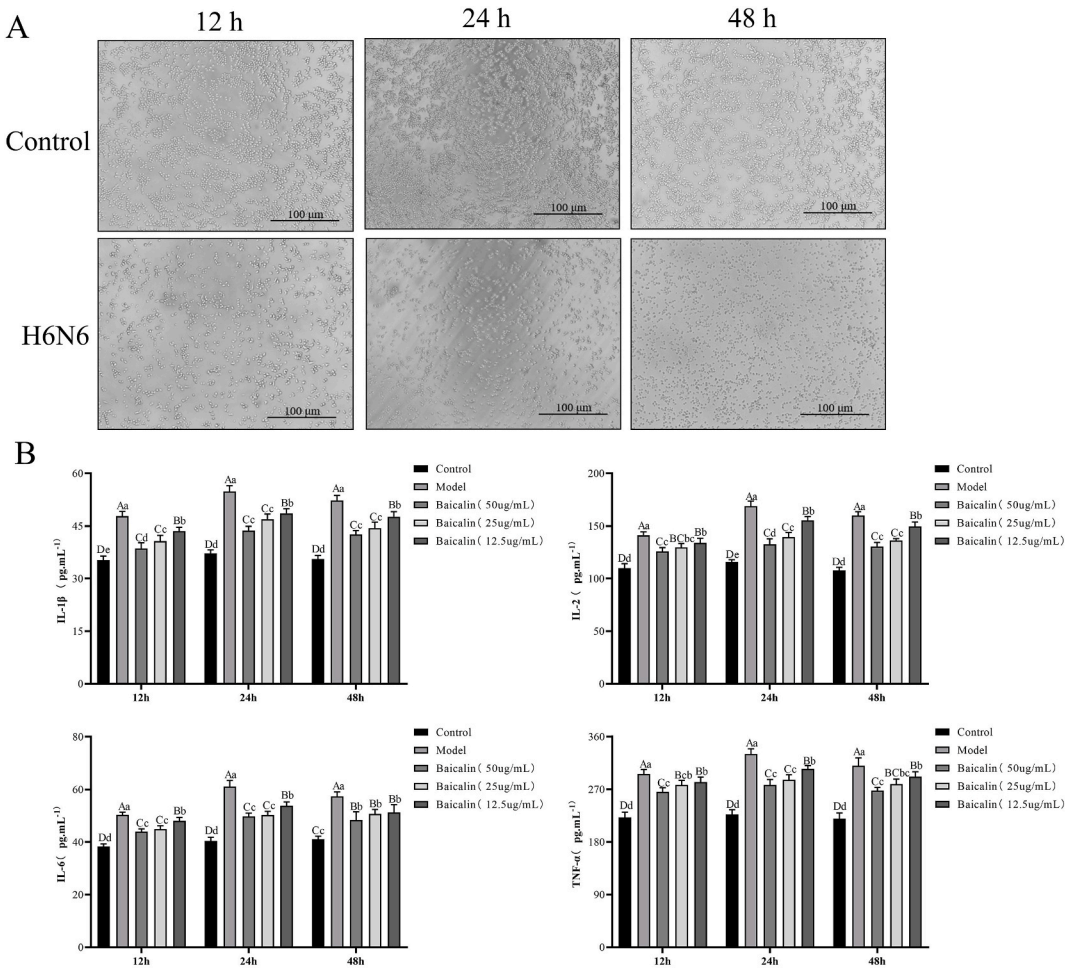

Fig. 3. Baicalin reduced H6N6-induced release of inflammatory factors in RAW264.7 cells. (A) Morphological changes in RAW264.7 cells infected with H6N6 at 12, 24, and 48 h were observed by light microscopy (scale bar = 100 μm). (B) Effects of baicalin on the levels of four inflammatory factors (IL-1β, IL-2, IL-6, and TNF-α) in RAW264.7 cells infected with the H6N6 virus. Inconsistent capital letters indicate significant differences ($P < 0.01$), consistent uppercase letters indicated no significant difference ($P > 0.01$), and inconsistent lowercase letters indicate significant differences ($P < 0.05$), and consistent letters indicate no significant difference ($P > 0.05$).

3.5. Fluorescence quantitative detection of key autophagy genes

To observe whether baicalin could regulate the mRNA expression of autophagy key genes, RAW264.7 cells were treated with the baicalin solution (50, 25, and 12.5 μg/mL) for 24 h. The mRNA transcription levels of LC3, beclin-1, ATG12, and ATG5 genes were detected by fluorescence quantitative PCR. The mRNA expression of LC3, Beclin-1, ATG12, and ATG5 in RAW264.7 cells infected with H6N6 was significantly increased ($P < 0.01$). Compared with the H6N6 infection group, the mRNA transcription levels of LC3, Beclin-1, ATG12, and ATG5 in the three baicalin treatment groups reduced to varying degrees ($P < 0.01$) and demonstrated a certain dependence (Fig. 5A–D). High-performance liquid chromatography (HPLC)-grade solvents of methanol (MeOH), acetonitrile (ACN), and formic acid (FA).

The H6N6 subtype AIV is a low pathogenic influenza virus that triggers only mild clinical manifestations. However, recent epidemiological investigations have demonstrated that the isolation rate of the H6N6 subtype AIV has gradually increased in poultry. The ease of rematching with other subtypes of viruses has allowed the new influenza viruses to cross the species barrier and infect mammals as well as pose a threat to human health [14,15].

Baicalin, a natural compound, is endowed with potent antiviral properties against the influenza virus [16]. Baicalin was found by Fan et al. to continuously inhibit the replication of MDV, directly affecting virus infectivity. In addition, baicalin can inhibit the viral mRNA, protein levels, and overall formation of plaque in a time-dependent manner [17]. In our study, the cell viability significantly increased after baicalin treatment. Furthermore, an analysis of CPEs revealed that all treatment groups could relieve the damage caused to RAW264.7 cells, as evident through H&E staining. Certain studies have identified cytokine-induced autophagy, which could be an important mechanism for the body to clear infections by pathogenic microbes [18]. The massive release of virus-induced

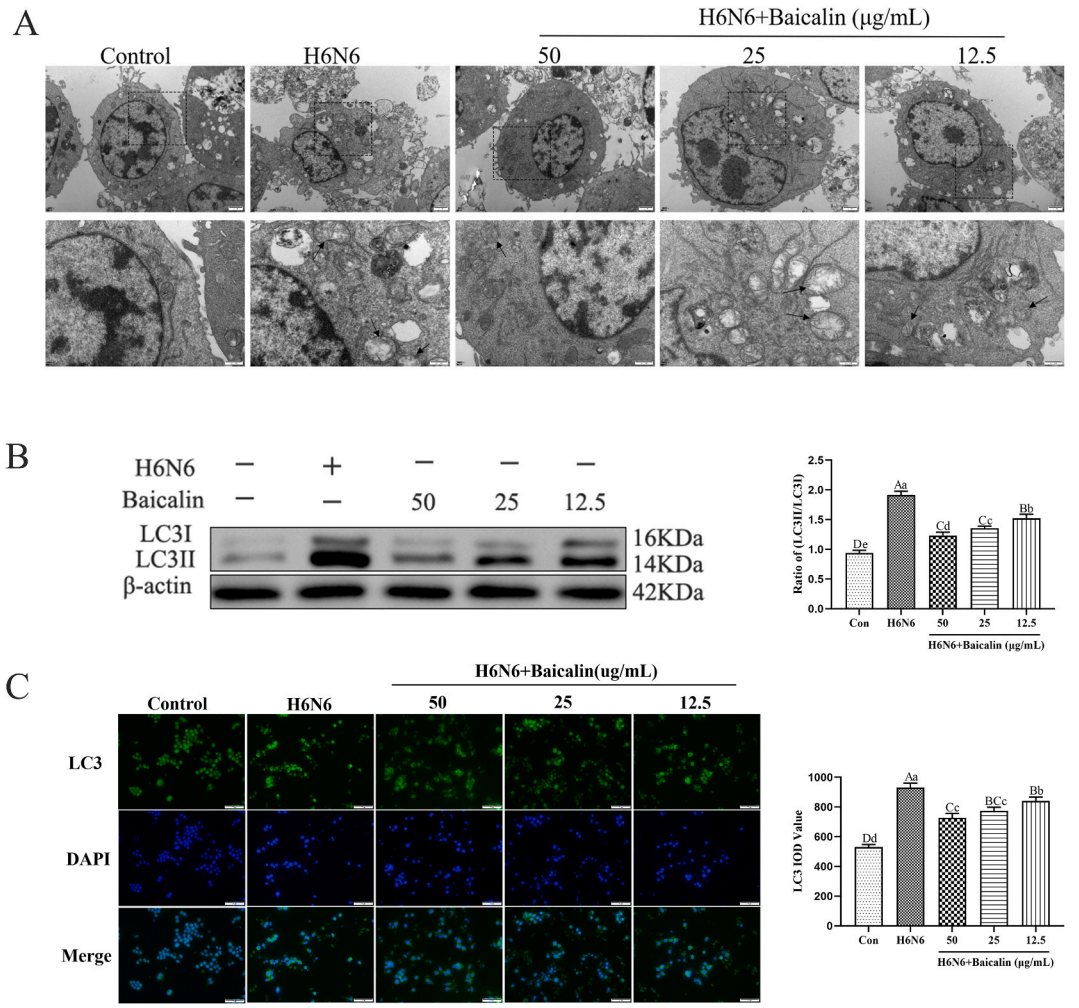

Fig. 4. Baicalin reduced H6N6-induced autophagy in RAW264.7 cells. (A). Transmission electron microscopy was used to detect the changes in the autophagosomes in RAW264.7 cells infected with H6N6 for 24 h. The black box was observed at low power (6000 ×), and the arrow was observed at high power (30,000 ×). The black arrows represent autophagosomes. (B). The expression of LC3II/I in RAW264.7 cells was detected by western blotting after 24 h of baicalin treatment and H6N6 influenza virus infection. (C) The changes in the LC3 protein in RAW264.7 cells treated with baicalin and infected with the H6N6 influenza virus were observed by immunofluorescence. An average of 3 views were taken for optical density measurement with an average of 3 cells in each view. Inconsistent capital letters indicate significant differences ($P < 0.01$), consistent uppercase letters indicated no significant difference ($P > 0.01$), and inconsistent lowercase letters indicate significant differences ($P < 0.05$), and consistent letters indicate no significant difference ($P > 0.05$).

inflammatory cytokines and chemokines is another important factor in the pathogenesis of influenza virus infection. Autophagy mediates the inflammatory response induced by the influenza virus and subsequently produces IL-1, IL-2, IL-6, TNF-α, and other inflammatory factors regulating autophagy [19]. Studies have found that autophagy and immune cells regulate each other, and autophagy can regulate the release of tumor necrosis factor, interleukin, epoxidase and other inflammatory factors, and participate in the occurrence and development of diseases [20]. Pro-inflammatory cytokines and the death ligand TNF-α can also act as inducers of autophagy and mediate autophagy levels [21]. There are research reports that the H9N2 influenza virus infection increased the production of TNF-α in A549 cells, whereas the 3-MA and Atg5 siRNA treatment significantly reduced the production of TNF-α [22]. In this study, the contents of IL-1β, IL-2, IL-6, and TNF-α in supernatants from the three treatment groups were found to be reduced by varying degrees, and the concentration of baicalin was dependent only on a certain range. The results demonstrated that baicalin significantly reduced the release of inflammatory factors and prevented the cellular damage caused by the H6N6 virus.

Because of its extensive antiviral activity, baicalin can inhibit influenza virus-induced autophagy [12,23]. Different subtypes of influenza viruses (H5N1 and H9N2) can induce autophagy [24,25]. The regulation of influenza A virus-induced autophagy is conducive to virus replication, and the inhibition of autophagy by drug treatment could be responsible for the decrease in the production of the influenza A virus [26]. LC3 was the only mature protein associated with the autophagy body that remained stable, and cytolysosome and autophagy–lysosome relative specificity, measure autophagosome amount of gold standard is the activity of LC3,

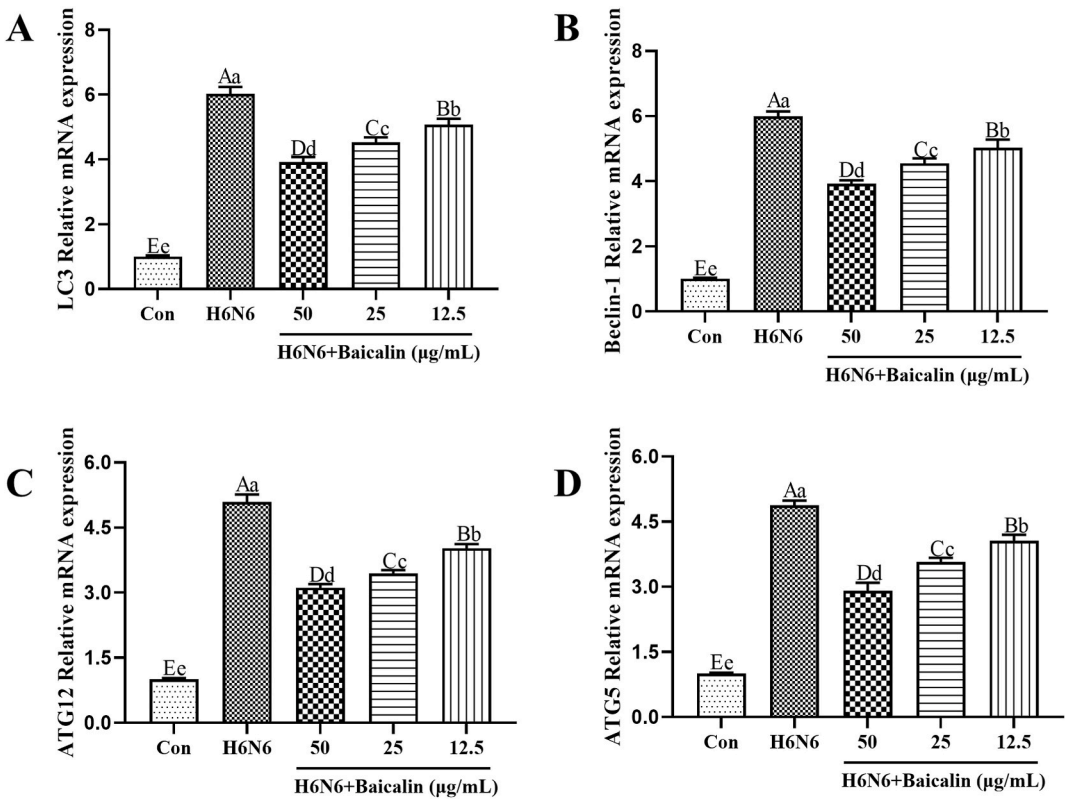

Fig. 5. The mRNA transcription levels of (A) LC3, (B) Beclin-1, (C) Atg12, and (D) Atg5 were detected in the RAW264.7 cells infected with H6N6 by real-time fluorescence. Inconsistent capital letters indicate significant differences ($P < 0.01$), consistent uppercase letters indicated no significant difference ($P > 0.01$), and inconsistent lowercase letters indicate significant differences ($P < 0.05$), and consistent letters indicate no significant difference ($P > 0.05$).

also observed under TEM in the process of the occurrence of autophagy and autophagy-lysosome is the gold standard of autophagy [27,28]. This study demonstrated that H6N6 could induce autophagy in RAW264.7 cells, and baicalin inhibited the expression of LC3 protein to a certain extent, thus affecting the level of autophagy. In addition, the number of autophagosomes in RAW264.7 cells infected with H6N6 avian influenza virus was significantly increased, as confirmed by TEM, and the number of autophagosomes in the baicalin treatment group was significantly decreased and the cell damage was reduced. The *beclin-1* gene [29], which encodes for a marker protein of autophagy and plays a decisive role in the activation of autophagy, can promote the fusion of lysosomes and autophagosomes. The position and the extent of activation of the *ATG5* gene in autophagy vesicles are regulated by the *beclin-1* gene. In classical autophagy, the cup-shaped isolation membrane, which is formed by the Atg12-Atg5-Atg16 complex, combines with PE to form autophagosomes and assembles on the membrane, and is finally fused with lysosomes to form autophagolysosomes, which regulate autophagy [30]. Studies have demonstrated that significantly increased expression of Atg12-Atg5 protein in mouse embryonic fibroblasts infected with *Salmonella*, which initiated LC3 lipid binding, led to the formation of the autophagosome membrane to phagocyte *Salmonella* [31,32]. In our study, RAW264.7 cells were infected with H6N6, following which a significant increase in the levels of LC3, Beclin-1, Atg12, and Atg5 mRNAs was observed. It was further confirmed that H6N6 induced autophagy in RAW264.7 cells. However, baicalin decreased the levels of Beclin-1, Atg12, and Atg5 mRNAs, indicating that it can effectively regulate the H6N6-induced autophagy of RAW264.7 cells.

4. Conclusion

Baicalin inhibited the release of cellular inflammatory factors, reduced cell damage, and down-regulated the expression of autophagy-related genes. The anti-viral activities of baicalin against H6N6 are likely related to its anti-autophagic properties. Understanding the relationship between baicalin-mediated inhibition of H6N6-triggered autophagy and inflammatory response could provide a new target to treat inflammation caused by the influenza virus. These findings will help further elucidate the pharmacological properties of baicalin against the influenza virus.

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Data availability statement

The data associated with our study has not been deposited into a publicly available repository, data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Xin Yang: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Junxian Li:** Methodology, Investigation, Formal analysis. **Chunlan Shan:** Project administration. **Xuqin Song:** Writing – review & editing. **Jian Yang:** Writing – review & editing. **Hao Xu:** Visualization, Validation, Software. **Deyuan Ou:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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