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Baicalin alleviated APEC-induced acute lung injury in chicken by inhibiting NF-κB pathway activation

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ABSTRACT

Bacterial pneumonia is a leading cause of death in the animal husbandry. Acute lung injury (ALI), most often seen as a part of systemic inflammatory process, characterized by progressive hypoxemia, edema, and neutrophil accumulation in the lung. Baicalin has been reported to inhibit inflammatory response, but its role in ALI remains unknown. The purpose of our study was to determine the protective effect and possible mechanism of baicalin against avian pathogenic *Escherichia coli* (APEC)-induced ALI in chicken. Chickens were conditioned with baicalin 1 week before intratracheally instilled with APEC. Then, chickens were sacrificed by CO_2 inhalation 12 h later and the lung tissues were collected for examining histopathological changes, wet/dry (W/D) ratio, myeloperoxidase (MPO) activity, levels of pro-inflammatory cytokines and activation of NF-κB signaling pathway. The results showed that pre-treatment of chickens with baicalin significantly alleviated the death rate, histopathological changes in lung tissues. The W/D ratio, MPO activity and production of cytokines, such as IL-1β, TNF-α, IL-6 of lung tissues were also decreased following treatment with baicalin. Furthermore, the mechanism responsible for these effects was attributed to the inhibitory effect of baicalin on nuclear factor-κB (NF-κB) signaling activation. These data thus support the application of baicalin as a potential medicine for the treatment of *E. coli*-induced ALI by regulating NF-κB signaling pathway.

1. Introduction

Bacterial pneumonia is the third most common cause of death in the world, which can lead to acute lung injury (ALI) [1,2]. ALI, an acute failure of the respiratory system, has been most often seen as part of a systemic inflammatory process, particularly systemic sepsis [3]. The pathogenesis of ALI mainly involves exaggerated pulmonary inflammation, ultimately leading to an impairment of the alveolar-capillary barrier and to the deterioration of gas exchange [4]. Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 play important roles in ALI [5]. NF-κB, a transcription factor, recognized as a common consensus DNA sequence and involved in the regulation of large numbers of target gene, including TNF-α, IL-1β, and IL-6 [6]. Evidence has also suggested that inhibiting the activation of NF-κB is responsible for the inhibition of the development of ALI [7]. *Escherichia coli* (*E. coli*), used as a surrogate for infectious exposure, induces experimental ALI when it is introduced into the airway [8]. Similarly, APEC can invade the respiratory tract and cause the disruption of the lung endothelial and epithelial barriers leading to the ALI in chicken. APEC belongs to a subgroup of extra-intestinal pathogenic *Escherichia coli* (ExPEC) and has been identified as a zoonotic pathogen, sharing similar phylogenic background and certain virulence genes with human ExPEC [9,10]. The APEC invasion begins in the upper respiratory tract, targets the air sac system, and subsequently progresses to septicemia and systemic infection targeting vital organs [11].

Despite the development of intensive care, ALI induced by bacteria or microbe infection remains not yet well-understood. Traditional Chinese medicine has a long history of use because of its potential complementary therapy and fewer adverse effects, and is currently used to treat patients with systemic inflammatory response syndrome [12]. Baicalin is an important medicinal ingredient isolated from dry roots of *Scutellaria baicalensis Georgi* that has been applied to treat upper respiratory infections [13]. Baicalin has been used as an anti-inflammatory agent in the treatment of a variety of inflammatory diseases, such as bronchitis, nephritis, hepatitis and asthma [14]. In this study, we explored the protective effects of baicalin on APEC-induced ALI in chicken. Our results showed that baicalin reduced the lung injury through inhibiting the NF-κB signaling pathway. Therefore, our study

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suggested that baicalin may be a potential therapeutic agent for APEC-induced ALI and provided a novel direction for treatment of chicken colibacillosis.

2. Materials and methods

2.1. Reagents and bacterial strain

Baicalin (purity > 99%) was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). APEC-O78 strain (CVCC1418) was purchased from the Chinese Veterinary Culture Collection Center (CVCC). ELISA kits were obtained from Lengton Bioscience Company (Shanghai, China).

2.2. Animals and experimental design

1-day-old male Hyline Brown healthy chickens were provided by Jilin Academy of Agricultural Sciences (Changchun, Jilin, China). A total of 60 chickens were randomly assigned to six groups including control group, APEC-O78 group, baicalin alone (200 mg/kg) group, APEC-O78 + baicalin (200, 100, 50 mg/kg) groups and each group included ten chickens. Baicalin dissolved in a solvent mixture containing DMSO was administrated by oral gavage 1 week before APEC-O78 infection. Then the lung injury models were induced by intratracheal inoculation with 2×10^9 CFU APEC-O78. Chickens were sacrificed with CO_2 inhalation, and samples were collected at 12 h after APEC administration. Throughout the entire experimental period, chickens were kept in cages under a 16/8 light/dark cycle and allowed ad libitum consumption of feed and water, and the average temperature was 24 ± 2 °C at day and night.

2.3. Death rate measurement

Counted the number of survivals and the number of deaths after APEC administration for 12 h. The death rate equaled that the number of deaths divided by total of each group.

2.4. Organ scoring

Lungs were aseptically removed after the death of chicken, and the severity of the macroscopic lesions attributed to *E. coli* was scored according to an organ lesion score described in Table 1 [15].

2.5. Histopathology imaging of lung tissues

Upper right lung lobes were removed and fully fixed with 10% buffered formalin (formaldehyde: sterile water = 1:9) for approximately 1 week. Then the specimens were dehydrated and embedded in paraffin followed by hematoxylin and eosin (H&E) staining. Finally, the sections were observed by two experienced pathologists by a light microscopy and randomly chose 10 microscopic fields (100×) per tissue sample. All sections were scored according to the criteria previously described [16]: 0, normal tissue; 1, minimal inflammatory change; 2, no obvious damage to the lung architecture; 3, thickening of the capillaries septae; 4, formation of nodules or areas of pneumonitis that distorted the normal architecture; and 5, total obliteration of the field.

Table 1
Description of organ lesion scoring.

Organ	Score	Description of organ lesions
Lung	1	Single small lesion, locally restricted (1/5 of the organ)
	2	Multiple, locally restricted small lesions and/or one bigger lesion (2/5 of the organ)
	3	Lesions cover about 1/2 of the organ
	4	Lesions cover about 4/5 of the organ
	5	Complete organ covered with lesions

2.6. Determination of lung wet/dry (W/D) ratio

The right middle lobes excised from the sacrificed chickens were weighed to obtain the 'wet' weight. Then, the lung tissues were incubated in an oven at 60 °C for 48 h to obtain the 'dry' weight. The lung wet weight was divided by its dry weight to supply the lung W/D weight ratio to determine the severity of pulmonary edema.

2.7. Myeloperoxidase (MPO) assay

MPO activity reflects accumulation of macrophages and neutrophils in the lung tissues. MPO activity in the lung tissues was measured by MPO kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, tissue was homogenized in 1 mL of 50 mmol/L potassium phosphate buffered saline (PBS, pH 6.0) containing 0.5% hexadecyltrimethylammonium hydroxide and centrifuged at 12,000 r/min at 4 °C for 20 min. 10 μL of the supernatant was transferred into PBS (pH 6.0) containing 0.17 mg/mL 3,3′-dimethoxybenzidine and 0.0005% H_2O_2. MPO activity of the supernatant was determined by measuring the H_2O_2-dependent oxidation of 3,3′-dimethoxybenzidine and expressed as units per gram of total protein (u/g). Total protein content in samples was analyzed using a BCA protein assay kit.

2.8. Inflammatory cytokines assay

The lung tissue was weighed and homogenized to obtain 10% homogenate with PBS. The levels of TNF-α, IL-6 and IL-1β in the supernatants of lung tissues were measured by ELISA kits (Lengton Bioscience Company, Shanghai, China). The standard curve was established according to the standards provided by the kits.

2.9. Western blot analysis

Total proteins were extracted from homogenized lung tissues with M-PER mammal protein extraction reagent (Thermo, USA) according to the manufacturer's instructions. The concentration of proteins was measured using a BCA kit and samples of equal amounts were fractionated on 12% SDS-polyacrylamide gel for electrophoresis. Subsequently, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% BSA for 3 h, followed by incubation with primary antibodies including anti-IκB rabbit polyclonal antibody (Sangon Biotech Company, Shanghai, China), anti-phospho- IκB rabbit polyclonal antibody (Sangon Biotech Company, Shanghai, China), anti-p65 rabbit polyclonal antibody (Bioss, Massachusetts, USA) and anti-phospho- p65 rabbit polyclonal antibody (Bioss, Massachusetts, USA) at 4 °C and anti-rabbit secondary antibodies (Bioworld Technology, Georgia, USA) at room temperature. The proteins bands were detected using an enhanced chemiluminescence (ECL) western blotting detection reagents (Millipore, Merck, Massachusetts, USA).

3. Results

3.1. Effect of baicalin on death rate induced by APEC

The data of death rate showed that treatment of baicalin alone has no effect on the survival of the animals. However, administration of APEC resulted in the death rate of the animals approximately 90%. And treatment with baicalin (200, 100, 50 mg/kg) markedly reduced the death rate induced by APEC (Fig. 1).

3.2. Effect of baicalin on pathomorphological change

After the euthanasia of chicken, the severe pathomorphological

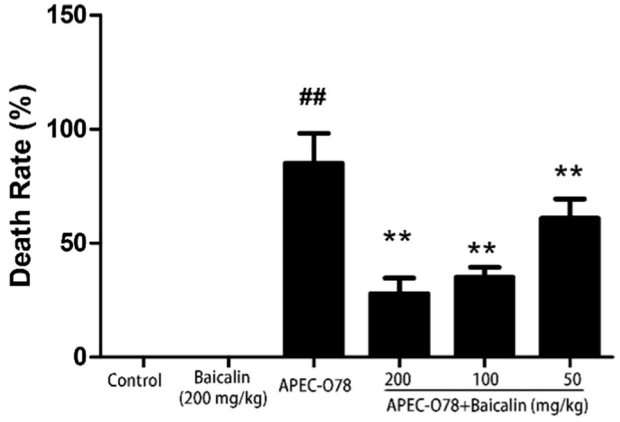

Fig. 1. The death rate of chicken.
After APEC administration for 12 h, the death rate was calculated by the number of survivals and death ratio. $^{\#}P < 0.01$ is significantly different from the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the APEC group.

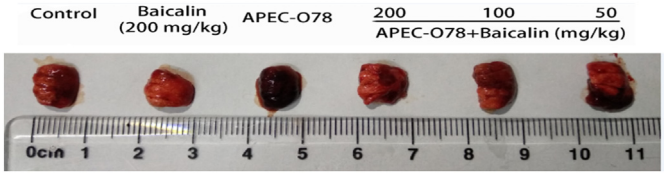

Fig. 2. Pathomorphological changes of the lung 24 h after infection with APEC. Control group (Score 0); Baicalin alone group (Score 0); APEC infection group (Score 5); APEC + Baicalin (200 mg/kg, score 0); APEC + Baicalin (100 mg/kg, score 1); APEC + Baicalin (50 mg/kg, score 3).

changes of lung were scored according to the Table 1. The lungs infected by APEC alone were observed completely covered with lesions. On the contrary, the groups of baicalin administration in advance including 200 mg/kg (score 0), 100 mg/kg (score 1), 50 mg/kg (score 3), have less lesions than APEC group (Score 5). These data clearly showed that baicalin (200, 100, 50 mg/kg) significantly extenuated the pathomorphological changes of lung after APEC infection (Fig. 2).

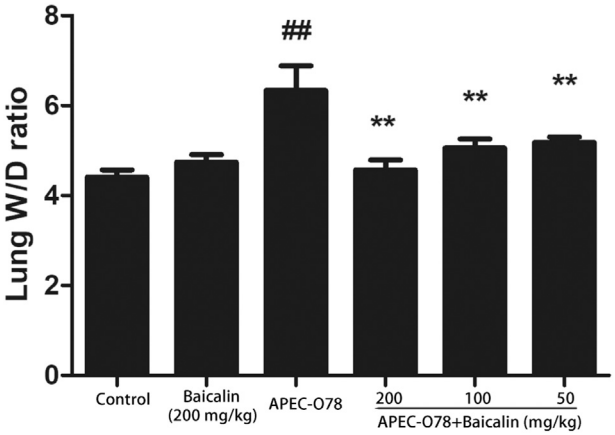

Fig. 4. Lung wet/dry radio. The lung wet/dry ratio was determined 12 h after APEC challenged. The values presented are the means ± SD (n = 10). $^{\#}P < 0.01$ is significantly different from the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the APEC group.

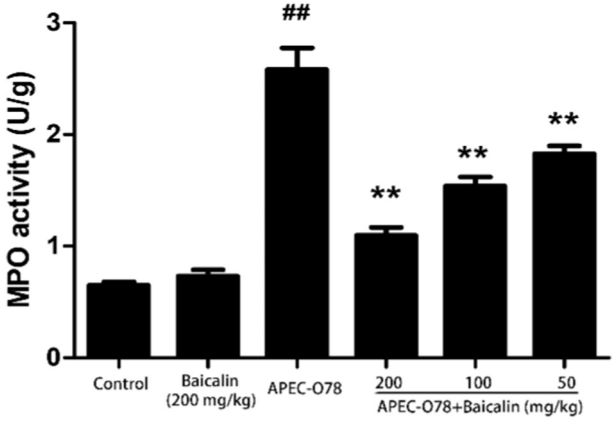

Fig. 5. MPO activity. The lung tissues were homogenized and centrifuged to collect the supernatant for the MPO activity measurement. The values presented are the means ± SD (n = 10). $^{\#}P < 0.01$ is significantly different from the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the APEC group.

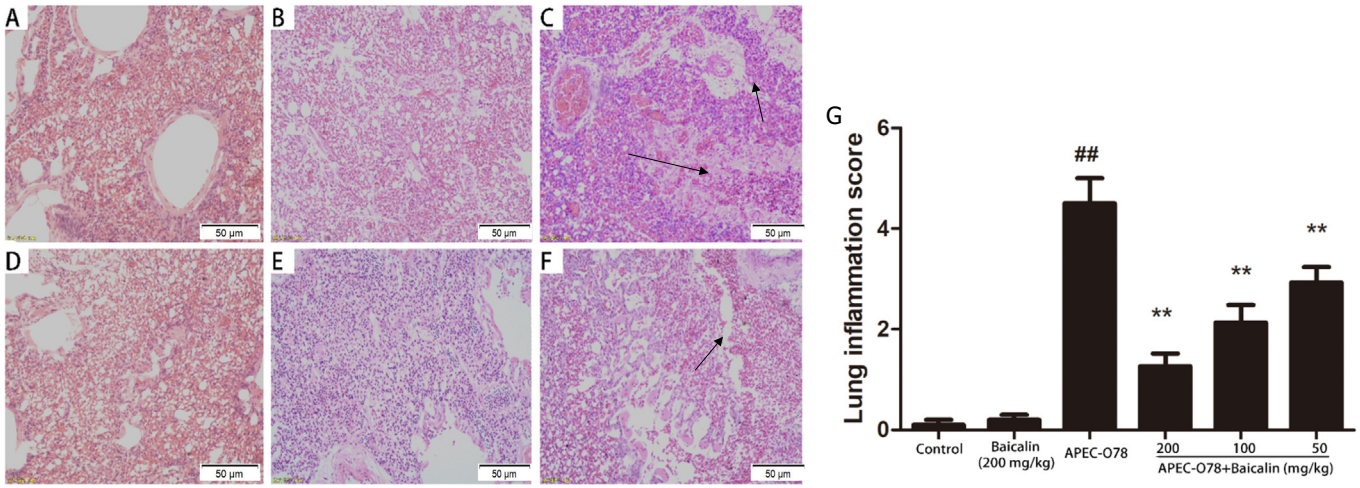

Fig. 3. Histological changes of lung tissues (HE, ×100). Nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Lung tissues histological changes from (A) control, (B) Baicalin alone, (D) APEC + Baicalin (200 mg/kg), (E) APEC + Baicalin (100 mg/kg), and (F) APEC + Baicalin (50 mg/kg) were determined by HE staining and lung inflammation score (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

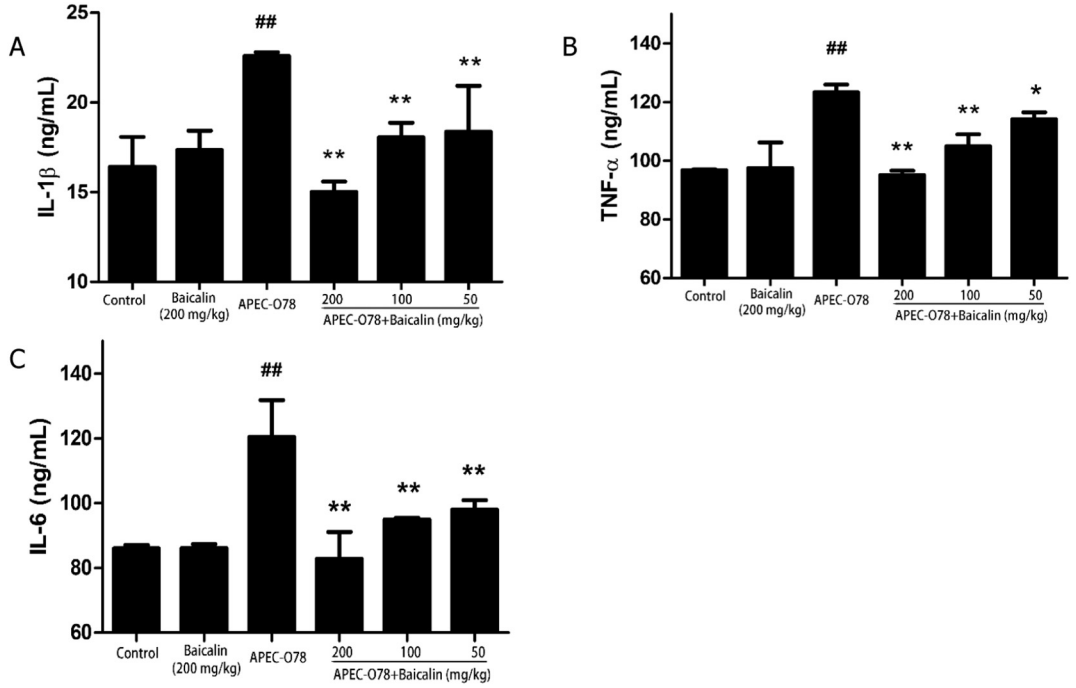

Fig. 6. Cytokines production in lung tissues. The supernatant of homogenized lung tissues were used to analyze the inflammatory cytokines IL-1β (A), TNF-α (B), and IL-6 (C). The values presented are the means ± SD (n = 10). $^{\#}P < 0.01$ is significantly different from the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the APEC group.

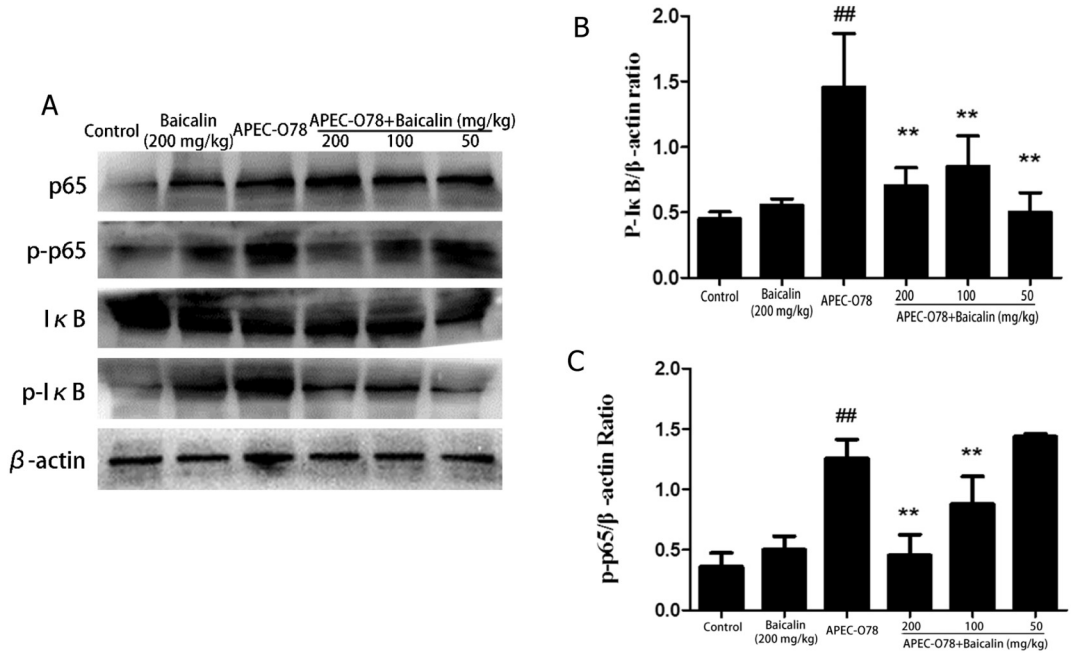

Fig. 7. The protein expression of NF-κB pathway. (A) NF-κB protein samples were analyzed by western blot with IκB, p-IκB, p65, p-p65 antibodies. β-Actin was used as a control. (B) Quantification of p-IκB proteins was determined by densitometry and has been normalized to β-actin. (C) Quantification of p-p65 proteins was determined by densitometry and has been normalized to β-actin. The values presented are the means ± SD (n = 10). $^{\#}P < 0.01$ is significantly different from the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the APEC group.

3.3. Effect of baicalin on APEC-induced histopathological changes in lung tissues

To assess the protective effect of baicalin on lung histopathological changes induced by APEC, H&E staining was performed. The results showed that there are no any damaged of lung tissues in the control group. However, obviously damaged of lung tissues, including capillaries wall hyperaemia and excessive neutrophil infiltration around the pulmonary vessels were shown in the lung tissues of the APEC treatment group. Treatment of baicalin inhibited the severity of pulmonary damages induced by APEC (Fig. 3).

3.4. Effect of baicalin on lung W/D ratio

Lung W/D ratio is usually used to assess the degree of lung edema. As shown in Fig. 4, treatment of baicalin inhibited the lung W/D ratio

induced by APEC.

3.5. Effect of baicalin on MPO activity in lung tissues

MPO activity, a marker of the accumulation of polymorphonuclear granulocytes (PMNs), was used to assess the inflammatory response in the lung in the present study. MPO activity significantly increased in the APEC group compared to the control group. However, MPO activity obviously reduced by baicalin (Fig. 5).

3.6. Effect of baicalin on the levels of inflammatory cytokines in lung tissues

The effect of baicalin on the levels of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in the lung were assessed by ELISA kits in the present study. Stimulation of APEC increased the levels of IL-1β, TNF-α, and IL-6, and these changes were attenuated by baicalin (Fig. 6).

3.7. Effect of baicalin on NF-κB signaling pathway

To explore the potential protective mechanism of baicalin on APEC-induced acute lung injury, the activation of the NF-κB signaling pathway was measured. As shown in Fig. 7, the expression of p-NF-κB p65 and p-IκB were elevated in the APEC group. However, treatment of baicalin (200, 100, 50 mg/kg) significantly inhibited the activation of phosphorylation of NF-κB p65 and phosphorylation of IκB.

4. Discussion

ALI, characterized by the pathogenesis of acute inflammation, causes significantly high morbidity and mortality worldwide, making a substantial impact on public health [4]. *E. coli*, a gram-negative bacillus, is one of the major cause of bacterial pneumonia [17]. In some researches, *E. coli* was used to induce the model of acute lung injury in mice and rats [18–20]. APEC first invades the upper respiratory tract and then, crosses the respiratory epithelia and induced the development of ALI [21]. Although many potential agents were investigated to cure this disease, the death rate from the disease remained high. Baicalin, a flavone glycoside extracted from *Scutellaria baicalensis Georgi*, has received extensive attention for its medicinal value. Some studies have proved that baicalin has anti-viral, anti-microbial, and anti-inflammatory activities [22–24]. In the present study, we assessed the protective effects of baicalin on APEC-induced ALI in chicken. we demonstrated that baicalin significantly relieved the symptoms of ALI induced by APEC, which can suppress lung wet/dry ratio, thin the intra-alveolar septa, weaken interstitial edema, decrease notable infiltration of prominent inflammatory cells (including alveolar macrophages and neutrophils), and elevate the survival rate of ALI chicken.

Neutrophils are the earliest immune cells recruited to the site of inflammation and exert antimicrobial effects by producing ROS, proteinases, and cationic peptides during ALI. However, over-production of these antimicrobial compounds into the extracellular space can injury host tissues [25]. Previous research suggested that inhibiting the number of PMN could relieve the severity of ALI [26]. MPO activity is serve as an indicator of neutrophil influx into tissues. In the present study, we found that baicalin significantly inhibited MPO activity induced by APEC. In addition, pro-inflammatory cytokines, such as TNF-α, and IL-1β play an important role in the development of ALI. These cytokines are produced mainly by monocytes and macrophages and they can recruit neutrophils into lung to induce the development of ALI [27]. Therefore, suppression the production of pro-inflammatory cytokines could be a potential strategy to treat *E. coli*-induced lung injury. In the present study, we found that treatment of baicalin significantly inhibited the levels of TNF-α, IL-1β and IL-6 of lung tissues during APEC-induced ALI.

NF-κB activation, which is required for the transcription and generation of pro-inflammatory mediators including TNF-α, IL-1β and IL-6 in the early phase of acute lung injury, has been implicated as a critical step in the pathogenesis of ALI [28]. To explore the potential protective mechanism of baicalin on APEC-induced ALI, the NF-κB signaling pathway was tested in the present study. The result showed that baicalin have an ability to inhibit the activation of NF-κB signaling pathway induced by APEC.

5. Conclusion

In summary, our results suggested that baicalin was able to decrease the death rate of model, lung W/D ratio, MPO activity, inflammatory cytokines production, and lung histopathological changes. Moreover, the protective effect of baicalin may be related to attenuation of inflammatory reactions and inhibition of the activation of NF-κB signaling pathway. These findings provide a powerful evidence that baicalin may be a potential medicine for preventing ALI induced by APEC.

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