Original Article

Immune Responses Elevation by Intranasal Administration of Chitosan Microparticles Adjuvanted Poly-Epitope against *Streptococcus pneumoniae* in BALB/c Mice

Sahar Sadeghi[1], Mojgan Bandehpour[1,3]*, Mostafa Haji Molla Hoseini[2], Bahram Kazemi[3]

[1]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Medical Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Cellular and Molecular Biology Research Center, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Received: 18 July, 2021; Accepted: 28 September, 2021

Abstract

Background: Designing inhaled vaccine formulations against respiratory pathogens like *Streptococcus pneumoniae* (*S. pneumoniae*) lead to more effective mucosal and local immunity in the upper respiratory tract. Choosing chitosan (the chitin de-acetylated derivative) as a biocompatible, biodegradable, and non-toxic biopolymer and a suitable mucosal adjuvant can help to stimulate both systemic cellular and humoral immunity, as well as local protection, is valuable. The purpose of this study is the determination the ability of the designed pneumococcal immunogenic polytope to evoke a bactericidal response when adjuvanted with chitosan microparticles.

Materials and Methods: We chose virulence proteins from *S. pneumoniae* (Pneumolysin, Neuraminidase, Zink-Metalloproteinase, Hydrolase) and designed a new multi-epitope construct by linking their individual predicted T and B cell epitopes. Intranasal immunization with PNEU protein and chitosan microparticle administered in BALB/c mice.

Results: Our formulation showed enhanced systemic IgG-2a, IgA, and mucosal IgA antibody concentrations, revealing significant humoral responses to the polytope. The polytope increases the number of IFN-γ-producing cells in the re-stimulation of splenocytes in the culture medium and a rise in the concentrations of IL-6, IL-17, and TNF-α along with the regulatory responses of IL-10 presented the capacity of the formulation to provoke immune responses. The bactericidal test ultimately confirmed the high efficacy of the vaccine in inhibiting the bacteria.

Conclusion: Immunological responses were significantly induced after intranasal administration of the *S. pneumoniae* computational predicted polytope accompanied by chitosan microparticles as a potent mucosal adjuvant. Bactericidal assay confirmed effective immune responses in *S. pneumoniae* inhibition.

Keywords: Poly-epitope, *Streptococcus pneumonia*, Chitosan micro-particles, Inhaled vaccine

***Corresponding Author:** Mojgan Bandehpour, Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Email: m.bandehpour@sbmu.ac.ir

Please cite this article as: Sadeghi S, Bandehpour M, Haji Molla Hoseini M, Kazemi B. Immune Responses Elevation by Intranasal Administration of Chitosan Microparticles Adjuvanted Poly-Epitope against *Streptococcus pneumoniae* in BALB/c Mice. Novel Biomed. 2022;10(1):17-26.

Introduction

Streptococcus pneumoniae as a respiratory pathogen is the central agent for pneumonia, meningitis, otitis media, and sepsis, especially in young children and elders[1]. This microorganism has about 105 serotypes classified based on their immunologically distinct capsules with different disease severity[2]. In the field of vaccination against respiratory pathogens, a desirable approach is inhaled administration of the vaccine to achieve more effective immunity in the upper respiratory tract in contradiction of invasive microorganisms. An inhaled vaccine formulation that could stimulate systemic cellular and humoral immunity and mucosal protection would be pretty appropriate. Since adjuvants play a significant role in vaccine immunogenicity, it is important to select the helpful adjuvant to accompany the antigens. A suitable mucosal adjuvant without side effects and causing allergies that can help to induce a proper immune response would be valuable. A widely studied compound, chitosan, is a biocompatible, biodegradable, and non-toxic biopolymer that can be used as microparticles and nanoparticles forms to exert its satisfactory mucosal immunity[3-8]. *With the help of its numerous virulence factors, Pneumococci* can colonize in the host's nasopharynx and evade the immune system and in proper conditions and sensitive groups (immune-deficient patients, elders, children,) cause infection or even invasive disease[9]. The rapid resistance rate to various antibiotics[10] among serotypes is a significant problem.

On the other hand, existing capsular polysaccharide-based (23-valent) and conjugate (7-,10-or 13-valent) vaccines against *pneumococcus* encounter several issues. They include serotype-specific protection (inadequate coverage), the prevalence of non-vaccine serotypes (serotype replacement), vaccine's shortage, unaffordable and is expensive, especially for developing countries (most affected ones), not eliciting immunological memory and low efficacy in at more risk populations such as newborns, elderly and patients with immune deficiency disorders[11]. Therefore, there is an urgent need to develop new vaccines lacking mentioned problems to replace. One alternative approach is assessing and identifying pneumococcal virulence proteins in all serotypes and play major roles in pathogenesis as new targets for anti-pneumococcal vaccination strategies[12]. To date of writing this article, no research has been found to investigate and evaluate the immune responses against pneumococcal poly-epitopes designed by immune-informatics accompanied by chitosan microparticles. In this study, we applied a poly epitope protein designed in our previous study[13]. It was made from virulence proteins from *S. pneumonia* (Pneumolysin, Neuraminidase, Zink-Metalloproteinase, Hydrolase). We considered their function and role during colonization and infection and then we designed a new multi-epitope construct by linking their individual predicted T and B cell epitopes. Three intranasal immunizations with chitosan microparticles administered at an interval of 3 weeks in different groups of BALB/c mice. Seven days after the last immunization, our formulation showed enhanced systemic IgG-2a, IgA, and mucosal IgA antibody concentrations, revealing humoral significant responses to the polytope. An increase in the number of IFN-γ-producing cells in the re-stimulation of splenocytes in the culture medium by the polytope and a rise in the concentrations of IL-6, IL-17, and TNF-α along with the regulatory responses of IL-10, presented the capacity of the formulation to provoke immune responses. The bactericidal test ultimately confirmed the high efficacy of the vaccine in inhibiting the bacteria. It concluded that B cell, CTL, and Th cell epitopes used in this poly-tope construct in the presence of chitosan microparticles as a potent mucosal adjuvant were able to induce mucosal and systemic antibody responses as well as T cell reactions.

Methods

To elevate mice's immune responses by vaccine candidate complex, we should prepare the PNEU recombinant protein and Chitosan microparticles as follows.

Antigens selection and construct preparation: Virulence proteins were chosen from *S. pneumoniae* based on various reviews and research literature. Complete amino acid sequences of proteins retrieved from the UniProtKB database in FASTA format. By considering haplotype "d" as a dominant allele in the

BALB/c mice population, we predicted MHC I and II restriction epitopes in the IEDB server (Immune Epitope Database and Analysis Resources) (www.iedb.org). To build the desired multi-epitope construction, selected high score epitopes linked to each other using GGGS, KFERQ, GPGPG, HHAA, and HHAL linkers. S-tag oligopeptide sequence was also added to the C-terminal of the construct by the GGGS linker. The Codon Adaptation Tool (JCAT) was used for reverse translation and codon optimization of the PNEU construct. The *NdeI* and *XhoI* restriction enzyme sites were added to the 5' and 3' sequences. The Codon Adaptation Tool (JCAT) was used for reverse translation, and codon optimization of the PNEU construct. The DNA construct was then ordered to be cloned to the expression vector pET26b.

Transformation of *E. coli* BL21 (DE3) strain by vectors and expression of the PNEU polytope: *E. coli* BL21 (DE3) was used as an expression host. It was transformed with the PNEU recombinant plasmid (pET26b) containing the poly-epitope construct and grown overnight at 37°C on Luria Bertani agar (LB agar) medium. The transformed colonies were used for the expression induction of the recombinant protein. To induce the expression of recombinant polytope, 0.5M IPTG (Merck) was added, and the culture was incubated for 4 hours. After that, the bacterial pellet was collected by centrifugation (4000 rpm, 10 min).

Confirmation of purified protein by SDS-PAGE and Western Blotting: Protein extraction was done by protein lysis buffer (Tris 50mM, Glycerol 50%, Triton x-100 0.1%, PMSF 1mM, and 2λ of PMSF) ultra-sonication. Expression was analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), so 20µL of the sample with 10µL of loading buffer (Bromophenol blue 0.25%, Glycerol 40%) was loaded on 12% SDS-PAGE gel. Coomassie blue stain (Merck) (0.25% (w/v) Coomassie blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid) was used for gel staining. Transmission of protein bands from gel to nitrocellulose membrane by transfer buffer in a western blot apparatus (APELEX) for one hour. Then, with the help of anti-S-tag antibodies (Abcam, UK) that were conjugated to alkaline phosphatase and in the presence of the enzyme-substrate (NBT-BCIP) (Sigma), the appearance of the desired protein bands occurred.

Protein purification by affinity chromatography: An affinity purification procedure was performed for the S-tagged protein. The prepared bacterial lysate was incubated with the S-protein agarose for 1 hour in the column. After washing the unbound compounds, elution was performed with 3M magnesium chloride. Purified protein was dialyzed in PBS, and their concentration was determined by the Bradford method. Purified protein confirmation was carried out by western blotting again.

Preparation of Chitosan microparticles for usage as adjuvant: To provide small chitosan microparticles, solid chitosan powder (C-3646, Sigma Chemical Co. St. Louis, MO) was suspended in sterile deionized water. The suspension solution was sonicated and isolated using 100-µm, 70-µm, and 40-µm filters (Cell Strainer, BD Falcon, Mexico). The chitosan microparticle (<40µm) was acquired by centrifugation at 2800g for 10 min; the pellet was freeze-dried. The obtained powder was weighted, and a suspension of 100 µg/100 µl concentration was obtained in distilled water, stilled, and stored at 4°C until use. Particle size and size distribution were checked using a laser particle size analyzer (Malvern Master Sizer, Malvern Instruments, Ltd., Worcestershire, UK). More than 83% of the particles were <40 µm in diameter. The micro-particle suspension was analyzed for the presence of endotoxin before use as an adjuvant, using the Limulus Amebocyte Lysate (LAL) kit (Cambrex). The chitosan without any endotoxin was present as an adjuvant.

Mice immunization and evaluation of their immunity system

Complex formulation and immunization of BALB/c mice: In compliance with all ethical principles of research on laboratory animals following the animal ethics guidelines of the Committee and the Code of Ethics IR.SBMU.RETECH.REC.1396.1165, we chose twenty BALB/c female mice (6-8-week old) divided into 4 groups. Mice in group P+CsMP were immunized with 50µg of the PNEU protein (P) mixed with 100µg chitosan microparticles (CsMP) in 40µl volume intranasal (20µl per nostril).

In the same way, mice in CsMP and P groups were immunized only with 100µg chitosan microparticle and 50µg of the PNEU polypeptide alone, respectively. The control group received 20µl 1X sterile PBS. At an

interval of 2 weeks, three immunizations were administered. Seven days after the third vaccination, mice were bled and sacrificed by cervical dislocation.

Specimens collection: The sera of experimental groups were collected from the blood and pooled to antibody and cytokine ELISA assay and for bactericidal antibody response tests. The nasal washes were collected from mice. After a sacrifice and cut off mice's lower jaws, a syringe needle was placed into the posterior opening of the nasopharynx, and 0.5 ml PBS was used to wash the nasal cavity. Then collected nasal wash was centrifuged (700g, 10 min.), and the supernatants were stored at -20°C until used for IgA antibody assay. Next, the spleen was dissected out from each animal; B and T lymphocytes were isolated from them by Ficol 70 (Sigma) and then cultured in RPMI (Biosera) (with penicillin 100u/mL and streptomycin 100µg/mL) at a known density of cells for each later immunological assay. Proliferation stimulation (by antigen + adjuvant) was performed 24h after cell culture. Liver tissues were removed for examination of any pathological changes following the use of each complex formulation. Liver tissues were kept in formalin until slide staining.

Spleen lymphocytes proliferation assay (MTT assay): MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was performed according to the standard protocol.[11] In brief, 100µl phenol red and serum-free RPMI medium containing 40000 lymphocytes were plated in each well of 96-well plate. After 24h incubation in 37°c, 10µg of the PNEU polytope and 10µg of the adjuvant were added to each well to stimulate the proliferation of lymphocytes. As a positive control, cells were stimulated with 2.5 µg/mL of phytohaemagglutinin (PHA), and for the negative control, PBS was added to wells. After 48h, 100 µL of MTT solution (5mg/mL) was added per well. After 4h of incubation, colored formazan crystals were dissolved with 100 µL of isopropanol. Optical density (OD) was read on an ELISA reader at 570 nm after 5 minutes. The mean values of the stimulation index (SI) with antigens were calculated for samples. The geometric mean values of the antigen-stimulated triplicates were identified and divided with the geometric mean values of the medium control triplicates to calculate the stimulation index (SI).

ELISA (Enzyme-Linked Immune Sorbent Assay): Serum IgG2a (eBioscience™ Mouse IgG2a ELISA Ready-SET-Go! ™ Kit), serum and nasal wash total IgA (Bioassay Technology Laboratory Mouse immunoglobulin A ELISA kit) and cytokines concentrations as well as the concentrations of cytokines secreted into the supernatants from stimulated splenocytes were quantified using ELISA kits for IL-6, TNF-α, IL-10, and IL-17 according to the manufacturer's (R&D Systems, DuoSet® ELISA development system, USA) instructions. Briefly, ELISA plates were coated with the capture antibody. After washing, plates were blocked by blocking solution. Then, 1/2 dilution of sera, culture supernatants, and antibody standards were added to wells, incubated for 2h. After washing, a detection antibody was used, and the conjugated enzyme was added. Twenty minutes after adding the substrate solution, a stop solution was used, and the ELISA reader read the plate's ODs at 450nm. The antibody concentrations were calculated using the standards and linear regression of the log-transformed readings.

IFN-γ ELISPOT assay: According to the Mouse IFNg ELISPOT kit (eBioscience), the ELISPOT assay was done in triplicate for each experimental group. In brief, a 96 well PVDF plate (Millipore) was coated with a capture antibody (anti-mouse IFN-γ) solution and incubated at 4°C overnight. The plate was washed 2 times and blocked with complete RPMI-1640 for one hour at room temperature. Then 1×10^6 spleen lymphocytes were added to each well and stimulated with 10 or 20µg of the PNEU polytopes and adjuvant. Positive controls stimulated by PHA. Plate incubated at 37°C in 5% CO2 for 48 hours. Cells were then removed by wash buffer, and the biotin-conjugated detection antibody was added to the wells and incubated for 2 hours at room temperature. The plate was washed 4 times with wash buffer, and Avidin-horseradish peroxidase solution was added and incubated for 45 minutes. After washing five times, the substrate solution was added to each well, and the plate was kept in the dark, up to 60 minutes for spot development. Then the plate was washed 3 times with distilled water and dried at room temperature. A loop counted spots, and the results were represented as the number of spot-forming cells per 300 thousand cells.

Serum bactericidal assay: Type 19 (serotype 19F)

pneumococcal strain, which supplies freeze-dried ampule, was purchased from Persian Type Culture Collection (Iranian Research Organization for Science and Technology (IROST)). The bacterial suspension was prepared with 300 µl sterile PBS, cultured on blood agar (trypticase soy agar with 5% sheep blood), and then incubated for 24 hours at 37°C in a candle jar. The following day, colonies were removed and diluted in HBSS (Hanks Balanced Salt Solution) with 1% BSA (Bovine Serum Albumin) to a concentration of 3×10^3 CFU/ml. Sera were also diluted at 1:100 and 1:1000 in HBSS with 1% BSA. 200µl of the bacterial solution was added to a tube mixed with 80µl of each diluted serum. Tubes were incubated for 30 minutes at 37°C with shaking. Naïve mouse serum was added (20µl) to each tube as the complement source, incubated for 30 min again. The suspensions were then poured on blood agar plates. As a control, suspension without serum was placed on agar. Mouse neutrophils were purified from mouse peripheral blood as phagocytic (effector) cells[12]. When the suspension was soaked on the plate at room temperature, 1×10^6/ml neutrophil cells in HBSS with 1% BSA were placed over the agar to cover it entirely. After soaking in agar, the plates were incubated for 12 hours at 37°C. The number of the bacterial colonies on each plate was counted the following day. Each group was repeated three times, and the results were reported as an average of the number of colonies for each group. This protocol is adapted with some modifications of Genschmer et al.'s paper[14].

Toxicity effect evaluation of the PNEU poly-epitope: For histopathology study, liver tissue samples fixation was performed with 10% neutral buffered formalin. Then, sections were embedded in paraffin and stained with Haematoxylin & Eosin. The histopathological examination was carried out as published in Cui et al. article[15].

Statistical analysis: Data are expressed in the mean ± standard error of the mean (SEM), and statistical analysis was performed using ANOVA (analysis of variance) with Tukey's post hoc test. (*P<0.05, **P<0.01, ***P<0.001).

Results

Preparation of PNEU polytope: Based on IEDB server predictions, epitopes from the aligned consensus sequences of the proteins with higher scores so strong binding affinity to MHC alleles were chosen. Figure 1 shows *S. pneumoniae* determinant epitopes fused with suitable linkers randomly.

The size and purity of the protein were confirmed by SDS-PAGE (~ 20 KDa) and western blot using an anti-S-tag monoclonal antibody (Fig. 2).

Evaluation of the immunized mice

Spleen lymphocytes proliferation assay by MTT: Cellular response stimulation has been indicated by elevated splenic cell expansion. Proliferation index (PI) was computed by [OD of test sample−O.D. of negative control /OD of negative control]. The mean values of the proliferation index in the P+CsMP group were 2.8±0.36 that was significantly higher than other groups. Positive controls in stimulation with PHA in each group showed a normal condition of cells in culture. Although group P had a significantly different mean PI value (P<0.001) from the control group, it also had a significant difference with P+CsMP group.

Antibodies and Cytokines concentrations evaluation by ELISA and IFN-γ ELISPOT assays: IgG-2a and IgA antibody concentration in mice sera were examined by ELISA assay [Figure 3A& B]. The assay was performed on pooled sera from five mice in each group. Similarly, the serum's concentration of secreted cytokines (IL-6, IL-10, IL-17, and TNF-α) was measured in the serum [Figure 4] and in the supernatants of splenocytes after exposure to the polytope. IgA concentration in nasal washes was also determined by ELISA assay. Immunized mice in P+CsMP produced significant (P<0.001) IgG-2a antibody response compared with the P group. Similar observations were also observed in animals' nasal wash and serum production of IgA. The CsMP and P alone groups elicited low levels of each cytokine. In contrast, the P+CsMP group showed high IL-6, TNF- α, IL-10, and IL-17 cytokine concentrations. Upper cytokine concentrations were observed in the P+CsMP groups for both sera and supernatant samples.

LYIELLRNLAGGGSSDHVDPYPYLAKKFERQAETYAAVELIESHSTKGPGPGDYRVVVDPVK
PAYSDGGGSYPQVKDVYVQHHAATSLRSGNFIGPGPGVGAMGLVVLPSAGAVGGGSAFGLL
TVGSLLLIGGGSYSTASYNALGPGPGGNKTRASLLVPKVDYGGGSPLVYISSVAYHHAAKQI
YYTVSVDAVKNPGGGSKETAAAKFERQHMDS

Figure 1. The sequence of the PNEU poly-tope. The epitopes were joined to each other using underlined linkers.

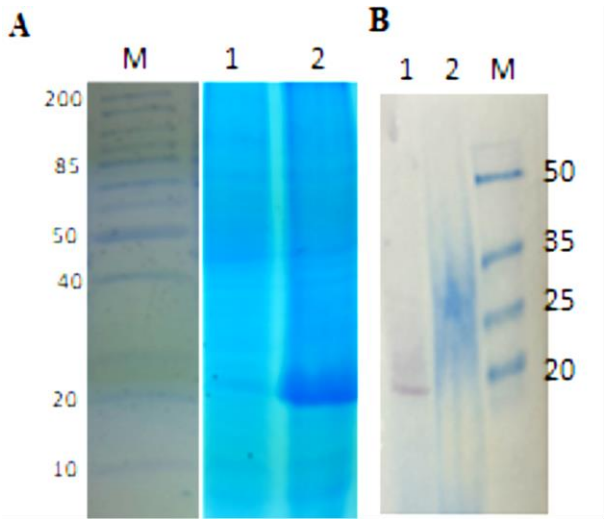

Figure 2. The PNEU recombinant protein expression analysis. (A) SDS-PAGE (12%) followed by an R250 Blue Coomassie stain. (B) Confirmation of expression by Western blot. M: Protein Molecular Weight Marker (KDa), Lane 1: Non-induced control, Lane 2: poly-tope protein.

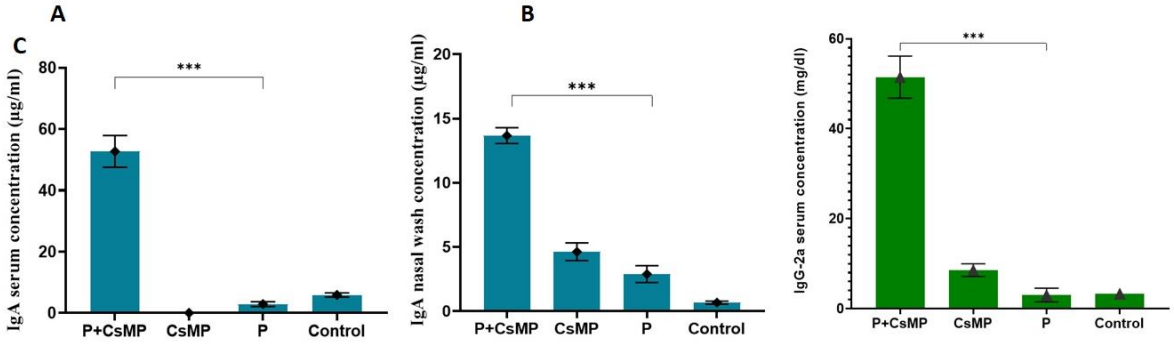

Figure 3. The ELISA assay on pooled sera from five mice in each group. A) Serum IgG-2a, B) serum IgA, and C) nasal wash IgA concentrations. (* P<0.05, ** P<0.01, *** P<0.001).

Cell-mediated immunity was assessed by detecting spots of IFN-γ secreting splenic cells stimulated by 10µg or 20µg of the polytope in culture. The mean number of spots was calculated in the triplicate wells. In both polytope concentrations, we apperceived a significant increase in the number of spots for P+CsMP group compared with other groups. Besides, the spot number of the P group for both polytopes concentrations was more than the CsMP alone and control groups (P<0.05) but not more than P+CsMP group (P<0.001).

Serum bactericidal assay: This complement and the phagocyte-dependent assay were used to evaluate the protective antibodies elicited against *pneumococci* protein epitopes in animal sera. Neutrophils as effector cells can phagocyte the bacteria on the surface of blood

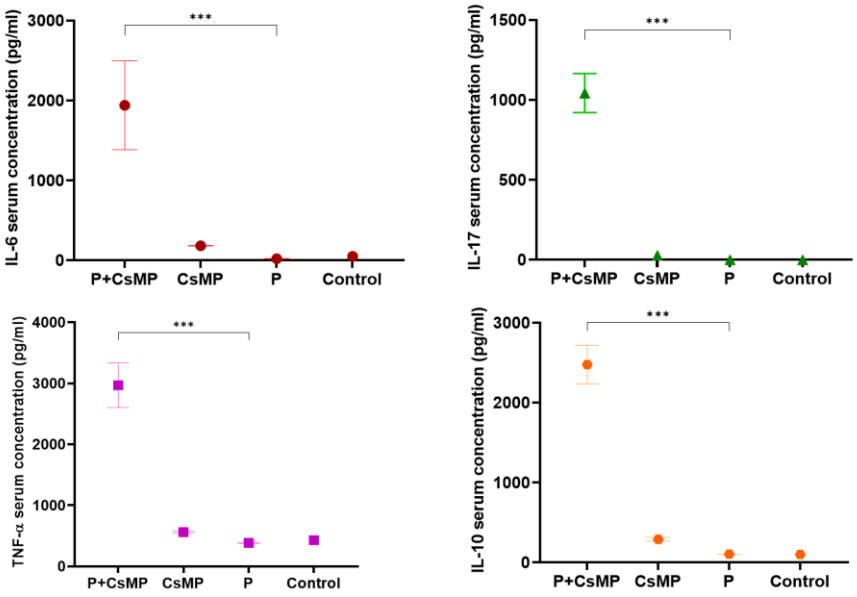

Figure 4. Serum cytokine concentrations analysis. IL-10, IL-17, IL-6, TNF-α serum concentrations were measured by ELISA on pooled sera from five mice in each group. (* P<0.05, ** P<0.01, *** P<0.001).

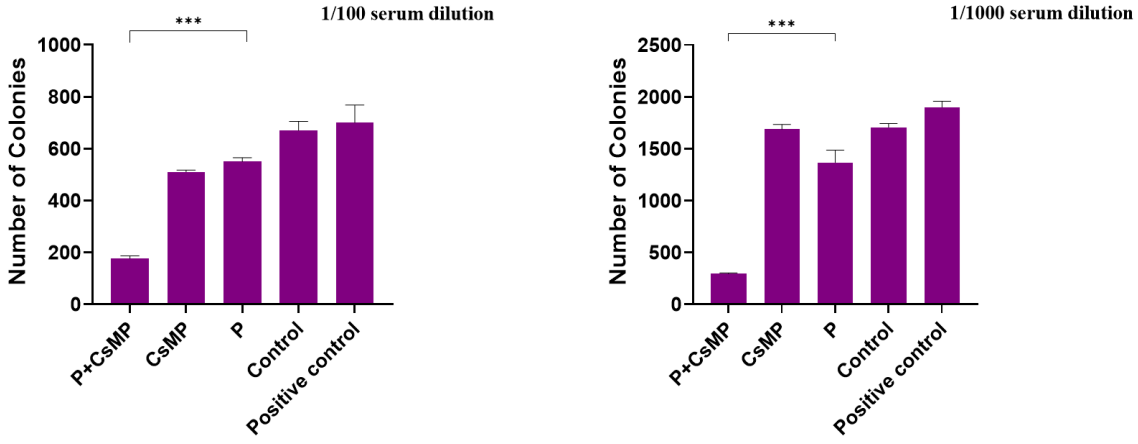

Figure 5. Comparison of Pneumococcal colony numbers on blood agar. A) 1/100, B) 1/1000 serum dilutions. (* P<0.05, ** P<0.01, *** P<0.001).

agar in the presence of serum protective antibodies, and naïve mice complement. As seen in Figure 5, sera obtained from P+CsMP group contain antibodies that can inhibit bacterial growth on the surface of the agar at both serum dilution 1/100 and 1/1000. This bactericidal effect is shown by reducing their bacterial colony numbers compared to the control group. Bacterial growth-inhibiting of P+CsMP group was significantly higher at 1/100 serum dilution (P<0.05).

Microscopically evaluation of polytopes effects: Histological examination of the liver tissues showed completely healthy hepatocytes and tissue structure and the control group.

Discussion

Anyway, a predicted poly-tope construct should be tested and confirmed by in vitro and in vivo experimental studies like immunological tests. In this

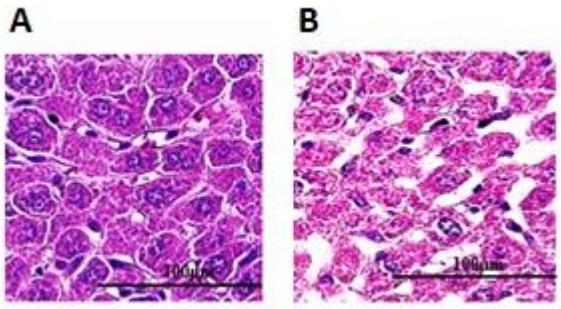

Figure 6. Microscopic view of the Hematoxylin & Eosin stained liver tissue sections (Bar= 100µm). There are no specific pathological changes in liver tissues of immunized mice with A) P+CsMP group, B) Control group.

study, we have developed a poly-tope construct named PNEU. The proliferation stimulation assay showed that combining chitosan with the polytope (complex) can effectively enhance proliferation compared to polytope alone. This complex has offered a further boost in proliferation index for P+CsMP group so that the formulation could induce proper immune responses against *S. pneumoniae* pathogen. Up to date, no study has evaluated chitosan microparticles in combination with pneumococcal antigens or vaccines. However, we found studies that had studied the administration of this polymer with antigens or vaccines of other bacterial and viral pathogens. In several studies, chitosan micro-particles have been studied both as carriers and adjuvants. Tetanus[16] and diphtheria[17] toxoids transported orally by chitosan microparticles resulted in IgA and IgG production, leading to both systemic and local protection against Clostridium tetani and Corynebacterium diphtheria, respectively. Micro-particles made with mannosylated chitosan have been shown to stimulate the immune responses in the intranasal delivery of Bordetella bronchiseptica antigens[18]. Bordetella bronchiseptica antigens against atrophic rhinitis in pigs[19], recombinant hepatitis B antigen in mice[20] and meningococcal C conjugate vaccine[21], when carried intranasal by a chitosan or its derivatives micro-particles, all of them showed similar results in stimulating the humoral immune responses (both mucosal and systemic) as well as cellular immune activities.

In our study, consistent with other studies, as demonstrated by enhanced IgG-2a and IgA concentrations in mice sera and IgA concentration in the nasal wash, it can be concluded that intranasal administration of PNEU adjuvanted protein resulted in increased antibody responses in vivo. IgG-2a antibody has the role of pathogen clearance[22]. IgA antibody can neutralize pathogens at their attack site. Consequently, inhalation vaccines against respiratory pathogens can inhibit the pathogen at the entry site with the help of secretory IgA antibodies. In our study, the cell proliferation was accompanied by increased concentration of IL-6 and TNF-α cytokines secretion in sera and supernatant of splenocytes. Considerably cytokine production was higher for the group treated with P+CsMP but not for those treated with CsMP or P only. Alongside these results, the IFN- γ ELISpot assay revealed that P+CsMP group significantly increased the numbers of IFN-γ-secreting cells compared to the rest of the groups. IFN-γ was secreted in response to the re-activation of cells in vitro. Upper levels of IFN-γ producing cells and TNF-α secretion in sera and supernatants observed in our main study groups confirmed other reports that showed chitosan and its derivatives could have overexpressed such cytokines in the mouse models[23]. Increasing in IFN-γ, induces antibody class switching to IgG2a.

Furthermore, an increase in the concentration of IL-10 in P+CsMP group is observed in our study. It was shown that the binding of chitin and chitosan to the surface receptors of macrophages causes the production of interleukin-10[24]. The microparticles size impacts the production of IL-10. Microparticles with a 40-70 micrometers induce TNF-α but not IL-10 generation from the TLR-2 pathway. Microparticles below 40 µm provoked the production of both of them by macrophages. The dectin-1 receptor and the Syk

signaling pathway had the main impact on the production of the IL-10 by microparticles below 40 micrometers[25]. Intranasal administration of vaccines promotes IL-17 secretion. Also, particles can stimulate Th-17 amplification by activating the TLR-2-MyD88 signaling pathway.

IL-17 causes secretory IgA transportation to the nasal cavity[26]. The augmented local and systematic IgA concentrations in P+CsMP group are justified by the presence and secretion of IL-17 cytokine, which is also confirmed by the increased IL-17 concentration in sera and supernatants of the mentioned group. Also, it is demonstrated that the cytokine IL-17 protects mice against pneumococcal colonization in a strain-dependent mode by neutrophil recruitment[27]. Thus, it is worthy of formulating vaccines prompting IL-17 responses[28]. Overall, it can be the result that small-sized chitosan microparticles could elicit a balanced Th1/Th2/Th17 response and be an efficacious adjuvant candidate for a wide range of vaccines. Bactericidal results revealed that antibodies against bacterial protein epitopes could kill the bacteria on a blood agar surface. Neutrophils and complements are necessary for this antibody-mediated growth-inhibiting and killing. A decrease in bactericidal effect was observed in less dilution of serum (1/1000), with lower concentrations of neutralizing antibodies. Our results show elevation of antibodies and cytokines and lymphocytes proliferation to our formulation. Because chitosan has more positive charges, we can say that chitosan has a higher affinity for the negatively-charged polypeptides, depots antigens, and presents them to the immune system more effectively. However, by the mucoadhesive property, chitosan causes antigen entrapment in mucosal tissue and enhances their uptake by nasal-associated lymphoid tissue[29]. Chitosan can depot and slow the release of antigens, increase the presentation of APCs (Antigen Presenting Cells) and produce more intense responses. For designing safe and efficient mucosal vaccines, chitosan microparticles are valuable candidates as they can properly overcome chemical and physical mucosal barriers[30].

Conclusion

PNEU protein accompanied by chitosan microparticles showed acceptable elicited humoral and cellular immune responses in BALB/c mice. Also, the bactericidal test showed the capacity of the formulation to inhibit the bacteria.

Acknowledgment

This research is a part of the student's thesis. The authors would like to thank Dr. Zarin Sharifnia and the Cellular and Molecular Research Center staff at Shahid Beheshti University of Medical Sciences. This study was funded by Shahid Beheshti University of Medical Sciences [Grant number 12747]. The authors declare no conflict of interests, and all of the raw data are available based on a request from the corresponding author.

References

1. Chiavolini D, Memmi G, Maggi T, Iannelli F, Pozzi G, Oggioni MR. The three extra-cellular zinc metalloproteinases of Streptococcus pneumoniae have a different impact on virulence in mice. BMC Microbiol. 2003;3:14.

2. Mellroth P, Daniels R, Eberhardt A, Rönnlund D, Blom H, Widengren J, et al. LytA, Major Autolysin of Streptococcus pneumoniae, Requires Access to Nascent Peptidoglycan. J Biol Chem. 2012;287:11018–29.

3. Daniels CC, Rogers PD, Shelton CM. A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens. J Pediatr Pharmacol Ther. 2016;21:27–35.

4. Wadhwani A, Khanna V. In Silico Identification of Novel Potential Vaccine Candidates in Streptococcus pneumoniae. J Informatics Data Min. 2016;1:1–8.

5. Haji Molla Hoseini M, Sadeghi S, Azizi M, Pouriran R. Immunomodulatory activities of chitin and chitosan microparticles. In: GOPI S, THOMAS S, PIUS A, editors. Handbook of chitin and chitosan: Chitin- and chitosan-based polymer materials for various applications. 3rd ed. Susan Dennis; 2020. p. 609–39.

6. Khan N, Jan AT. Towards Identifying Protective B-Cell Epitopes: The PspA Story. Front Microbiol. 2017;8:1–8.

7. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: Transmission, colonization and invasion. Nat Rev Microbiol. 2018;16:355–67.

8. AlonsoDeVelasco E, Verheul AFM, Verhoef J, Snippe H. Streptococcus pneumoniae: virulence factors, pathogenesis, and vaccines. Microbiol Rev. 1995;59:591–603.

9. Sharapova Y, Suplatov D, Švedas V. Neuraminidase A from Streptococcus pneumoniae has a modular organization of catalytic and lectin domains separated by a flexible linker. FEBS J. 2018;285:2428–45.

10. Harish Prashanth KV, Tharanathan RN. Chitin/chitosan: modifications and their unlimited application potential—an overview. Trends Food Sci Technol. 2007;18:117–31.

11. Verma A, Prasad KN, Singh AK, Nyati KK, Gupta RK, Paliwal VK. Evaluation of the MTT lymphocyte proliferation assay for the diagnosis of neurocysticercosis. J Microbiol Methods. 2010;81:175–8.

12. Swamydas M, Luo Y, Dorf ME, Lionakis MS. Isolation of Mouse

Neutrophils. Curr Protoc Immunol. 2015;110:3.20.1-3.20.15.
13. Sadeghi S, Bandehpour M, HajiMollaHosseini M, Kazemi B. Designing and expression of an immunogenic poly-epitope protein from Streptococcus pneumoniae. TPPS.2021
14. Genschmer KR, Accavitti-Loper MA, Briles DE. A modified surface killing assay (MSKA) as a functional in vitro assay for identifying protective antibodies against pneumococcal surface protein A (PspA). Vaccine. 2013;32:39–47.
15. Cui L-M, Zhang K, Ma D-J, Liu S-P, Zhang X-W. Protein expression under sustained activation of signal transducer and activator of transcription-3 in diethylnitrosamine-induced rat liver carcinogenesis. Oncol Lett. 2014;8:608–14.
16. Ahire VJ, Sawant KK, Doshi JB, Ravetkar SD. Chitosan Microparticles as Oral Delivery System for Tetanus Toxoid. Drug Dev Ind Pharm [Internet]. 2007 Jan 25;33(10):1112–24. Available from: doi: 10.1080/03639040701377847
17. Van Der Lubben IM, Kersten G, Fretz MM, Beuvery C, Verhoef JC, Junginger HE. Chitosan microparticles for mucosal vaccination against diphtheria: Oral and nasal efficacy studies in mice. Vaccine. 2003;21(13–14):1400–8.
18. Jiang HL, Kang ML, Quan JS, Kang SG, Akaike T, Yoo HS, et al. The potential of mannosylated chitosan microspheres to target macrophage mannose receptors in an adjuvant-delivery system for intranasal immunization. Biomaterials. 2008;29(12):1931–9.
19. Kang ML, Jiang HL, Kang SG, Guo DD, Lee DY, Cho CS, et al. Pluronic®F127 enhances the effect as an adjuvant of chitosan microspheres in the intranasal delivery of Bordetella bronchiseptica antigens containing dermonecrotoxin. Vaccine. 2007;25(23):4602–10.
20. Jaganathan KS, Vyas SP. Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant Hepatitis B antigen administered intranasally. Vaccine. 2006;24(19):4201–11.
21. Baudner BC, Verhoef JC, Giuliani MM, Peppoloni S, Rappuoli R, Del Giudice G, et al. Protective immune responses to meningococcal C conjugate vaccine after intranasal immunization of mice with the LTK63 mutant plus chitosan or trimethyl chitosan chloride as novel delivery platform. J Drug Target. 2005;13(8–9):489–98.
22. Dorosti H, Eslami M, Negahdaripour M, Ghoshoon MB, Gholami A, Heidari R, et al. Vaccinomics approach for developing multi-epitope peptide pneumococcal vaccine. J Biomol Struct Dyn. 2019;37:3524–35.
23. Fang L, Wolmarans B, Kang M, Jeong KC, Wright AC. Application of Chitosan Microparticles for Reduction of Vibrio Species in Seawater and Live Oysters (Crassostrea virginica). Appl Environ Microbiol. 2015;81:640–7.
24. Da Silva CA, Chalouni C, Williams A, Hartl D, Lee CG, Elias JA. Chitin Is a Size-Dependent Regulator of Macrophage TNF and IL-10 Production. J Immunol. 2009;182:3573–82.
25. Da Silva CA, Pochard P, Lee CG, Elias JA. Chitin Particles Are Multifaceted Immune Adjuvants. Am J Respir Crit Care Med. 2010;182:1482–91.
26. Da Silva CA, Hartl D, Liu W, Lee CG, Elias JA. TLR-2 and IL-17A in Chitin-Induced Macrophage Activation and Acute Inflammation. J Immunol. 2008;181:4279–86.
27. Ritchie ND, Ritchie R, Bayes HK, Mitchell TJ, Evans TJ. IL-17 can be protective or deleterious in murine pneumococcal pneumonia. PLOS Pathog. 2018;14:e1007099.
28. Moffitt KL, Gierahn TM, Lu YJ, Gouveia P, Alderson M, Flechtner JB, et al. TH17-based vaccine design for prevention of streptococcus pneumoniae colonization. Cell Host Microbe. 2011;9:158–65.
29. Kang ML, Cho CS, Yoo HS. Application of chitosan microspheres for nasal delivery of vaccines. Biotechnol Adv. 2009;27:857–65.
30. Li M, Wang Y, Sun Y, Cui H, Zhu SJ, Qiu H-J. Mucosal vaccines: Strategies and challenges. Immunol Lett. 2020;217:116–25.