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Original Article

Production and Purification of CAMP-Sialidase Chimer Protein as a Vaccine Candidate for Acne and Its Immunization in the Animal Model

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Abstract

Introduction: Acne vulgaris affects ~85% of young adults aged 12-25 years. Regarding the critical role of *Propionibacterium acnes* in the pathogenesis of acne and current therapeutic failures, developing efficient acne vaccines are appreciated. Accordingly, designing a chimeric protein from CAMP and sialidase parts of *Propionibacterium acnes* and evaluating its immunogenicity in mice model as an acne vaccine candidate was aimed in this study.

Materials and Methods: CAMP-sialidase recombinant gene expression was carried out through cloning in the vector pET28a and transferring to *E. coli* BL21DE3. The protein was purified using Ni-NTA column and its concentration was measured. The recombinant protein was injected in test and control mice groups. Then, antibody titration and challenge test were made to determine the immunogenicity.

Results: After successful expression and purification, the protein band was observed at a molecular weight of 65 kDa. Western blotting confirmed the purified protein. The results of serum ELISA indicated the IgG titer was 1:204800 and 1:1600 against the recombinant protein and inactivated *P. acne,* respectively. Although there was no change in test mice, inflammation happened in 50% of the control group.

Conclusions: The current study demonstrated that the CAMP-Sialidase recombinant protein can appropriately induce humoral antibody. However, more evaluations need to introduce it as an acne vaccine candidate.

Keywords: Acne, *Propionibacterium Acnes*, CAMP-Sialidase Recombinant Protein, Acne Vaccine
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Introduction

Acne is one of the first three common skin diseases in the world.[1] Approximately, 85% of people aged 12 to 25 years experience some degree of acne in their life.[2] Acne is a multi-factorial disease mainly caused by the increased production of sebum in the skin (associating with the production of comedones and production of substrates for acne), excessive follicular keratinization (the blockage of the pilosebaceous canal that causes acne scars to spread), and finally abnormal bacterial function (*Propionibacterium acnes*, a Gram positive anaerobic bacterium that resides as a member in the human skin normal flora).[2,3-8,10-16] Regarding the burden of the acne disease and more failure of therapeutic measures, developing an appropriate acne vaccine is appreciated. In this regard, as studies have demonstrated a critical role of *P. acnes* in the pathogenesis of acne disease, the pathogen is now considered as a good candidate for *ance* vaccine as a preventive strategy. The best vaccine targets investigated to now are extracellular enzymes of the *P. acnes*, parts of the membrane or cell wall, and cell membrane bound proteins.[5] Kim in a study in 2008 demonstrated that some antibodies produced against *P. acnes* resulted in tissue damage.[17] He concluded that all antibodies produced against *P. acnes* are not useful and may even exacerbate the disease. In 2008, Nakatsuji et al. showed that recombinant sialidase- vaccinated mice produced antibody against acne infection.[18] In another study, Bambery et al. studied acne disease, its prevalence, causes, and interactions between *P. acnes* and toll like receptors (TLRs).[19]

In 2011, Nakatsuji et al. showed that inflammation caused by *P. acnes* is reduced in CAMP factor- vaccinated mice that elicited antibody.[4] In 2013, Bilott et al. showed that new treatments should be made using by molecules involved in immune system activation such as TLRs and protease receptors and microbial peptides as an alternative to topical antibiotics.[5] Considering the high prevalence of this disease, antibiotic resistance, and lack of an effective vaccine, in this project, we tried to use a chimeric construct (a combination of both sialidase protein and CAMP factor) and administration in a mouse model as a vaccine candidate for acne disease.

Materials and Methods

Culture of P. acnes

Lyophilized *P. acnes* (PTCC 6919) was purchased from the Pasteur Institute. After being transferred to the research lab, 1 ml of sterilized BHI was added and incubated for an hour at 37 °C. Then, 150 µl of the sample was cultured linearly on blood agar in the anaerobic condition at 37 °C using gas-pak for three days.

Design and Construction of Recombinant Chimeric Structure

Two parts of *CAMP* and *sialidase* genes were selected which encode 300 and 230 carboxyl terminal amino acids, respectively. An appropriate linker consisting of four repeats from the EAAAK linker sequence was used as a third piece interfacing between the two pieces for more flexibility and efficient separation. Physicochemical parameters of the chimeric structure were determined using the protparam online server for amino acid composition and molecular weight, proccaleexpas software for flexibility and polarity, and clustalW software for identifying conserve sequences. The chimeric gene was designed for cloning and expression in the *Escherichia coli* bacterial host. In order to simplify the purification, the His-tag was added to the end of the construct.

Expression of the CAMP-Sialidase Recombinant Protein

In order to express the CAMP-Sialidase gene, the BL21DE3 strain of the *E. coli* colonies carrying the recombinant plasmid pET28a was used. First, 50 µl bacteria were inoculated in 5 ml LB containing kanamycin (40 µg/ml) and incubated at 37 °C with overnight shaking. Then, kanamycin (40 µg/ml) and a 5 ml bacterial culture sample were added to 100 ml of new culture medium and incubated for 3 h in a shaker-incubator (37 °C, 150 rpm) until the absorbance at 600 nm reached about 0.7. Further, 1 ml IPTG was added at a concentration of 1 mM to 100 ml culture medium and the culture was placed in an incubator shaker for overnight to express the desired gene.

Purification of Recombinant Proteins

Due to the use of the 6His-tagged sequence, Ni-NTA column was used for purification. The medium was centrifuged (6000 rpm) at 4 °C for 10 min. After separation of the precipitate, the supernatant was discharged and lysis buffer and denaturation B buffer (1:3 ratio) was added to the sediment and sonicated. Upon completion of sonication, the sample was centrifuged for 15 min at 13000 rpm and the supernatant was collected. The supernatant was passed through the column and the output was collected. Then the column was washed with 20 and 250 mM imidazole and 20 mM MES buffer, and finally the output was collected.

Gene Expression Analysis Using Polyacrylamide Gel

At first, the samples were expressed and purified then were electrophoresed on SDS-PAGE 12% gel and was concentration of protein was determined by the Bradford method.

Confirming Expressed Protein with Western Blot

Ten µl of each sample along with loading buffer were loaded into SDS-PAGE 12% gel electrophoresis and electrophoresis performed at 100 voltage. After electrophoresis, the gel was removed and the Wattman filter paper was placed on the sponge. The nitrocellulose paper, which was embedded in an electroblotting buffer for at least 30 min, was placed on the gel and followed by another wattman filter paper on nitrocellulose paper. The sandwich was placed inside the tank and the transfer operation was carried out for 2 h at the voltage of 100 V, and then was floated in blocking buffer 5% (milk powder in PBST) overnight. After washing with PBST, the anti-histidine solution at 1:1000 dilution was poured on each paper cut of nitrocellulose and shacked for an hour at 37 °C. After washing, DAB solution was poured onto nitrocellulose paper and after the advent of bands, the reaction was inhibited.

Mice Immunization

For the administration of recombinant CAMP-Sialidase protein, Balb/c mice were divided into two groups (five mice /group). Twenty micrograms of the recombinant CAMP-Sialidase protein plus complete Freund's adjuvant (in the first injection) and incomplete Freund adjuvant (in following injections) was used. PBS was injected for the control group. Three injections were made every two weeks and blood sampling was done after each injection to keep at -20 °C.

Immunization of Mice with Heat-killed P. acnes

A group of five mice were also considered as positive control groups and vaccinated with heat-inactivated *P. acnes*. In order to inactivate *P. acnes*, *P. acnes* colonies were cultured and dissolved in PBS until the absorbance reached 0.3 (600 nm). Then, 5 ml of the culture was centrifuged at 5000 rpm for 10 min and the sediment was dissolved in 500 µl PBS and incubated at 70 °C for 45 min. The solution was mixed with complete Freund's adjuvant in the first injection

and incomplete Freund adjuvant in subsequent injections and injected to each mouse at 200 µl. Injections were performed three times every two weeks and after each blood sampling.

Determination of IgG Responses to Recombinant Proteins Using Indirect ELISA

Five micrograms of CAMP-Sialidase recombinant protein was coated into the ELISA wells. Also, 100 µl of blocking buffer (5% skim milk/PBST) were loaded into all wells. Then, the serum titration was performed in the wells and the conjugated mice antibody were added (diluted 1/800). The TMB substrate was added and after stopping the reaction by 2M H_2SO_4, the absorbance was read at a wavelength of 450 nm. Washing procedure with PBST buffer was done three times after each ELISA step.

Bacterial Challenge

To investigate immunization in mice, one month after the last injection, 10^7 CFU of *P. acnes* was diluted in a PBS buffer and injected into one of the ears of the immunized mice and control group. Also, PBS was injected to the other ear as the control. Then, in a 40-day period, the thickness (by millimeter ruler) and ear inflammation in mice were daily examined.

Statistical Analysis

T-test was used to analyze the control and test groups. *p* values≤ 0.05 was considered significant.

Results

The Expression of the CAMP-Sialidase Gene

After induction and expression of the CAMP-Sialidase gene, the resulting cell precipitate was dissolved in PBS. The cells were lysed and centrifuged. The resulting precipitate was dissolved in denaturation buffer B and centrifuged after one hour at room temperature. The supernatant was electrophoresed on 12% SDS-PAGE gel and examined for the desired band. The protein band was observed at a molecular weight of 65 kDa (Figure 1).

Purification of CAMP-Sialidase Protein Using Ni-NTA Column

Recombinant pET28a plasmid bacteria were cultured on a large scale (100 ml of medium) and under optimal conditions, the recombinant CAMP-Sialidase gene was induced. Following the collection of cells and after sonication and centrifugation, the supernatant was used to purify the recombinant protein on the nickel column. The protein band was purified using a separator buffer (Figure 2).

Western Blotting

SDS-PAGE containing non-induced bacteria and purified protein were transferred on nitrocellulose paper. A specific 65 kDa band represented the desired protein (Figure 3).

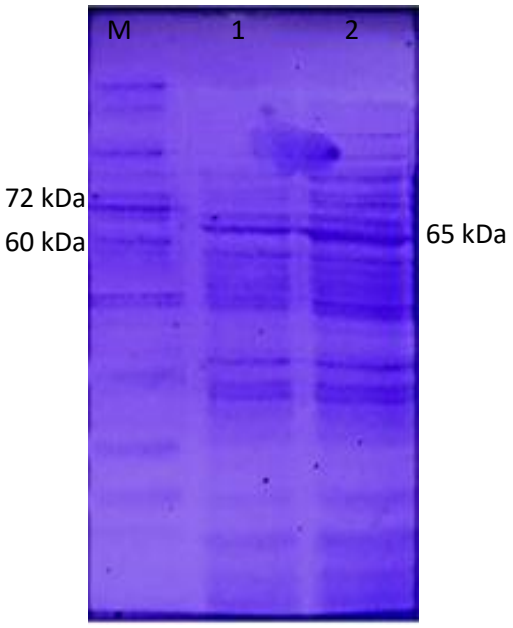

Figure 1. Gel Electrophoresis Before and After Induction. M) Protein size marker, 1) Non-induced sample, 2) The sample dissolved in the urea buffer.

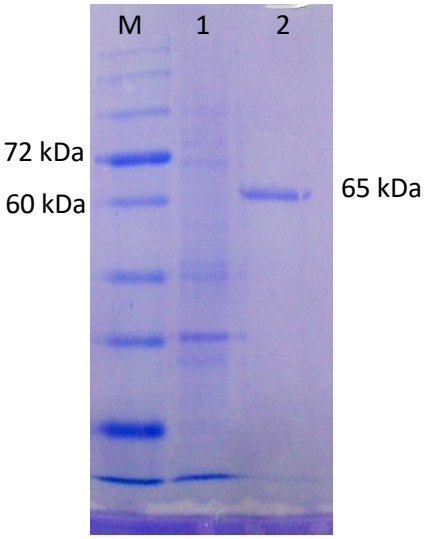

Figure 2. Purification of CAMP-Sialidase Recombinant Protein Using Ni-NTA Column. M) Protein size marker, 1) Flow through, 2) Elution buffer containing 250 mM imidazole.

Antibody Induction After CAMP-Sialidase Recombinant Protein Injection in Mice

Twenty µg of CAMP-Sialidase recombinant antigen was injected to Balb/c mice three times (two weeks intervals) and blood samples were taken every time. Then, the induction of systemic immune response was investigated using ELISA method.

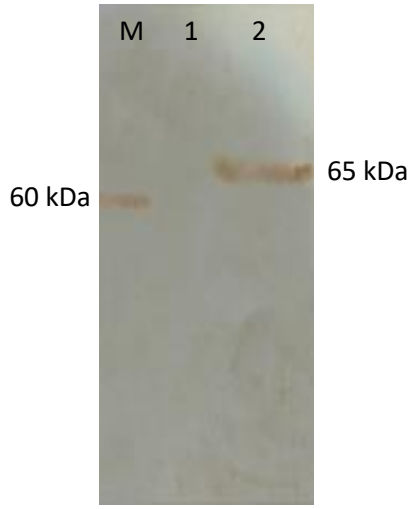

Figure 3. Western Blotting for Protein Confirmation. M) Protein size marker, 1) Non-induced as control sample, 2) The CAMP-Sialidase chimeric protein purified by Ni-NTA column.

Serum IgG Titration

The results of ELISA on serum samples indicated that induction of the systemic response occurred and specific serum IgG titer against CAMP-Sialidase protein increased. The results indicated that the antibody titer is 1:204800 (Figure 4).

Antibody Response Induction After Inactivated Bacteria Injection in Mice

The results of serum ELISA indicated the systemic response induction and increased serum IgG titers against inactivated bacteria. The results indicated that the antibody titer is 1:1600 (Figure 5).

Challenge Test

To determine the level of immunity after the recombinant protein injection, the exposure test was performed. Although no change was observed in test mice, inflammation happened in 50% of the control group (Figures 6 a & b).

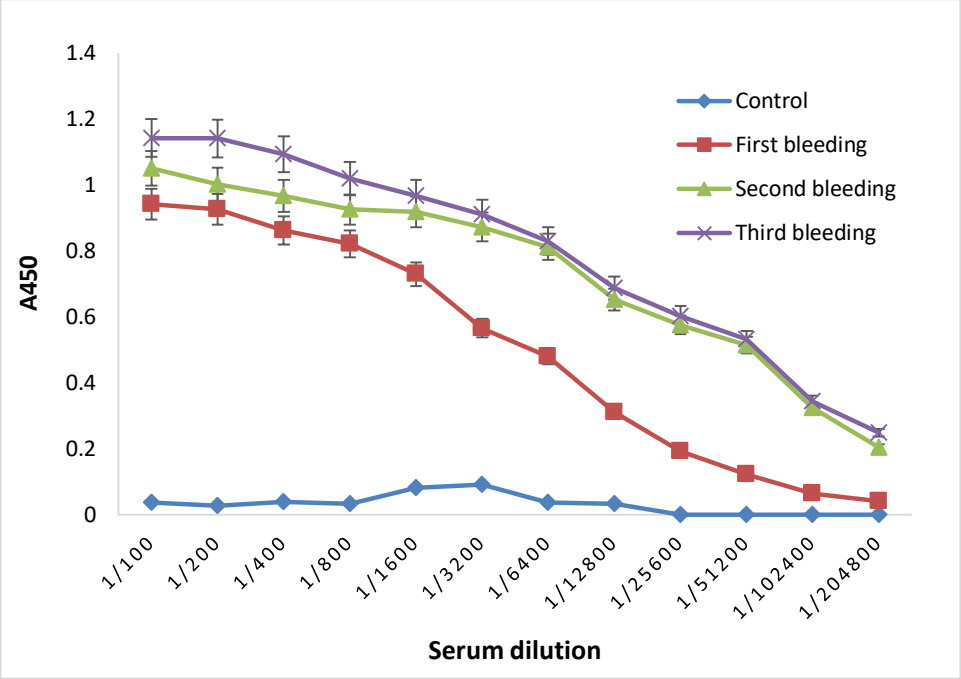

Figure 4. Test Group Serum IgG Titration.

Discussion

At present, the important problem associated with the *P. acnes* vaccination is to identify the best target for the vaccine that can produce protective antibodies.[5] Nakatsuji et al. demonstrated that the ears of the mice were swollen and their skin was frying 24 h after the inactivated bacterial injection.[3] In this study, tissue infusion showed that the *P. acnes* injection caused a significant increase in inflammatory cells. To produce an adequate antibody against *P. acnes*, mice were immunized by the heat-killed *P. acnes* injection in the nose, three times in a one-week interval. A week after the last inoculation, serum was collected. Data from SDS-PAGE and western blot showed that two main combinations (approximately 64 and 250 kDa) of the *P. acnes* have a better immune response than antibodies extracted from heat-treated whole bacteria. Also, nasal administration suggests that inactivated vaccines based on *P. acnes* are suitable for mucosal immunity. In order to determine the immunity generated by vaccination with the heat-killed *P. acnes*, the immunized mice were challenged intraperitoneally with *P. acnes* and the increase in the thickness of the ear was observed. This increased ear thickness significantly decreased

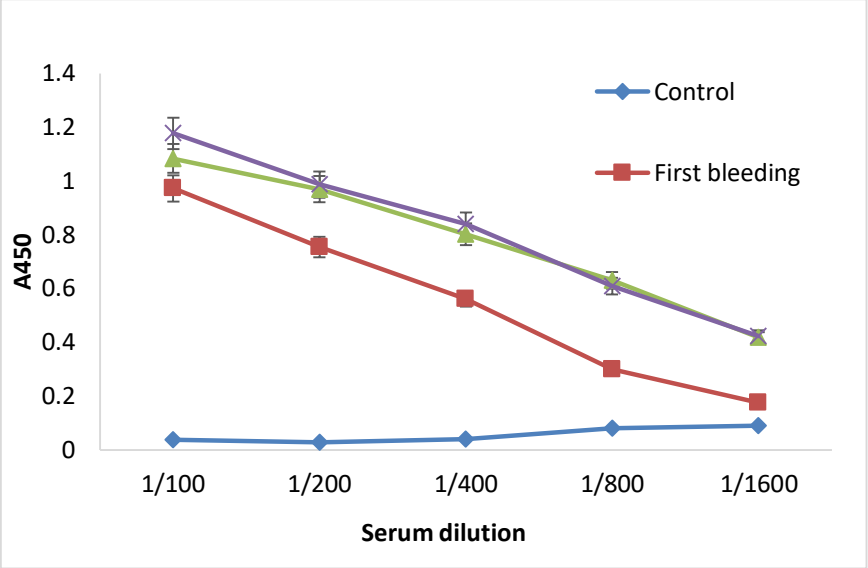

Figure 5. Positive Control Group Serum IgG Titration.

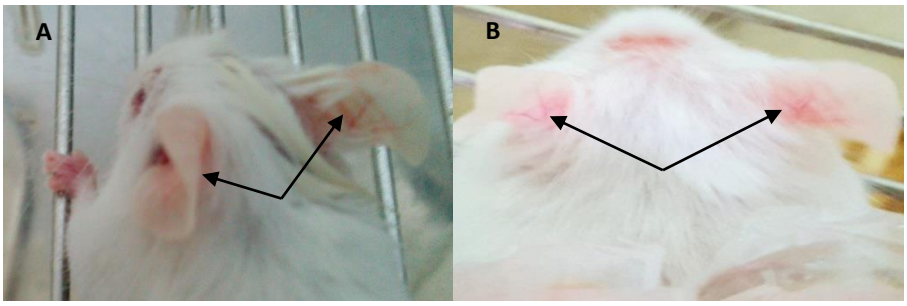

Figure 6. Inflammation in the Mice Ear of the Test (A) and Control (B) Groups.

decreased when the mice were immunized by inactivated *P. acnes* in both stages (days 1 and 7). The increase in ear thickness of *P. acnes* immunized mice completely reduced after 22 days of the challenge, suggesting that vaccination by inactivated *P. acnes* reduces bacterial progression and improves inflammation. Nakatsuji et al. concluded that acne vaccines would lead to new treatment for acne diseases. The data from this study showed that purified recombinant sialidase breaks down the sialoglycoconjugates and sialic acid have been released. After immunization with recombinant sialidase, *P. acnes* was injected subcutaneously into the left ear of mice immunized with sialidase or GFP while PBS was injected into the right ear as the control. The ear thickness was measured periodically (71 days) until it completely diminished. Treatment with purified sialidase for the first two hours did not affect the life of the sebocyte cells. After treatment with sialidase in the first two hours, sebocytes were exposed to *P. acnes* culture overnight. *P. acnes* induced 20% cell death in treated sebocytes in the control group, while cell death was significantly higher in the sialidase treated sebocytes (about 34%). This suggests that treatment with sialidase increases the specificity and susceptibility of the sebocytes to *P. acnes*. These results, in addition to the fact that sialidase is a peripheral protein with LPXTG motif, represents sialidase as a potentially valuable candidate to create a vaccine against acne vulgaris. To evaluate the immunogenicity of sialidase, mice were vaccinated with the killed *P. acnes* for nine weeks. Recombinant sialidase, GFP, and the cell lysate of *P. acnes* were subjected to the western blot analysis. A large number of proteins with a molecular weight greater than 50 kDa, showed an immune response to mice serum which was obtained from the immunized treated mice with heat-killed *P. acnes*.[18] In this study, the mice were immunized with recombinant sialidase or GFP by Freund's adjutant. In the western blot analysis, antibody production was observed in the serum of immunized mice two weeks after the last immunization. When the purified sialidase reacted with the serum of sialidase immunized mice, a strong band of 53 kDa was observed, suggesting that sialidase produced an immune response in immunized mice. In this study, the mice were also immunized with the recombinant sialidase, in addition to inactivated *P. acnes*. The results showed that sialidase

should not be immunogenic if immunization is carried out with killed *P. acnes*; while sialidase is immunogenic if the immunization is carried out with the recombinant protein. Consequently, in acne patients, it can effectively prevent the progression and recurrence of the disease by generating a strong antibody response against *P. acnes* sialidase. Acne vaccines use *P. acnes* sialidase instead of killed *P. acnes* as an immunogenic agent, and it reduces the probability of adverse effects. Therefore, sialidase-based acne vaccine may be more specific while reducing undesirable effects. Nekatsuji et al. demonstrated that the immunization of mice with the CAMP factor provides protective immunity against *P. acnes* showing that the CAMP factor plays a role in the *P. acnes* induced inflammation. To evaluate the immunogenicity of the CAMP factor, CAMP factor intranasally immunized mice along with UV-deactivated *E. coli* which were compared with a control group (GFP control protein).[4] Fourteen days after immunization, a western blot analysis revealed raised IgG titers, but it was not observed in GFP-immunized mice. ELISA analysis showed a significant increase in the antibody titer at 14 and 21 days after immunization. Twenty-one days after immunization, the IgG antibody titer in the serum of the immunized mice was more than 100,000 while the antibody titer in the control group was less than 100. To determine the immunogenicity of the CAMP factor in *P. acnes* in vivo, recombinant CAMP factor and GFP were injected subcutaneously into the mice's ear. Injection of the CAMP factor for 24 h caused a significant increase in the ear thickness, and this represents the role of the CAMP factor in inflammation. In this study, we used a combination of two parts of the CAMP protein and *P. acnes* sialidase as a vaccine candidate in the test and control mice groups. The recombinant protein was injected subcutaneously into the intervention group in three steps. Then, the live bacteria were injected into the ear of both test and control groups. Finally, by measuring the antibody titer in the intervention group, the final immunogenicity was examined. We also examined the ears of the mice for any inflammation. In addition, a vaccine group was considered for further investigation and comparison for which the inactivated bacteria were injected in three stages and the antibody titer was measured. The antibody titer was 1.204800 in the intervention group and 1.1600 in the vaccine group. This dramatic and significant difference in antibody titer indicated a higher specificity and efficacy of the recombinant protein. Mice ear was apparently inflamed in 50% of the control group. Live bacterial injection in the recombinant protein immunized group did not cause inflammation in the ear of mice, and the antibody titer increased after each blood collection. Therefore, it seems that this recombinant protein is extremely suitable as a vaccine candidate with no side effects. More experiments on this recombinant protein could be necessary to more evaluate the clinical efficacy of the recombinant protein for acne disease.

Conclusion

In general, killed bacteria have non-specific immunity, but the CAMP and sialidase factors, as well as the CAMP-sialidase chimeric protein work in a specific way. In our study, by putting together parts of the genome that promote pathogenesis, the specificity of the CAMP-sialidase protein increased.

Authors' Contributions

KM was involved in planning the research, and drafted the manuscript, AA was involved in planning the research and proof the manuscript, JS was involved in planning the research and proof the manuscript, PZ help to calculate the experimental data, JA designed and supervised the project, performed the analysis and designed the figures. All authors participated in the critical revision of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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