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Abstract

Background: Alcohol extract from the root of *Urena lobata L.* (ULL) had broad spectrum antimicrobial activity. Studies *in vitro* have shown that ULL aqueous extract has antibacterial effect on *S. aureus*, and the combination therapy of the ULL aqueous extract with cefazolin sodium showed additive effect.

Materials and Methods: The mice underwent nasal inhalation with *S. aureus*, a subset of mice were intra-gastric gavage with ULL and/or intravenous injection cefazolin sodium twice daily. After being exposed to *S. aureus* for 5 days, 10 days and 14 days respectively, the white blood cells count (WBC), neutrophils absolute value (NEU) and the neutrophil percentage (NEU%) in peripheral blood, as well as the levels of serum immunoglobulin (Ig) G and IgM were determined using commercial kits. The colony count of *S. aureus*, the levels of interleukin (IL) -6 and IL-10 of mice lung tissue were detected, and the pathological changes of lung tissue were examined using H & E staining.

Results: ULL significantly protected against *S. aureus* pneumonia, as evidenced by the remarkable decrease in the rate of *S. aureus* colony count/lung weight, WBC, NEU and NEU% in peripheral blood, as well as the attenuation of lung histopathological damage. Additionally, ULL+cefazolin could have markedly reduced the rate of *S. aureus* colony count/lung weight when compared with cefazolin. Furthermore, ULL and ULL+cefazolin both could significantly decrease the serum levels of IgG and IgM, and the levels of IL-6, IL-10 in mice lung tissue.

Conclusion: This study first demonstrated that ULL may have potential use as a therapeutic agent for *S. aureus* pneumonia, and the roles of IgG, IgM, IL-6 and IL-10 in ULL protection against *S. aureus* pneumonia remain to be further studied.

Keywords: *Staphylococcus aureus*; pneumonia; antibacterial effect; *Urena lobata L.*; immunoglobulin; interleukin

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common pathogens which causes pyogenic infection in humans. The pyogenic infections caused by *S. aureus* can be found in a variety of systems such as pneumonia, pericarditis, osteomyelitis, pyelonephritis, and renal abscess. Some diseases caused by *S. aureus* can endanger human life such as bacteremia and endocarditis. Additionally, *S. aureus* has a high infection rate on clinic, and the progress of these diseases caused by *S. aureus* infection is fast. Moreover, the resistance rate of *S. aureus* to drugs is high (Li et al., 2004). Traditional herbal medicines are used in many countries (Jeon et al., 2014). Additionally, traditional herbal medicines have a history of over a thousand years for anti-infection therapy (Kwon et al., 2008). They have many effective ingredients (Lu et al., 2006; Xiong et al., 2013) and can be applied to multiple targets, so they have a wide range of pharmacological effect, and do not easily generate drug resistance (Yang et al., 2010). The study of traditional

herbal medicine for antimicrobial has become a new research direction, that will provide a new way to solve the problem of bacterial drug resistance. It has been reported that the combination therapy of Chinese herbal medicine and beta-Lactams antibacterial drugs can improve the treatment effect of the beta lactam antibiotic (Liu et al., 2000), reducing bacterial resistance. *Urena lobata L.* (ULL) mainly originated in Guangxi, Yunnan, Guizhou. ULL can clear away heat and toxic material. The ULL aqueous extract contained many kinds of chemical components with antibacterial activity, such as rutin and kaempferol (Jia et al., 2011). Moreover, rutin and kaempferol also could enhance the antibacterial activity of the other antimicrobial agents (Arima et al., 2002; Lim et al., 2007). There was report that alcohol extract from the root of ULL had broad spectrum antimicrobial activity (Mazumder et al., 2001). Studies *in vitro* have shown that ULL aqueous extract has antibacterial effect on *S. aureus*, and the combination therapy of the ULL aqueous extract and cefazolin sodium showed additive effect (Qin et al., 2013). By now, *in vivo* studies about antibacterial activity of the ULL aqueous extract and the combined antibacterial effect of the ULL aqueous extract with the antibacterial drugs have not been reported. Hence, we established mice model of pneumonia infected by *S. aureus*, and study the antibacterial effects of the ULL aqueous extract and that of ULL combined with cefazolin on *S. aureus*.

Materials and methods

Animal

Kunming (KM) mice weighing 24±2 g was taken from the Experimental Animal Center of Guangxi Medical University (Guangxi, China). The experiment was conducted according to the protocols approved by the Institutional Ethical Committee of Guangxi Medical University (approval no.: 201409208).

Drugs and reagents

Cefazolin sodium for injection (Batch number H13020668), hydrocortisone tablets (Batch number 12071531) and cyclophosphamide injection (Batch number H14023686) were obtained respectively from Hebei pharmaceutical co., LTD., Yang Zi Jiang pharmaceutical group co., LTD., and Shanxi Pude pharmaceutical co., LTD. The ELISA kits of IL-6 and IL-10 were purchased from Wuhan biological engineering co., LTD. IgG and IgM kit were purchased from Shanghai Zhicheng biological technology co., LTD. The continuous spectrum ELISA (Plus Spectra Max 384) was purchased from Hong Kong molecular instrument co., LTD.

The preparation of ULL aqueous extract

The medicinal herbs of ULL were gathered in Guangxi Guigang on October 2013, and identified as the aerial part of *Urena Lobata L.* (ULL) by Professor Cai Yi in the Guangxi University of Chinese Medicine. After soaking for 30 minutes in distilled water (weight 10 times the amount of ULL), the ULL medicinal materials was boiled for 1 h, and filtered. An equal amount of distilled water was add to the dregs of a decoction, and boiled for 1 h. Finally, the filtrate was merged, and enrichment to 2 g/ml (per milliliter liquids is equivalent to 2 grams of dried medicinal herbs), stored at 4°C for further research.

The preparation of bacterial suspension:

The standard strain of *S. aureus* (CC29213) was provided by the clinical laboratory of people's hospital in the Guangxi Zhuang Autonomous Region, and preserved in the clinical microbiology laboratory of the first affiliated

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hospital in Guangxi Medical University. The *S. aureus* strain was recovered in the nutrient broth at 37°C for 24 h, and switched to culture in MH AGAR plate, incubated in the incubator for 24 h at 37 °C. Finally, the colonies of *S.aureus* were collected from the AGAR, and diluted with sterile saline to 3.3 McFarland turbidity units (1×10^9 CFU/ml).

Experimental design and drug treatment

Male and female mice were half and half, Yes it is. Water and food were provided ad libitum. After 1 week of acclimatization period, the animals were divided randomly into the following seven groups (table 1) (n = 32/group). All drugs were given twice per day.

Table 1: Experimental design and drug treatment

| Group | Drugs | administration method | time |
|---|--|---|----------------------------|
| (I) The normal control group (naïve) | the same volume of saline | intragastric gavage | day 1 to 14 |
| (II) The <i>S. aureus</i> group (inf. Model) | the same volume of saline | intragastric gavage | day 1 to 14 |
| (III) The cefazolin group (Cefazolin) | cefazolin sodium (0.5 g/kg) | intramuscular injection | day 1 to 14 |
| (IV) The ULL high dose group (ULL HD) | ULL (40 g/kg) | intragastric gavage | day 1 to 14 |
| (V) The ULL middle dose group (ULL MD) | ULL (20 g/kg) | intragastric gavage | day 1 to 14 |
| (VI) The ULL low dose group (ULL LD) | ULL (10 g/kg) | intragastric gavaged | day 1 to 14 |
| (VII) The ULL+ cefazolin group (ULL+ Cefazolin) | ULL (20 g/kg) cefazolin sodium (0.5 g/kg) | intragastric gavaged intramuscular injection | day 1 to 14 day 1 to 14 |

• Before the experiment, mice in each group received hydrocortisone (25 mg/kg) in subcutaneous injection. Six hours later, mice received cyclophosphamide (25 mg/kg) by gavage once daily for 3 days. Twenty-four hours after the last treatment, mice in each group (II to VII) received *S. aureus* suspension liquid by nasal inhalation under ether anesthesia (Rodriguez - Martinez et al., 2008; Saini et al., 2008). The control group received nasal inhalation with the same volume of saline. After 24 h exposed to *S. aureus*, mice in each group were received the corresponding drugs. The doses of ULL were adopted according to the acute toxicity experiment results in our previous research (Xiao et al., 2015), and the dose of cefazolin sodium was converted from its adult clinical dose.

Specimen collection and testing indicators

During the experiment, the general conditions of mice were observed, including food and water intake, mental state, etc. After being exposed to *S. aureus* for 5 days, 10 days and 14 days, blood samples of all mice were collected from eyeball, the count of white blood cells (WBC) and neutrophils (NEU), neutrophil percentage (NEU%) in blood were detected using automatic blood cell analyzer, the levels of serum IgG and IgM were determined using ELISA. After exposed to *S. aureus* for 5 days (n=10/group), 10 days (n=11/group) and 14 days (n=11/group), mice were sacrificed, a part of the right lung tissue were weighed and made into homogenate at aseptic conditions, the levels of IL-6 and IL-10 of lung tissue were determined using ELISA. All performed procedures were strictly in accordance with

the kit requirements.

Lung tissue homogenate colony culture: The other part of the right lung tissue was weighed and made into homogenate at aseptic conditions, cultured in MH AGAR plate and incubated for 24 h at 37°C in incubator. Finally, the bacterial colony was counted. The colony count of lung tissue = (The number of colonies × diluted multiples)/lung tissue weight. Hematoxylin and eosin (H & E) staining: Lung fragments were fixed with 10% formalin, and then cut into sections. Finally, the sections were stained with H & E reagents for pathological histological examination.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows. Differences between groups were assessed using analysis of variance, and differences between two groups were assessed by T-test. These quantitative data were presented as the means ± SE. A *P*-value < 0.05 was considered to be statistically significant.

Results

The general conditions of mice

The general conditions, food and water intake of mice in the normal control group were not abnormal. The intake of water and food decreased, spirit was a bit poor, the activity decreased and body weight was lost in mice which exposed to *S. aureus*.

Effects of ULL on the colony count of *S. aureus* in mice lung tissue

The rate of *S. aureus* colony count/lung weight in the *S. aureus* group all increased significantly when compared with the normal control group (*P*<0.05) at 3 time points. The rate of *S. aureus* colony count/lung weight in ULL groups all decreased significantly when compared with *S. aureus* group (*P*<0.05) at 3 time points. Moreover, the rate of *S. aureus* colony count/lung weight both decreased significantly with the increase of the ULL dose and with the extension of time (*P*<0.05).

The rate of *S. aureus* colony count/lung weight decreased significantly in both the ULL+ cefazolin group and the cefazolin group when compared with the *S. aureus* group (*P*<0.05) at 3 time points. The rate of *S. aureus* colony count/lung weight in the ULL+ cefazolin group all decreased significantly when compared with the cefazolin group (*P*<0.05) at 3 time points. See Figure 1.

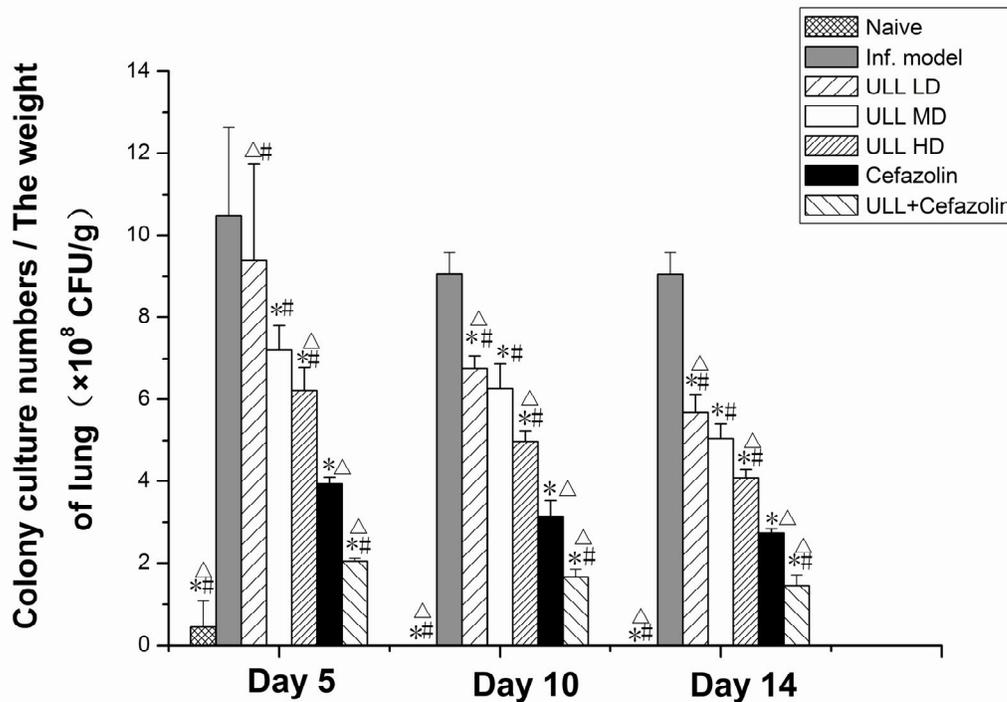
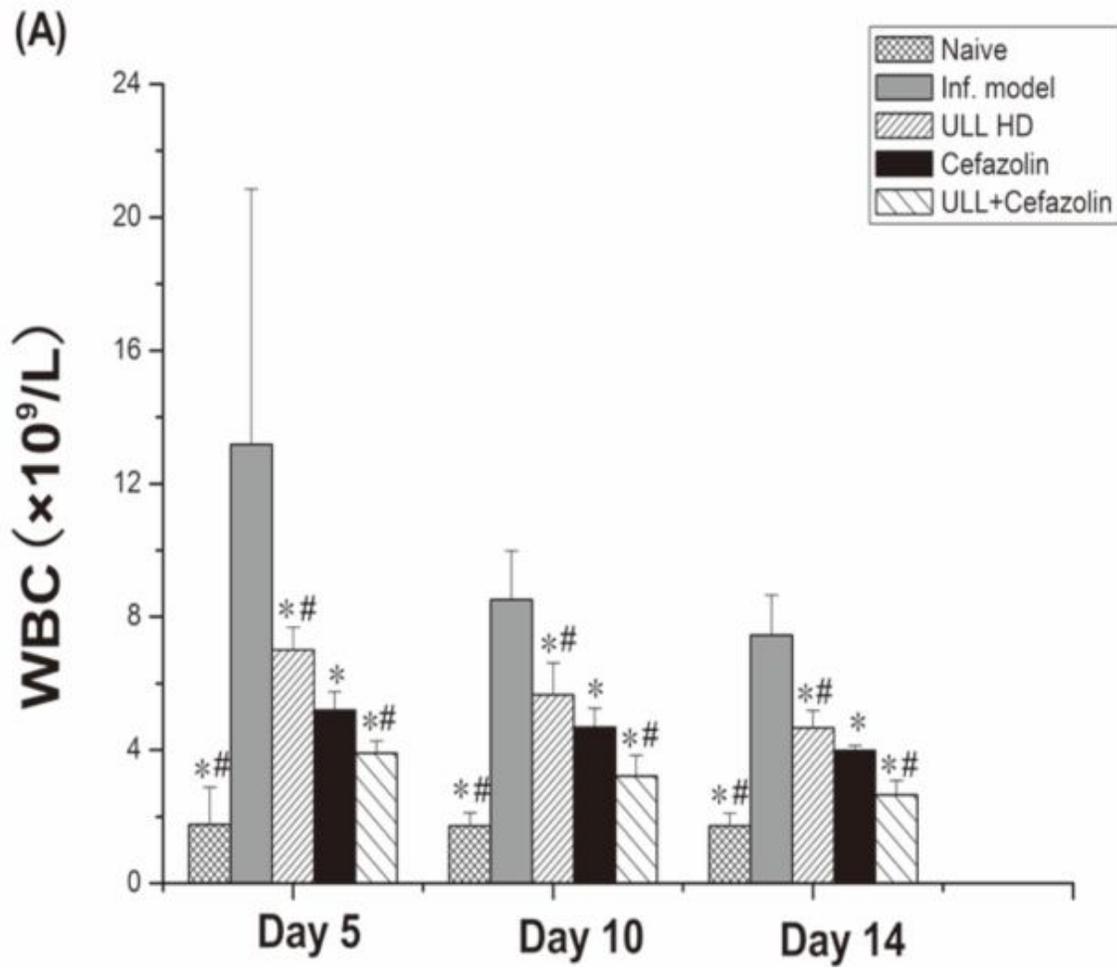


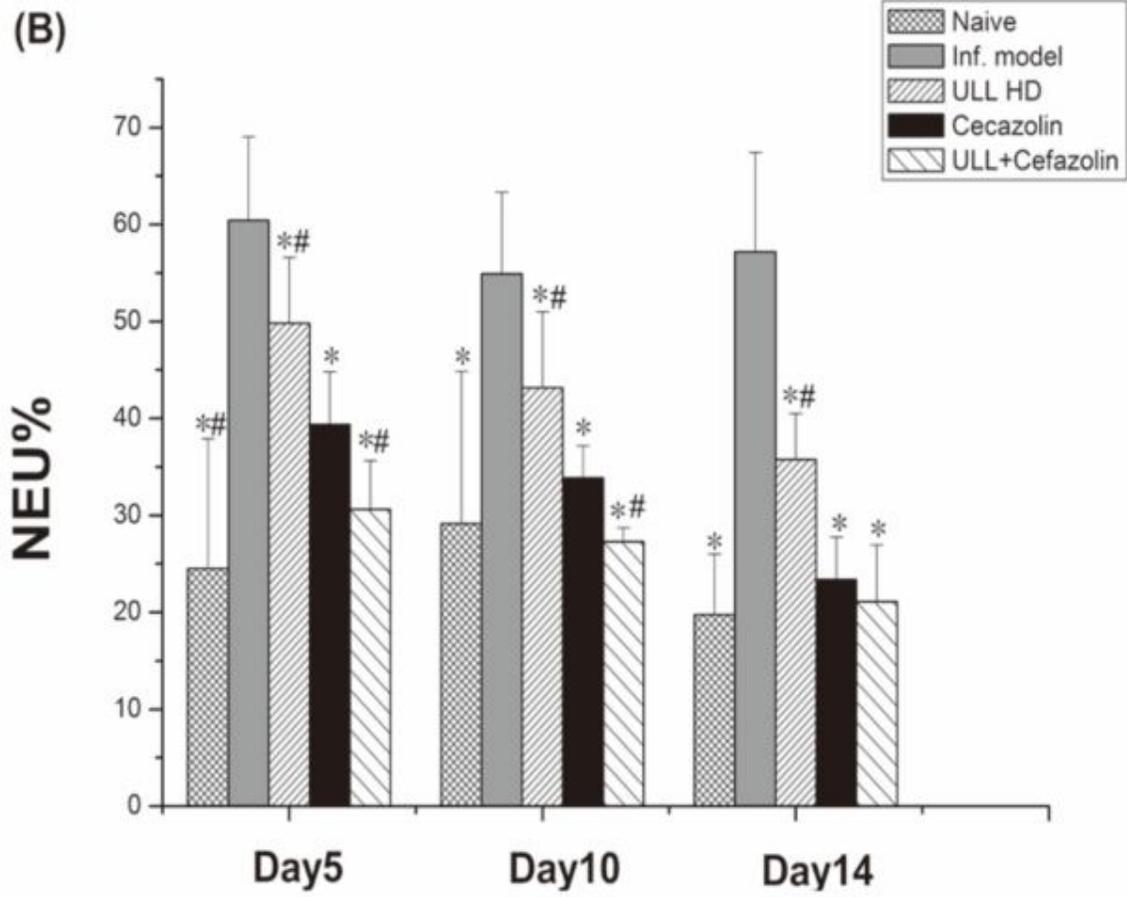
Figure 1: The effect of ULL on the rate of *S. aureus* colony count/lung weight in mice lung tissue. The results are presented as the means \pm SE. * $P < 0.05$ when compared with Inf. Model; $\Delta P < 0.05$ when compared with ULL MD; # $P < 0.05$ when compared with the cefazolin group. Naïve: the normal control group; Inf. Model: the *S. aureus* group; ULL HD: the ULL high dose group; ULL MD: the ULL middle dose group; ULL LD: the ULL low dose group.

Effects of ULL on the WBC, NEU and N% in mice Peripheral blood

The WBC, NEU and NEU% in the *S. aureus* group all increased significantly when compared with the normal control group ($P < 0.05$) at 3 time points. The WBC, NEU and NEU% in the ULL high dose group, the ULL+ cefazolin group and the cefazolin group all decreased significantly when compared with *S. aureus* group ($P < 0.05$) at 3 time points. The WBC, NEU and NEU% in these 3 treatment groups all decreased significantly with the extension of time ($P < 0.05$). See Figure 2.

The WBC, NEU and NEU% in the ULL+ cefazolin group all significantly decreased when compared with the cefazolin group ($P < 0.05$) at 3 time points, except that at day 14 the NEU% was no significant difference between these two groups.





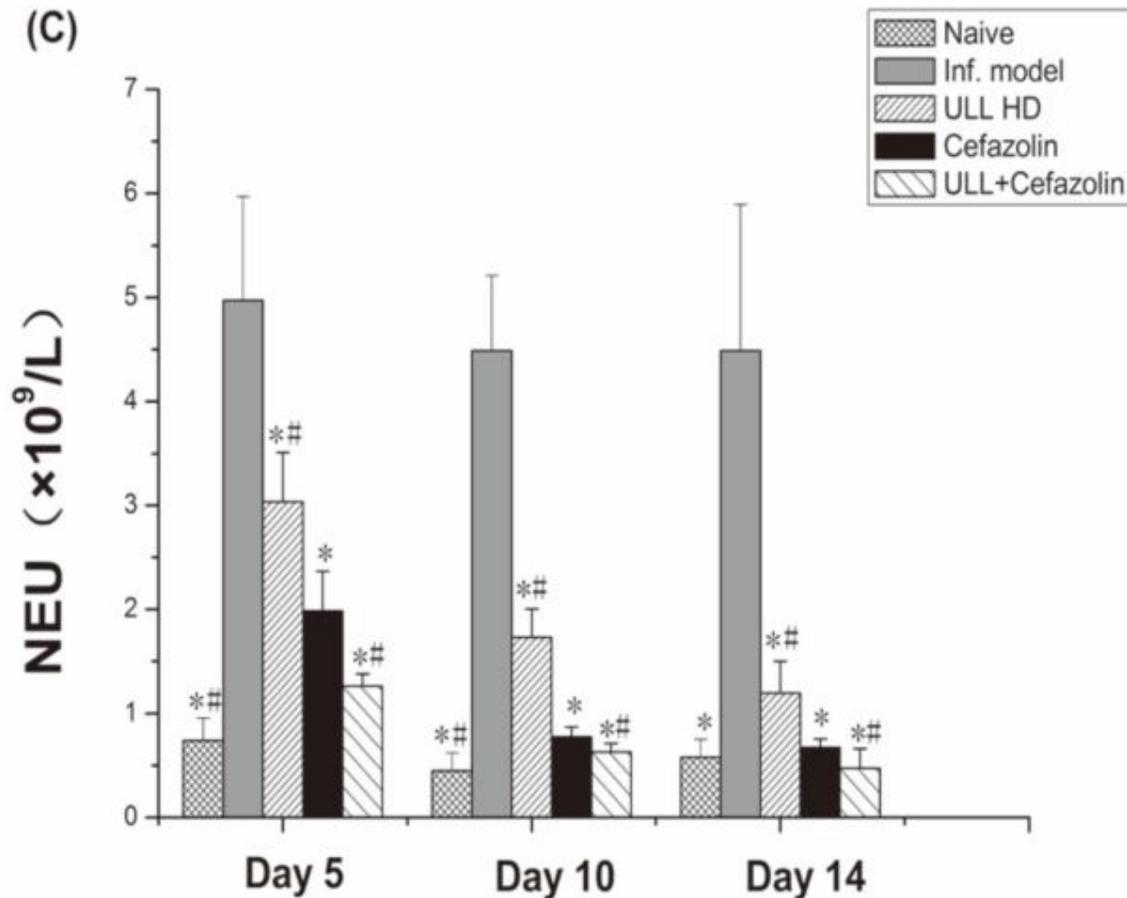


Figure 2: The effect of ULL on the WBC, NEU and NEU% in peripheral blood. The results are presented as the means \pm SE. * $P < 0.05$ when compared with the Inf. Model, # $P < 0.05$ when compared with the cefazolin group. Naïve: the normal control group; Inf. Model: the *S. aureus* group; ULL HD: the ULL high dose group.

Effects of ULL on the IgG, IgM in mice peripheral blood

As showed in Figure 3, the levels of IgG and IgM in mice peripheral blood in the *S. aureus* group at 3 time points all increased significantly when compared with the normal control group ($P < 0.05$). In addition, the levels of IgG and IgM in the ULL high dose group, the ULL+ cefazolin group and the cefazolin group all decreased significantly when compared with *S. aureus* group ($P < 0.05$) at 3 time points. The level of IgM in these 3 treatment groups all decreased significantly with the extension of time ($P < 0.05$). The level of IgG in the ULL+ cefazolin group and the cefazolin group all decreased significantly at day 10 and day 14 when compared with day 5 ($P < 0.05$). The levels of IgG and IgM in the ULL+ cefazolin group all significantly decreased when compared with the cefazolin group ($P < 0.05$) at 3 time points.

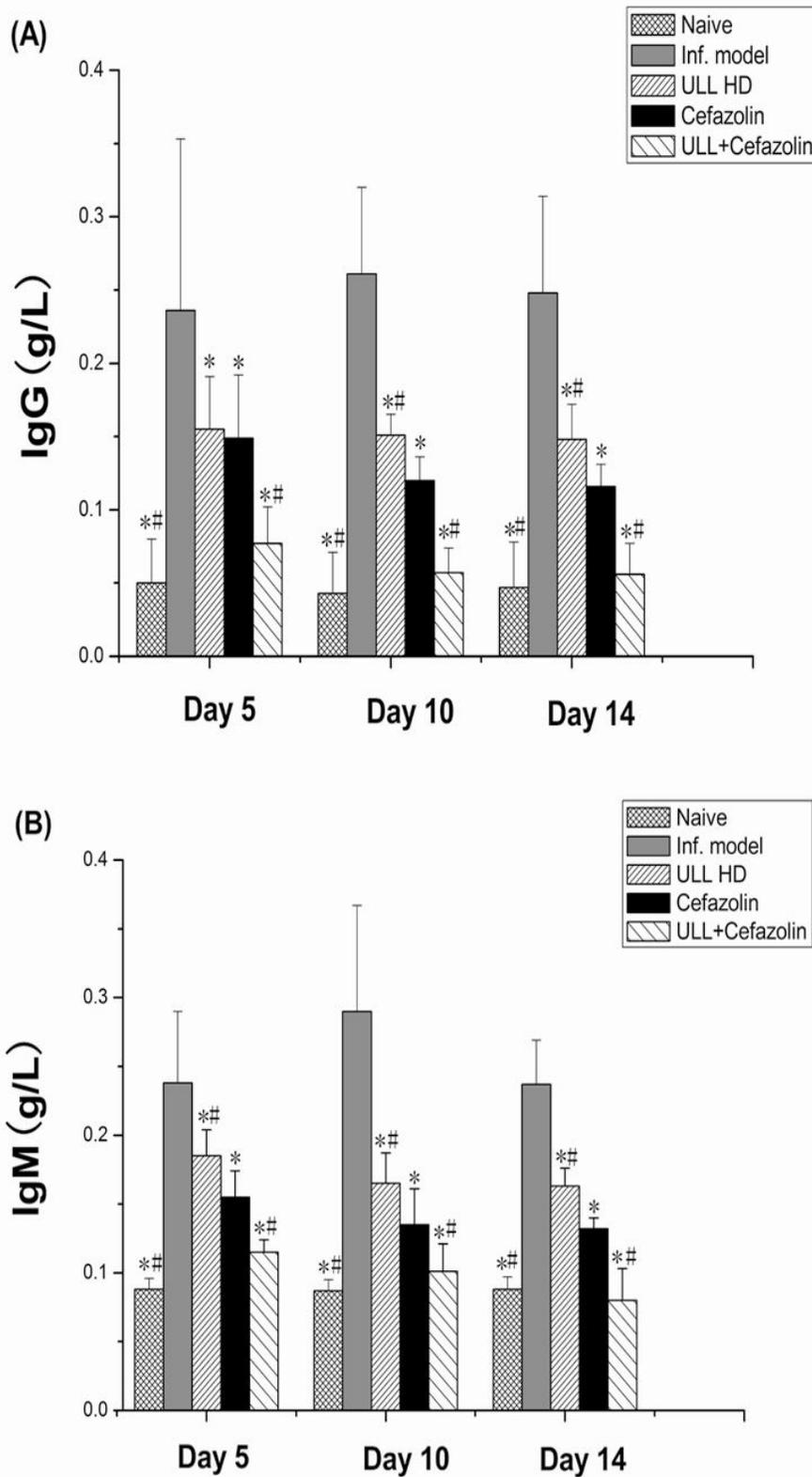


Figure 3: The effect of ULL on IgM and IgG level in blood. The results are presented as the means \pm SE. * $P < 0.05$ when compared with the Inf. Model group, # $P < 0.05$ when compared with the cefazolin group. Naïve: the normal control group; Inf. Model: the *S. aureus* group; ULL HD: the ULL high dose group.

Effects of ULL on the IL-6, IL-10 in mice lung tissue

As showed in Fig.4, the levels of IL-6 and IL-10 in mice lung tissue in the *S. aureus* group all increased significantly when compared with the normal control group ($P<0.05$) at day 5 and day 14. Moreover, they were all decreased significantly in the ULL high dose group, the ULL+ cefazolin group and the cefazolin group when compared with the *S. aureus* group ($P<0.05$). In addition, the levels of IL-6 and IL-10 in these 3 treatment groups all decreased significantly with the extension of time ($P<0.05$), they were all significantly decreased in the ULL+ cefazolin group when compared with the cefazolin group ($P<0.05$).

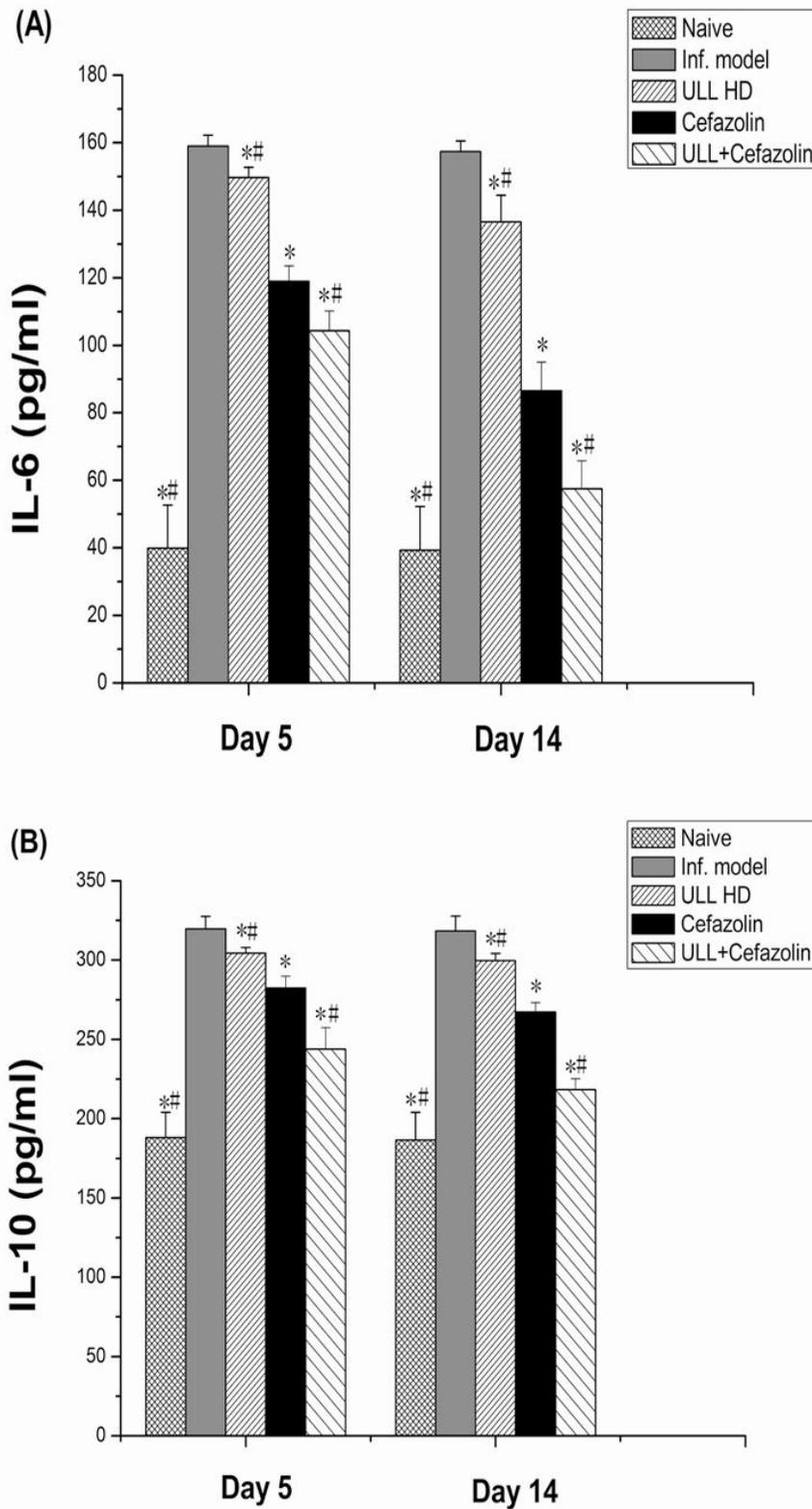
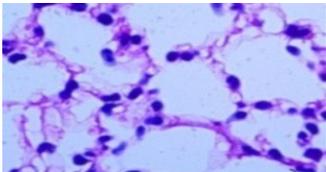
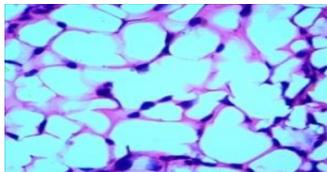
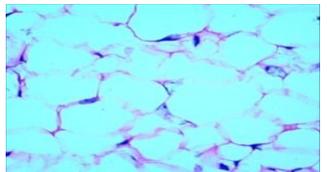
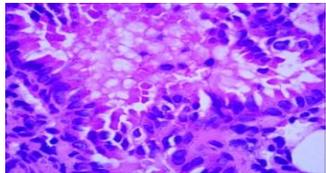
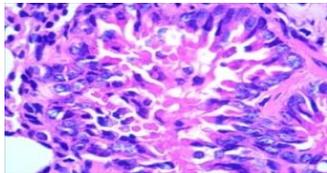
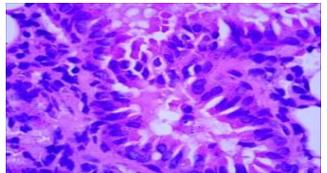
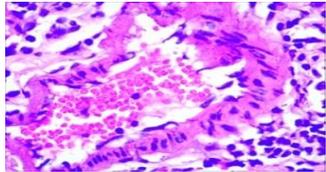
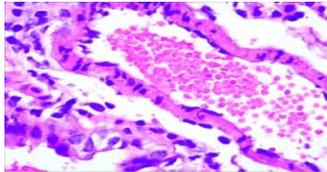
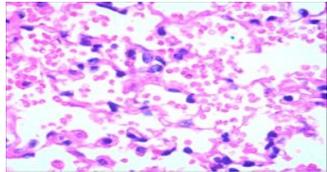
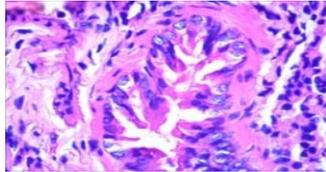
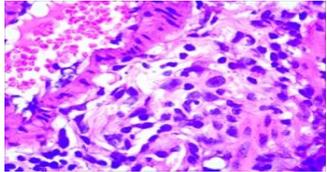
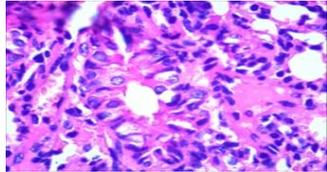
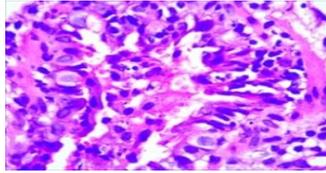
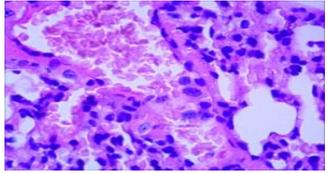
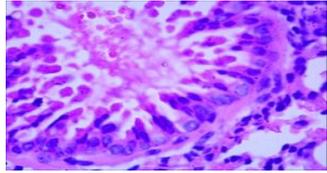
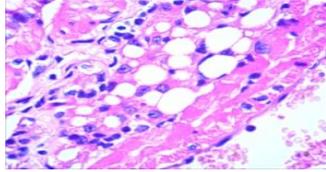
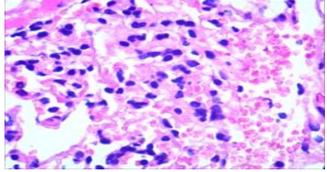
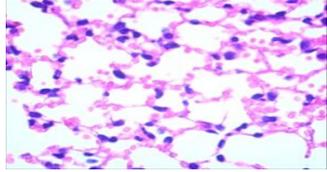


Figure 4: The effect of ULL on IL-6 and IL-10 level in lung tissue. The results are presented as the means \pm SE. * $P < 0.05$ when compared with the Inf. Model group, # $P < 0.05$ when compared with the cefazolin group. Naïve: the normal control group; Inf. Model: the *S. aureus* group; ULL HD: the ULL high dose group.

Effects of ULL on pathological examination of lung tissue

The structure of mice trachea and alveolar in the normal control group were intact, no inflammatory secretions could be seen. In the *S. aureus* group, inflammatory exudates and bacterial colonies in mice bronchial lumen, as well as angiectasis and hyperemia in pulmonary interstitial could be seen, and there were a large number of inflammatory cells infiltration in alveolar space and alveolar walls. Additionally, the alveolar normal structure was disappeared in the *S. aureus* group. The pathological damages of mice lung tissue in all treatment groups were improved when compared with the *S. aureus* group, and all improved along with the treatment time extension. Furthermore, the pathology damages of lung tissue in ULL groups were significantly improved along with the increase in the ULL dosage. The ULL+ cefazolin group significantly decreased the lung tissue pathology damages when compared with the cefazolin group. See Figure 5.

| | Day 5 | Day 10 | Day 14 |
|------------|---|--|---|
| Naïve |  |  |  |
| Inf. Model |  |  |  |
| ULL HD |  |  |  |
| ULL MD |  |  |  |
| ULL LD |  |  |  |
| Cefazolin |  |  |  |

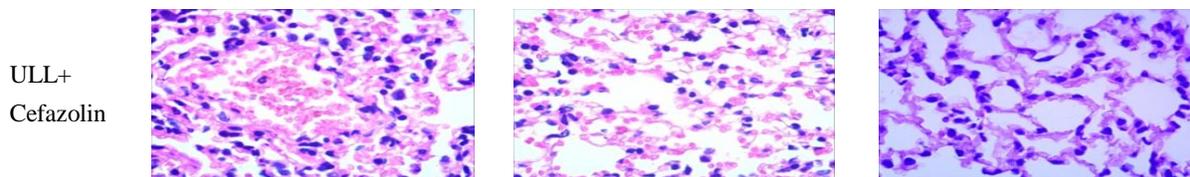


Figure 5: Histomorphological examination of mice lung tissue (H&E staining, 400×). Naïve: the normal control group; Inf. Model: the *S. aureus* group; ULL HD: the ULL high dose group; ULL MD: the ULL middle dose group; ULL LD: the ULL low dose group.

Discussions

mice model of pneumonia infected by *S. aureus*

S. aureus is a worldwide distributed pathogen which can produce serious diseases in many species, and it can cause infections in lung tissue, soft tissue and bloodstream, etc. Neutrophils can rapidly mobilize to sites of infection to help host defense and remove bacteria by phagocytosis, which form the first line of host defense against bacterial pathogens. Local immunization or infection recruit neutrophils from the blood to lymph nodes which are close to the local injection of *S. aureus* (Kamenyeva et al., 2015). In this experiment, the rate of *S. aureus* colony count/lung weight, the WBC, NEU and NEU% of peripheral blood in the *S. aureus* group all increased significantly when compared with the normal control group at 3 time points. There were inflammatory exudates and bacterial colonies in the mice bronchial lumens, a large number of inflammatory cells infiltrated in alveolar walls, and the normal structure of alveolar disappeared in the *S. aureus* group. These results demonstrate that the mice model of pulmonary infected by *S. aureus* was made success.

ULL has therapeutic effect on *S. aureus* pneumonia in mice

ULL, a member of the Malvaceae family, has been used as a traditional medicinal plant in China and India. The plant is a popular folk medicine used as febrifuge, diuretic, and also as a remedy for cough, dysentery and dropsy. It exhibited a variety of biological activities, including anti-inflammatory, antioxidant, anti-proliferative, and antibacterial activities (Mazumder et al., 2001; de las Heras et al., 1998; Pieme et al., 2010), anti-yeast activity (Gao et al., 2015). In our experiment, the rate of *S. aureus* colony count/lung weight, the WBC, NEU and NEU% in mice peripheral blood all decreased significantly after treatment with ULL. Moreover, these indicators decreased more significantly with the increase of ULL doses, and the longer duration of treatment. In addition, ULL could obviously improve the lung tissue pathological damage. Furthermore, the antibacterial activity of ULL combined with cefazolin to *S. aureus* pneumonia in mice significantly increased when compared with ULL or cefazolin. These results demonstrate that ULL has therapeutic effect on mice pneumonia infected by *S. aureus*. According to reports, dihydroxy benzoic acid (Jia et al., 2011), and flavonoid glycosides such as kaempferol have been isolated from the aerial parts of ULL (Jia et al., 2010). These compounds have the pharmacological effects such as antibacterial. In addition, ULL was found to possess significant antioxidant activity (Lissy et al., 2006), inhibit lipid peroxidation, scavenges hydroxyl and superoxide radicals. But it needs further study that whether the antioxidant activity of ULL is related to the effect of ULL on *S. aureus* pneumonia.

The effect of ULL on IgM and IgG level in blood

Natural immunoglobulin (Ig) M antibodies have been reported to protect against microbial infections. A monoclonal natural anti-keratin antibody IgM could bind directly to methicillin-resistant *S. aureus* (MRSA) and

mannose-binding lectin, it promoted the activation of the complement system in MRSA infected mice and played an important role in the anti-MRSA immune response (Joost et al., 2011). Patients showed significantly higher titers of IgM and IgG than controls, and patients with deep or severe infections of *S.aureus* showed higher titers than those with superficial or mild disease (Pujato et al., 2015). IgG, another natural Ig, is the highest amount of immune globulin in constitute of serum antibodies, it is the main force of the body against infection of pathogenic microorganisms. IgG can promote mononuclear macrophage phagocytosis to invading pathogenic microorganisms. One of IgG mAbs from mice induced by cell walls of *S. aureus* exhibited life-saving effects in mouse models of sepsis, and had a curative effect in pneumonia model caused by community-acquired MRSA strain (Ohsawa et al., 2015). IgG induced by wall teichoic acid (WTA) protects from infection with MRSA, its protective mechanisms are mediated in part by complement activation and opsonization and clearance of pathogens (Takahashi et al., 2013). Serum anti-WTA-IgG is a real trigger for the induction of classical complement-dependent opsonophagocytosis against *S.aureus* (Jung et al., 2012). There are a lot kinds of IgG in alveolar epithelial cells, they can reduce or prevent the occurrence of pneumonia (Riberdy et al., 1999; Oran et al., 2003). Our results showed that the levels of IgG and IgM in peripheral blood increased significantly in mice with *S. aureus* pneumonia. In addition, ULL, cefazolin and ULL+ cefazolin could decrease significantly the levels of IgG and IgM, and decreased significantly the IgM level with the extension of time. ULL combined with cefazolin can further reduce the level of IgG and IgM. However, it is still not clear that whether IgG and IgM take part in the protection of ULL against *S. aureus* pneumonia or not.

The effect of ULL on IL-6 and IL-10 level in mice lung tissue

IL-6 is a kind of inflammatory cytokine with multiple immune functions. There are reports that IL-6 plays an anti-inflammatory role during lipoteichoic acids-induced pulmonary inflammation, while it plays a proinflammatory role in peptidoglycan-induced acute lung inflammation (Leemans et al., 2002). Impaired clearance of pulmonary *S. aureus* was accompanied by altered cytokine expression such as decreased levels of IL-6, keratinocyte-derived chemokine (KC) in bronchoalveolar lavage (Olszewski et al., 2007). Intracellular killing and *in vivo* clearance of *S. aureus*, as well as resistance to *S. aureus* sepsis were significantly increased, and the serum levels of cytokines such as IL-6, IL-1 β were significantly higher in Olfm4 and gp91phox double-deficient mice than those in WT mice (Liu et al., 2013). Galarmin efficiently protects mice against lethal MRSA infection, and it significantly increased and modulated the levels of IL-10, IL-6 and KC in both peritoneal lavage fluid and blood, and also leukocyte counts, IgM and IgG in blood (Durgaryan et al., 2012). These reports showed that IL-6, IL-10 could successfully enhance immune defense against *S. aureus*. In our study, the levels of IL-6 and IL-10 in mice lung tissue increased significantly in mice with *S. aureus* pneumonia. In addition, they decrease significantly after ULL, cefazolin and ULL+ cefazolin treatment and with the extension of time. Moreover, ULL combined with cefazolin can further reduce the level of IL-6 and IL-10. However, it is still not clear that whether IL-6 and IL-10 take part in the protection of ULL against *S. aureus* pneumonia or not. In a word, ULL treatment could reduce the rate of *S. aureus* colony count/lung weight and NEU in lung tissue, and it could gradually improve the lung tissue damage. The antibacterial effect of ULL combined with cefazolin is better than that of ULL or cefazolin. These results show that ULL may have potential use as a therapeutic agent for *S. aureus* pneumonia. It needs further study to elucidate that whether ULL protects against mice *S. aureus* pneumonia through regulating the productions of IL-6, IL-10, IgG and IgM.

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Disclosure of conflict of interest

There are not any conflicts of interest to disclose among the authors.

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