Original article

Protective effect of lithium chloride on pulmonary injury caused by *Actinobacillus pleuropneumoniae* via inhibition of GSK-3β-NF-*κ*B-dependent pathway

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Abstract

Porcine contagious pleuropneumonia (PCP) is a very serious respiratory disease which is difficult to prevent and treat. In this study, the therapeutic effects of lithium chloride (LiCl) on PCP were examined using a mouse model. A mouse model of PCP was established by intranasal infections with *Actinobacillus pleuropneumoniae* (App). Histopathological analysis was performed by routine paraffin sections and an H-E staining method. The inflammatory factors, TLR4 and CCL2 were analyzed by qPCR. The expression levels of p-p65 and pGSK-3ß were detected using the Western Blot Method. The death rates, clinical symptoms, lung injuries, and levels of TLR-4, IL-1ß, IL-6, TNF-α, and CCL2 were observed to decrease in the App-infected mice treated with LiCl. It was determined that the LiCl treatments had significantly reduced the mortality of the App-infected cells, as well as the expressions of p-p65 and pGSK-3ß. The results of this study indicated that LiCl could improve the pulmonary injuries of mice caused by App via the inhibition of the GSK-3β-NF-κB-dependent pathways, and may potentially become an effective drug for improving pulmonary injuries caused by PCP.

Key words: porcine contagious pleuropneumonia, mouse model, lithium chloride, toll-like receptor 4, GSK-3β-NF-κB-dependent pathways

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Introduction

Porcine contagious pleuropneumonia (PCP) is a type of fibrin hemorrhagic necrotizing pleuropneumonia caused by *Actinobacillus pleuropneumonia* (App) infections. It is considered to be a very serious respiratory disease characterized with high infectivity and fatality rates. PCP has led to declines in productivity and economic losses in the pig industry in many countries (Pereira et al. 2018, Sassu et al. 2018).

It has been confirmed that innate immunity plays an important role in the protection and pathogenesis of an infectious disease. For example, when invading microorganisms and inflammatory cells are recognized (including polymorphonuclear cells), neutrophils and macrophages are recruited to the infection sites (Kumar 2018). These inflammatory phagocytes not only directly kill the invading bacteria, but also produce chemokines, various proinflammatory cytokines (such as interleukin (IL)- 6, IL-1β, tumour necrosis factor (TNF)- α), and anti-inflammatory cytokines (such as IL-10) for the purpose of regulating antimicrobial immunity (Kumar 2018, Kumar 2020). However, if not controlled, these inflammatory mediators often elicit overly robust responses which may be conducive to bystander tissue damage (Kumar 2018, Kumar 2020). The recognition processes of invading microorganisms are carried out by the interactions between the pattern-recognition receptors (PRRs) (such as Toll-like receptors (TLRs)) and the pathogen-associated molecular patterns (PAMPs). After the TLRs have recognized microbial PAMPs, some molecules (such as NF-κB) are activated to mediate the production of proinflammatory cytokines and other cytokines (Paramel et al. 2015, Kumar 2018). Some components of the NF-κB signaling system become novel therapeutic targets in various diseases (Kumar 2018). The TLR4/NF-κB pathways are important signal pathways for inducing innate immunity. It has been reported that the TLR4/NF-κB pathways are involved in the pathogenesis of PCP (Li et al. 2016, Li et al. 2018). Therefore, tight regulation of the TLR4//NF-κB pathways will mediate host inflammatory responses and provide a possible way to control the lung inflammation caused by PCP (Makola et al. 2020) and Francisella infection (Zhang et al. 2009).

Lithium chloride (LiCl) is a type of mood stabilizer which has been used for many years to treat such diseases as bipolar disorder. At the present time, research evidence has shown that LiCl may also have antiviral and antibacterial effects (Chen et al. 2013, Li et al. 2018, Snitow et al. 2021). For example, some research regarding the mechanism underlying the therapeutic efficacy of LiCl has indicated that several molecules are its targets, including glycogen synthase kinase (GSK)-3β, toll-like receptor (TLR) 4, and nuclear factor-κB (NF-κB). Li et al. (Li et al. 2016) and Makola et al. (Makola et al. 2020) reported that LiCl inhibited LPS-induced TLR 4 expressions and pro-inflammatory cytokine production. Chen found that LiCl promoted host resistance against *Pseudomonas aeruginosa* keratitis by suppressing GSK3β, as well as the subsequent production of inflammatory factors which promote bacterial clearance (Chen et al. 2013). GSK-3 is involved in many intracellular functions (Jope et al. 2007, Jope et al. 2017, Liu et al. 2018, Leinie et al. 2020). For example, GSK-3 is critical in promoting or repressing NF-κB activity, which suggests that it may also play a regulatory role in inflammation (Medunjanin et al. 2016, Hoeflich et al. 2000). In animal models of sepsis examined in previous research investigations, it was determined that treatments with GSK-3 inhibitors could suppress NF-κB-dependent proinflammatory cytokine expressions and prevent organ damage and endotoxin shock (Hoffmeister et al. 2020, Makola et al. 2020). In recent years, LiCl has become a potential candidate for regulating chronic inflammation due to its interference in inflammatory factors, and such transcription factors as NF-κB and GSK-3β (Reuter et al. 2010). Zhang found that the inhibition of GSK3β resulted in significant reductions in the production of pro-inflammatory cytokine IL-6, IL-12, and TNF-α, as well as significant increases in the production of the anti-inflammatory cytokine IL-10. It is known that GSK3β regulates the cytokine responses induced by the *Francisella tularensis* LVS by differentially affecting the activation of transcription factors NF-κB and CREB (Zhang et al. 2009). Previous research findings have indicated that the inhibition of GSK3β by LiCl in vivo had effectively suppressed inflammatory responses in mice infected with *F. tularensis*b LVS and conferred obvious survival advantages (Zhang et al. 2009).

This study's preliminary research (Hu et al. 2015) and references (Brogaard et al. 2015, Li et al. 2016, Li et al. 2018) showed that NF-κB was involved in the lung injuries of mice infected with *Actinobacillus pleuropneumonia*. This study focused on the detection and analysis of the pharmacological effects of LiCl in an intranasally App-infected mouse model.

Materials and Methods

Bacterial strains, cell strains, and cultures

The *Actinobacillus pleuropneumonia* serotype I Changsha strain was isolated from pig lungs with PCP. Half the lethal dose (LD$_{50}$) of this strain to mice was 1×10^5 CFU/mL (Hu et al. 2015). It was then grown in Trypticase Soy Broth (TSB) (Qingdao Rishui Bio-

technology Co. Ltd., China) supplemented with 15 µg/ml of beta-nicotinamide adenine dinucleotide trihydrate (Beijing Dingguo Changsheng Biotechnology Co. Ltd., China) and 10% newborn calf serum (Gibco, Burlington, VT), at 37°C in 5% CO_2 conditions.

The RAW264.7 cells were kindly provided by Professor Jianming Su and cultured at 37°C under 5% CO_2 conditions in DMEM (Beijing Solarbio Science and Technology Co. Ltd., China), and supplemented with 10% FCS (Gibco, Burlington, VT, US); 100 U/ml of penicillin (Beijing Dingguo Changsheng Biotechnology Co. Ltd., China); and 100 mg/ml of streptomycin (Beijing Dingguo Changsheng Biotechnology Co. Ltd., China).

Cytotoxicity tests and the effects of LiCl on App-infected cells

Cytotoxicity tests

RAW264.7 cells were added to 96-well plates with LiCl (diluted with DMEM; without serum) at end concentrations of 0, 10, 20, 40, 60, 80, 100, 120 ,160, 200, and 300 mM in triplicate. After incubation for 48 hours at 37°C, the cell viability was calculated.

Effects of LiCl on App-infected cells

RAW264.7 cells were first incubated with App (App: cell =1: 100) at 37°C for one hour, and then treated with 60 and 80 mM of LiCl, respectively. As a control, cells were also infected with the same dosages of App without LiCl. Subsequently, the protective efficacy of LiCl was evaluated using the cell viability values. Moreover, the cell morphology was observed using an IX71 Olympus inverted microscope.

Cell viability

Following the App infections and treatments with or without LiCl, the RAW 264.7 cells were flushed with culture medium in the 96-well plates. The culture medium was then aspirated. At that point, the numbers of live and dead cells within the culture medium were directly calculated after staining with trypan blue.

Protective efficacy of LiCl in mice

The *in Vivo* experiment was carried out as described (Chen K et al. 2013, Hu et al. 2015) with slight improvement. In brief, in the current investigation, in order to examine the LiCl interference in-vivo, 40 four-week BALB/C mice obtained from the Hunan SJA Lab Animal facilities were equally divided into the following four groups: Control group; LiCl treated group; infected group, and LiCl treated + App infected group (*n* = 10 in each group). For LiCl treatment, LiCl (in PBS) was received by tail vein injection at a dosage of 110 µg/g body weight (2,200 µg/mouse) at one day post-infection (Chen et al. 2013). The mice in the infected group and LiCl treated + App infected group, respectively, received intranasal inoculations of a 0.25 ml diluent containing 1×10^6 CFU/mL ($10 \times LD_{50}$) App (Hu et al. 2015). The mice in the control group were intranasally inoculated with equal volumes of normal saline (0.9% wt/vol NaCl). The mice were sacrificed 48 hours after post-inoculation (Hu et al. 2015). The right lungs of the mice were collected for RNA extraction, and the left lungs of the mice were used for histopathological analysis. All of the animal experiments and procedures were performed in accordance with the Biological Studies Animal Care and Use Committee of Hunan Province, P. R. C. and aproved by Hunan Agricultural University's Local Animal Care Committee, document number: 2021(100). The mice were housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22±1°C. Standard mouse food and tap water were given ad libitum. The mice were sacrificed after treatment with an anesthetic CO_2.

RNA purification and real-time PCR

Total RNA was extracted from cell cultures and lung tissues using Trizol Reagent obtained from the TaKaRa Biotechnology (Dalian) Co. Ltd., China. Reverse transcription was performed from 1 µg of total RNA for each sample using 5×TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (AT341) supplied by the Beijing TransGen Biotech Co. Ltd. (China) according to the manufacturer's instructions. Real-time PCR amplifications were also performed using 2×TransStartR Tip Green qPCR Super-Mix (AQ141) acquired from the Beijing TransGen Biotech Co. Ltd. (China) in a final volume of 10 µg, which contained a 1 µg cDNA template from each sample. The primers used in this study are listed in Table 1. The cycling conditions were 95°C for 30 seconds, followed by 40 cycles at 94°C for 5 seconds, and at 60°C for 30 seconds. The levels of the gene expressions in the present study were analyzed using the "Delta-delta method" for relative quantification. The expressions of the selected genes were normalized to that of the reference β-action gene using the following equation:

$$2^{-[\Delta Cq \, sample \, - \, \Delta Cq \, control]} = 2^{-\Delta\Delta Cq}$$

The genes were then further converted into relative mRNA expressions (Hu et al. 2015).

Western Blot analysis

In the present study, total protein was extracted from the cell cultures using an EpiQuik Whole Cell

Table 1. q-PCR primer sequences and product length.

Genes	Primers (5'→3')	Product length
β-actin	F: 5'TTCCTTCTTGGGTATGGAAT3' R: 5'GAGCAATGATCTTGATCTTC3'	178bp
IL-1β	F: 5'GCCTCGTGCTGTCGGACCCA3' R: 5'TCCAGCTGCAGGGTGGGTGT3'	199bp
IL-6	F: 5'GCTGGTGACAACCACGGCCT3' R: 5'AGCCTCCGACTTGTGAAGTGGT3'	107bp
TNF-α	F: 5'ATCCGCGACGTGGAACTGGC3' R: 5'TTGGGGACCGATCACCCCGA3'	159bp
CCL 2	R: 5'CCAGCAAGATGATCCCAATGA 3' F: 5'TCTCTTGAGCTTGGTGACAAAAAC 3'	189bp
TLR-4	F: 5' CTGAGCAAACAGCAGAGGAAGAAC 3' R: 5' ATAGCAGAGCCCCAGGTGAGC 3'	124bp

Extraction Kit (AmyJet Scientific). The protein concentrations in the extracts were measured using the Bradford Assay Method. As previously described, equal amounts of the extracts were separated by SDS-PAGE, and then transferred onto nitrocellulose membranes for immunoblot analysis (Hu et al. 2015). The antibody to pGSK-3ß (ser 9), p- p65, and ß-actin was purchased from Cell Signaling Technology (CST).

Histopathological observations

Histopathological analysis was performed using a routine paraffin sectioning method. In summary, the extracted lungs of the experimental mice were fixed in 10% neutral buffered formalin for 24 hours, and then processed routinely and embedded in paraffin wax. Tissue sections (3 to 4 µm) were stained with hematoxylin and eosin (HE) and observed using conventional light microscopic examination techniques.

Statistical analysis

The statistical analysis was performed using a one-way analysis of variance. The error bars represent the standard error of means. A p value of < 0.05 was considered to be statistically significant.

Results

LiCl ameliorated App-induced lung injuries in mice

In the control group and the LiCl treated groups, no deaths and no significant clinical symptoms were observed. However, the App-infected mice showed sig-nificant clinical symptoms, including slight lameness, loss of appetite or even death (5/10), body contractions, shortness of breath with convulsions, depression, and severe coma and dyspnea before death. The dead mice showed evidence of pneumonia and pleurisy, and the trachea and bronchi were observed to be filled with foamy blood-colored mucus secretions. The lungs became congested, and bleeding with alveolar interstitial oedema and hemorrhagic pneumonia were observed near the hilum of the lung tissue. In contrast, no deaths and only slight clinical symptoms were observed in the App-infected mice following LiCl treatments. From a histopathological perspective, in the control and LiCl treated groups, the lungs showed no changes (Figs. 1A and 1B). However, acute hemorrhagic pneumonia, hemorrhage, neutrophils, and lymphocyte infiltration were evident in the App-infected mice (Fig. 1C). Moreover, in regard to the App-infected mice, after receiving treatments with LiCl, only slight lung injury and some evidence of inflammatory cell infiltration were detected (Fig. 1D).

LiCl down-regulated the expressions of TLR4 in the lung tissue of App-infected mice

The mRNA expression levels of TLR4 were measured in the lung tissue of the experimental mice in this study. The results indicate that the App-infected mice showed higher TLR4 expression levels when compared with the control group. Moreover, the LiCl treated mice displayed significantly decreased TLR4 expression levels in their lung tissue (Fig. 2).
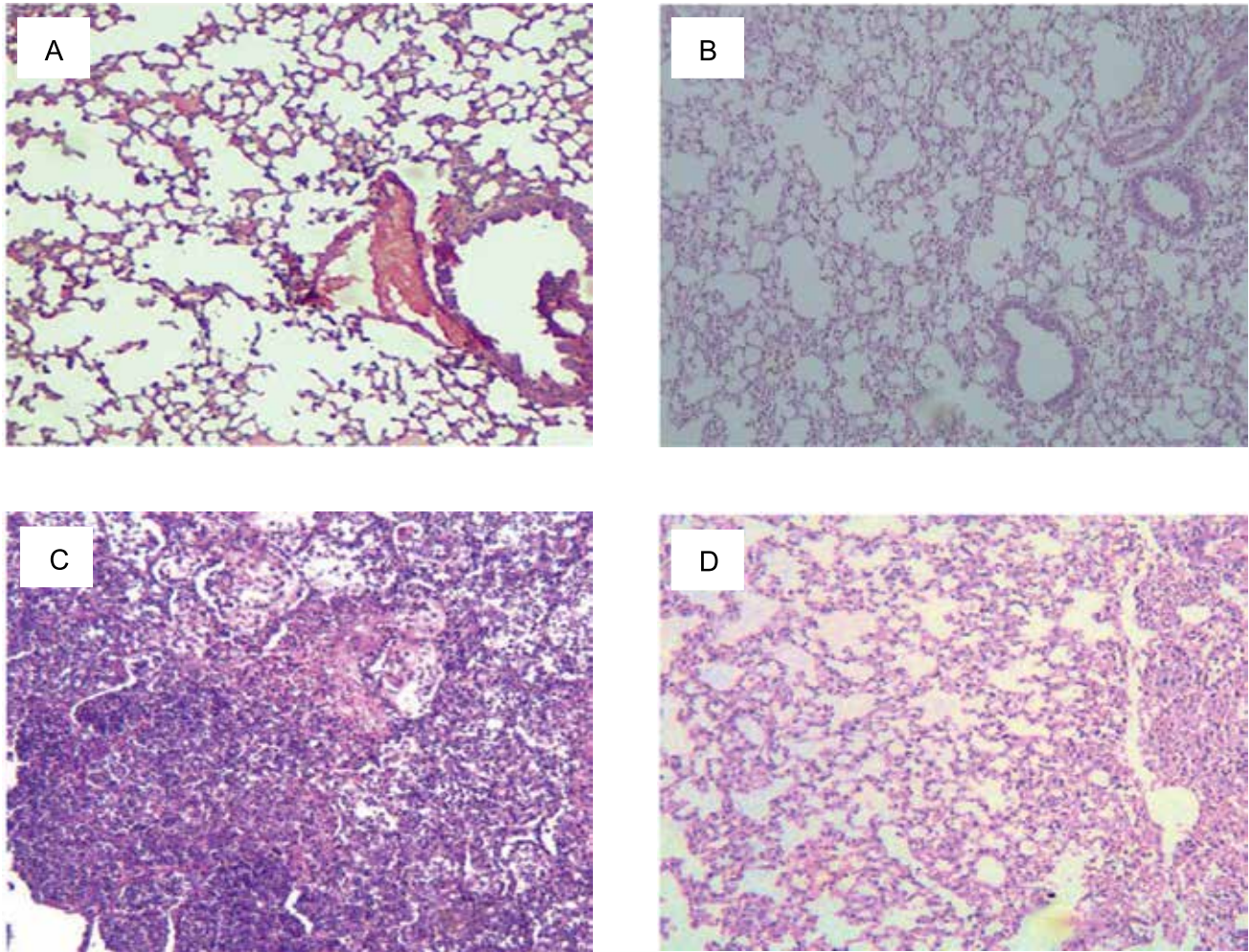
Fig. 1. LiCl reduced lung injury inApp-infected mice (x100);
(A) no lesions were observed in the lung tissues of control mice; (B) no lesions were observed in lung tissues of LiCl treated mice; (C) damaged lung after App infection. Alveolar cavities were filled with red blood cells, neutrophils and lymphocyte infiltration in the lung of infected mice; (D) LiCl reduced lung injury of App infected mice. Some inflammatory cell infiltration in the lung of infected mice with LiCl treatment was observed.

LiCl down-regulated the expressions of pro-inflammatory factors and CCL2 in the lung tissue of App-infected mice

The results of this study revealed that the IL-1β, IL-6, TNF-α, and CCL2 expressions were markedly increased in the infected group when compared with the control group (Fig. 3). However, following the LiCl treatments, the IL-1β, IL-6, TNF-α, and CCL2 expressions were observed to be markedly decreased. Therefore, the data results demonstrate that the LiCl had played a key role in the anti-pathogenesis of the pleuropneumonia caused by App (Fig. 3).

LiCl decreased the death rates of the App-infected cells

This study found that the 80 mM and the more than 80 mM LiCl treatments caused high mortality rates in the RAW264.7 cells (Fig. 4). In addition, the 60 mM LiCl treatments significantly decreased the mortality rates of the App-infected cells (Figs. 5 and 6).

LiCl decreased the expression levels of NF-κB and pGSK-3ß

It was found that the GSK-3ß levels were markedly increased in the infected group when compared with the control and LiCl-treated mice groups. Following the LiCl treatments, the GSK-3ß levels of the infected cells were observed to be markedly decreased (Figs. 7A and 7B). In addition, the p-p65 levels were found to be markedly increased in the infected-cells when compared with those of the control and LiCl-treated cells. It was found that, following the LiCl treatments, the p-p65 levels of the infected cells were markedly decreased and were still higher than those of the control and LiCl- treated cells (Figs. 7A and 7B).
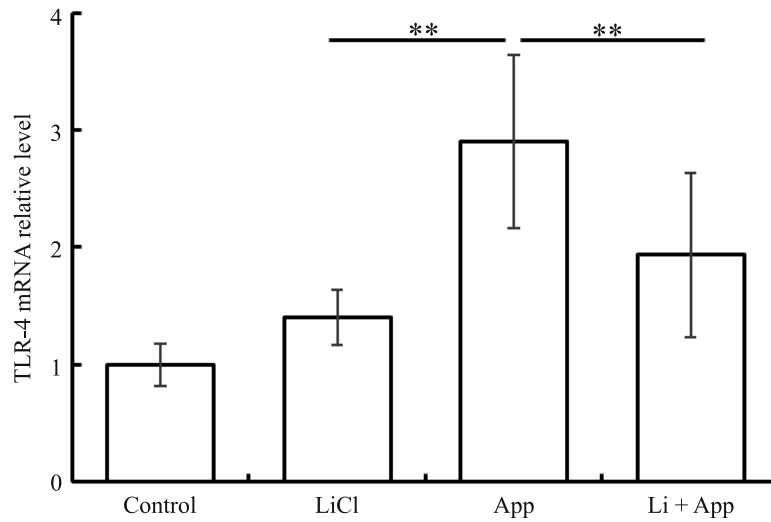
Fig. 2. Lithium down-regulates the production of TLR-4 in lungs of App-infected mice with and without LiCl treatment.
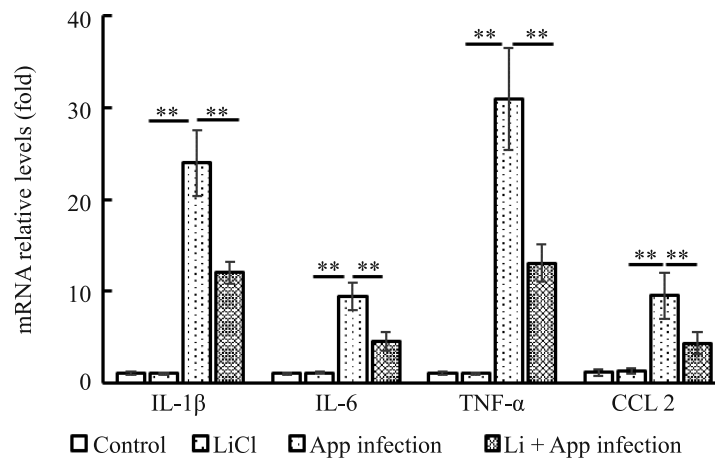

Fig. 3. Lithium down-regulates the production of IL-1ß, IL-6, TNF-α and CCL 2 in the lungs of App-infected mice with and without LiCl treatment. (** $p<0.01$)
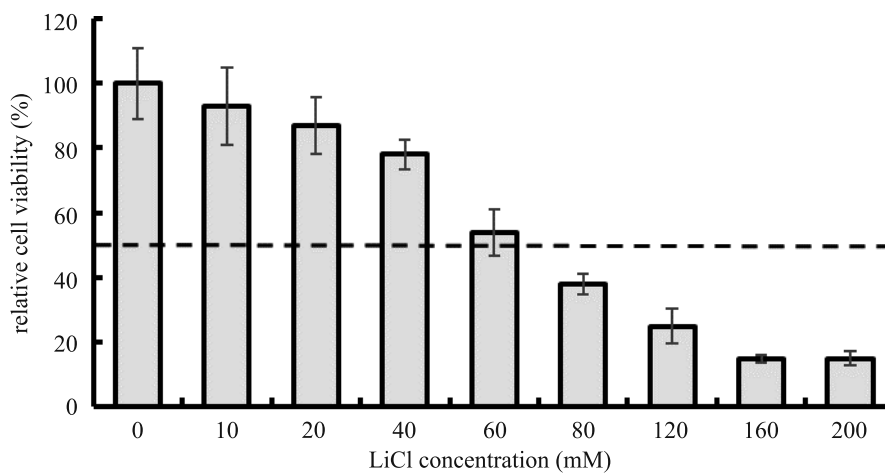

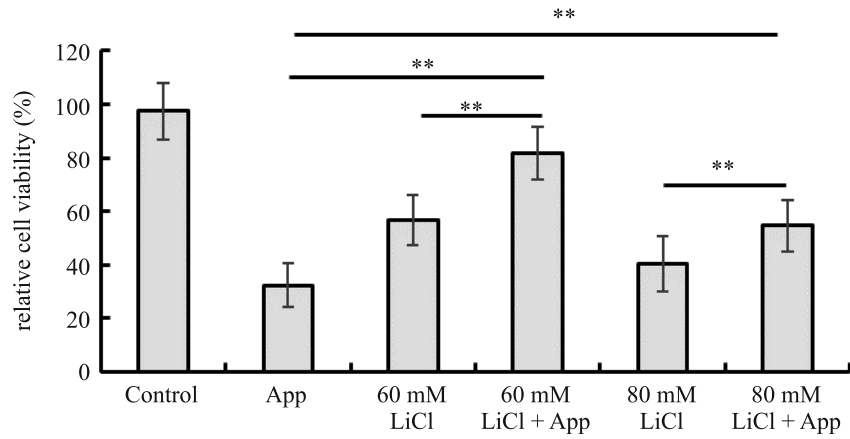Fig. 4. Cell viability after the treatment of a series concentration of LiCl.

Fig. 5. Cell viability of App- infected cells with and without LiCl treatment. (** p<0.01)
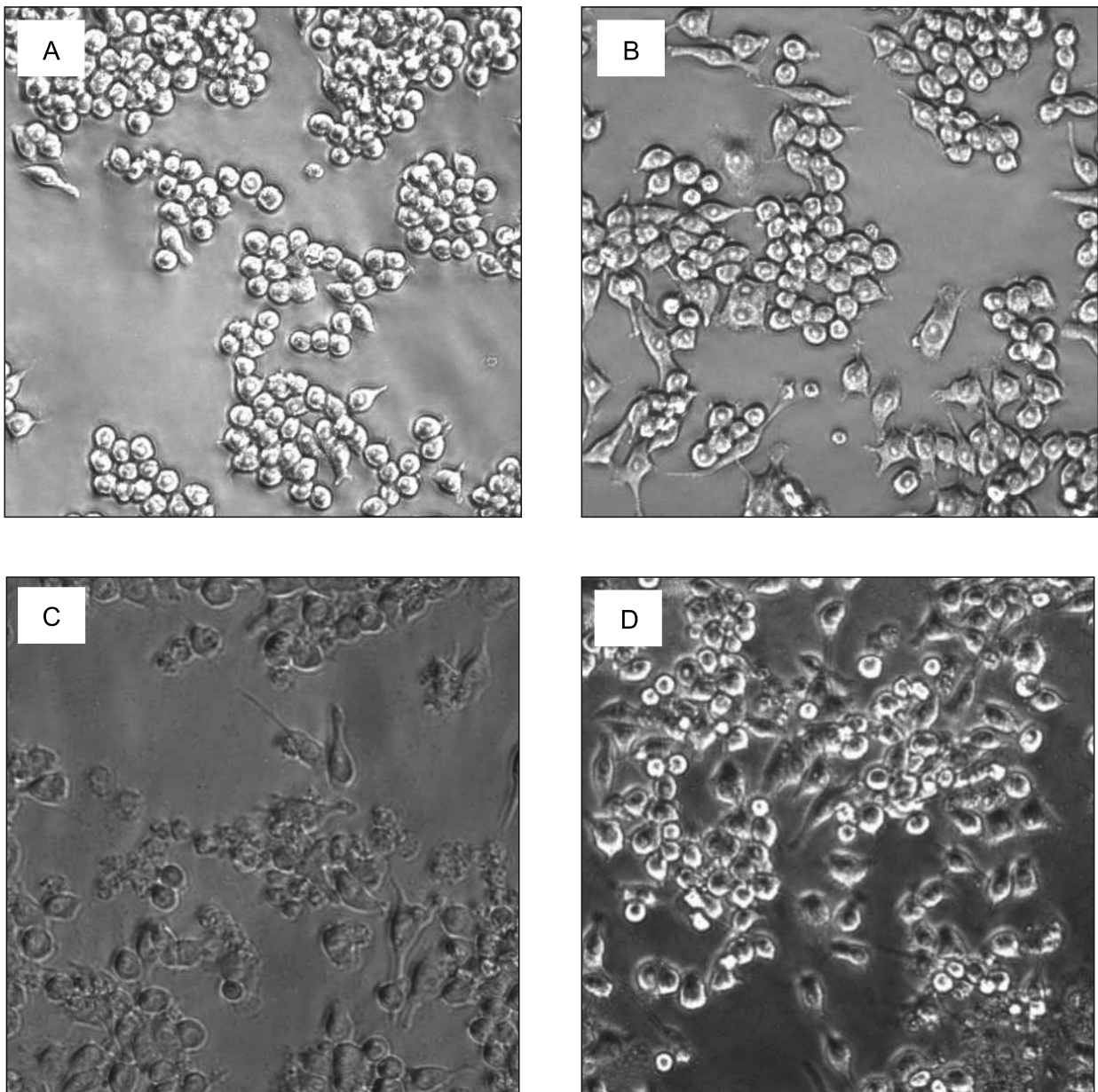

Fig. 6. Cell viability increased after treatment with 60 mM LiCl (x40);
(A) normal cells; (B) Cells with LiCl treatment; (C) App- infected cells; (D) App- infected cells with LiCl treatment.
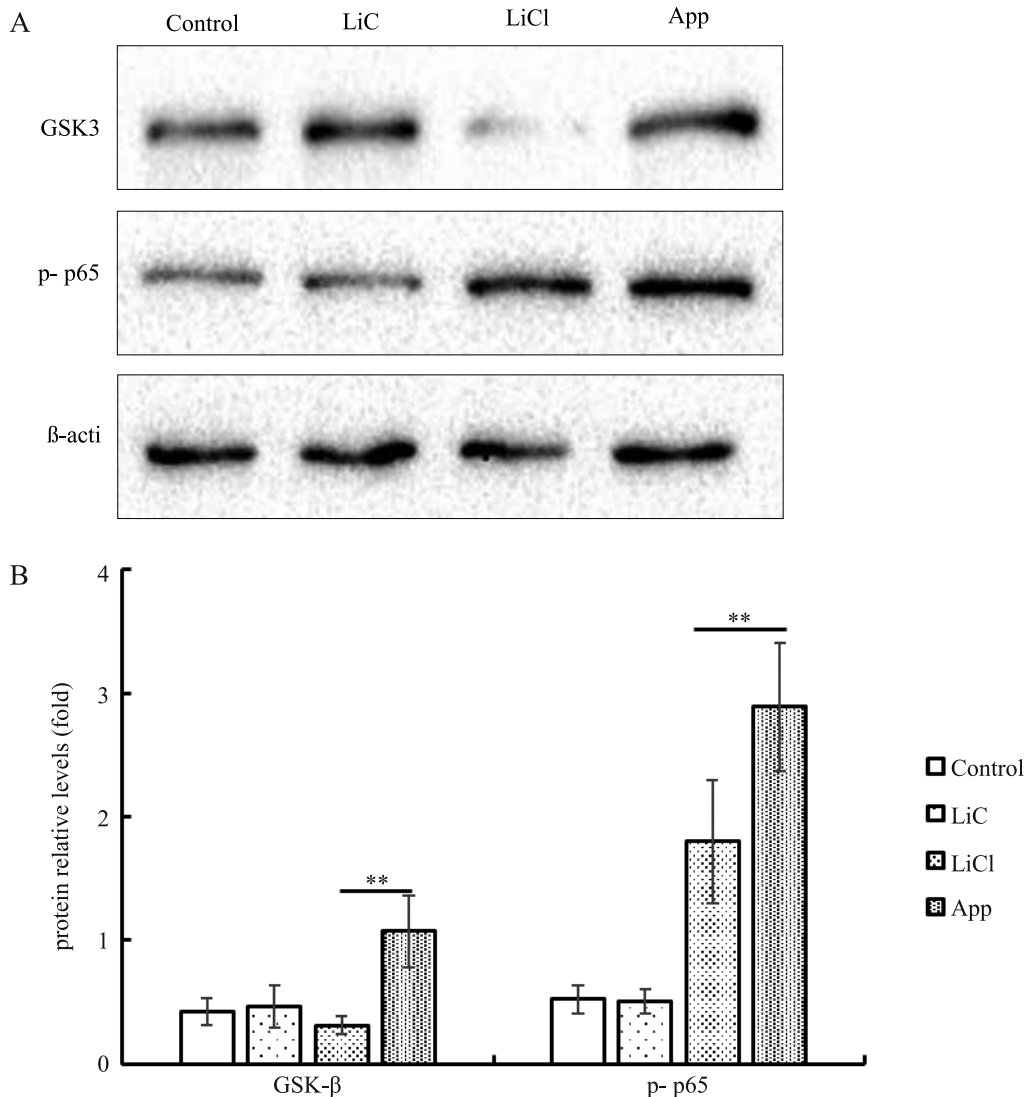
A

B

Fig. 7. Lithium chloride decreased the expression of p-p65 and GSK-3ß
(A) Detection of GSK-ß and p-p65 protein levels by western blot. (B) GSK-ß and p-p65 protein levels. (** P<0.01)

Discussion

Lung injuries caused by porcine contagious pleuro-pneumonia (PCP) are currently difficult to prevent and treat. Therefore, reducing the related mortality and morbidity caused by PCP remain critical issues (Sassu et al. 2018). Recent study results have shown that LiCl treatments can reduce organ and tissue injuries caused by viral and bacterial infections (Chen et al. 2013, Zhao et al. 2020). LiCl can not only suppress the production of proinflammatory cytokines, but also increase anti--inflammatory cytokines, thereby providing important survival advantages. LiCl has also shown the ability to attenuate organ injuries for animals in sepsis models (Dugo et al. 2005, Hoffmeister et al. 2020, Makola et al. 2020). Therefore, based on those research findings, it is expected that LiCl will become a potential drug for PCP treatments. In the present study, it was found that

the death rates, clinical symptoms, and lung injury degrees were reduced. In addition, the expression levels of p-p65, IL-1ß, IL-6, TNF-α, and CCL2 were observed to decrease in App-infected mice following LiCl treatments. It was suggested that LiCl treatments ameliorated the pulmonary injuries of App-infected mice by suppressing the production of proinflammatory cytokines.

In regard to the mechanism of LiCl alleviating organ damage, it has been documented that the LiCl actively reduced the expression levels of toll-like receptors, such as TLR4, GSK-3β, NF-*κ*B, and others (Chen et al. 2013). TLRs are a family of proteins which recognize different pathogen-associated-molecular patterns from bacteria, viruses, and fungi. TLRs are known to recognize molecular products derived from all the major classes of pathogens, resulting in the activation of NF-*κ*B, production of several NF-*κ*B-dependent

cytokines, and chemo-attractants which recruit phagocytic cells to clear infections (Song et al. 2007). It has been documented that the toll-like receptor 4/NF-κB-mediated pathways are involved in activating porcine alveolar macrophages by *Actinobacillus pleuropneumoniae* and PCP pathogenesis (Brogaard et al. 2015, Li et al. 2016, Li et al. 2018). In the present study, it was found that the App-infected mice showed higher lung TLR4 expression levels when compared with the control mice. Also, the App-infected mice with LiCl treatments showed significantly decreased lung TLR4 expression levels in their lung tissue.

Some studies have investigated the role of GSK in live bacterial infections. Zhang et al. found that glycogen synthase kinase-3ß (GSK3ß) inhibition suppressed inflammatory responses to Francisella infections (Zhang et al. 2009) and protected mice from tularemia (Li et al. 2016, Boren et al. 2017). It has been found that GSK-3 plays a key role in promoting or repressing NF-κB activity. After NF-κB is activated, its p50/p65 subunits translocate from the cytoplasm to the nucleus. Target gene transcription then becomes initiated, including proinflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteases (MMPs), and inducible nitric oxide synthase (iNOS) (Dugo et al. 2005). In another related study, Raghavendra reported that NF-κB plays a critical role in inflammatory responses to *Staphylococcus aureus* (GAS). It was observed that following treatments with various GSK-3β inhibitors in GAS-infected RAW 264.7 cells, the ratio of nuclear translocation of NF-κB subunits decreased, and the levels were even lower than the non-infected group (Raghavendra et al. 2014). Furthermore, in an animal model of sepsis, treatments with GSK-3 inhibitors were found to suppress NF-κB- dependent proinflammatory cytokine expressions and provide protection from organ injury and endotoxin shock (Dugo et al. 2005, Martin et al. 2005, Woodgett et al. 2005, Oviedo-Boyso et al. 2011). In this study, it was found that the cell viability of the App-infected RAW 264.7 cells increased, and GSK-3ß levels markedly decreased following the LiCl treatments. The p-p65 levels of the App-infected cells were markedly increased when compared with the control group, and the p-p65 levels of the infected group with LiCl treatments were observed to be markedly decreased. Therefore, based on this study's findings, it was deduced that the LiCl had lowered the GSK-3ß expression levels and the nuclear translocation of p-p65. In addition, regarding the cell viability of App-infected RAW 264.7 cells with LiCl treatments, it was assumed that this was due to the lower NO production of the App-infected cells caused by the LiCl. It has been reported that App-infected animals and streptococcus infected macro-phages have shown NO increases. Following the LiCl treatments, the streptococcus infected macrophages showed lower NO production and higher cell viability (Benga et al. 2009, Chang et al. 2013).

Overall, it was deduced in this study that the lithium chloride had ameliorated the pulmonary injuries caused by *Actinobacillus pleuropneumoniae* via the inhibition of the GSK-3β- NF-κB-dependent pathway. Therefore, it was considered to be a potentially effective drug for ameliorating PCP pulmonary injuries.

Acknowledgements

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