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A Therapeutic Oral Vaccine Candidate against *Propionibacterium acnes*: Preparation and Immunological Evaluation of Nanoparticles Encapsulated Sialidase-CAMP Fusion Protein

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HIGHLIGHTS

- This study evaluated chitosan- nanoparticulated fusion protein as an acne oral vaccine.
- The Sia-CAMP protein was encapsulated in chitosan nanoparticles and induced immune response in mice.
- Although subcutaneous immunization has induced better systemic immunity, oral administration could induce both systemic and mucosal immune responses.

Abstract: Acne Vulgaris is a common skin disease caused by *Propionibacterium acnes*, an anaerobic microbiota of human skin that plays a vital role in the pathology of acne. The aim of this study was to prepare nanoparticles containing an acne recombinant protein and determine its ability as an oral acne vaccine in mice. The recombinant Sialidase-CAMP gene was expressed and purified in a prokaryotic host. The chitosan nanoparticles containing the recombinant protein were prepared, encapsulated, and administered by both oral and subcutaneous routes to Balb/c mice. Sera IgA and IgG and stool IgA titers were measured by ELISA, and the immunized mice were challenged against *P. acnes*. A 65 kDa recombinant protein was confirmed by SDS-PAGE and western blot. The size and zeta potential of nanoparticles were 80 nm and +18 mV, respectively. After oral immunization, the serum IgG and IgA titers were 1:3200 and 1:16, respectively, and the stool IgA titer was 1:8. In the subcutaneous route, the serum IgG titer was 1:51200. Immunized mice showed no inflammation in the ear of challenged mice. It is the first study that examines a chitosan-nanoparticulated acne fusion protein as an applicable acne vaccine candidate with appropriate

immunogenicity potential. Further studies are required to validate the clinical usefulness of this vaccine candidate.

Keywords: *Propionibacterium acnes*; Sialidase-CAMP fusion protein; chitosan nanoparticle; Acne vaccine

INTRODUCTION

Acne Vulgaris is a multifactorial disease mostly contributed by the proliferation of Gram-positive anaerobic *Propionibacterium acnes*. It is a common skin disease experienced by approximately 85% of individuals in 18-25 years of their life, causing both physical and psychological complications, including scar tissue, depression, anxiety, and low self-confidence (1). The main treatment for acne is retinoic acids and antibiotic treatments. Due to long-term treatment periods, antibiotic administration can disrupt the integrity of skin and intestinal resident normal microbiota as well as dominating antibiotic-resistant bacteria in patients. Also, due to various side effects such as dry skin, epistaxis, photosensitivity, increased serum lipids, and myalgia, no adequate treatment is yet available for acne (2, 3). In this regard, vaccine development may be considered at least as a supplementary treatment for acne disease. But, despite the importance of vaccine therapy in the clinical treatment of many diseases, limited studies have been done on acne vaccine therapy. For years, the heat-killed *Propionibacterium acnes*, isolated from the same acne patient's skin lesions, has been used as a traditional supplemental treatment (4). Currently, AcneVac is a commercially available vaccine in Europe as a capsule containing three standard strains of killed *P. acnes* (5). Since recombinant bacterial vaccines are a suitable alternative to traditional vaccines, some new vaccine approaches containing the bacterial surface antigens were used as an acne vaccine candidate in recent years. In some studies, Nakatsuji and coauthors proposed and evaluated the immunogenicity properties of two *P. acnes* surface proteins as the potential acne vaccine candidates (6, 7). They separately examined the immunogenicity of sialidase enzyme (a 502 kDa cell wall attached protein of *P. acnes*) and CAMP factor (a 267 kDa hemolytic protein of *P. acnes*) as subcutaneous vaccine candidates, demonstrating their capacity to reduce bacterial-induced inflammation *in vivo* that can neutralize *P. acnes in vitro* (6, 7). In a previous study, we *in silico* designed a chimeric protein vaccine candidate that contained immunogenic parts of the sialidase and CAMP factor linked by four repeated EAAAK linkers (the Sia-CAMP fusion protein) (8). We showed that this fusion protein could make a strong immune response by immunoinformatic evaluation. In this study, we tried to investigate the immunogenicity potential of this fusion protein in a mice model by oral immunization. For years, oral immunization with vaccines has been examined against bacterial infections. Although being potentially immunologic, cost-benefit, applicable, and painless, only a few mucosal vaccines are available. Nanoparticulation with chitosan and other polysaccharides is the main strategy used for oral vaccine development. Chitosan, a natural linear polysaccharide made of D-glucosamine and N-acetyl-D-glucosamine units, is a suitable biopolymer for various biomedical applications including nonvaccine development (9). Regarding the advantages of oral vaccine, we aimed to express, purify, and immunologically evaluate the chitosan nanoparticulated Sia-CAMP fusion protein in mice model through the oral and injection routes as a therapeutic acne vaccine candidate.

MATERIAL AND METHODS

Mice and ethics statement

The 6-8-week-old female BALB/c mice were purchased from Razi Institute (Karaj, Iran) and maintained in polypropylene cages at 20–22 °C, 12-h light/dark cycles. All experimental procedures on animals were approved by the local ethical committee of Baqiyatallah University of Medical Sciences.

Expression, purification, and western blotting

In the previous study, a synthetic construct containing the C-terminal amino acids of the sialidase and N-terminal amino acids of the Camp factor was designed, and linked by a rigid EAAAK linker. Then, the recombinant Sia-CAMP gene sequence along with the EcoR1 and Hind III restriction sites sequences at 3' and 5' ends of the construct was synthesized with codon optimization for a prokaryotic host (8). After cloning in pET28a (+), the "pET28a /Sia-CAMP" construct was transformed and expressed in the *E. coli* BL21 DE3 host. For gene expression induction, IPTG (1mM) was added to the nightly cultured bacteria containing kanamycin (20 µg/mL) (10). After centrifugation (6000 RPM, 25min), the bacteria were sonicated, centrifuged, and the pellet was dissolved in lysis buffer (8 M urea, 0.1 NaH₂PO₄, and 0.01 M Tris). After centrifugation, the supernatant was run on 12% SDS-PAGE (11). For protein purification, the cell lysate supernatant was

loaded on the Nickel-nitrilotriacetic acid (Ni-NTA) affinity column using a buffer containing imidazole 500 mM as the eluting buffer. Finally, the protein concentration was determined by Bradford assay, and western blotting was done by standard protocols (10, 11).

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by ionic gelation. A solution of 2 mg/mL chitosan in 2% acetic acid and 1 mg/mL sodium polyphosphate solution were prepared. The sodium tripolyphosphate solution (5 mL) was added dropwise to 5.7 mL of chitosan solution containing the antigen, centrifuged for 40 minutes at 13,000 rpm, and the supernatant was separated (12, 13). The morphological properties of Sia-CAMP recombinant protein-loaded chitosan nanoparticles were examined using a scanning electron microscope (SEM). Also, the loading efficiency was calculated based on the difference in the amount of the initial protein added to the chitosan solution and the final amount of the residue in the supernatant through the following formula:

$$\text{LE (Loading efficiency)} = \frac{\text{Total amount of protein} - \text{Free protein}}{\text{Total amount of protein}} \times 100 \quad (1)$$

Also, the size of nanoparticles was determined using the DLS device model Malvern.

Mice Immunization with the antigen-loaded chitosan nanoparticles

Three mice groups (5 mice/group) were used to compare the systemic and mucosal immune responses against the Sia-CAMP fusion protein. For the systemic immune response evaluation, 20 µg of Sia-CAMP loaded nanoparticles were injected subcutaneously into the first mice group, followed by three booster doses within two weeks intervals. For the assessment of mucosal immune responses, 100 µg of Sia-CAMP loaded nanoparticles were gavaged to the second group with an additional three booster doses within two weeks intervals. As a control group, the third group was orally received nanoparticle loaded Bovine Serum Albumin in the same manner. The groups of immunized and control mice are shown in Table 1.

Mice Immunization with heat-inactivated *P. acnes*

To compare the immunization of nanoparticles loaded with the fusion protein as well as the heat-inactivated *P. acnes*, another group of five mice were immunized with heat-inactivated *P. acnes*. Freeze-dried *P. acnes* (PTCC 6919) was prepared from the Pasteur Institute of Iran (Tehran, Iran) and re-confirmed with standard microbiological methods. To prepare the inactivated *P. acnes*, a PBS dissolved colony suspension (10^7 CFU/mL) was centrifuged, washed three times, and heated at 65 °C for 45 min. After treatment, no bacterial growth was confirmed by culture in specific culture media. Then, the suspension was subcutaneously injected with the complete Freund's adjuvant (for the first injection) and incomplete Freund's adjuvant (three boosters; 2 weeks interval) into mice. The groups of immunized and control mice are shown in Table 1.

Determination of the antibody titer by indirect ELISA

Two weeks after each booster, peripheral blood was obtained from the retro-orbital plexus of all immunized and control mice groups. The serum titers of IgG and IgA against the Sia-CAMP fusion protein were measured by indirect ELISA using the standard protocol (14). Briefly, 96-well ELISA plates (NUNC, Denmark) were coated with the Sia-Camp fusion protein (1 µg/100 µl/well) in the carbonate-bicarbonate buffer (37 °C, 2h). The plates were washed with PBST (4X) and blocked with skim milk solution 2% (w/v) in PBST. Then, the plates were incubated with a serial dilution of 1:100 to 1:100000 for IgG and 1:2 to 1:32 for IgA (37 °C, 1h). Finally, anti-mouse IgG and IgA- HRP conjugated antibodies (Sigma, USA) were subjected to the plates. The sera collected from mice before immunization was used as negative control. For secretory IgA determination, fecal samples were collected two weeks after each booster from orally immunized mice. The PBS buffer containing cocktail protease inhibitor (Sigma, USA) was added to mice feces, roughly vortexed, and centrifuged 14000 rpm for 10 min. Finally, the supernatant was subjected to the Sia-CAMP protein-coated plate (15).

Challenge

For checking the immunization, 10^7 CFU of *P. acnes* was diluted in PBS buffer and subcutaneously injected into one of the ears of all immunized and control mice groups. Besides, PBS was injected into the

other ear, as the injection control. Then, during 40 days, the thickness and inflammation of the ear were recorded by micro-caliper until the swelling of the ear almost dropped.

Statistical analysis

The statistical analyses were carried out with SPSS statistical software (version 20). Data obtained from the antibody detection and also protection was analyzed using the two-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

RESULTS

Expression and purification of the recombinant protein

After induction of the chimeric gene in optimized condition (1 mM IPTG after 3h at 37°C), the protein content was run on 12% SDS-PAGE showing a complete expression of Sia- CAMP fusion (Figure 1). The majority of the fusion protein was expressed as a soluble protein, and following Ni-NTA affinity chromatography, the Sia-CAMP fusion was purified with a weight of 65 KD with high purity at 500 mM imidazole elution. Then, the protein expression was confirmed by western blotting due to having a His-tag sequence, with the desired band sited in the correct weight position with no other bands in the column control (Figure 2).

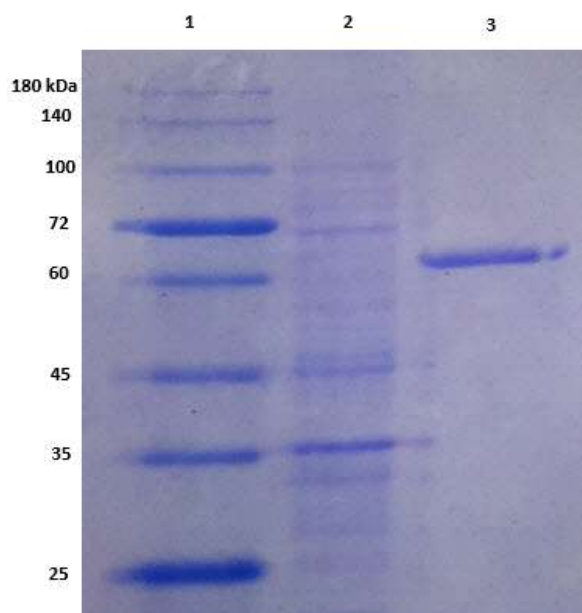


Figure 1. Gel electrophoresis after expression and purification of the protein using NI-NTA. Lane 1: protein weight marker; lane 2: Control or non-induced sample; lane 3: wash column with 500mM imidazole.

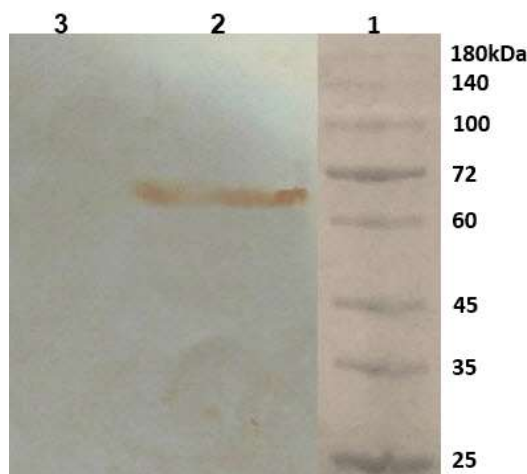


Figure 2. Confirmation of the recombinant protein by Western blotting. Column 1: Protein Molecular Marker; Column 2: The desired protein; Column 3: Control Sample (No IPTG).

Preparation of chitosan nanoparticles and protein loading rate

After the chitosan nanoparticle preparation by the ion gelation method and size measurement, the result of DLS indicated the production of chitosan nanoparticles with an average size of 85 nm. Zeta potential of the nanoparticles containing the recombinant protein was +18 mV. Also, after optimizing nanoparticles preparation, the loading rate was estimated at 88%. Besides, as shown in Figure 3, analyzing SEM images showed that the average diameter of the produced monodispersed spherical nanoparticles containing Sia-CAMP protein was found to be about 90 nm (Figure 3).

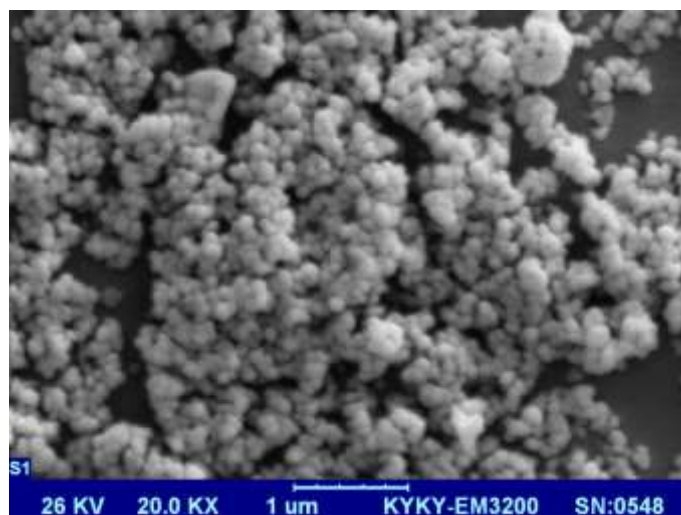


Figure 3. SEM images of Sia-CAMP fusion protein loaded chitosan nanoparticles.

Measurement of antibody titer

The IgG antibody titers were measured in serum samples for all groups. Also, the IgA antibody was tittered in both serum and stool samples of immunized and control mice groups. When the protein was injected with chitosan nanoparticles, after each booster, the IgG antibody titer increased significantly, from 1: 6400 to 1: 12800 in the first booster, and finally to 1: 51200 in the second booster. Chitosan nanoparticles have also been shown to be an appropriate adjuvant and could induce high titers of systemic IgG in mice (Figure 4, A). In comparison, the mucosal administration of chitosan nanoparticulated protein could pass the protein through the intestinal barrier and induced a strong systemic immune response of IgG with a titer of 1: 32000 (Figure 4, B). Also, it stimulated the mucosal immune system and increased serum IgA titers to 1:32 (Figure 4, D). Measurements of secretory IgA in feces also showed the induction of mucosal responses in mice up to the 1:8 titer (Figure 4, E). In contrast, the heat-inactivated *P. acnes*, which was injected with incomplete and complete Freund's adjuvant, induced only a systemic immune response, and IgG titers reached 1:102400 in the third booster (Figure 4,C). Furthermore, the mean IgG titer was compared between injection and oral administration routes of the nanoparticulated fusion protein with that of heat-inactivated bacteria (Figure 5). The mice immunized with heat-inactivated *P. acnes* showed higher IgG titers rather than the control group ($p < 0.003$). Also, in the mice group immunized with antigen-loaded nanoparticles, although the IgG titers were lower than mice immunized with heat-inactivated *P. acnes*, they had a significantly higher IgG titer against the control group ($p < 0.003$). The nanochitosan could show an adjuvanticity property during the injection and oral administration routes of the fusion protein and induced systemic immune responses in mice.

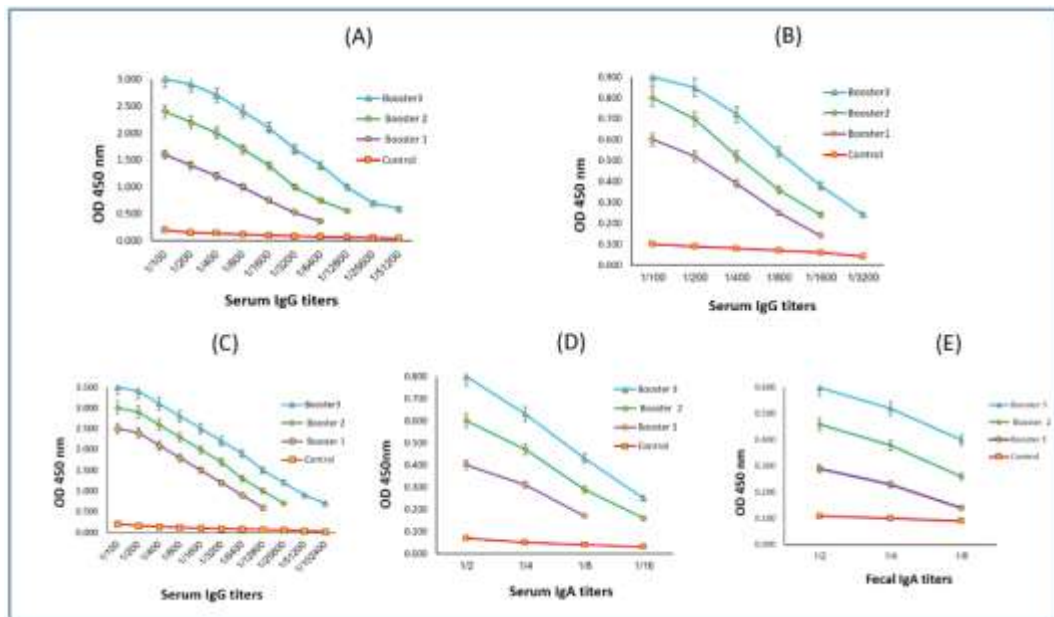


Figure 4. IgG and IgA antibody titers against Sia-CAMP fusion protein, and heat-inactivated *P. acnes* that were measured in Balb/c mice. (A) Serum IgG antibody titer after injection of antigen-loaded nanoparticles. (Test samples include antigen-loaded nanoparticles and control samples include antigen-free nanoparticles). (B) Serum IgG antibody titer after oral administration of the antigen-loaded nanoparticles. (The test samples included antigen-loaded nanoparticles and control samples include antigen-free nanoparticles). (C) Serum IgG antibody titer after the standard vaccination with the heat-inactivated bacteria. (D) Serum IgA antibody titer after oral administration of antigen-loaded nanoparticles. (E) Secretory IgA antibody titer after oral administration of antigen-loaded nanoparticles. Data expressed as mean analyzed sera/mice group. Values are significantly different from the control at $p < 0.001$.

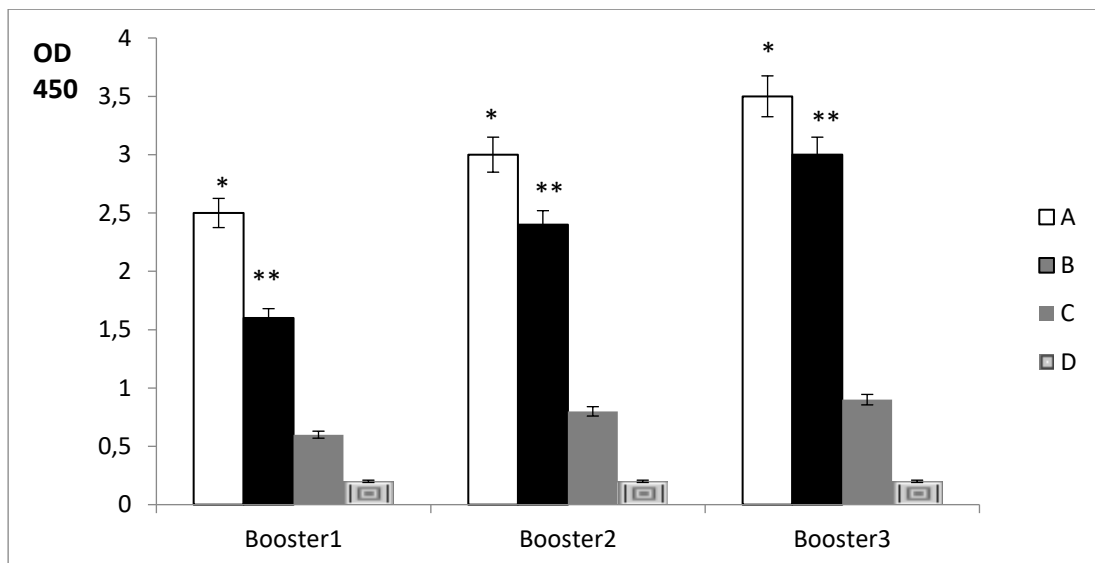


Figure 5. Comparison of serum IgG titers (dilution 1:100). The column includes heat-inactivated *P. acnes* subcutaneous injection (A), antigen-loaded nanoparticles subcutaneous injection (B), antigen-loaded nanoparticles oral administration (C), antigen-free nanoparticles oral administration (control) (D). Data expressed as mean analyzed sera/mice group. * and **: Values significantly different from the control at $p < 0.003$.

The challenge result

After two months of the last immunization, the protection level of the fusion protein was determined by challenging immunized mice with live *P. acnes*. The results showed that there is no inflammation, induration, and redness in the ear of both immunized mice groups, but the inflammation was induced in 50% of the control mice group. The results of the challenge with 10^7 CFU of *P. acnes* are shown in Table 2.

Table 2. The mice group challenged with 107CFU of *P. acnes* injected into mice ear.

Mice group	Ear thickness and Inflammation
Nanoparticulated fusion protein (injection)	No
Nanoparticulated fusion protein (oral)	No
Killed <i>P. acnes</i> (injection)	No
Control	Yes (50%)

DISCUSSION

Deficiencies and problems due to current therapeutic managements for acne disease, especially in severe acne vulgaris cases warrant developing new therapeutic strategies, including vaccine therapy. Although some efforts to achieve a therapeutic *P. acnes* vaccine, not such a satisfactory vaccine has been introduced to date, maybe due to the multifactorial nature of acne disease as well as various heterogeneous bacterial virulence factors (16, 17). Accordingly, the present study sought to introduce a chimeric protein antigen encapsulated in chitosan nanoparticle being able to induce local as well as systemic immune responses against *P. acnes*. Chimeric vaccines, with utmost attention in infectious disease prevention, have some benefits such as increased safety, cost-benefit, and less antigenic competition, making them appropriate vaccine candidates in many studies (18, 19). Therefore, in our recent bio-computational study, we introduced a Sialidase-CAMP chimeric vaccine candidate, based on two separated Nakatsuji studies (6, 7), and postulated that it could be able to induce immune responses (8). Now, in the present study, this chimeric construct was cloned, expressed, and used in a nanoparticle formulation. We aimed to evaluate the immunogenicity of this chimeric construct through oral administration. Oral vaccines, as a non-invasive route, remove disadvantages associated with the injection route, including pain, discomfort, and safety biohazard. Chitosan is a highly biocompatible, bioavailable, biodegradable, non-toxic material. It has antimicrobial properties as well as good adhesion strength, making it be approved as a Generally Recognized as Safe (GRAS) material, according to United States Food and Drug Administration (USFDA) (20). Accordingly, we used chitosan nanoparticles for oral administration to protect Sia-CAMP protein from enzymatic degradation of the gastrointestinal tract (21-23). Also, the ion gelation method was used to prepare chitosan nanospheres. The advantages of this method include the absence of harmful organic solvent, high encapsulation, and also controlled releasing (24). In addition, the chitosan solution with pH 5.5 was used for making chitosan nanospheres, because the chitosan molecule is more open at this pH and have more active sites for the formation of hydrogen bonds with protein molecules (25). Using 1 mg/mL chitosan for nanoparticulation, the size of the nanoparticle was about 80 nm, with a zeta potential of +18 mV, and a loading rate of 88%. Other studies have indicated a maximum of 100 nm as the optimum size of nanoparticles, making the antigens easily transfer from the injection site to the lymph nodes. In the innate immune system, antigen-presenting cells (APCs) such as dendritic cells and macrophages as the first line of immune defense cells could more efficiently uptake the nanoparticle below the 100 nm particle size. The size, shape, and elasticity of particle affect the immunogenicity properties and potential. Particles smaller than 40 nm induce cell-mediated immunity (TH1 mediated responses), while particles equal or larger than 100-nanometer could stimulate humoral immunity and antibody production (26). In comparison, Bagheripour and coauthors used chitosan nanoparticles as a carrier for the recombinant BoNT/E binding protein by the oral route. In this study, the size of the nanoparticle and the loading efficiency rate was 285 nm and 91%, respectively (23). Jesus and coauthors used Poly- ϵ -caprolactone/chitosan nanoparticles as carriers of the hepatitis B antigen recombinant protein. The size of the nanoparticle and the zeta potential was 201 nm and +18.6 mV, respectively, with a loading rate of 96 percent (27). Zolfagharnia and coauthors used nanoparticles composed of thiolated methylated pyridinyl chitosan as a new strategy for buccal drug delivery of insulin. The size of the nanoparticles and the zeta potential were 268 nm and +28.3 mV, respectively, and a loading rate of 91+2.6 percent (28). Nesalin and coauthors used chitosan nanoparticles with various concentrations as a carrier for the zidovudine. The size of the nanoparticles and the zeta potential were approximately 342–468 nm and 20.4 to 37.08 mV, respectively (13). In this study, two routes (oral and subcutaneous routes) were used for

administration. In both groups, immunized Balb/c mice showed high titers of serum IgG. For injected mice, the raised antibody titer suggested that nanoparticles can act as an adjuvant to induce immune responses. In orally immunized mice, nanoparticulation could protect against gastrointestinal degradation. The IgG titers were 1: 51200 and 1:3200 in subcutaneous and oral administration, respectively. Also, the vaccine mice group immunized with inactivated bacteria gave an IgG titer of 1:102400. In 2008, Nakatsuji and coauthors intranasally immunized mice with heat-killed *P. acnes* and found that the most antibodies are induced against two major antigens of *P. acnes*, including sialidase and CAMP factor (7). Later in another study, they showed that the mice had low protection against *P. acnes* when immunized with inactivated whole bacteria. They reported that sialidase was immunogenic in mice vaccinated with a recombinant sialidase (7). In the Nakatsuji study in 2011, the recombinant CAMP factor was administered by both intranasal and subcutaneous routes. Twenty-one days after immunization, the IgG titer was higher than 100,000 in the sera of immunized mice. According to two Nakatsuji studies, we aim to test whether the recombinant Sia-CAMP encapsulated fusion protein could elicit the mucosal immunity against *P. acnes*. In the present study, the IgG titer of the recombinant Sia-CAMP injected subcutaneously was approximately equal to the anti-CAMP IgG titer in the Nakatsuji study. Of note, they used the CAMP factor in conjunction with Freund's adjuvant (6), and in the present study, the chitosan nanoparticle played the role of the adjuvant. However, in the oral administration of the Sia-CAMP protein, the IgG antibody titer was lower. Also, in orally immunized mice, IgA titers in serum and fecal samples were 1:16 and 1:8, respectively, suggesting inducing the mucosal responses. The stool IgA titer was lower than the serum, which may be due to proteolytic degradation of antibodies before adding anti-protease (29). Unfortunately, no other study is available for the comparison of our data. In this study, to determine the immunity induced by recombinant Sia-CAMP protein, the ears of the vaccinated mice were subcutaneously challenged with live *P. acnes*. By examining the mice visually, ear inflammation was seen in 50% of the control group. The live bacteria injected into the mice group, immunized with the recombinant protein, did not affect the mice ears, and inflammation was not observed. Therefore, it seems that this recombinant protein is a suitable vaccine candidate, though it needs more clinical and immunological evaluations. In 2008, Nakatsuji and coauthors challenged the ear of the vaccinated mice with heat-killed *P. acnes* intradermally with living *P. acnes*. They showed that the bacterial progression might be suppressed by using inactivated *P. acnes*-based vaccines. This vaccination could also facilitate the recovery of *P. acnes*-induced inflammation (7). In another study in 2008, Nakatsuji and coauthors elicited a detectable antibody by sialidase-immunized mice. These antibodies could neutralize the *P. acnes* cytotoxicity *in vitro* and induce the production of *P. acnes* interleukin-8 (IL-8) in human sebocytes. Also, this treatment blocked the increase in ear thickness and also prevented the release of pro-inflammatory macrophage inflammatory protein (MIP-2) cytokine. A study by Wang and coauthors showed that mutations in the CAMP factor could significantly decrease the bacterial colonization and subsequent inflammation in mice. In addition, vaccination with CAMP factor could induce the production of MIP-2 (macrophage inflammatory protein-2) in mice. Using *ex vivo* acne model, they concluded that *P. acnes* CAMP factor is an essential source of inflammation in acne vulgaris and suggested that CAMP factor may be considered as a candidate target in acne vaccination (30). In another study by Agak, they showed that the functions of T helper 17 (Th17) cells are different between healthy skins versus acne associated *P. acnes* strains. They observed that acne-associated *P. acnes* strains induce higher IL-17 levels than healthy skin strains due to inducing different phenotype and function of Th17 cells. They finally suggested that acne disease-causing strains may generate Th17 cells that may contribute more efficiently to acne pathogenesis (31). Interestingly, recent work revealed that there are different phylogenetic of *P. acnes* strains, and these phylotypes can induce different immunological responses. In this regard, Yang and coauthors reported that acne-associated *P. acnes* phylotypes induced 2-3 folds higher levels of IFN- γ and IL-17 in peripheral blood mononuclear cells compared with healthy phylotypes. Meanwhile, healthy skin *P. acnes* strains are associated with induced 2-4 folds higher levels of IL-10 (32). This studies more emphases on immunological complexity of acne vulgaris disease.

CONCLUSION

It is the first study to design and evaluate chitosan- nanoparticulated fusion protein as an acne oral vaccine. Our results indicate that the Sia-CAMP fusion protein was expressed correctly and was encapsulated successfully in the chitosan nanoparticles, and has been able to induce a systemic and topical immune response in mice model. Although subcutaneous immunization has induced better systemic immunity, oral administration can induce as the therapeutic vaccine both systemic and mucosal immune responses. It seems that this approach can be a supplemental therapeutic method-along with other medications- for the management of severe acne diseases.

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