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Lithium chloride inhibits infectious bronchitis virus-induced apoptosis and inflammation

Xingyun Liu, Xinyu Chang, Qin Wu, Jun Xu, Lu Chen, Ruiting Shen, Xiaolin Hou *

Livestock and Poultry Healthy Breeding Research Sub-center of Biochip Beijing Engineering Research Center, Department of Veterinary Medicine, Beijing University of Agriculture, Beijing, People's Republic of China

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ABSTRACT

Avian infectious bronchitis (IB) was caused by infectious bronchitis virus (IBV), a coronavirus, which leads to enormous economic losses in the poultry industry. Studies have shown that lithium chloride (LiCl) is a good virus inhibitor. Through cell culture, virus infection, and RT-qPCR, we found that LiCl could down-regulate the apoptosis-related genes Caspase-3 and Bax, up-regulate Bcl-2, and down-regulate the inflammatory-related genes (NF-κB, NLRP3, TNF-α, and IL-1β) via inhibiting virus replication. Finally, clinical trials showed that LiCl could inhibit IBV-induced apoptosis and inflammatory in chicken embryos as well as reduce the mortality and deformity rate of chicken embryos. The results showed that LiCl has antiviral activity against IBV and clinical effects. Further studies are required to explore the exact action mechanism of LiCl on IBV-induced apoptosis and inflammation.

1. Introduction

Avian infectious bronchitis virus (IBV) belongs to the genus *Gammacoronavirus*, family *Coronaviridae*, in the order *Nidovirales*, and it possesses a single-stranded positive-sense RNA genome surrounded by a lipid envelope, which is approximately 27.6 kb in size [1,2]. IBV is an important pathogen causing acute and highly contagious respiratory diseases, which can cause great economic loss to the poultry industry [3]. In addition to the serious respiratory diseases caused by IBV, some IBV strains are nephropathogenic, causing nephritis [1,4–6]. Studies have found that IBV strains can be isolated from the digestive tract, fallopian tube, testis, cloaca, and liver of diseased chickens [7]. The contagious disease caused by IBV infection was first reported in the United States in 1931 [8], the virus was first cultured in chicken embryos in 1937 by Beaudette and Hades. Due to the high recombination and mutation rate of IBV, the genotype and serotype of IBV have become more and more diversified and complicated in recent years, thus causing the poor cross-protection effect of IBV vaccine [9–11]. In recent years, new strains of IBV have appeared constantly, and the poor cross-protection effect of vaccines has resulted in the lack of effective prevention and treatment methods for IBV, which reflects the importance of studying new antiviral drugs.

LiCl is used as a mood-regulating drug, and it has been proved that LiCl alleviates the symptoms of acute mania and depression [12,13], LiCl has also been used to treat Alzheimer's disease [14]. Recently, LiCl has been reported to have an antiviral effect on various viruses [15–24], Although Harrison et al. have confirmed the inhibitory effect of LiCl on IBV replication in cells [19], whether LiCl has inhibitory effects on IBV-induced apoptosis and inflammation has not been reported yet.

In this study, we confirmed the inhibitory effects of LiCl on IBV infection-induced apoptosis and inflammation not only in cells, but also in chicken embryos. In addition, research data showed that LiCl could decrease the mortality of IBV-infected chicken embryos. These data suggest that LiCl may be a potential compound for the treatment of IBV infection-induced apoptosis and inflammation.

2. Materials and methods

2.1. Reagents

LiCl (Solarbio, Beijing, China) with the molecular weight of 42.30, was diluted in sterile water at 1000 mM as the stock solution, filter-sterilized, and stored at - 20 °C for use. When used, the stock solution was diluted with serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) and then treated cells. Thiazolyl blue tetrazolium bromide (MTT) (Solarbio, Beijing, China), Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Shanghai, China).

* Corresponding author.
E-mail address: hxlsx@163.com (X. Hou).

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Abbreviations

Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma-2
BHK cell	Baby hamster kidney cell
CAM	Chorioallantoic membrane
CPE	Cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
IBV	Infectious bronchitis virus
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LiCl	lithium chloride
MTT	Thiazolyl blue tetrazolium bromide
NF-κB	Nuclear factor kappa-B
NLRP3	Nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3
PBS	Phosphate buffered solution
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SPF	Specific-pathogen-free
$TCID_{50}$	50% tissue culture infectious dose
TNF-α:	Tumor necrosis factor-α

2.2. Cells and viruses

BHK cells, a baby hamster kidney cell line, were obtained from Kunming cell bank of Type Culture Collection Chinese Academy of Sciences (Yunnan, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS) (Gibco, Australia) at 37 °C in an incubator containing 5% CO_2.

IBV Beaudette strain, a strain of embryo-culture IBV adapted to Vero cells [25], was kindly provided by Sichuan agricultural university. We propagated it in the allantoic cavity of 10-day-old specific-pathogen-free (SPF) chicken embryos 0.2 mL per sample (Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd) for 48 h at 37 °C, then kept the chicken embryos in 4 °C and collected the allantoic fluid, directly stored in −80 °C after the aseptic examination. The collected viruses were adapted to BHK cells after three passages.

2.3. Cytotoxic assay

The cellular cytotoxic effect of LiCl on BHK cells was determined using the MTT assay [26]. Briefly, BHK cells were seeded into 96-well cell culture plates (Corning Incorporated, USA) and grown to 80%–90% confluence, then washed with phosphate-buffered solution (PBS) three times, the cells were treated with different concentrations of LiCl for 48 h at the incubator. The supernatant was discarded, washed with PBS three times, and each well (96-well plate) was incubated with 20 μL of MTT reagent (5 mg/mL) at 37 °C for 4 h. After the MTT was sucked out, 150 μL of DMSO was added to each well, the plate was incubated at 37 °C in the dark for 10 min. The OD value was measured with a microplate reader at 490 nm, the cell survival rates were calculated according to the following equation: (OD_{490} drug)/(OD_{490} control) × 100%. The experiments were performed in triplicate.

2.4. Virus titration and infection

Virus titers were calculated based on the 50% tissue culture infectious dose ($TCID_{50}$) [27], and the cells were inoculated into 96-well plates. When the cells reached 80%~90% fusion, the cells were infected with a series of 10-fold dilutions of the virus sample, 8 wells in each gradient, with 0.1 mL of virus per well. After incubation at 37 °C for 1h, the cells were washed twice with PBS, 0.2 mL of maintenance medium was added to each well, and incubated for 48 h, recording the number of cytopathic wells per dilution gradient. The viral titer was calculated as $TCID_{50}$ by the Reed and Muench methods. The virus titer was $10^{5.6}$ $TCID_{50}$/mL.

2.5. Cells treatment

To determine the effect of LiCl on IBV Beaudette-infected BHK cells, the BHK cells cultured on 6-well plates were treated with different concentrations of LiCl for 36 h at 37 °C after infecting with 10 $TCID_{50}$ IBV for 1 h. The antiviral effect of LiCl was evaluated by cytopathic observation and the relative mRNA expression levels of the IBV-N gene.

2.6. Chicken embryos treatment

The 9-day-old SPF chicken embryos were randomly divided into four groups (n = 13): mock-infected group, IBV-infected group, high concentration LiCl group (50 mM), and low concentration LiCl group (15 mM). Each embryo was injected with 0.1 mL 100 $TCID_{50}$ virus solution through the allantoic cavity. After incubation for 1 h, each group was injected with different concentrations of LiCl. At 3 d.p.i, three chicken embryos were dissected in each group, the allantoic fluid and chorioallantoic membrane (CAM) were collected. The virus load in the allantoic fluid and the mRNA levels of inflammatory factors in CAM were detected by RT-qPCR. The embryo growth was observed up to the 9th day, and the daily embryo death was recorded. The survival curves were plotted with GraphPad 8.0. Finally, compared the growth of embryos in each group.

2.7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total cellular RNA was extracted using the RNApure Tissue&Cell Kit (CWBio, China), according to the manufacturer's instructions. The total RNAs were reverse transcribed into cDNAs using the HiFi-MMLV cDNA Kit (CWBio, China). All primer pairs for RT-qPCR detection are listed in Table 1 for BHK cells and Table 2 for chicken embryos. These primers were synthesized by Sangon Biotech Co., Ltd.(Shanghai, China). RT-qPCR amplifications were carried out on an ABI 7500 Fast-time PCR system (Applied Bio-systems, USA) using UltraSYBR Mixture (Low ROX) (SYBR Green I) (CWBio, China), according to the instructions of the manufacturer. The amplification system was 1 μL cDNA, 0.5 μL forward and reverse primers, 12.5 μL UltraSYBR Green (Low ROX) and 10.5 μL ddH_2O, and the final volume of each system was 25 μL.The reaction parameters were as follows: 95 °C for 10 min, followed by 40 two-step amplification cycles consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. The amplification specificity of each assay was confirmed by melting curve analysis carried out at 60–95 °C. Relative fold expressions were analyzed using the $2^{-\Delta\Delta Ct}$

Table 1
Sequences of primer used for real-time PCR assays to detect gene expression.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
β-actin	CCACCATGTACCCAGGCATT	AGGGTGTTAAACGCAGCTCA
IBV-N	GGTTGCTGCTAAGGGTGCTGATAC	TCAGGTCCGCCATCCGAGAATC
TNF-α	AAGGGAGAGTGGTCAGGTTGC	CAGAGGTTCAGTGATGTAGCG
IL-1β	GCAGGCAGTATCACTCATTGT	GGCTTTTTTGTTGTTCATCTC
NF-κB	GACACGACAGAATCCTCAGCATCC	CCACCAGCAGCAGCAGACATG
NLRP3	GCCGTCTACGTCTTCTTCCTTTCC	CATCCGCAGCCAGTGAACAGAG
Caspase-3	TTAATAAAGGTATCCATGGA	TTAGTGATAAAAATAGAGTT
Bcl-2	CCGTCGTGACTTCGCAGAGATG	ATCCCTGAAGAGTTCCTCCACCAC
Bax	AAGCGGCTCACCTCCTTCCTC	GCTGCGGATTGCCTGAGTGTC

Table 2
Sequences of primer used for real-time PCR assays to detect gene expression.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
β-actin	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC
IBV-N	GGTTGCTGCTAAGGGTGCTGATAC	TCAGGTCCGCCATCCGAGAATC
TNF-α	CCCAGTTCAGATGAGTTGCCCTTC	GCCACCACACGACAGCCAAG
IL-1β	CAGAAGAAGCCTCGCCTGGATTC	GCCTCCGCAGCAGTTTGGTC
IL-6	GAGGTTGGGCTGGAGGAGGAG	TCTCGCACACGGTGAACTTCTTG
iNOS	GTGGTATGCTCTGCCTGCTGTTG	GTCTCGCACTCCAATCTCTGTTCC

method and β-actin Ct values as internal references in each sample.

2.8. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) from three independent experiments performed in triplicate. The statistical analyses were conducted using Student's t-test in GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). A p-value was considered significant as the following: *p < 0.05; **p < 0.01; ***p < 0.001.

3. Results

3.1. LiCl cytotoxicity on BHK cells

The cytotoxicity of LiCl to BHK cells was detected by MTT assay. The result showed that within the range of 25 mM LiCl, compared with the control group, the cell viability was >90% and cell morphology have no significant change (Fig. 1). Thus, 5–20 mM LiCl was selected in the subsequent assays in vitro.

3.2. LiCl inhibited IBV replication

To investigate the replication of IBV in BHK cells, the CPE of BHK cells infected with IBV was observed at different times. The main characteristic of BHK cells CPE included cells' shape changed from spindle to round shape, or the cells gather or fall off to form visible plaque. The IBV-infected BHK cells developed cytopathic effects at 24 h and the lesions began to intensify significantly at 36h (Fig. 2A). The viral load in BHK cells was measured by RT-qPCR. It was found that when the BHK cells were infected at 36 h, the intracellular virus load got the highest (Fig. 2B). Next, the effect of LiCl on the replication of IBV in BHK cells was investigated. The inhibitory effect of LiCl on IBV was determined by observing CPE and RT-qPCR. We found that 5 mM and 20 mM LiCl inhibited viral replication compared to mock-treated cells, especially when the concentration of LiCl was 20 mM (Fig. 2C and D). The results indicated that LiCl inhibited the replication of IBV in a dose-dependent manner.

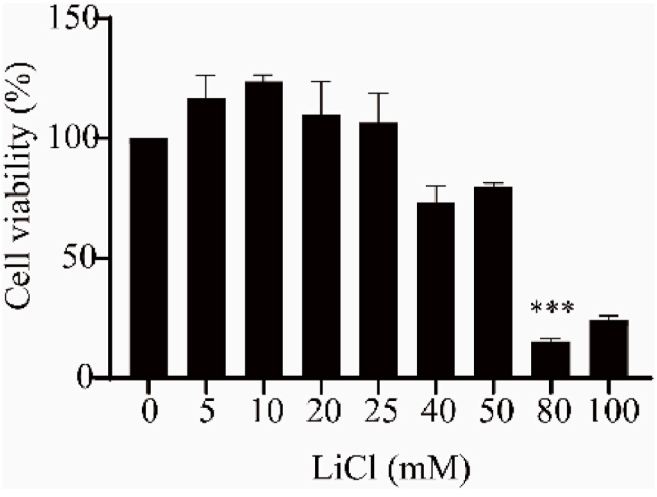

Fig. 1. Cytotoxic effect of LiCl on BHK cells. MTT assay was used to detect the cytotoxicity of LiCl to BHK cells. The survival rates of BHK cells were given at different concentrations of LiCl, and cell survival rates of more than 90% were considered to be the maximum non-toxic concentration of LiCl. Data are shown as the mean ± SEM of three independent experiments.

3.3. LiCl inhibited IBV-induced cell apoptosis

IBV infection could induce BHK apoptosis. To investigate the effect of LiCl on IBV-induced apoptosis of BHK cells. The mRNA levels of apoptosis-related genes, including Caspase-3, Bax, and Bcl-2 were determined using RT-qPCR. Compared with the IBV infection group, the mRNA levels of Caspase-3 and Bax reduced significantly in LiCl treatment groups. In contrast, the mRNA expression levels of Bcl-2 genes increased in a dose-dependent manner in LiCl treatment groups. (Fig. 3A). These results indicated that LiCl alleviated IBV-induced BHK cell injury by inhibiting apoptosis.

3.4. LiCl suppressed IBV-induced cell inflammation

IBV infection could induce the release of pro-inflammatory factors in BHK cells. To investigate the effect of LiCl on IBV-induced BHK cell inflammation. The changes of NF-κB, NLRP3, TNF-α, and IL-1β expression in BHK cells were determined using RT-qPCR. The data showed that LiCl significantly blocked the expression of NF-κB, NLRP3, TNF-α, and IL-1β (Fig. 3B). These data demonstrated that LiCl inhibited inflammatory responses in IBV infected cells.

3.5. LiCl inhibited IBV-induced apoptosis and inflammation in chicken embryos

Next, to further clarify the inhibition effect of LiCl on IBV infection-induced apoptosis and inflammatory, transcript levels of inflammation and apoptosis-related genes in CAM were measured using RT-qPCR after being infected with IBV and treated with LiCl for 72 h. As expected, the mRNA levels of IBV-N in the chorioallantoic membrane (CAM) were significantly decreased in the LiCl-treated groups (Fig. 4A). We then compared alterations of genes related to inflammation and apoptosis in each group of chicken embryos. Treatment with LiCl gradually decreased expressions of apoptosis-related genes Caspase-3 and BAX, on the contrary, the levels of BCl-2 were increased in a dose-dependent manner (Fig. 4B). We further compared alterations of inflammation-related genes(NF-κB, NLRP3, TNF-α, IL-1β, and IL-6) in chicken embryos infected with IBV (Fig. 4C), transcript levels of these genes were significantly decreased in a dose-dependent manner. Besides, LiCl treatment significantly increased the survival rate of chicken embryos (Fig. 4D and E). As shown in Fig. 4D, at 3 d.p.i, the survival rate of chicken embryos in the IBV group was 0%, at 9 d.p.i, the survival rate was 30.77% in the high concentration LiCl group (50 mM) and 23.07% in the low concentration LiCl group (15 mM). Collectively, these findings demonstrated that LiCl could inhibit apoptosis and inflammation responses in IBV-infected chicken embryos and reduce the mortality of chicken embryos.

4. Discussion

LiCl has been proved to be an effective immunomodulatory drug [28]. LiCl also has antiviral activity against varieties of DNA and RNA viruses. However, the current research has not found that LiCl has a direct virucidal effect on coronaviruses. Studies have shown that LiCl generally acted during the coronavirus replication phase and had no direct effect on the attachment of coronaviruses to cell receptors or the entry of viruses into cells, moreover, LiCl has been shown to play a role in the replication phase of IBV [19,29]. In this study, we explored the

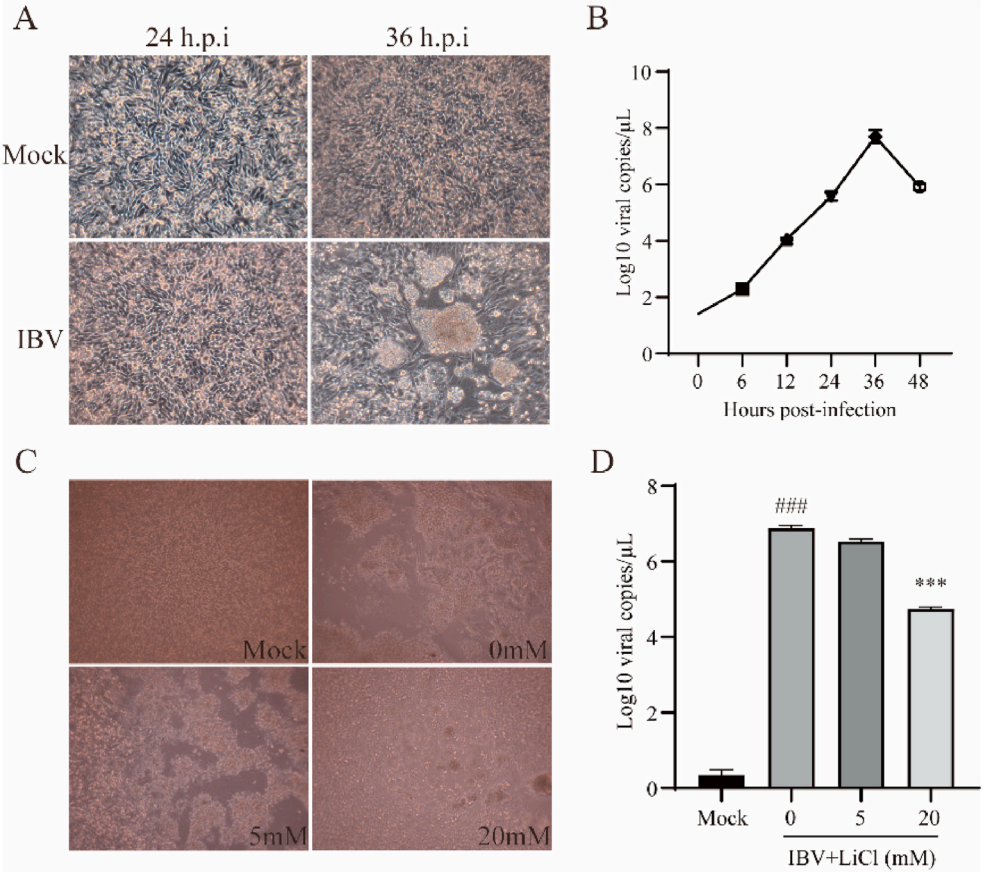

Fig. 2. LiCl inhibited cytopathy and IBV replication. The BHK cells were with 10 $TCID_{50}$ IBV. (A) The CPE of BHK cells infected with IBV at 24 h.p.i and 36 h.p.i. (B) The intracellular viral copies at specified times after BHK cells infected with IBV. (C) Therapeutic effects of different concentrations of LiCl on IBV-induced CPE. (D) The viral copies in cell lysates with different concentrations of LiCl. Viral RNA was extracted and quantified with RT-qPCR. The data are expressed as mean ± SEM of 3 independent experiments. ###p < 0.001, compared with the mock group. ***p < 0.001, compared with the IBV infection group.

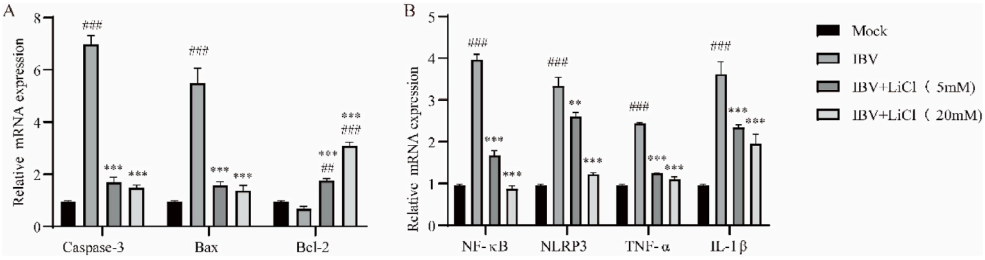

Fig. 3. LiCl inhibited IBV-induced apoptosis and inflammation in vitro. The BHK cells were mock-treated or treated with different concentrations LiCl for 36 h after infection with 10 $TCID_{50}$ for 1h. Total RNA was subsequently extracted from cell lysates. (A) The mRNA levels of Caspase-3, Bax and Bcl-2 were assessed by RT-qPCR. (B) The mRNA levels of NF-κB, NLRP3, TNF-α and IL-β were assessed by RT-qPCR. The data were expressed as mean ± SEM of 3 independent experiments. ###p < 0.001, compared with the mock group. ***p < 0.001, compared with the IBV infection group.

effect of LiCl on IBV-induced apoptosis and inflammation, The chicken embryos experiment was further clinically verified the inhibitory effect of LiCl on apoptosis and inflammation induced by IBV infection.

Apoptosis and inflammatory response are the pathogenic mechanism of virus-induced diseases, which can interfere with virus replication, and are the internal defense mechanism of virus-host cells [30]. IBV infection and replication can induce apoptosis [31,32] and inflammation. In this study, LiCl can down-regulate the pro-apoptosis genes Caspase-3 and Bax, and up-regulate the anti-apoptosis gene Bcl-2, thus inhibiting the IBV-induced apoptosis and alleviating the damage of BHK cells. LiCl inhibits IBV-induced inflammatory reaction by down-regulating inflammatory-related genes (NF-κB, NLRP3, TNF-α, and IL-1β). The data indicated that LiCl inhibited IBV-induced apoptosis and inflammation in vitro.

So far, the clinical application of LiCl to inhibit IBV-induced diseases has not been reported, and we explored the effects of LiCl on IBV-infected chicken embryos. According to the analysis of the transcription levels of apoptosis-related genes, IBV could induce apoptosis in chicken embryos, and the inflammation-related genes increased significantly after IBV infected chicken embryos, accompanied by the death of massive chicken embryos. The dead chicken embryos showed pathological states such as congestion and malformation. It has been suggested that LiCl could reduce chicken embryos death induced by IBV infection via inhibiting apoptosis and inflammation. However, the exact mechanism of LiCl on IBV-induced apoptosis and inflammation needs to be defined.

In conclusion, this study has demonstrated that LiCl could inhibit the apoptosis and inflammation induced by IBV infection, and reduce the mortality of clinical infection. According to the cell experiment and chicken embryos experiment, we have preliminarily verified that LiCl may be a potentially effective drug against IBV-induced disease, further studies are required to determine the clinically antiviral effect of LiCl.

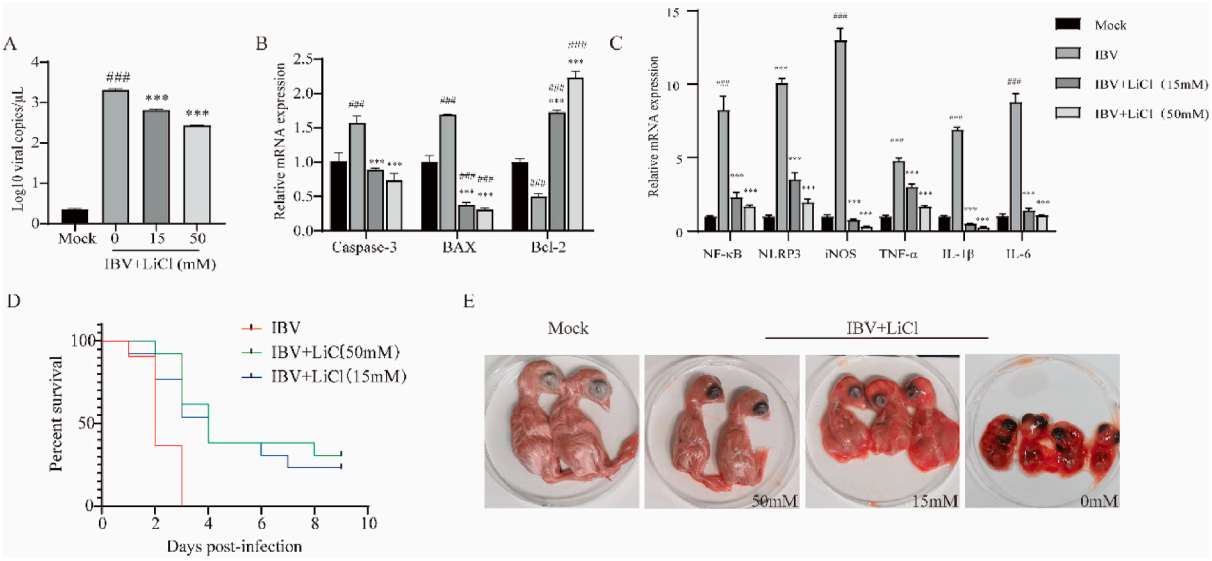

Fig. 4. LiCl inhibited IBV-induced apoptosis and inflammation in chicken embryos Chicken embryos infected with 100 $TCID_{50}$ IBV were injected with different concentrations of LiCl for treatment. Allantoic fluid and CAM were collected at 72 h.p.i after infection. (A) The levels of IBV-N in the allantoic fluid were analyzed by RT-qPCR. (B–C) The levels of apoptosis and inflammation-related genes in CAM were measured by RT-qPCR. (D) The survival rate of chicken embryos was recorded every day until the 9th day. (E) The embryo's growth of each group on the 9th day. The data were expressed as mean ± SEM of 3 independent experiments. ###p < 0.001, compared with the mock group. ***p < 0.001, compared with the IBV infection group.

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CRediT authorship contribution statement

Xingyun Liu and **Xinyun Chang:** Software, Data curation, experimentation, Writing-Original draft preparation, xingyun Liu and Xinyu Chang contribute equally. **Jun Xu:** Methodology,Data curation. **Lu Chen**, **Ruiting Shen**, and **Qin Wu:** Visualization, Investigation, Validation. **Xiaolin Hou:** Conceptualization, supervision, Writing- Reviewing and Editing.

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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