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Comparative Study on the Immunomodulatory Effects of Antimicrobial Peptide Sublancin and Astragalus Polysaccharide in Mice (Postprint)

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Abstract

The present study aimed to compare the immunomodulatory effects of antimicrobial peptide Sublancin and Astragalus polysaccharide on mice. Sixty healthy female BALB/c mice aged 4-6 weeks were selected and randomly divided into 6 groups (n=10 per group). Mice in each group were intragastrically administered the following substances for 7 consecutive days, once daily, at a volume of 0.2 mL per mouse: blank control group, administered normal saline; challenge group, administered normal saline; 12.0 mg/kg BW Astragalus polysaccharide group, administered 12.0 mg/kg BW Astragalus polysaccharide solution; 48.0 mg/kg BW Astragalus polysaccharide group, administered 48.0 mg/kg BW Astragalus polysaccharide solution; 1.0 mg/kg BW sublancin group, administered 1.0 mg/kg BW sublancin solution; 2.0 mg/kg BW sublancin group, administered 2.0 mg/kg BW sublancin solution. Except for the blank control group, the remaining 5 groups were challenged with Salmonella typhimurium at a concentration of 1×10^9 CFU/mL via gavage at a dose of 200 L per mouse 24 hours after the final administration. At 3 and 24 hours post-challenge, 5 mice were randomly selected from each group for collection of peripheral blood, spleen, and cecal contents to measure serum cytokine levels, T lymphocyte subset CD4+ and CD8+ counts in splenocytes, Salmonella counts in intestinal contents, and other indicators.

The results showed that at 3 hours post-Salmonella challenge, compared with the challenge group, serum interleukin-6 (IL-6) levels were significantly decreased in the 12.0 and 48.0 mg/kg BW Astragalus polysaccharide groups and the 2.0 mg/kg BW sublancin group (P<0.05); serum interleukin-10 (IL-10) levels were significantly increased in the 1.0 mg/kg BW sublancin group (P<0.05); serum tumor necrosis factor- (TNF-) levels were significantly decreased in the 12.0 and 48.0 mg/kg BW Astragalus polysaccharide groups and the 1.0 and 2.0

mg/kg BW sublancin groups (P<0.05), while serum interleukin-25 (IL-25) levels showed no significant changes (P>0.05); serum monocyte chemoattractant protein-1 (MCP-1) levels were significantly decreased in the 48.0 mg/kg BW Astragalus polysaccharide group and the 2.0 mg/kg BW sublancin group (P<0.05); the CD4+/CD8+ ratio in splenocytes was significantly increased in the 48.0 mg/kg BW Astragalus polysaccharide group and the 1.0 and 2.0 mg/kg BW sublancin groups (P<0.05); Salmonella counts in intestinal contents showed no significant changes in the 12.0 and 48.0 mg/kg BW Astragalus polysaccharide groups and the 1.0 and 2.0 mg/kg BW sublancin groups (P>0.05), but a decreasing trend was observed.

At 24 hours post-Salmonella challenge, compared with the challenge group, serum IL-6 levels were significantly decreased in the 2.0 mg/kg BW sublancin group (P<0.05); serum TNF- levels were significantly decreased in the 48.0 mg/kg BW Astragalus polysaccharide group and the 2.0 mg/kg BW sublancin group (P<0.05); serum MCP-1 levels were significantly decreased in the 12.0 and 48.0 mg/kg BW Astragalus polysaccharide groups and the 2.0 mg/kg BW sublancin group (P<0.05). Additionally, at 24 hours post-Salmonella challenge, compared with the blank control group, serum IL-10 levels were significantly increased in the 2.0 and 48.0 mg/kg BW Astragalus polysaccharide groups and the 1.0 and 2.0 mg/kg BW sublancin groups (P<0.05).

Based on these results, it can be concluded that appropriate doses of antimicrobial peptide sublancin and Astragalus polysaccharide both exert favorable immunomodulatory effects on mice; compared with Astragalus polysaccharide, antimicrobial peptide sublancin provides more comprehensive regulation of immune function in Salmonella-infected mice.

Full Text

Comparative Study on the Immunomodulatory Effects of Antimicrobial Peptide Sublancin and Astragalus Polysaccharide in Mice

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Abstract: This experiment was conducted to compare the immunomodulatory effects of antimicrobial peptide sublancin and Astragalus polysaccharide on immune function in mice. Sixty healthy female BALB/c mice aged 4-6 weeks were randomly divided into six groups with ten mice per group. All groups received continuous intragastric administration for seven days at a daily dose of 0.2 mL per mouse as follows: blank control group received physiological

saline; challenge group received physiological saline; 12.0 mg/kg BW Astragalus polysaccharide group received 12.0 mg/kg BW Astragalus polysaccharide solution; 48.0 mg/kg BW Astragalus polysaccharide group received 48.0 mg/kg BW Astragalus polysaccharide solution; 1.0 mg/kg BW sublancin group received 1.0 mg/kg BW sublancin solution; and 2.0 mg/kg BW sublancin group received 2.0 mg/kg BW sublancin solution. Except for the blank control group, all other five groups were challenged with *Salmonella typhimurium* at a concentration of 1×10 CFU/mL (200 L per mouse) 24 hours after the final intragastric administration. At 3 and 24 hours post-challenge, five mice were randomly selected from each group to collect peripheral blood, spleen, and cecal contents for detection of serum cytokine levels, T lymphocyte subsets CD4 and CD8 counts in splenocytes, and *Salmonella* counts in intestinal contents.

The results showed that at 3 hours post-challenge, compared with the challenge group, serum interleukin-6 (IL-6) levels were significantly reduced in the 12.0 and 48.0 mg/kg BW Astragalus polysaccharide groups and the 2.0 mg/kg BW sublancin group ($P<0.05$). The 1.0 mg/kg BW sublancin group showed significantly increased serum interleukin-10 (IL-10) levels ($P<0.05$). Serum tumor necrosis factor- (TNF-) levels were significantly reduced in both Astragalus polysaccharide groups and both sublancin groups ($P<0.05$), while serum interleukin-25 (IL-25) levels showed no significant changes ($P>0.05$). Serum monocyte chemoattractant protein-1 (MCP-1) levels were significantly reduced in the 48.0 mg/kg BW Astragalus polysaccharide group and the 2.0 mg/kg BW sublancin group ($P<0.05$). The CD4 /CD8 ratio in splenocytes was significantly increased in the 48.0 mg/kg BW Astragalus polysaccharide group and both sublancin groups ($P<0.05$). Although *Salmonella* counts in intestinal contents showed no significant differences among the treatment groups ($P>0.05$), a decreasing trend was observed.

At 24 hours post-challenge, compared with the challenge group, the 2.0 mg/kg BW sublancin group showed significantly reduced serum IL-6 levels ($P<0.05$). The 48.0 mg/kg BW Astragalus polysaccharide group and the 2.0 mg/kg BW sublancin group showed significantly reduced serum TNF- levels ($P<0.05$). Serum MCP-1 levels were significantly reduced in both Astragalus polysaccharide groups and the 2.0 mg/kg BW sublancin group ($P<0.05$). Additionally, at 24 hours post-challenge, serum IL-10 levels were significantly higher in both Astragalus polysaccharide groups and both sublancin groups compared with the blank control group ($P<0.05$). These results indicate that appropriate doses of both antimicrobial peptide sublancin and Astragalus polysaccharide exert beneficial immunomodulatory effects in mice, with sublancin demonstrating more comprehensive immunomodulatory effects than Astragalus polysaccharide in *Salmonella*-infected mice.

Key words: antimicrobial peptide; sublancin; Astragalus polysaccharide; mice; immune function

Antimicrobial peptides (AMPs) represent evolutionarily ancient anti-infectious polypeptides that constitute an essential component of the innate immune system across diverse organisms, from prokaryotes to humans, forming the first line of defense against pathogenic microbial invasion. AMPs exhibit broad-spectrum antimicrobial activity and have become a research hotspot as antibiotic alternatives due to their low propensity for inducing resistance. Recent studies have demonstrated that AMPs possess diverse immunomodulatory functions beyond direct antimicrobial activity, including regulation of inflammatory responses, chemotaxis of immune cells, promotion of cell differentiation, and activation of innate and adaptive immune responses, which are closely associated with resistance to pathogenic infections and immune-related diseases. Research has shown that AMPs can enhance antigen-specific humoral and cellular immune responses and improve vaccine efficacy, demonstrating promising applications in human and animal health and disease prevention.

Sublancin is an antimicrobial peptide isolated and identified by Hansen' s research team at the University of Maryland from *Bacillus subtilis* fermentation products. It is a cationic peptide composed of 37 amino acids with two disulfide bonds, with an amino acid sequence of GLGKAQCAALWLQCASGGTIGCGGGAVACQNYRQFCR and a molecular mass of approximately 3,875.74 Da. Sublancin is extremely stable, tolerating pH ranges from 1.5 to 9.5 and high temperature environments, and can inhibit the budding growth and reproductive division of Gram-positive bacteria. Our laboratory' s previous studies have shown that immunomodulatory function is an important aspect of sublancin' s ability to protect mice and broilers against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and *Clostridium perfringens* infections, with doses of 1.0 and 2.0 mg/kg demonstrating significant effects in mouse experiments. The Chinese Ministry of Agriculture has approved three immunomodulatory agents: Astragalus polysaccharide, sheep placenta transfer factor, and echinacea, among which Astragalus polysaccharide is currently the most widely used. Therefore, this study used Astragalus polysaccharide as a control to compare the immunomodulatory effects of antimicrobial peptide sublancin and Astragalus polysaccharide in mice, providing a theoretical basis for the application of sublancin as an immune enhancer in animal production.

1.1 Reagents and Experimental Animals

Salmonella typhimurium was purchased from the China Center of Industrial Culture Collection (CICC 22956) and stored in freeze-dried form in ampoules. The strain was activated for 2–3 generations to restore viability before use and diluted with physiological saline to a concentration of 1×10 CFU/mL under aseptic conditions. Astragalus polysaccharide was obtained from Beijing Addison Biological Technology Co., Ltd., with main components from *Astragalus* and a specification of no less than 450 mg Astragalus polysaccharide per gram. Solutions were prepared at concentrations of 2.04 mg and 8.16 mg per 2 mL based on mouse body weight. Antimicrobial peptide sublancin was provided

by the National Feed Engineering Technology Research Center with a purity of 99.6%, prepared as solutions containing 0.17 mg and 0.34 mg per 2 mL based on mouse body weight.

Additional reagents included anhydrous ethanol and xylene from Sinopharm Chemical Reagent Co., Ltd.; EDTA antigen retrieval buffer (pH 8.0), phosphate-buffered saline (PBS), 3% hydrogen peroxide, bovine serum albumin (BSA), hematoxylin staining solution, hematoxylin differentiation solution, hematoxylin bluing solution, neutral balsam, primary antibodies (CD4 and CD8), diaminobenzidine (DAB) chromogen kit, and secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-rabbit] from Beijing Kangjiahongyuan Biotechnology Co., Ltd. and KPL. Mouse ProcartaPlex kits were from Thermo Fisher.

Specific-pathogen-free (SPF) female BALB/c mice aged 4–6 weeks were purchased from Beijing Huafukang Bioscience Co., Ltd. The experiment was conducted in the Mouse Nutrition and Metabolism Room of the Ministry of Agriculture' s Supervision, Inspection and Testing Center for Feed Efficiency and Safety Evaluation (Beijing). The mouse facility was computer-controlled for temperature, humidity, and lighting, with temperature maintained at 18–22 °C, relative humidity at 35%–55%, and a 12-hour light-dark cycle. Mice had free access to feed and water.

1.2 Experimental Design

Sixty healthy female BALB/c mice aged 4–6 weeks were randomly divided into six groups of ten mice each. All groups received daily intragastric administration for seven consecutive days at 0.2 mL per mouse as follows: blank control group received physiological saline; challenge group received physiological saline; 12.0 mg/kg BW Astragalus polysaccharide group received 12.0 mg/kg BW Astragalus polysaccharide solution; 48.0 mg/kg BW Astragalus polysaccharide group received 48.0 mg/kg BW Astragalus polysaccharide solution; 1.0 mg/kg BW sublancin group received 1.0 mg/kg BW sublancin solution; and 2.0 mg/kg BW sublancin group received 2.0 mg/kg BW sublancin solution. Except for the blank control group, all other five groups were challenged with *Salmonella typhimurium* at 1×10 CFU/mL (200 L per mouse) 24 hours after the final administration. Samples were collected from five randomly selected mice per group at 3 and 24 hours post-challenge.

1.3 Sample Collection

Blood samples (200–300 L per mouse) were collected from the orbital sinus of five randomly selected mice from each group into 1.5 mL sterile centrifuge tubes. After standing at room temperature for 2 hours, samples were centrifuged at 3,000 rpm for 10 minutes at 4 °C. The supernatant serum was then aspirated under aseptic conditions, aliquoted, and stored at -80 °C for subsequent analysis. Following blood collection, mice were euthanized by cervical dislocation, and

spleens were aseptically removed for section preparation. Cecal contents were also collected under sterile conditions.

1.4 Detection Indicators

1.4.1 Serum Cytokine Levels Magnetic bead mixtures, wash buffer, quality control samples, and standards were prepared, and serum samples were diluted accordingly. Then, 25 L of blank, standard, quality control, or diluted serum samples (1:400) were added to respective wells of a 96-well plate, followed by 25 L of magnetic bead mixture. The plate was sealed and incubated overnight at 4 °C on a plate shaker in the dark. After three washes to remove unbound components, 50 L of detection antibody was added to each well and incubated for 1 hour at room temperature (20-25 °C) with shaking in the dark. Following another wash, 50 L of streptavidin-conjugated phycoerythrin (PE) fluorescent dye was added and incubated for 30 minutes at room temperature in the dark. Finally, after three washes, 100 L of sheath fluid was added and incubated for 10 minutes at room temperature in the dark to resuspend the magnetic beads. Serum levels of interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-25 (IL-25), interferon- (IFN-), tumor necrosis factor- (TNF-), and monocyte chemoattractant protein-1 (MCP-1) were measured using a Magpix instrument.

1.4.2 T Lymphocyte Subsets CD4 and CD8 Counts and Ratio in Splenocytes Mouse spleen paraffin sections were prepared according to the method of Wang Qingwei, and immunohistochemistry was used to detect CD4 and CD8 lymphocyte subset counts and ratios in splenocytes.

1.4.3 Salmonella Counts in Intestinal Contents Cecal contents (0.1 g) were collected from mice at 3 and 24 hours post-challenge, dissolved in 900 L physiological saline, and serially diluted to 10^{3}, 10 , and 10 levels. Then, 100 L aliquots were plated on Salmonella-Shigella (SS) agar and incubated at 37 °C for 24 hours before colony counting.

1.5 Statistical Analysis

Data were analyzed using one-way ANOVA with the General Linear Model (GLM) procedure in SAS 9.4. Significant differences identified by ANOVA were further analyzed using LSD for multiple comparisons. Results are expressed as means and standard error of the mean (SEM), with $P<0.05$ considered statistically significant.

2.1 Effects of Antimicrobial Peptide Sublancin and Astragalus Polysaccharide on Serum Cytokine Levels in Mice

As shown in Table 1 , at 3 hours post-challenge, serum IL-6, IL-10, IFN- , TNF- , and MCP-1 levels were significantly increased in the challenge group compared with the blank control group ($P<0.05$). Compared with the challenge group,

12.0 and 48.0 mg/kg BW Astragalus polysaccharide and 2.0 mg/kg BW sublancin significantly reduced serum IL-6 and IFN- levels (P<0.05). Astragalus polysaccharide at 12.0 and 48.0 mg/kg BW significantly decreased serum IL-10 levels (P<0.05), whereas 1.0 mg/kg BW sublancin significantly increased serum IL-10 levels (P<0.05). Both Astragalus polysaccharide groups and both sublancin groups significantly reduced serum TNF- levels (P<0.05), while 12.0 mg/kg BW Astragalus polysaccharide and 1.0 mg/kg BW sublancin had no significant effect on serum MCP-1 levels (P>0.05). Overall, both sublancin and Astragalus polysaccharide modulated immune function in *Salmonella*-infected mice, with 1.0 mg/kg sublancin showing the best effect.

As shown in Table 2 , at 24 hours post-challenge, serum IL-6, IL-10, IFN- , TNF- , and MCP-1 levels were significantly increased in the challenge group compared with the blank control group (P<0.05). The 2.0 mg/kg BW sublancin group significantly reduced serum IL-6, IFN- , TNF- , and MCP-1 levels (P<0.05). The 1.0 mg/kg BW sublancin group had no significant effect on cytokine levels except for IFN- (P>0.05). The 12.0 mg/kg BW Astragalus polysaccharide group significantly reduced serum MCP-1 levels (P<0.05) but had no significant effect on other cytokines (P>0.05). The 48.0 mg/kg BW Astragalus polysaccharide group significantly reduced serum IL-10, TNF- , and MCP-1 levels (P<0.05). Overall, sublancin showed slightly better immunomodulatory effects than Astragalus polysaccharide at 24 hours post-challenge in *Salmonella*-infected mice.

2.2 Effects of Antimicrobial Peptide Sublancin and Astragalus Polysaccharide on T Lymphocyte Subsets CD4 , CD8 Counts and Ratio in Mouse Splenocytes

As shown in Tables 3 and 4 , the CD4 /CD8 ratio in splenocytes was significantly reduced in the challenge group compared with the blank control group at 3 hours post-challenge (P<0.05), while CD4 counts were significantly reduced at 24 hours post-challenge (P<0.05). Compared with the challenge group, 48.0 mg/kg BW Astragalus polysaccharide and both sublancin doses significantly increased the CD4 /CD8 ratio at 3 hours post-challenge (P<0.05), restoring it to levels 接近 the blank control group.

2.3 Effects of Antimicrobial Peptide Sublancin and Astragalus Polysaccharide on Salmonella Counts in Mouse Intestinal Contents

As shown in Table 5 , no *Salmonella* was detected in the blank control group at 3 hours post-challenge, while the challenge group showed significantly increased *Salmonella* counts (P<0.05). Although there were no significant differences in *Salmonella* counts among the treatment groups (P>0.05), numerical reductions were observed in the 2.0 mg/kg BW sublancin group and the 48.0 mg/kg BW Astragalus polysaccharide group compared with the challenge group.

3 Discussion

Xu Xin reported that *Salmonella* infection in mice promotes expression of pro-inflammatory cytokines (such as IL-6 and TNF-) in peripheral blood and reduces the CD4 /CD8 ratio in splenocytes, leading to decreased immune capacity. Based on the detected indicators including serum cytokine levels, CD4 and CD8 counts and CD4 /CD8 ratio in splenocytes, and intestinal *Salmonella* counts, this study successfully established a *Salmonella* infection model in mice.

Numerous studies have demonstrated that Astragalus polysaccharide exerts broad effects on specific and non-specific immunity, as well as cellular and humoral immunity. As a biological immunomodulator, it primarily acts through several pathways: activating macrophages, promoting T cell transformation, activating cytotoxic T (Tc) cells, and inducing various immune factors such as interferon (IFN), interleukin-2 (IL-2), and tumor necrosis factor (TNF). Lin Aihua et al. demonstrated that Astragalus significantly enhances T lymphocyte proliferation induced by concanavalin A (ConA), showing clear immunoenhancing effects. Jiang Ruixue et al. confirmed that Astragalus polysaccharide at 3.0 mg/kg significantly enhances innate and cellular immune function in mice, making it an excellent immunomodulator. Additionally, Li Shuyi reported that Astragalus polysaccharide can enhance innate, cellular, and humoral immune functions in mice.

Research indicates that Astragalus polysaccharide promotes anti-inflammatory factor secretion. Antimicrobial peptides such as -defensin 2, hLF1-11, and the cationic synthetic peptide IDR-1018 can activate innate immunity and initiate adaptive immunity by inducing differentiation of dendritic cells and macrophages. LL37 can directly act as a chemoattractant for neutrophils, monocytes, mast cells, and T helper (Th) cells, or stimulate host cells to release chemokines for neutrophils and monocytes, enabling rapid accumulation at inflammatory sites to exert protective functions and eliminate inflammation. Besides direct chemotactic activity, LL37 can also exert indirect chemotactic effects by stimulating innate immune cells to release chemokines and cytokines through receptor-dependent mechanisms. Yang Qing reported that appropriate doses of sublancin can induce mixed Th1 and Th2 cell immune responses in ovalbumin (OVA)-immunized mice, enhancing both humoral and cellular immunity. The present results show that at 3 hours post-infection, sublancin and Astragalus polysaccharide differentially increased serum IL-10, IFN- , and MCP-1 levels compared with the blank control group. IL-10 is an important anti-inflammatory cytokine that inhibits dendritic cell maturation and IL-12 production, facilitating Th2 immune responses, while IFN- promotes Th1 immune responses by upregulating transcription factor T-bet. MCP-1 is a crucial immune cell chemokine. These results suggest that during acute infection, both sublancin and Astragalus polysaccharide favor IL-10 expression, while sublancin exhibits more comprehensive effects than Astragalus polysaccharide in promoting Th1 and Th2 immune responses and immune chemotaxis.

Excessive activation and amplification of innate immunity can damage the host, but antimicrobial peptides can inhibit excessive inflammatory responses by suppressing bacterial product-induced production of harmful cytokines and inhibiting transcription of pro-inflammatory cytokine genes (such as IL-6 and TNF-), thereby preventing endotoxemia. Astragalus polysaccharide can simultaneously promote anti-inflammatory factor secretion and inhibit pro-inflammatory factor secretion. Our previous studies showed that antimicrobial peptide Microcin J25 significantly reduced serum IL-6 and TNF- levels in weaned piglets, thereby enhancing innate immune function. The present study demonstrates that at 3 hours post-infection, both Astragalus polysaccharide and sublancin significantly reduced serum IL-6 and TNF- levels; at 24 hours post-infection, sublancin significantly reduced serum IL-6 and TNF- levels, while Astragalus polysaccharide only significantly reduced TNF- levels. Both agents also differentially reduced serum IFN- and MCP-1 levels, indicating that they can inhibit pro-inflammatory cytokine gene transcription during acute infection, with sublancin providing more comprehensive effects than Astragalus polysaccharide at 24 hours post-infection.

T lymphocytes mediate cellular immune functions, including helper functions of Th cells and cytotoxic effects of Tc cells that directly kill target cells. T lymphocytes recognize antigens associated with major histocompatibility complex (MHC) class I or II molecules through receptors. T cells expressing receptors that recognize MHC class II-associated antigens express CD4 , while those recognizing MHC class I-associated antigens express CD8 . Interactions between CD4 /CD8 and MHC molecules are critical for T cell activation and antigen response. Xu Xin reported that echinacea polysaccharide significantly increased the CD4 /CD8 ratio in splenocytes of *Salmonella*-infected mice, thereby enhancing immune capacity. Zhang Ruiqi et al. demonstrated that Astragalus polysaccharide increased the CD4 /CD8 ratio in mouse peripheral blood, improving immune function. In this study, at 3 hours post-infection, 48.0 mg/kg BW Astragalus polysaccharide and both sublancin doses significantly increased the CD4 /CD8 ratio in splenocytes, indicating that both agents can enhance splenocyte immune function during acute infection.

At 3 hours post-infection, detection of intestinal *Salmonella* counts revealed no significant differences among groups, though numerical reductions were observed in both sublancin and Astragalus polysaccharide groups.

4 Conclusion

1. Comparison between blank control and challenge groups confirmed successful establishment of the *Salmonella* infection mouse model.
2. Appropriate doses of sublancin and Astragalus polysaccharide inhibited pro-inflammatory cytokine production, promoted anti-inflammatory IL-10 production, and enhanced innate immune function in *Salmonella*-infected mice. Both agents also increased the CD4 /CD8 ratio in splenocytes, thereby enhancing cellular immune function.

3. Compared with Astragalus polysaccharide, antimicrobial peptide sublancin demonstrated more comprehensive immunomodulatory effects in *Salmonella*-infected mice.

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