Luteolin Binds Streptolysin O Toxin and Inhibits Its Hemolytic Effects and Cytotoxicity

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INTRODUCTION

GAS is a Gram-positive bacterium that causes a variety of diseases ranging from pharyngitis, erysipelas, and cellulitis to more severe, life-threatening diseases such as streptococcal toxic shock syndrome and necrotizing fasciitis. Additionally, GAS infection can also cause severe immune-mediated diseases such as acute glomerulonephritis, rheumatic fever, and rheumatic heart disease (Martin and Green, 2006; Steer et al., 2007; Walker et al., 2014). Current research reports that nearly 50,000 people die of GAS infection annually (Nelson et al., 2016).

As a human adapted pathogen, GAS is protected by a number of cell surface-bound and secreted virulence factors that contribute to its versatility as a pathogen and help it to subvert host innate immune defenses including phagocytosis, complement deposition, antibody opsonization,
antimicrobial peptides, and neutrophil killing mechanisms (Mitchell, 2003; Walker et al., 2014). Among them, 
Streptolysin O (SLO), an extracellular product produced by almost all GAS strains that belongs to the cholesterol-
dependent cytolysin (CDC) proteins, is a conserved family of β-
barrel PFTs, is one of the most important virulence factors in the 
GAS arsenal (Shewell et al., 2014; Lukoyanova et al., 2016). PFTs 
are one type of important virulence factors including α-
Hemolysin (Hla), Pneumolysin O (PLY), Listerialysin O 
(LOO), and Suyllisin (SLY), etc. (Los et al., 2013; Dal Peraro 
and van der Goot, 2016). The SLO monomer binds to the host 
cell membrane through the conserved region of the C-terminal 
domain 4 (D4), then forms a protein multimer to create a 
barrel structure that enables it to penetrate the host cell, 
resulting in cell lysis and subsequent organ system dysfunction 
during invasive infections (Tweten, 2005; Rossjohn et al., 2007; 
Mozola and Caparon, 2015; van Pee et al., 2017). Additionally, 
during GAS infection SLO mediates the transfer of NAD+ 
glycohydrolase SPN into the host cell cytosolic compartment, 
in which it exerts its toxic effects (Bricker et al., 2002). Studies 
have shown that SLO plays a key role in related disease 
phenotypes and outcomes caused by GAS infection, therefore, 
specific anti-toxin treatments are expected to impact the clinical 
outcome (Clarke et al., 2021). Thus, screening for inhibitors of 
SLO would facilitate the development of anti-SLO treatments for 
infections.

With the increasing resistance of bacteria to antibiotics, more 
and more researchers are developing new antibacterial drugs by 
targeting virulence factors such as hemolysin (Jiang et al., 2019; 
Lu et al., 2019; Vite et al., 2020). Chinese herbal medicines are rich 
in resources and have various components. Starting from a 
foundation of Chinese herbal medicines and then focusing on 
their active ingredients may have broad prospects in the 
development of new antibacterial drugs against bacterial 
virulence factors. In clinical treatment of tonsillitis and other 
diseases caused by GAS-infection, Traditional Chinese Medicines 
with “clear away heat and toxic material” are often used, such as 
Tanacetum parthenium, Trollius chinensis, Taraxacum officinale, 
Scutellaria barbata, Schizonepeta tenuifolia, Satureja parvifolia, 
Platycodon grandiflorum, etc. These medicines all contain 
flavonoid compounds. Flavonoids, also known as polyphenols, 
have been widely studied because of their good bacteriostasis, 
including the inhibition of multi-drug resistant bacteria (Biharee 
et al., 2020). Luteolin is a member of the flavonoid family of 
natural compounds, which are commonly found in many types of 
plants used for medicine and food such as natural herbal drugs, 
vegetables, fruits, luteolin-rich herbal extracts have been widely 
used as new traditional herbal medicines and possesses numerous 
biological effects including anti-inflammatory, anti-oxidant, anti-
hypertensive, anti-cancer activities (Lin et al., 2008; Lopez- 
Lazaro, 2009). Additionally, luteolin has been proved to have 
antibacterial activity against many types of bacteria (Lv et al., 
2009; Siriwong et al., 2015; Guo et al., 2020) and can eliminate 
antibiotic resistance in multidrug-resistant Trueperella pyogenes 
(Zhang et al., 2019). Recent studies indicate that luteolin can 
inhibit the formation and apoptosis of macrophage foam cells, 
can suppress activation of the NLRP3 inflammatory corpuscle 
and promote polarization of macrophages towards the M2 
phenotype (Chen et al., 2014; Zhang et al., 2016).

As mentioned earlier, the SLO protein is a key virulence factor 
of GAS and plays an important role in its infection process. 
Therefore, we tested the hypothesis that luteolin may be an 
effective compound against SLO protein-related functional 
activities. Here, we showed that luteolin binds SLO with high 
affinity through a non-conventional binding site located in 
domains 1 and 3. This binding activity inhibits SLO 
cytotoxicity in vitro in HEp-2 and also significantly reduces its 
hemolytic effects in red blood cells (RBCs). These results support 
the hypothesis that luteolin protects cells from SLO toxicogenic 
effects. In addition, we also verified the anti-infection effect of 
luteolin in vivo through a mouse infection model.

MATERIALS AND METHODS

Bacterial Strain, Cell and Culture Conditions

Group A Streptococcus (ATCC 700294) was selected for use in this 
study. The selected GAS strain was cultured at 37°C in Todd-Hewitt 
brøth amended with 0.2% yeast extract (THY) (BD, United States). 
Human laryngeal carcinoma epithelial type-2 cells (HEp-2) were 
purchased from ATCC and cultured in Dulbecco’s Modified Eagle 
Medium (DMEM) (Sigma, United States), supplemented with 10% 
fat bovine serum (FBS) (Gibco, United States), and 100 μg/ml of 
penicillin and streptomycin (Solarbio, China). Cells were cultured at 
37°C under 5% CO2 and counted using the Invitrogen Countess II 
chamber (Thermo Fisher, United States).

Luteolin (purity ≥ 98%) was purchased from Shanghai yuan ye 
Bio-Technology Co., Ltd. (Shanghai, China) and dissolved in 
distilled water to make stock solutions of various concentrations. 
The luteolin solution was filter sterilized through a 0.22 μm 
microfiltration membrane.

Construction, Expression, and Purification of Proteins

Primers were designed to amplify the GAS gene encoding SLO 
using DNA extracted from GAS strain M1 as the template; the 
primers used are listed in Table 1. BamH I and Sac I restriction 
enzymes were used to digest pET28a prokaryotic expression 
vector and the amplified SLO gene, were then cloned into the vector 
using a Gibson assembly strategy (NEB). To express the 
recombinant protein, the pET28a-SLO vector was transformed into 
Escherichia coli BL21 (DE3). PLY, LLO, SLY were 
constructed in the same way (strains used were Streptococcus 
pneumoniae TIGR4, Listeria monocytogenes 10403S, and 
Streptococcus suis 05ZYH33; primers are listed in Table 1). 
The cells were cultured at 37°C until absorbance at OD600 
reached 0.6–0.8, SLO, PLY, LLO expression was induced with 
0.5 mM IPTG, and cells were harvested after growing for an 
additional 4 h at 25°C or overnight at 16°C. SLY was induced with 
1 mM IPTG and harvested after growing 4 h at 28°C. The 
harvested cells were resuspended in washing buffer consisting of 
20 mM PB, 20 mM imidazole, and 0.5 M NaCl (pH 7.4), and 
lysed by sonication. The cell lysate was centrifuged at 8,000 × g for
10 min, and the supernatant was loaded onto a HisTrap HP column. The recombinant protein was bound to a Nickel-affinity chromatography column, and the column was flushed with wash buffer consisting of 20 mM PB, 0.5 M imidazole, and 0.5 M NaCl (pH 7.4). The His-tagged protein was eluted in elution buffer containing 20 mM PB, 0.5 M imidazole and 0.5 M NaCl (pH 7.4). The purified protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Hemolytic Activity Assay**

RBCs hemolysis assay was modified with reference to previous protocols (Dong et al., 2018; Dong et al., 2021). Briefly, SLO (0.1 μg/ml) was added to the solution with or without luteolin; Sheep RBCs were harvested and added to the solution to a final concentration of 2% (v/v). The whole mixture was incubated for 30 min at 37°C and centrifuged at 5,000 rpm to obtain the OD₅₄₀ of the supernatants. Samples treated with 0.1% Triton-X 100 were fully hemolytic and used as positive controls. The final hemolysis ratio was expressed as: [OD₅₄₀(samples)/OD₅₄₀(positive controls)] × 100%. In addition, the hemolytic activity of SLY, PLY, and LLO proteins was measured by the same way. To evaluate the effect of luteolin on the hemolytic activity of natural SLO protein, GAS culture supernatant was harvested with centrifugation at 10,000 rpm for 5 min. Subsequently, 10% (v/v) GAS culture supernatant was added to the hemolysis buffer with or without luteolin for 30 min prior to detection, and the hemolysis efficiency was determined using the same method performed with SLO protein.

**Molecular Docking**

The small molecule ligand was set to be flexible, and the protein receptors were rigid. Luteolin was docked into SLO (PDB code: 4hsc), SLY (PDB code: 3hvn), PLY (PDB code: 5cr6), and LLO (PDB code: 4cdb) 3D X-ray structures, using the docking program AutoDock 4.0 (AutoDock program, The Scripps Research Institute, La Jolla, CA) (Morris et al., 2009; Hu et al., 2010). PyMOL and Ligplus programs were used to visually analyze the docking conformation.

**Molecular Dynamics Simulation and Calculation of the Binding Free Energy.**

A Molecular dynamics simulation (MD simulation) of the SLO-luteolin complex was carried out using the Gromacs 5.0.4 package to explore the binding mode of the complex. The protein used ff14SB force field parameters, the small molecule ligand used gaff general force field parameters, and the atomic charge of AM1-BCC was calculated by the ANTECHAMBER module. The binding free energy of the complex was calculated by the MMPBSA.py module (Jogalekar et al., 2010; Vorontsov and Miyashita, 2011).

**Surface Plasmon Resonance Analysis**

The affinity and kinetics of luteolin to SLO, SLY, PLY, LLO were measured by surface plasmon resonance (SPR) at 25°C on a REICHERT 4SPR (Reichert, United States) using carboxymethyl dextran hydrogel surface sensor chips. Proteins were dissolved in 10 mM sodium acetate (pH 4.5) and immobilized on the chip with 6,000 response units (RU) at a flow rate of 10 μl/min. Luteolin was serially diluted in PBST buffer (PBS containing 0.005% Tween 20) to concentrations of 0.125, 0.25, 0.5, 1, 2, and 4 μg/ml. Each of the six concentrations was injected at a flow rate of 25 μl/min for 3 min; for dissociation, the flow rate was set at 25 μl/min for 5 min. To regenerate channels, 10 mM glucopyranoside (Sigma-Aldrich St. Louis, MO, United States) was injected for 90 s at a flow rate of 25 μl/min, followed by injection of PBST buffer for 15 s until the RU reached the original reading. All injections were performed at 25°C. Reference cell values and signal from buffer injection controls were subtracted, and the sensorgram traces were fitted to a 1:1 Langmuir binding model using the data analysis program Scrubber 2.0 (BioLogic Software, Canberra, Australia) to calculate ka, kd, and KD values. The figures were made using Prism (GraphPad Software, Inc.).

**Determination of Protein Secondary Structure**

MMS measurements were conducted using the automated AQS3 Pro system (RedshiftBio) with AQS3 analytics software. Solutions of SLO protein and protein + luteolin, each at 0.8 mg/ml, were prepared in PBS. All samples and their corresponding buffer blanks were preloaded into a 24-well plate in a pairwise manner. The samples and buffers were degassed using a built-in well plate degasser for 30 min. An automated testing protocol, including all reference buffer and sample measurements, was set up in the acquisition software in triplicate for each experiment.

**Determination of Melting and Aggregation Temperatures**

Thermal and colloid stabilities of SLO proteins before and after luteolin treatment were evaluated using the Uncle/UNIt system...
(Unchained Labs, Pleasanton, CA, United States). The barycentric mean (BCM) and static light scattering (SLS) at 266 nm were used to monitor the evolution of protein structures in response to increasing the temperature from 25°C to 95°C at a rate of 0.5°C/min. The label-free fluorescence from intrinsic aromatic amino acid residues excited by the inset excitation wavelength of 266 nm was collected for melting temperature (the midpoint of unfolding event, Tm) calculation, while SLS at 266 nm was monitored, respectively, to determine the temperature where SLO protein starts to aggregate (the starting point of aggregation event, Tagg).

Oligomerization Analysis

Purified SLO (1 mg/ml) incubated with different concentrations of luteolin was incubated at 37°C for 30 min. Purified SLO without the luteolin was used as a control. An equal volume of each sample was incubated at 40°C for 10 min in 1 × SDS-PAGE loading buffer without β-mercaptoethanol (β-ME). Equal quantities of bacterial protein were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with rabbit polyclonal antibody raised against Streptolysin O (1:500; Abcam, United Kingdom), and probed with Goat anti-Rabbit IRDye®680RD (1:5,000, LI-COR, United States). The protein bands were visualized using an Odyssey SA (LI-COR, United States).

Cell Viability Assay and Protection Experiment

To test the protection effect of luteolin, a cell culture model of HEp-2 cells were established. And first, the effect of luteolin on the viability of HEp-2 cells was determined by a cell counting kit-8 (CCK-8) assay according to the manufacturer’s description (MCE, United States). Cells were seeded at a density of 1 × 10⁴ into 96-well plates with or without different concentrations of luteolin, and DMEM alone and the CCK-8 reagent system, with or without cells, were used as the control group and blank group, respectively. After incubation at 37°C for 24 h, 10 μl of CCK-8 solution was added to each well and cells incubated for 3 h more, after which the absorbance at OD450 was measured using a multifunctional enzyme labeling instrument (Thermo Fisher, United States).

For determination of IC50 values, dose-response (1, 2, 4, 8, and 16 μg/ml SLO) and time-course (0.5, 3, and 6 h) experiments were performed. Briefly, HEp-2 cells were seeded in flat-bottomed 96-well plates at a concentration of 1 × 10⁴ cells/well. After 24 h, confluent cell monolayers were placed in serum-free medium and treated with 3 μg/ml SLO with or without luteolin (8 μg/ml). After 6 h, cells were stained with the LIVE/DEAD™ Cell imaging Kit (Thermo Fisher Scientific, United States) following the manufacturer’s instructions. Cells were immediately analyzed and acquired using the FV1000 confocal microscope (Olympus, Japan) at ×40 magnification.

Mice Nebulized Lung Delivery Infection Model

Wild-type (WT) C57BL/6 female mice (6 weeks old) were purchased from Charles River (Beijing, China). The mice were maintained and bred under Specific pathogen-free (SPF) conditions in the animal facilities of the Academy of Military Medical Sciences. For pulmonary infection, mice were anesthetized and then 50 μl of PBS containing a 1 × 10⁸ CFU dose of GAS was inhaled via a nebulized lung delivery. After infection, mice were injected intraperitoneally (i.p.) with 100 μl of luteolin at a dose of 10 mg/kg body weight (Zhang et al., 2021) and luteolin was additionally administered at 24 h intervals for 12 days. The blank control group was not treated with GAS and was separately isolated; the positive control group was given the same volume of PBS according to the same procedure after infection. Each experimental group contained 10 mice, and the survival rate was recorded within 12 days and observed every 24 h.

Hematoxylin-Eosin Staining of Infected Lung Tissue

For histopathological analysis, mice were sacrificed by cervical dislocation 7 days after infection, each group containing multiple lung tissue samples was fixed in with 4% formaldehyde, stained with hematoxylin and eosin, and observed by OlympusBX53 microscope.

Data Analysis

Results are shown as mean ± standard deviation (SD) derived minimally from three independent experiments. A paired two-tailed Student’s t-test, one-way or two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test was used to test for significant differences using GraphPad Prism 8.0 software (San Diego, California, United States). Differences were considered statistically significant when p < 0.05: *p < 0.05, **p < 0.01, and ***p < 0.001.

RESULTS

Luteolin Inhibits the Hemolytic Activity of SLO

Luteolin has been shown to have antibacterial activity against several bacterial species. Previous studies have shown that luteolin exhibited anti-GAS activity with an MIC = 128 μg/ml (Siriwong et al., 2015). We found the protective effect of luteolin to be significantly lower than the MIC against SLO-induced cytotoxicity by hemolysis tests. First, a dose-response analysis
of the SLO protein (Figure 1A) and GAS culture-supernatant (Figure 1B) hemolytic effects in RBCs was performed. Results showed hemolytic release to be proportional to the concentration of SLO or the percentage of supernatant added, reaching up to 80% hemolysis in RBCs treated with 0.1 μg/ml SLO or 10% (v/v) supernatant, compared to the negative control. SLO or supernatant was incubated with different concentrations of luteolin for 30 min, compared with luteolin-untreated cells exposed to SLO or supernatant, and we found that luteolin likewise inhibited the percentage of hemolytic release in a dose-dependent manner (Figures 1C,D).

Analysis of the Interactions and Affinity Between Luteolin and SLO
Molecular docking technology is to predict the possible binding mode and conformation in the binding site between small molecules (also known as ligands) and protein receptors through the interaction between them, and to estimate the affinity for this specific interaction (Alonso et al., 2006). MD simulations carry out a more intensive conformational search than molecular docking methods do and provide a more accurate representation of protein motions (Morra et al., 2010). Therefore, in order to explore the mechanism of interaction between SLO and luteolin, we employed molecular docking and MD simulation to analyze the SLO—luteolin complex. Luteolin was found to bind to protein domains 1 and 3 of the SLO protein (Figure 2A). The binding site is mainly composed of β pleats, surrounded by helical structures on both sides. It is a semi-open binding site, which has a certain hydrophilicity. The binding energy between luteolin and SLO was −7.2 kcal/mol. The binding force mainly included hydrogen bonding, hydrophobic interactions, and van der Waals forces, and the interacting residues involved mainly included Gly396, Asp167, Asp230, Tyr344, Tyr429, Ile229, Tyr222, and Tyr174 (Figure 2B). The MM-PBSA method was used to calculate the interaction free binding energy to determine the contribution of amino acid residues that promote the binding of luteolin to SLO. The results confirmed a strong interaction between THR-170, TYR-429, PRO-430, and luteolin (Figure 2C), the binding energies of which were −1.9793, −1.544, −1.4026 kcal/mol, respectively.
To verify the molecular interaction between luteolin and streptolysin O, SLO protein was immobilized on a SPR chip and luteolin was used as the mobile phase to study the interaction between them. The results (Figure 2D) showed that luteolin had a high affinity for the SLO protein, with a KD of 0.372 μM (Table 2).

The 2nd derivative MMS spectra of these samples were overlaid in Figure 2E and the secondary structure composition (%) were compared in Figure 2F. The results clearly show that there was no significant difference in protein structure before and after small molecule binding. Taking the average value of three repeated tests, using the SLO protein as the standard to calculate the similarity, the similarity between the luteolin-SLO complex and SLO protein alone was 97.27%. These results show that luteolin binding did not significantly change the secondary structure of the SLO protein.

**Luteolin Increases the Stability of the SLO Protein and Inhibits Its Polymerization.**

To further investigate the interaction mechanism between luteolin and SLO, we further analyzed the MD simulation results of SLO protein and luteolin. The root-mean-square-deviation (RMSD) curve represents the fluctuation of SLO protein conformation. It can be seen in Figure 3A, that for the small molecule—protein complex containing the ligand (luteolin), the fluctuation of protein conformation during the simulation process was significantly smaller than that of the system containing only protein. These results indicated that binding of luteolin may stabilize the SLO protein’s conformation. Next, we analyzed the root-mean-square-fluctuation (RMSF) (Figure 3B) to explore the conformational changes of side chain residues before and after luteolin binding. We found that there were similar amino acid residue fluctuations between the two groups. And we used the UNcle multi-parameter...
high-throughput protein stability analysis system to detect changes in SLO protein stability before and after luteolin treatment. The results showed that the melting temperature (Tm) of the SLO protein treated with luteolin (Tm = 43.66°C) increased relative to that of the untreated SLO protein (Tm = 41.25°C) (Figure 3C). The small size and unfolded aggregates of the protein were observed at 266 nm by static light scattering (SLS). The aggregation temperature (Tagg) of the treated SLO protein was 42.49°C, while the of the untreated protein (Tagg) was 38.62°C (Figure 3D). These results suggest that luteolin may help maintain the conformational stability of SLO protein and prevent aggregation. In addition, oligomerization analysis was conducted to determine whether luteolin affects the toxicity of SLO proteins by affecting their pore-forming ability. Early evidence suggests that oligomers of hemolysin proteins loaded into SDS-PAGE are less stable than monomers (Lv et al., 2020). Therefore, the protein concentration (100 μg/ml) used in oligomeric analysis is much higher than that used in the hemolysis assay. The purified SLO protein was mixed with luteolin at different concentrations, and those without added compounds were used as positive controls.
As shown in Figures 3E,F, oligomerization decreased in response to high concentrations of luteolin. Compared with hemolysis tests, hemolysis activity was almost completely inhibited by luteolin at the same concentration (2 μg/ml), but the presence of SLO protein oligomers could still be detected, likely due to the higher protein concentration used in this test. We know that the formation of protein oligomers is a coordinated change in conformation of the protein domain from the α-helices to the insertion of two β-sheets into the membrane to form a β-barrel structured pore in the membrane (Heuck et al., 2003). Therefore, we speculate that luteolin might play a role in inhibiting SLO toxicity by affecting stability of the protein structure, thus inhibiting the SLO protein oligomerization.

**Luteolin Binds to Other CDC Family Proteins and Inhibits Their Hemolytic Activity**

Molecular docking results showed the binding mode of luteolin with SLY, PLY, and LLO proteins (Figure 4A). We compared the important residues resulting from the combination of SLO and luteolin with the amino acid sequences of other CDCs. Interestingly, we found no conserved residues between them. But the SPR results (Figure 4B) showed that luteolin also had high affinities for SLY, PLY, and LLO proteins, with affinity constants of 0.554, 0.613, and 0.437 μM (Table 2), respectively. Additionally, our results indicated that luteolin also inhibited the hemolytic activity of these proteins (Figure 4C), and luteolin concentrations that completely inhibited...
the hemolytic activities of SLY, PLY, and LLO proteins were 8, 4, and 4 μg/ml, respectively.

**Luteolin Protects HEp-2 Epithelial Cells From SLO-Induced Cytotoxicity**

In order to neutralize the toxicity of SLO without inducing cytotoxicity, preliminary experiments were carried out on HEp-2 cells with different concentrations of luteolin to determine the optimal concentration to use. The results showed that the effective concentration of luteolin lower than 16 μg/ml could be used for toxicity test intervention without affecting cell viability (Figure 5A).

Next, dose-response and time-course analyses of SLO cytotoxicity in HEp-2 cells grown in FBS free medium was performed. Cells treatment with SLO caused a dose- and time-
dependent decrease in cell survival (Figure 5B). On the basis of the IC50 values (3 μg/ml) calculated, we treated HEp-2 cells with 3 μg/ml of SLO for 6 h. HEp-2 cells exposed to 3 μg/ml SLO and co-incubated with tolerated doses of luteolin for 6 h showed increased cell viability compared with luteolin-untreated cells (Figure 5C), the results showed that the cell viability was increased by 26.9%, 40.0%, 50.0%, 59.4%, and 61.4%, respectively. Moreover, we compared the concentrations of luteolin and found that luteolin of 8 μg/ml was the lowest effective concentration to still completely resist the toxicity of SLO protein. In addition, we investigated whether luteolin could protect HEp-2 cells from SLO dependent permeability. Intact living cells were visualized by staining with FITC fluorescent cell dye solution (green signal); dead cells were visualized using Texas Red stain, which mainly produces nuclear red fluorescence in cells with damaged cell membranes, but is not taken up by living cells.

**FIGURE 6 |** Luteolin protects mice against GAS pneumonia. (A) The influence of luteolin on the mortality of GAS-infected mice. GAS, GAS + luteolin, and Control groups of mice inhaled 1 × 10⁸ CFU dose of GAS via a nebulized lung delivery, and the mortality of mice was supervised for 12 days. Data are representative of three independent experiments (n = 10 mice in the per group) and are shown as mean ± SD (Kaplan-Meier tests). *p < 0.05 versus control. (B,C) Pathologic and histopathological changes in lung tissue. Lung tissue from GAS-infected mice (7 days after infection). Lung tissue was stained with H&E (original magnification ×200).
cells. The results showed that the SLO protein induced cell permeability, and the presence of luteolin in the culture medium inhibited SLO induced permeability (Figure 5D).

**Luteolin Provides Protection Against GAS Pneumonia in Mice**

Based on the above-presented experimental conclusions, we further verified whether luteolin has a similar protective effect in mice infected with GAS. First, mortality due to pneumonia caused by GAS was monitored every 24 h after infection over a 12-day time course. The survival curve is shown in Figure 6A. Compared with the healthy control group, the survival rate of mice infected with GAS was 10%. However, the survival rate increased to 50% when infected mice were treated with luteolin. Pathological analysis of lung tissue was performed on each group of mice to evaluate the mitigating effect of luteolin on lung injury. Pathological examination revealed that lung tissue from infected mice was dark red and showed severe congestion and edema. In contrast, mice treated with luteolin showed a light pink lesion with focal infection (Figure 6B). Pathological assessment of lung tissue sections showed severe destruction of lung tissue and inflammatory cell infiltration in infected mice. After luteolin treatment, lung tissue damage was reduced, and the inflammatory response was relieved (Figure 6C). The results of this experiment indicate that luteolin treatment can improve the pulmonary inflammatory injury induced by GAS, delay and decrease the aggravation of the disease.

**DISCUSSION**

At present, drug resistance of pathogenic bacteria is becoming increasingly widespread due to the extensive, massive, long-term, and non-standard use of antibiotics, producing one of the largest medical challenges of the 21st century (Group et al., 2012; Shulman et al., 2012; Hedin et al., 2014; Yu et al., 2020). As a result, the failure rate for treating related infectious diseases is increasing (Cohen, 2004). Several new antimicrobial strategies against not only GAS but other bacteria, including interfering with bacterial toxicity and/or intercellular signaling pathways, have been identified through further research and understanding (Rasko and Sperandio, 2010). For example, human serum albumin can bind *C. difficile* TcdA and TcdB toxins, reduced bacterial toxin dependent infection, and helped protect the host cell (Di Bella et al., 2015; di Masi et al., 2018). Honokiol inhibited the secretion and hemolytic activity of Hla, which reduced the Hla-induced inflammatory response and the damaging effects of *Staphylococcus aureus* infection on cells (Guo et al., 2019). Quercetin significantly reduced PLD-induced hemolytic activity, cytotoxicity, and cell damage by suppressing the formation of oligomers and alleviated the pathological damage to lung tissue and the release of cytokines in mice infected with *Streptococcus pneumoniae* (Lv et al., 2020). These compounds have different modes of action than traditional bactericidal or bacteriostatic chemicals, which expands the arsenal of antibacterial treatments to a certain extent.

In this study, we discussed the detoxification mechanism of the small plant-based ligand luteolin against the SLO protein, one of the key virulence factors of GAS. SLO induces eukaryotic cell lysis, resists neutrophil phagocytosis and clearance of GAS, inhibits neutrophil oxidative burst, and blocks neutrophil degranulation, interleukin-8 secretion, and reactivity, thereby contributing to the pathogenesis of streptococcal infection (Uchiyama et al., 2015), and thus has increasingly becoming a promising potential drug target for the treatment of streptococcal infection (Arzanlou and Bohlooli, 2010; Turner et al., 2020).

Some studies have demonstrated that luteolin has increased antibacterial effects relative to many commonly used compounds, and can have a synergistic effect when combined with ceftazidime (Siriwong et al., 2015). Additionally, it also has obvious bacteriostatic effects on *Bacillus subtilis, Staphylococcus aureus, Pseudomonas fluorescens, Escherichia coli, Enterobacter cloacae, and Trueperella pyogenes* (Lv et al., 2009; Guo et al., 2020; Geng et al., 2021; Qian et al., 2021). At present, luteolin has been used in food packaging films to exert long-term antioxidant and antibacterial activities during food storage (Bi et al., 2021). Scientists are constantly trying various methods to increase the bioavailability of luteolin, one example is the use of luteolin-loaded Methoxy poly(ethylene glycol)-poly(lactide) micelles that can significantly promote bacterial clearance while reducing inflammatory infiltration in a mouse model of pulmonary infection caused by *Klebsiella pneumoniae* (Miao et al., 2021).

Recent studies have demonstrated that luteolin can alleviate olfactory dysfunction after COVID-19, which provides further support for the clinical application of luteolin (D’Ascanio et al., 2021). In addition, through bioinformatics analysis such as TCGA-RNA-seq and network pharmacology, the researchers concluded that luteolin may play a clinical role in anti-PC and 2019-nCoV disease by regulating the activities of core genes (MPO and FOS) (Ye et al., 2021).

However, we demonstrated that luteolin binds the SLO protein at domain 1 and 3 with high affinity. These domains consist of several α-helices and a number of loops surrounding a core β-sheet (Feil et al., 2014), and conformational changes are key to the formation of protein polymers and pores (Hotze et al., 2012). The results of this study further demonstrate that luteolin strengthened the conformational stability of the SLO protein and inhibited the formation of its polymers, thus weakening its pore-forming activity. We also carried out point mutations on residues involved in SLO binding to luteolin, purified the protein, and then verified the hemolytic functional experiment. At the same time, we carried out experiments on the interaction between luteolin and the point mutant protein, but we did not find obvious changes in protein function and intermolecular interaction (data not shown). Therefore, we speculate that the mutation of a single residue may not play a key role and may require the joint action of multiple residues. Meanwhile, luteolin inhibited the hemolysis activity of the SLO protein in a concentration-dependent manner. Luteolin also played a protective role by preventing SLO induced permeability of epithelial cell membranes.

Meanwhile, our experiments verified that luteolin also interacted with other CDC family proteins, including SLY,
PLY, and LLO, and inhibited their hemolytic activity. Although the binding residues among them are not very consistent through further analysis by molecular docking, luteolin has high affinities for SLY, PLY, and LLO proteins. Previous studies had shown that luteolin can play a targeted role against L. monocytogenes mediated infection by inhibiting translation of the LLO protein and reducing its expression, however, luteolin was not found to affect the release of PLY (Wang et al., 2019). However, our study demonstrated that there are certain interactions between luteolin and CDC family proteins that can inhibit some of their functional activities, thus enriching the antibacterial targets of luteolin. Moreover, the results of in vivo experiments concluded that luteolin intervention could improve the inflammatory damage of mice with pulmonary infection induced by GAS and improve the survival rate. This may also provide a preliminary scientific basis for the treatment of clinically relevant infectious diseases.

Studies on in vivo infection showed that cytotoxicity of the SLO protein could protect GAS from phagocytic killing by host cells and enhance its virulence, which was significantly related to the pathogenicity of GAS and severity of disease (Sierig et al., 2003; Zhu et al., 2017). Our results show that luteolin can significantly inhibit the pathogenicity of GAS, improve damage associated with inflammation. These results may be related to the interaction between luteolin and SLO protein. In conclusion, luteolin may be a potential SLO-targeting, indirectly antibacterial compound, which reduces the selection pressure against bacteria and the probability of drug resistant bacteria evolving. The very promising medicinal value of luteolin lays a foundation for further study of its anti-virulence mechanisms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal center of the Academy of Military Medical Sciences.

AUTHOR CONTRIBUTIONS

TG and PL performed the experiments, analysed the data, and wrote the manuscript. YZ, WH, DK and QL contributed to the preparation of recombinant proteins. ZW, LD, and ZW played an important role in the preparation of experiments. HJ, YJ and LS designed the study and revised the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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