Inhibitory Effect of the Traditional Chinese Medicine Ephedra sinica granules on Streptococcus pneumoniae Pneumolysin

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Streptococcus pneumoniae (S. pneumoniae) is an opportunistic pathogen that causes pneumonia, meningitis and other invasive diseases, with high mortality and morbidity among children under five years of age, especially in developing countries. Antibiotics are considered to be an effective strategy for the treatment of S. pneumoniae infection. However, with the emergence and spread of antibiotic resistance around the world, such a strategy is increasingly being threatened by increased medical costs, extended hospitalization, disability and even death. Thus, new treatment strategies or candidates are urgently needed to meet such challenges, especially for antibiotic-resistant S. pneumoniae infections.

Several strategies exist to replace antibiotics in the treatment of S. pneumoniae infection: vaccines, antimicrobial peptides, phage and natural compounds. Pneumococcal vaccines, including pneumococcal conjugate vaccine (PCV) and pneumococcal polysaccharide vaccine (PPV), are an important promising treatment strategy for pneumococcal disease. All PCVs are safe and well tolerated. However, the use of PCV7 led to an increased risk of severe lung disease, especially lung necrosis and parapneumonic empyema. This problem increased uncertainty around the clinical application of PCV10, PCV13 and PPV23. In addition, the purification and mass production difficulties indicate that antimicrobial peptides and bacteriophages are a long way from being commercially available. Plant products (natural compounds) have been used as clinical drugs throughout human history and are the main source of health care for approximately 70% of the world’s population. Recently, as an alternative strategy to fight bacterial infections, plant products have attracted great interest. Some plant products have been reported as promising candidates for the treatment of bacterial infections due to specifically targeting bacterial virulence factors (such as pore-forming toxins, sortase and secretion systems) rather than affecting the viability of bacteria. Such a strategy has been shown to reduce or even neutralize the pathogenicity of bacteria without putting significantly evolutionary pressure on the target bacteria. Thus, fewer resistant subgroups may proliferate following the treatment of bacterial infections with plant products.

Ephedra sinica, a gymnosperm shrub widely distributed across Central and Eastern Asia, is one of the oldest medicinal herbs with a divergent wind and cold effect for the treatment of colds, coughs, pneumonia and rheumatism. Modern medical studies have suggested that E. sinica has potential antineuroinflammatory, antioxidant, antiproliferative, antimicrobial, proapoptotic, anticancer, anti-inflammatory, antiphotoaging, diabetes mellitus treatment and arthritis treatment effects. Although antibacterial activity (only with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) data) has been reported, the antibacterial mechanism of Ephedra sinica granules (ESG), especially against S. pneumoniae, has not been elaborated. Here, the potential inhibitory effect of ESG on pneumolysin (PLY) activity and S. pneumoniae virulence was examined, the findings of which may partially elaborate the therapeutic effect by which ESG inhibits S. pneumoniae infection.

MATERIALS AND METHODS

Bacteria and Reagents S. pneumoniae strain D39 se-rotype 2 (NCTC 7466) was kindly provided by Professor...
Jian Huang from Zunyi Medical University (Zunyi, China) and was cultured in a 37°C shaker using Todd–Hewitt broth (THB, HOPEBIO Co., Ltd., Qingdao, China). *Ephedra sinica* granules (ESG), the prescription drug on the market (batch number: 19037174), was commercially obtained from Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Jianyin, China) and dissolved in dimethyl sulfoxide (DMSO, St. Louis, MO, U.S.A.). The major constituents (ephedrine hydrochloride and pseudoephedrine hydrochloride) in ESG were determined using HPLC, which was provided by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Jianyin, China). The HPLC was performed based on the “Pharmacopoeia of the People’s Republic of China (2015)” and the HPLC profile was provided by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Jianyin, China). (A) Standard comparison with ephedrine hydrochloride and pseudoephedrine hydrochloride. (B) HPLC chromatogram of ESG. (C) Blank control. The methanol–0.092% phosphoric acid solution (containing 0.04% triethylamine and 0.02% di-α-butylamine) (1.5: 98.5) was used as the mobile phase to detect the major constituents with the wavelength of 210 nm.

**Purification of Recombinant PLY** The vector (PET-28a-PLY with BamHI and XhoI endonuclease sites) for recombinant PLY expression is held in our laboratory. PLY purification was performed as previously described. Briefly, *Escherichia coli* BL21 harboring a recombinant vector was induced with IPTG (0.2 mM) at 16°C for 18 h, harvested by centrifugation (5000 rpm for 30 min), and lysed by sonication. Following centrifugation (18000 rpm for 60 min), the supernatant was loaded onto a Ni-NTA agarose column, and PLY was eluted with elution buffer (phosphate buffered saline (PBS) containing 200 mM imidazole, pH 7.4) and concentrated using a Millipore Amicon filter (30 kDa cutoff) for desalting.

**Hemolysis Assay** One microliter of purified PLY (0.2 mg/mL) was preincubated with various concentrations of ESG (0, 4, 8, 16 and 32 µg/mL) in 965 µL of PBS for 30 min at 37°C. Following the addition of 25 µL of defibrinated sheep red blood cells, the reactants were mixed and incubated for 10 min at 37°C. After centrifugation at 3000 × g for 5 min, the supernatant was measured at OD543 nm, and the hemolytic activity was measured by comparing each sample to the positive control (100% hemolysis). A sample treated with PBS or 1% Triton X-100 was used as a negative control or a positive control, respectively.

**Anti-*S. pneumoniae* Activity** *S. pneumoniae* strain D39 was cultured in THB with various concentrations of ESG (0, 4, 8, 16 and 32 µg/mL) at 37°C. Bacterial growth was determined by monitoring the optical density of each sample every 30 min at 600 nm. Additionally, the minimum inhibitory concentration of ESG against *S. pneumoniae* strain D39 was examined according to a previous study.

**Immunoblot Analysis** *S. pneumoniae* strain D39 was cultured in THB with various concentrations of ESG (0, 4, 8, 16 and 32 µg/mL) at 37°C. Following centrifugation (3000 rpm for 10 min) of the cultures, the supernatant was removed and boiled with Laemmli sample buffer for 10 min and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins of interest were transferred to a polyvinylidene difluoride (PVDF) membrane, labeled with a monoclonal antibody against pneumolysin (1:1000, Abcam, Cambridge, U.K.) and a horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibody (1:2000, Proteintech), and detected by a Tanon-4200 imaging system (Tanon, Shanghai, China) using an enhanced chemiluminescence (ECL) reagent (Thermo Scientific, Rockford, IL, U.S.A.). The equal histidine (His)-tagged protein (NDM-1) was added into each of the bacteria culture as the loading control, which was also detected using Western-blotting analysis.

**Oligomerization Analysis of PLY** PLY was preincubated with various concentrations of ESG (0, 4, 8, 16 and 32 µg/mL) at 37°C for 1 h. After 5× SDS-PAGE loading buffer without β-mercaptoethanol was added, the mixture was incubated at 50°C for 10 min. Then, 20 µL of each sample was separated by 6% SDS-PAGE, and the oligomerization of PLY was analyzed by immunoblot analysis as described above. The equal His-tagged protein (NDM-1) was added into each of the sample as the loading control.

**Cell Infection Assays** A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% trypsin-EDTA and 10% fetal bovine serum. The cells were harvested every 30 min after 5×10^5 cells were seeded into a 6-well plate (Nunc). After 24 h, the media were removed, and the cells were washed with PBS three times. Then, the cells were infected with 1×10^5 CFU/mL of *S. pneumoniae* strain D39 and cultured in DMEM supplemented with 1% fetal bovine serum at 37°C for 1 h. After infection, the supernatant was removed, and PBS was added to the wells. Then, the media were replaced with fresh DMEM supplemented with 1% fetal bovine serum and 1% trypsin-EDTA. The cell counts were measured by counting the number of cells in each well using a counting chamber (Coulter). The percentage of infected cells was calculated using the formula: 

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\text{Percentage of infected cells} = \left( \frac{\text{Number of infected cells}}{\text{Total number of cells}} \right) \times 100\%
\]

**Fig. 1. HPLC Profile of ESG**

The major constituents (ephedrine hydrochloride and pseudoephedrine hydrochloride) in ESG was determined using HPLC, which was provided by Jiangyin Tianjiang Pharmaceutical Co., Ltd. (Jianyin, China). (A) Standard comparison with ephedrine hydrochloride and pseudoephedrine hydrochloride. (B) HPLC chromatogram of ESG. (C) Blank control. The methanol–0.092% phosphoric acid solution (containing 0.04% triethylamine and 0.02% di-α-butylamine) (1.5: 98.5) was used as the mobile phase to detect the major constituents with the wavelength of 210 nm.
with 10% fetal bovine serum (FBS), trypsinized, seeded in 96-well plates at a density of $2 \times 10^4$ cells per well, and incubated overnight. Then, the cells were cocultured with ESG preincubated with PLY for 5 h at 37°C. Cells treated with 1% Triton X-100 or DMEM were used as positive or negative controls, respectively. Following a centrifugation step (1000 rpm for 10 min), the lactic acid dehydrogenase (LDH) level in the supernatants was examined by a cytotoxicity detection kit (Roche, Mannheim, Germany) according to a previous study. Additionally, the viability of infected A549 cells was observed under a confocal laser scanning microscope (Olympus, Tokyo, Japan) using live/dead (green/red) reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions.

**Mouse Infection Assays** BALB/c mice (female, 6–8 weeks, 20–22 g) were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, China) and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the mouse infection assays were approved by the ethics committee of Changchun University of Chinese Medicine.

*S. pneumoniae* strain D39 was cultured in THB at 37°C until the OD600 nm reached 0.4 (mid-logarithmic phase), collected by centrifugation (1000rpm for 10 min) and mixed three times with PBS. The mice were divided into three groups, and each mouse was transnasally infected with 1.5×10⁸ colony forming units (CFUs) of bacteria, except for the healthy control group. Then, the mice were subcutaneously administered ESG (40mg/kg) or DMSO every 8h. For survival analysis, mouse death was observed for 72h ($n = 10$). For other mouse infection assays ($n = 8$), the lungs from sacrificed mice were used for gross pathological analysis by camera or histopathological analysis by hematoxylin–eosin staining under light microscopy at 48h postinfection. Furthermore, the bronchoalveolar lavage (BAL) fluid from other mice was collected and centrifuged. The levels of cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-6) in the supernatants were determined using mouse enzyme-linked immunosorbent assay (ELISA) kits (Biolegend) according to the manufacturer’s recommendations.

**Statistical Analysis** The data were expressed as the mean ± standard deviation (S.D.) ($n \geq 3$) and analyzed by GraphPad Prism 6.0 (GraphPad Software) using two-tailed independent-sample $t$-test and one-way ANOVA followed by Tukey’s post hoc multiple comparison test. *, $p < 0.05$ and **, $p < 0.01$.

**RESULTS**

**ESG Directly Inhibits PLY-Mediated Hemolytic Activity** PLY, which is one of the most important virulence factors involved in *S. pneumoniae* pathogenicity, has hemolytic activity. Here, using a hemolysis assay, we found that the hemolytic activity of PLY was significantly inhibited in the presence of ESG (Fig. 2A). Almost no hemolytic activity was observed in the sample treated with 16$\mu$g/mL ESG, and the IC₅₀ for this inhibition level was 1.17$\mu$g/mL. Then, we further determined whether ESG affects *S. pneumoniae* growth or PLY production. As shown in Fig. 2B, the growth curve indicated that ESG treatment had no visible influence on *S. pneumoniae* growth at the concentrations required for the inhibition of PLY activity. Additionally, the MIC of ESG against *S. pneumoniae* D39 was greater than 256$\mu$g/mL. The addition of ESG to *S. pneumoniae* cultures did not affect the production of PLY (Fig. 2C). Taken together, our results demonstrate that ESG treatment does not influence the growth of *S. pneumoniae* and PLY production, but such treatment could...
directly affect the hemolytic activity of PLY in a concentration-dependent manner.

**ESG Reduces PLY Oligomerization** PLY is a water-soluble monomer that binds to the target cell membrane where it oligomerizes to form circular pores, causing cell lysis and death. Therefore, we speculated that ESG may prevent the oligomerization of PLY monomers, causing the loss of the PLY pore-forming activity. An oligomerization analysis assay showed that PLY oligomers formed in the sample without ESG treatment (Fig. 3). As expected, PLY oligomerization was visibly reduced following treatment with various concentrations of ESG (Fig. 3). The oligomerization inhibition tendency was similar to the hemolytic activity inhibition tendency. Together, our results suggest that ESG inhibits the activity of PLY by inhibiting PLY oligomerization.

**ESG Alleviates PLY-Induced A549 Cell Damage** To further validate the effect of ESG on PLY-induced cytotoxicity, A549 cells were co-incubated with ESG pretreated PLY. The degree of cell damage was semiquantitatively and quantitatively examined by live/dead (green/red) analysis and LDH release analysis, respectively. Consistent with the above results, most cells died following incubation with PLY, as indicated by the red fluorescence (Fig. 4A). However, the red fluorescence intensity visibly decreased, and the green fluorescence intensity increased in the sample treated with ESG suggesting that ESG treatment markedly alleviates A549 cell damage induced by PLY (Figs. 4B, C). In addition, almost no red fluorescence was observed in the cells treated only with 32 µg/mL ESG, indicating that ESG is not cytotoxic at concentrations below 32 µg/mL (Fig. 4D). Consistent with these results, the level of LDH in the supernatants from the A549 cells incubated with PLY was significantly reduced in the presence of ESG in a dose-dependent manner (Fig. 4E). Taken together, our results establish that ESG treatment reduces PLY-induced damage in A549 cells.

**ESG Protects Mice against *S. pneumoniae* Pneumonia** To further verify that the *in vitro* protection occurs *in vivo*, mice were infected with *S. pneumoniae* to construct a pneumonia model. As shown in Fig. 5A, most of the infected mice died at 72h postinfection, and no mice died in the healthy control group; however, the mortality of infected mice that received 40 mg/kg ESG was decreased (Fig. 5A). The physical and pathological examination of the lungs of infected mice revealed marked redness, swelling and congestion in the gross pathological analysis and substantive lesions and inflammatory cell infiltration in the histopathological analysis (Figs. 5B, C). As expected, this pathological damage was visibly alleviated in infected mice treated with ESG (Figs. 5B, C). Furthermore, to evaluate the degree of inflammation in infected mice, the levels of the cytokines TNF-α and IL-6 in the BAL were determined by ELISA. Consistent with the pathological damage analysis, the levels of both TNF-α and IL-6 were significantly reduced in infected mice following treatment with ESG (Fig. 5D). Taken together, our results establish that ESG treatment systemically protects against *S. pneumoniae* pneumonia in mice.
S. pneumoniae has evolved a variety of virulence-associated proteins to facilitate the establishment of infection, among which PLY is a major virulence determinant. When S. pneumoniae invades the body, PLY is released into the cytoplasm in large quantities and forms a pore structure in the cell membrane, causing the loss of important metabolites and cellular components and causing cell death. This process triggers the production and release of proinflammatory chemokines and cytokines, including IL-1β, CXC-motif and CC-motif chemokines, and type 1 interferons, which trigger inflammatory responses and mucus secretion and facilitate S. pneumoniae transfer to a new host. Zafar et al. reported that PLY promotes mucosal inflammation and is required for pup-to-pup transmission of S. pneumoniae. In addition, PLY inhibits cilia-mucus clearance in the human lung, separates tight junctions between cells (promotes tissue penetration), and exposes new sites of pneumococcal attachment. The classical complement pathway is also activated by PLY release from bacteria through a domain similar to the Fc component of immunoglobulin G (IgG), thereby reducing serum op-

Fig. 5. ESG Protects Mice from S. pneumoniae Pneumonia

Mice were transnasally infected with $1.5 \times 10^8$ CFUs of S. pneumoniae strain D39 and subcutaneously administered E. sinica or DMSO. (A) The survival of infected mice with the indicated treatments was observed continuously over 72 h. At 48 h postinfection, the lungs from mice were used for gross pathological analysis (B) and histopathological analysis (C). (D) The cytokine (TNF-α and IL-6) levels in BAL fluid were determined by mouse ELISA kits. Data are expressed as the mean ± S.D., and the significance was analyzed using *t*-test. **, $p < 0.01$. (Color figure can be accessed in the online version.)
sonic activity.\textsuperscript{23} Thus, PLY is an indispensable and important virulence factor for \textit{S. pneumoniae} and an ideal target for the development of anti-infective agents for the treatment of \textit{S. pneumoniae} infections.

Here, we found that treatment with the traditional Chinese medicine ESG significantly inhibited the pore-forming activity of PLY at relatively low ESG concentrations ranging from 1 to 16\textmu g/mL, indicating that ESG is an effective PLY inhibitor. Furthermore, treatment with ESG did not affect the viability of \textit{S. pneumoniae} or the production of PLY. These results suggested that ESG treatment would put milder selective pressure on \textit{S. pneumoniae} than traditional antibiotics based on bactericidal and bacteriostatic activity.\textsuperscript{15} Consistent with this hypothesis, almost no bacterial resistance to ESG has been reported. In China, ESG has been used effectively to treat upper respiratory tract infections for thousands of years, but the mechanism of action of this drug has not been elucidated.\textsuperscript{17}

Our further results indicated that ESG treatment reduces the oligomerization of PLY, subsequently inhibiting the activity of PLY. As expected, the cell injury induced by PLY was significantly alleviated following ESG treatment. Furthermore, ESG provided systemic protection against \textit{S. pneumoniae} in mice. Thus, the data presented in this study demonstrate that ESG is a promising agent for the treatment of \textit{S. pneumoniae} infection. Although various biological activities have been reported for ESG,\textsuperscript{23} which may also contribute to the antibacterial effects, our results partially elucidate the mechanism of ESG action against pneumococcal disease.

In summary, the results presented here demonstrate the viability of ESG as an effective drug for the clinical treatment of \textit{S. pneumoniae} infections via the targeting of a bacterial virulence factor rather than antibacterial activity. Furthermore, the therapeutic effect will be improved through the targeting of virulence factors and bacteria when ESG is used in combination with antibiotics. These data pave the way for the development of traditional Chinese medicines combined with antibiotics for the treatment of bacterial infections.

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Conflict of Interest The authors declare no conflict of interest.

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