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ORIGINAL RESEARCH

Protective Efficacy of the OprF/OprI/PcrV Recombinant Chimeric Protein Against *Pseudomonas aeruginosa* in the Burned BALB/c Mouse Model

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Background: *Pseudomonas aeruginosa* infection is the major cause of death in burn patients. Thus, in this study, a chimeric vaccine harboring the $OprF_{185-350}$–$OprI_{22-83}$–$PcrV$ was designed and expressed in *Escherichia coli*. The immunogenicity of the recombinant chimer, OprI, OprF, and PcrV was studied in a burned mouse model.

Methodology: Recombinant proteins including the proposed chimer, OprF, OprI, and PcrV were expressed in the *E.coli*. Mice were immunized with the purified recombinant proteins, and the antibody titre was estimated in the sera obtained from immunized mice. Immunized and control mice were challenged with 2, 5, and $10xLD_{50}$ of the *P. aeruginosa* strains (PAO1, PAK, and R_5), and microbial counts were measured in the skin, liver, spleen, and kidney of the studied mice.

Results: Results showed that the antibody titre (total IgG) was significantly increased by injection of 10 µg of chimeric protein in the experimental groups compared to the control groups. The antibody survival titre was high until 235 days after administration of the second booster. The survival rate of the mice infected with $10xLD_{50}$ was significantly increased and the number of bacteria was reduced, especially in the internal organs (kidney, spleen, and liver) compared to the mice immunized with any of the OprF, OprI, and PcrV proteins alone.

Conclusion: The findings of our study revealed that the chimeric protein is a promising vaccine candidate for control of the *P. aeruginosa* infection.

Keywords: burned, chimeric protein, *Pseudomonas aeruginosa*, vaccine

Introduction

Pseudomonas aeruginosa (PA) is the most common germ-negative pathogen causing opportunistic infection in the human, particularly in the immunosuppressed patients.[1] The Intensive Care Units (ICUs), the respiratory tract infection and burn wards in the hospital are the main places where PA infection can spread.[2] Burns and respiratory infections can quickly lead to systemic infection with a mortality rate between 38% and 70%.[3] However, the treatment of PA infection is difficult due to high prevalence of drug resistance and limited therapeutic options.[4] Therefore, rapid and timely control of the infection is the most effective approach to prevent the PA infection in burn wounds.[5] Although, treatment with the antibiotics is an important strategy, medication resistance by *Pseudomonas aeruginosa* has made it difficalt. Carbapenem antibiotics are used to treat the Multidrug-Resistant Pseudomonas (MDRP). With the prevalence of the carbapenem-resistant pseudomonas (CRP), the scientists have attempted to produce an appropriate vaccine or develop other safety

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treatments.[6,7] So far, several vaccine candidates have been introduced against the PA.[8–11] However, no license has been issued yet.[12] The presence of several pathways used by the bacteria to cause infection is a major challenge for the development of the vaccine against the PA. Thus, the vaccines that block one or two routes are not able to have the appropriate protective effect. PA has the Type III Secretion System (T3SS) through which directly affects the host cells' function.[13] The PA V-antigen (PcrV) is an extracellular molecule of T3SS that kills the epithelial cells and immune cells by injecting a toxic protein. Promising results have been reported regarding the antibody (Fab) raised against the recombinant PcrV such that it decreased the inflammation and injury in the patients with cystic fibrosis and chronic infections caused by PA.[14] Outer membrane protein I (OprI) is another important surface lipoprotein playing a vital role in the PA, particularly making it resistant to antimicrobial peptides.[15,16] There is a highly promising vaccine for PA (NCT01563263), which is currently in the Phase III of clinical trial and consists of the OprI and OprF proteins. As an adjuvant, OprI launches the Toll-Like Receptor 2(TLR2)/Toll-Like Receptor 4(TLR4) pathway to improve the function of the immune system.[17–19] According to the analysis of the immunogenicity, distribution, and critical role of the OprF, PcrV, and OprI in the pathogenesis of PA, and based on bioinformatics calculations, it was hypothesized that a combination of these proteins as a chimer may provide significant immunity and could provide the protection against the infection. Therefore, in this study, a chimeric vaccine harboring the OprF $_{185–350}$ - OprI $_{22–83}$ - PcrV was designed and expressed in the *E.coli*. The immunogenicity of the recombinant chimer was studied in the mouse model of burn.

Materials and Methods
Ethics Statement
All the animal studies were performed in accordance with the protocols provided by the Animal Care Committee of the Shahed University (Tehran, Iran). All the decisions of this committee were made in compliance with the UK Animals Scientific Procedures Act 1986 and related guidelines including the EU Directive 2010/63/EU and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 8023, modified in 1978). We tried our best to minimize the animal suffering.

Animals, Microorganisms, and Cultures
Six to eight-weeks old male BALB/c mice, weighing 22–25 grams were purchased from the Razi Vaccine and Serum Research Institute (Alborz province, Karaj, Iran). There were eleven mice in each group in all the performed tests. The microorganisms, namely *Pseudomonas aeruginosa* strains of PAO1 (ATCC$_{15692}$) and PAK (ATCC$_{25102}$) used in this study were obtained from the microbial collection of the Faculty of Basic Sciences, Shahed University (Tehran, Iran). *Pseudomonas aeruginosa* R$_5$, an aggressive and antibiotic-resistant hospital strain was collected from the Faculty of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran). Luria-Bertani (LB) medium, Nutrient Agar (NA), and nutrient broth (all purchased from Merck, Germany) were used for routine culture of all the bacterial strains. All the chemical materials and solvents used in this study were purchased from the Merck Company (Germany).

Cloning, Expression, and Purification of the Recombinant OprF, OprI, PcrV, and OprF$_{185–350}$–OprI$_{22–83}$–PcrV Chimer
The reading framework of *OprI* and *PcrV* genes for protein production was isolated and amplified by the primers listed in Table 1 and was cloned on the pET28a expression vector, in the frame with a T7 promoter, kanamycin-resistant gene, and the N-terminal six-His-tagged sequence. *HindIII* and *EcoRI* (Fermentas, Lithonia) restriction sites were located at the 5′ end *PcrV* and *OprI* genes, respectively. The OprF clone was gifted by the research Laboratory, in the Department of Cell Biology, Faculty of Basic Sciences, Shahed University (Tehran, Iran). The OprF-OprI-PcrV chimeric gene was

Table 1 Primers Used for Amplification of Open Reading Frame Sequences of the OprI and PcrV

Primer Name	Sequence (5′ to 3′ Direction)	Enzyme	Tm
OprI-Forward	CACGGAATTCATGAACAACGTTCTGAAATTC	*EcoRI*	59.1
OprI-Reverse	CCTAAAGCTTTTACTTGCGGCTGGCTTTT	*HindIII*	60.1
PcrV-Forward	CACGGAATTCATGGAAGTCAGAAACCTTA	*EcoRI*	61.5
PcrV-Reverse	ACCTAAGCTTCTAGATCGCGCTGAGAATG	*HindIII*	58.7

Abbreviation: Tm, melting temperature.

designed according to the genetic code of *pseudomonas aeruginosa* PAO1 $ATCC_{15692}$ strain in the National Center for Biotechnology Information (NCBI). The gene was analyzed through the prospective bioinformatics analysis and was synthesized on the pET28a vector by the Biomatik Company (Canada). All the clones were cultured on the LB medium with kanamycin 70 ug/mL (Sigma-Aldrich, Germany) and were expressed in the *E.coli* BL-21 (DE3) strain (Novagen, Darmstadt, Germany) using 1 mM Isopropyl β-D-1-Thiogalactoside (IPTG) at OD_{600} of 0.6 as an inducer. After 6 h of incubation at 37°C and 200 rpm, centrifugation was done at 4000 g, 4°C for 15 min. The cell pellets were suspended in the Tris-EDTA buffer (Tris 1 M, EDTA 0.5 M) and were sonicated three times for 45 s at 200 W, cycle 0.5 s with 1 min resting on ice in each interval. The protein solutions were obtained at 17,500 g, 4°C for 20 min, and the supernatant containing recombinant proteins were purified with Ni-NTA agarose column (Qiagen, USA) in a natural condition using the imidazole gradient. Purified recombinant proteins were analyzed using the Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) and were approved by the Western blotting. For Western blot analysis, the antigens were separated by the SDS-PAGE electrophoresis and were transferred onto the nitrocellulose membrane under semi-dry condition. Horseradish Peroxidase (HRP)-conjugated anti-His antibody (diluted 1:10,000) (Abcam, Cambridge, MA, USA) was used to detect the recombinant proteins.

Immunization of the Mice and Determination of the Antibody Titre

Different mice groups were immunized with 10 μg of each of the recombinant proteins subcutaneously on days 0, 14, and 28. The first injection was performed with complete and two boosters with incomplete Freund's adjuvant (Sigma-Aldrich, Germany). Burned mice were contaminated with the bacteria and burned mice without any contaminations were kept as two non-immune control groups. Blood samples were collected from the eyes of the mice before the second and third injections and the antibody titre of the sera was measured by the Enzyme-Linked Immunosorbent Assay (ELISA). For this purpose, 96-well flat-bottom plates were coated with 3 μg/well of each antigen in the carbonate–bicarbonate buffer (pH 9.6) at 4°C overnight. Then, the plates were washed three times with Phosphate-Buffered Saline (PBS) containing 0.05% of Tween 20 (PBST) and were blocked using the 5% skim milk (w/v) in PBST at 37°C for 45 min. After washing, serial

dilutions of sera were added in duplicates and were subsequently incubated at 37°C for 1–2 h. The plates were washed and then, goat anti-mouse IgG-HRP (diluted 1:10,000) (DNAbiotech Co., Tehran, Iran) was added (100 uL/well). Following 1 h incubation of the plates at 37 °C and washing with the Phosphate-Buffered Saline with Tween® detergent (PBST), 3, 3′, 5, 5′- Tetramethylbenzidine (TMB) (Pasteur Institute, Tehran, Iran) solution was added and incubated in the dark for 20 min at room temperature. The 3M sulfuric acid (H2SO4) was used to stop the reaction followed by absorbance measurement at 450 nm on the ELISA plate reader.

Burn Infection

All the animals (weighing 20–25 g) were anesthetized with 200 μL of anesthetic drug (10 μL of xylazine, 20 μL of ketamine, and 170 μL of Double-Distilled Water (DDW)) (all purchased from Sigma-Aldrich Company, Germany) prior to burning.[20] The right side of the waist and lower part of the body of all the mice were shaved using the shaving cream (Depi, Iran). The cylindrical probe made of iron alloy with a diameter of 22 mm, a length of 100 mm, and a weight of 165 g was heated to the temperature of 104°C and was applied on the shaved part of the animals for 8 s to create the 3rd-grade burns. Mice immediately received the peritoneal injection with 500 μL of 0.9% saline and 100 μL of acetaminophen (3.25 gr/mL) to prevent them from being shocked and feeling the pain.

Determination of LD_{50}, $2xLD_{50}$, $5xLD_{50}$, and $10xLD_{50}$ Doses

Bacterial count was measured by estimating the OD_{600} using two-beam spectrophotometer (UV-Vis model T80Plus - PG, United Kingdom) and the graph was plotted using the Excel 2016 software. Different concentrations (10, 10^2, 5×10^2, 10^3, 2×10^3, and 10^4) of *P. aeruginosa* strains (PAO1, PAK, and R5) were injected subcutaneously at the burn center of all the mice to obtain the LD_{50} dose.

The results were analyzed using the SPSS software version 20, and LD_{50}, $2xLD_{50}$, $5xLD_{50}$, and $10xLD_{50}$ doses were determined. The test was performed three times individually.[21]

Burned Mice Challenged with *P. aeruginosa*

Fourteen days after the third injection, burned immunized mice were challenged with the subcutaneous injection of bacteria at doses of $2xLD_{50}$, $5xLD_{50}$, and $10xLD_{50}$ at the burn center. They were kept for one month after burning until the wound was healed.[20]

Microbial Counting of the Mouse Tissues

Twelve hours after subcutaneous injection at a dose of $10xLD_{50}$ from each bacteria, three challenging mice from each group were sacrificed. Skin, liver, spleen, and kidney samples were homogenized in 4 mL of sterile PBS. Serial dilutions of homogeneous samples were prepared in sterile PBS and 100 μL of the diluted samples was placed on the Nutrient Agar (NA) and was incubated for 24 h at 37°C. The colonies were counted and the number of Colony Forming Units (CFU) from each sample in grams of tissue (CFU/g) was calculated.[20,21]

Bacterial Resistance

Bacterial resistance of *P. aeruginosa* strains including PAO1, PAK, and R_5 was measured according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016).[22]

Statistical Analysis

The Graph Pad Prism software (version 6) and Excel 2016 were used to draw the charts, graphs, and data concepts. Data were analyzed by the Analysis of Variance (ANOVA) and *t*-test using the SPSS software (version 20). Mean, standard deviation, and significance level were calculated and a *p*-value of <0.05 was considered as statistically significant.

Results

Characterization of the OprF, OprI, PcrV, and Chimeric Protein

Expression and purification of the OprF, OprI, PcrV, and the chimeric protein consisting of OprF $_{185-350}$, OprI $_{22-83}$, and PcrV were analyzed on the SDS-PAGE (Figure 1A, C, E, and G), and were verified by the Western blotting (Figure 1B, D, F, and H). The molecular weight of the recombinant proteins of OprF, OprI, PcrV, and the chimer was equal to 38, 8.4, 32, and 66 kDa, respectively.

Antibody Titer

The ELISA was used to determine the antibody titres raised against the recombinant proteins. Figure 2B, C, and D show
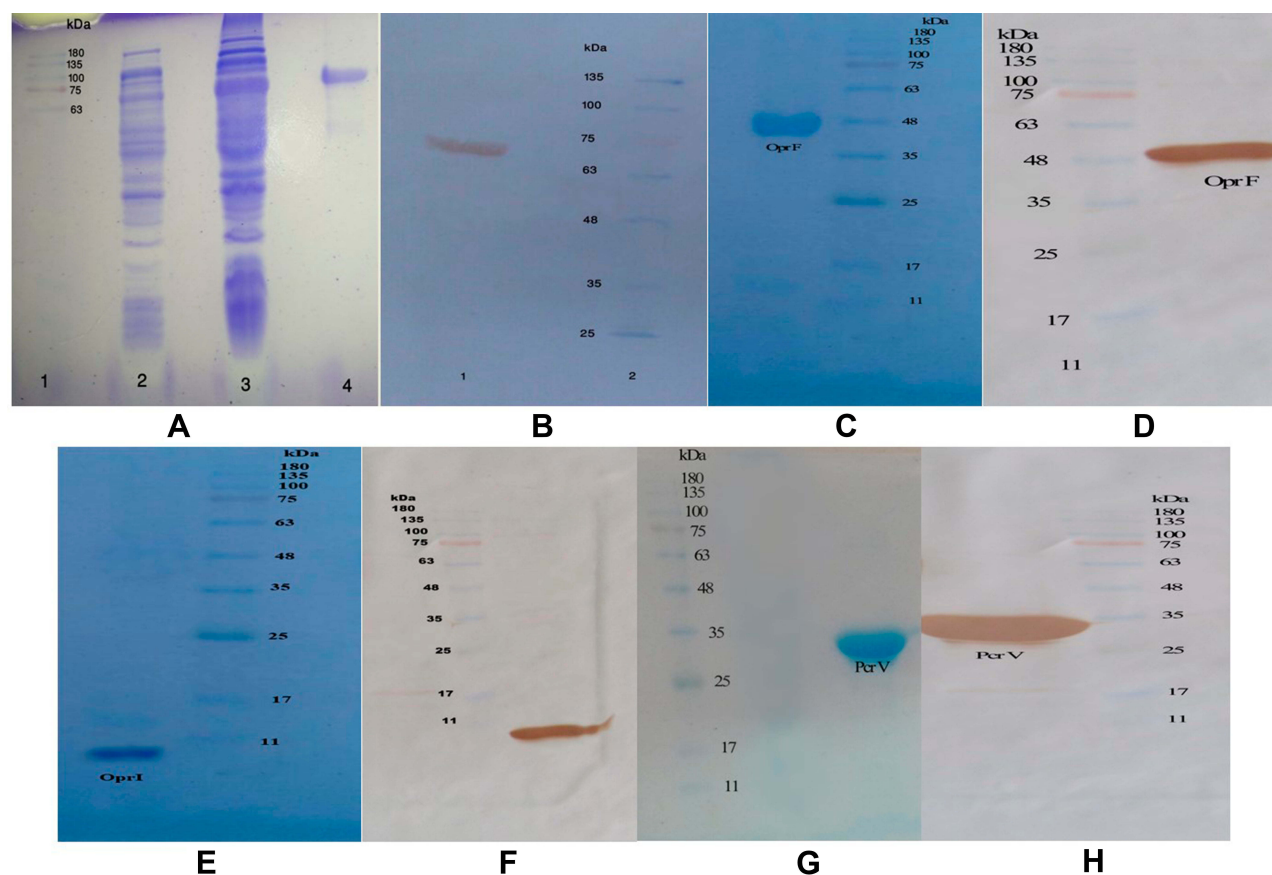
Figure 1 (**A**) Expressed and purified chimeric protein in the SDS-PAGE. The gel (12% w/v) was stained with the Coomassie blue G-250. Lane 2, pellet of the uninduced bacteria with IPTG; lane 1, standard protein size marker (kDa); lane 3, pellet of IPTG-induced bacteria; lane 4, Purification of the chimeric protein on the SDS-PAGE. (**B**) Confirmation of the chimeric protein through the Western blotting. (**C**) The OprF protein purified in the SDS-PAGE (12% w/v gel). (**D**) The OprF protein approved by the Western blotting. (**E**) The OprI protein purified in the SDS-PAGE by 9% w/v gel. (**F**) Confirmation of the OprI protein through the Western blotting. (**G**) The PcrV protein purified in the SDS-PAGE by 12% w/v gel. (**H**) The PcrV protein approved by the Western blotting.
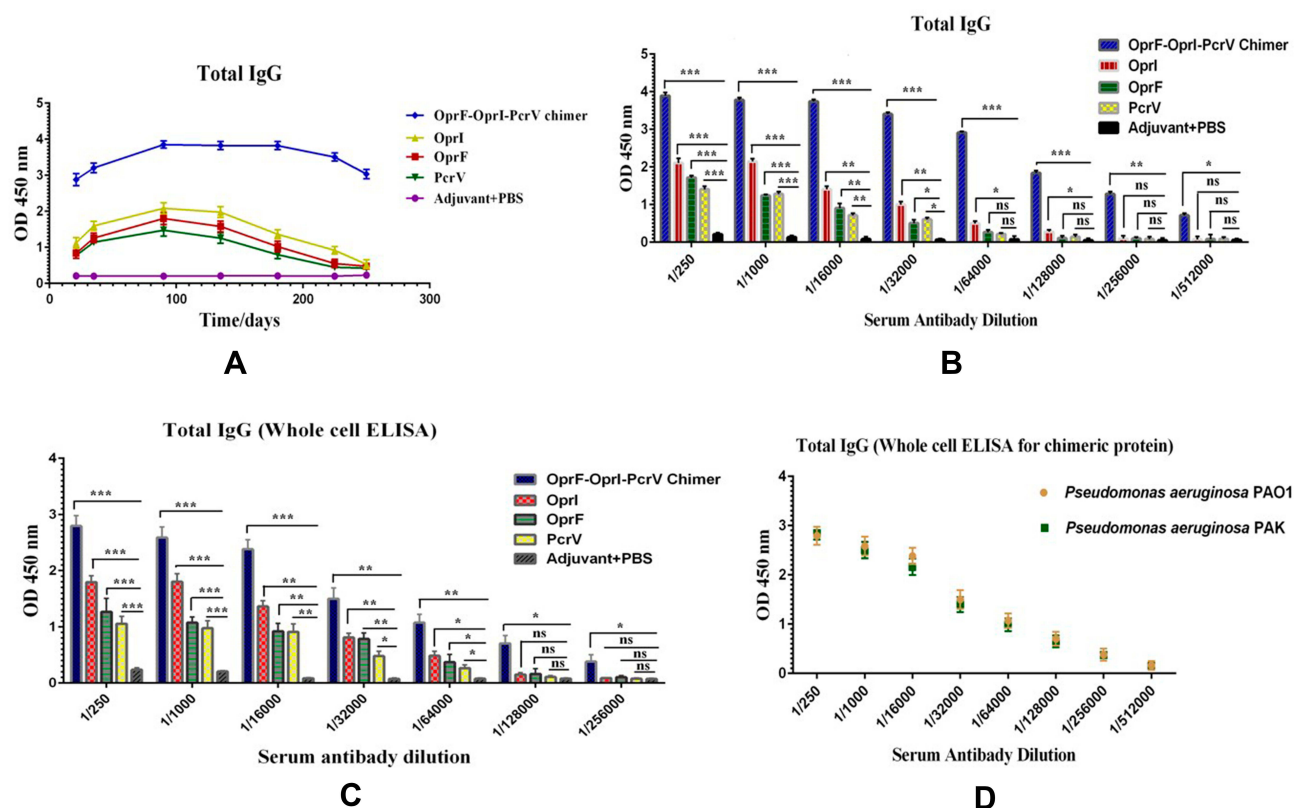
Figure 2 ELISA assay. (**A**) Total IgG level in the mice immunized with the chimeric and recombinant proteins at different times. (**B**) The Total IgG bar chart after administration of the second booster in serial dilution antibody for chimeric and recombinant proteins. (**C**) The binding power of specific antibodies to the *Pseudomonas aeruginosa* PAO1 whole-cell in the chimeric and recombinant proteins. (**D**) Comparison of the whole-cell ELISA results between two reference strains of *Pseudomonas aeruginosa*. *$p< 0.05$, **$p< 0.01$ and ***$p< 0.001$.
Abbreviation: Ns, not significant.

the related results. The durability of the antibody titres was also estimated (Figure 2A). The chimeric protein exhibited better immunogenicity and durability compared to other three recombinant proteins. No significant differences were observed in the results of the whole-cell ELISA for *P. aeruginosa* strains of PAO1 and PAK.
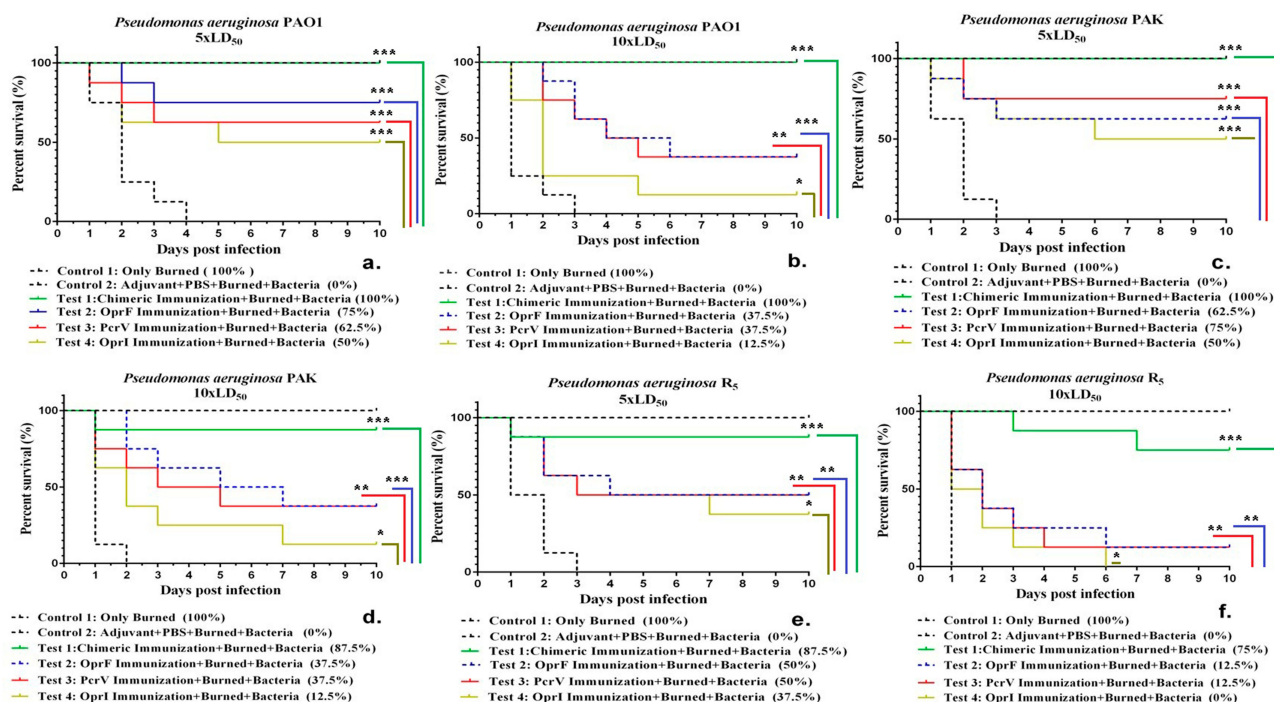
LD_{50} and Animal Challenges

Table 2 shows the results regarding the animal challenges at a dose of LD_{50} for each bacteria. The immunized mice were challenged with $2xLD_{50}$, 5xLD50, and $10xLD_{50}$ doses of *P. aeruginosa* strains of PAO1, PAK, and R_5 to determine the protective potency of our recombinant proteins. A significant ($p< 0.05$) improvement was observed in the survival rate of the mice vaccinated with the chimeric protein compared to those immunized with any of OprI, PcrV, and OprF proteins (Figure 3). The survival rate of the mice immunized with the OprI protein was much lower than that of other recombinant proteins at the doses of 5xLD50 or 10xLD50. Totally, 75% of the mice immunized with the chimeric protein successfully survived against the $10xLD_{50}$ of R_5 resistant strain (Figure 3F). The mice immunized with the chimeric protein survived after 3 days of severe infection caused by the 5xLD50 of PAO1, PAK, or R_5 resistant

Table 2 The Amounts of Inoculation Doses of Death by the *Pseudomonas aeruginosa* Strains of PAO1, PAK, and R_5 (in the Burned Mice)

Bacteria	Dose	CFU
Pseudomonas aeruginosa PAO1	LD_{50} $2xLD_{50}$ $5xLD_{50}$ $10xLD_{50}$	$1.4×10^3$ $2.8×10^3$ $7×10^3$ $1.4×10^4$
Pseudomonas aeruginosa PAK	LD_{50} $2xLD_{50}$ $5xLD_{50}$ $10xLD_{50}$	$1.7×10^3$ $3.4×10^3$ $8.5×10^3$ $1.7×10^4$
Pseudomonas aeruginosa R_5	LD_{50} $2xLD_{50}$ $5xLD_{50}$ $10xLD_{50}$	$6×10^2$ $1.2×10^3$ $3×10^3$ $6×10^3$

Abbreviation: CFU, cloning forming unit.

Figure 3 Comparison of survival rate in the two control groups (including non-immune mice that were only burned and those received adjuvant with PBS) and the mice immunized with four vaccine candidates (chimeric protein, OprI, OprF, and PcrV) that were challenged with burn wound infections by the *P. aeruginosa* strains of PAO1, PAK, and R_5. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.
Abbreviation: ns, not significant.

strains. However, the mice immunized with each of the proteins tolerated severe infection for 6 days after receiving the $5xLD_{50}$ of the bacteria.

Bacterial Burden

As shown in Figure 4A and B, the mice immunized with the recombinant proteins showed significantly lower bacterial loads in the skin, liver, spleen, and kidney compared to the control groups 12 h after infection. However, the bacterial count in the mice immunized with the chimeric protein was much lower in different organs than that of the three individual proteins. Our results exhibited that immunization with the chimeric protein could reduce the bacterial local colonization and systematic spreading simultaneously.
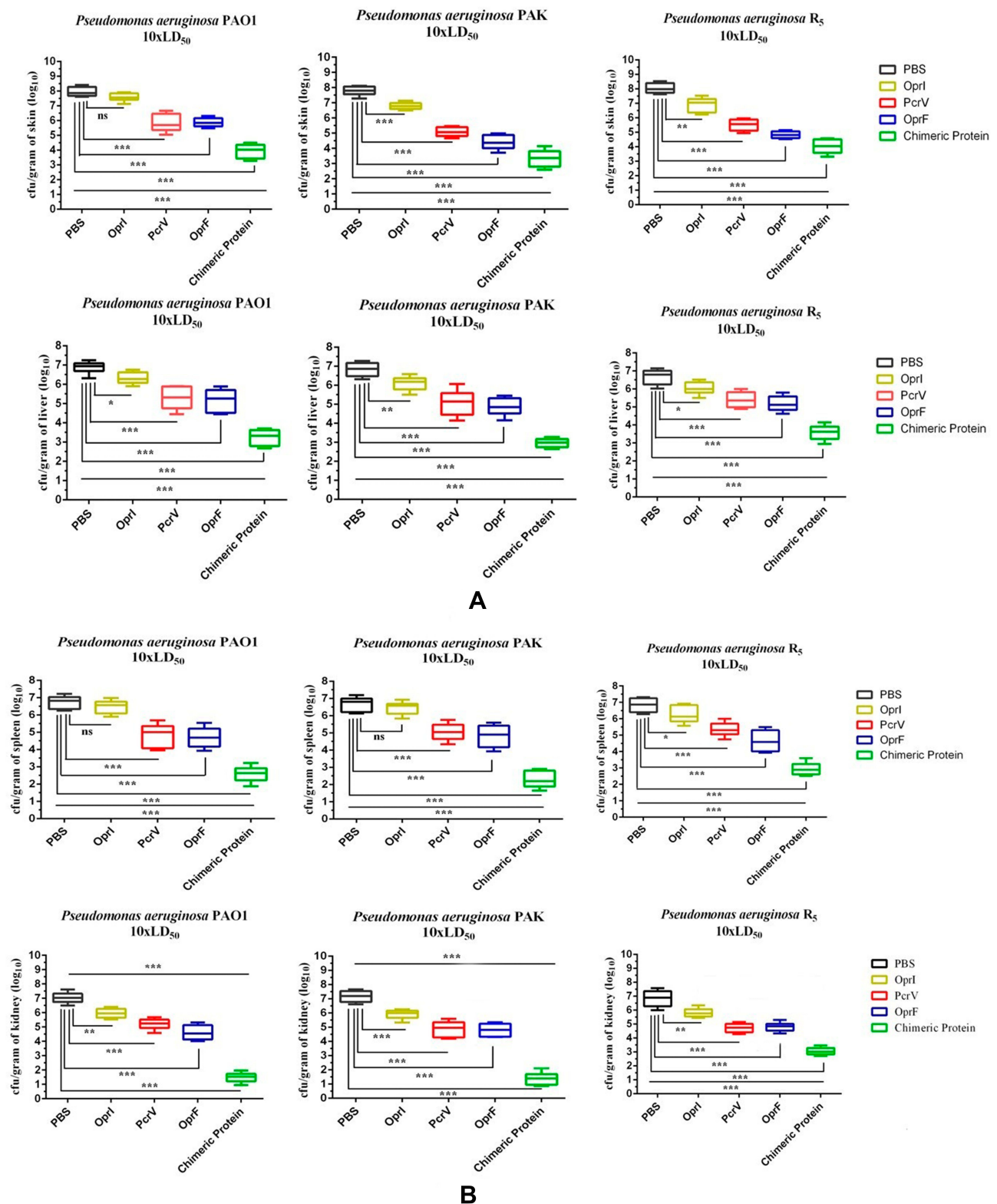
Bacterial Resistance

Disc diffusion method (CLSI-2016) was used to evaluate the resistance to the *P. aeruginosa*.[22] The R_5 hospital strain showed resistance to ciprofloxacin 5 μg and imipenem 10 μg. However, the growth inhibition zone was equal to 28 mm in the imipenem disk for the *P. aeruginosa* PAO1 and it was equal to 30 mm for the *P. aeruginosa* PAK. For the ciprofloxacin (5 μg), the growth inhibition zone was

equal to 34 and 37 mm for the PAO1 and PAK strains, respectively (Figure 5).

Discussion

Currently, there are several proteins available as a vaccine candidate for protection against the Pseudomonas bacteria. Some of these proteins have undergone research tests and some are under clinical trials.[23,24] Among these, OprI, OprF, and PcrV are of considerable importance, since they are expressed in most of the pathogenic strains of PA and play an essential role in the pathogenesis of PA.[24–26] In the present study, these proteins were selected and produced to investigate their potency as new vaccine candidates against PA infection. Several reports have indicated that the antibodies raised against the OprF, OprI, and PcrV could provide protection against the PA infection.[14,27–29] Active immunization with the OprI and OprF,[30–32] and passive immunization with the PcrV[14,27–29] have been shown to protect the animals challenged with the PA.[10,32] The three antigens selected in this study are conserved among different PA isolates, and as a result, one can expect a cross-protective effect when challenged with different clinical isolates. Previous studies have revealed that single subunit vaccines are mostly inadequate in protecting against the infections. Therefore, in this study, the

Figure 4 Bacterial loads in the skin and liver (**A**) spleen and kidney (**B**) of the mice immunized 12 h after infection with the $10xLD_{50}$ CFU of PAO1, PAK, and R_5. *$p< 0.05$, **$p< 0.01$ and ***$p< 0.001$.
Abbreviation: ns, not significant.
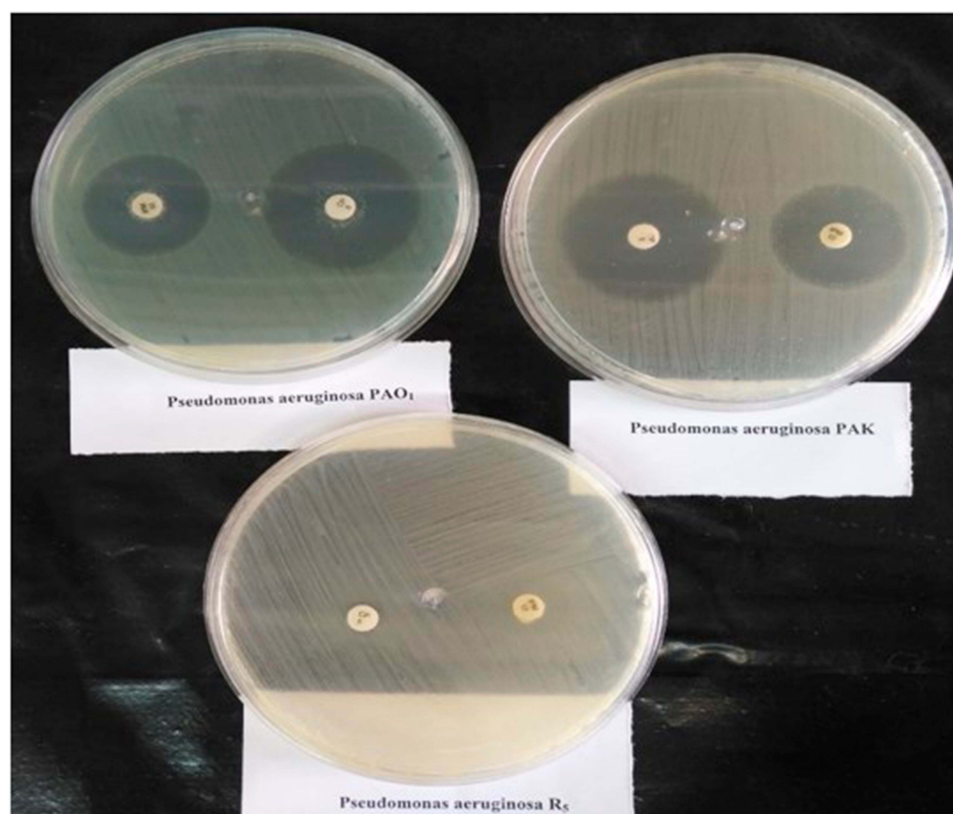
Figure 5 Bacterial resistance tested by the disc diffusion method on the *Pseudomonas aeruginosa* strains of PAO1, PAK, and R_5. R_5 hospital strain was resistant to ciprofloxacin 5 μg and imipenem 10 μg.

immunogenic regions of the three proteins were selected and designed to develop a chimeric protein using the bioinformatics tools. The antigenic and immunogenic MHC class I, MHC class II, and B cell epitopes present in the OprI and OprF proteins were identified by the reverse vaccinology approach. The selected epitopes were listed based on their antigenicity and toxicity potentials in order to design our chimeric vaccine. The immunogenicity of the chimeric protein was compared with each of the OprI, OprF, and PcrV proteins alone (Figure 1C–H). The anti-chimer antibodies could protect against the potential infections by the $10 \times LD_{50}$ of *P. aeruginosa* strains (PAO1, PAK, and R_5) in the mouse model of burn wound. The survival rate of the mice immunized with the chimeric protein was increased compared to other recombinant proteins applied alone against infections by all the strains (Figure 3). Hydrophobicity is considered as an important virulence factor of various pathogens. Factors increasing the hydrophobicity can enhance the adhesion of bacteria to intestinal epithelial cells and thereby facilitate the invasion. Adhesion to the epithelial cells has been found to be associated with the hydrophobicity of the *Streptococcus pyogenes* strain, and sub-inhibitory concentrations of rifampicin have been shown to

lower the hydrophobicity and reduce the cell adhesion.[33] The hydrophobicity of the bacterial surface acts as opsonin, creates the phagocytic capacity, and leads to the development of the complement system and IgG opsonization of *Salmonella enterica* serovar Typhimurium.[34] Calvinho et al have reported that fast-growing strains of *Staphylococcus aureus* exhibited high surface hydrophobicity and consequently high pathogenicity compared to slow-growing hydrophilic strains.[35] The engulfment of the bacteria by the phagocytic cells as a result of treatment with the antibiotics occurs due to increased bacterial surface hydrophobicity. In a study, hydrophobicity and phagocytic killing of the PA were increased when treated with sub-inhibitory concentration of aztreonam, whereas low exposures to inhibitory concentrations caused similar effects on other Gram-negative bacteria such as *E. coli, Serratia marcescens, Klebsiella pneumoniae*, and *Salmonella typhimurium*.[36] On the other hand, the increase in the bacterial surface hydrophobicity seems to fulfill the lack of opsonization and leads to competent phagocytosis. In this regard, non-opsonized hydrophobic strains of *Bacteroides buccae, Porphyromonas gingivalis, Staphylococcus saprophyticus*, and *Fusobacterium nucleatum* have been shown to be readily phagocytosed by

the Polymorphonuclear leukocytes (PMNs), whereas the phagocytic killing of hydrophilic strains required human serum opsonization.[37,38] Thus, it is assumed that the hydrophobicity confirmed by the bioinformatics analysis of the selected recombinant proteins consisting of the $OprF_{185-350}$, $OprI_{22-83}$, and PcrV increases the phagocytic ability of the PMNs by increasing the bacterial aggregation and may facilitate a rapid and competent host response. It has been reported that the antibodies act against the PcrV, OprI, and OprF virulence factors of PA speculate and interfere with the early stages of its pathogenesis, as well as later stages such as systemic dissemination to the organs.[39] Our results showed that resistance against pathogenic bacteria was started from the skin and more bacteria were killed in this area. None of PA strains (PAO1, PAK, and R_5) were found in the internal organs at the dose of $2xLD_{50}$. Bacteria were able to cross the damaged skin to a low extent at the doses of $5xLD_{50}$ and $10xLD_{50}$ (Figure 4A and B). The OprI, OprF, and PcrV proteins cause humoral and cellular immunity.[23] OprI increases the production of Interleukin 4 (IL-4), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interferon gamma (IFN-Y), Tumor Necrosis Factor-alpha (TNF-α), and Immunoglobulin G (IgG)[3,18] involved in the humoral and cellular immunity function. Rau et al have shown that the OprI can act as an adjuvant.[18] In our study, when the OprI was used along with the PcrV and OprF proteins, the antibody rise was significantly increased compared to any of the antigens used alone (Figure 2A–C). The antibody titre (IgG total), the concentration, and shelf-life of the antibody produced against the PA were much higher in the chimeric protein-immunized mice than the single proteins (Figure 1A and B). Weimer et al[40] have shown the synergistic effect of the OprI and OprF proteins in the rise of IgG titre, which seems to be a property of the OprI adjuvant.[18] The neutralization power of the antibody produced by the chimeric protein was much higher than any of the single proteins (Figure 2C). This means that the chimeric protein could attach to the whole-cell with high affinity. This was approximately the same in the whole-cell ELISA results for both strains *P. aeruginosa* of PAO1 and PAK (Figure 2D) attributing to the presence of the conserved domains of the selected proteins in all the three strains studied in our research. Zhang et al have reported that the immune induction occurred due to the presence of OprF in their proposed vaccine.[21] Besides increasing the IgG antibody titre, OprF also increases the production of the Interleukin 1 beta (IL-1β), Interleukin 2 (IL-2), IL-4, IL-6, Interleukin 12 (IL-12), Interleukin 17 (IL-17), INF-Y, and TNF-α.[23,41] Chuang Wan et al have shown that the $PcrV_{NH}$

protein not only increases the levels of IgG_1, IgG_{2a}, and IgG_{2b} but also increases the titre of cytokines including IL-4, IL-17, and IL-1β, suggesting the humoral immunity. The $PcrV_{NH}$ protein is composed of the N-terminal (1–127 amino acid) and the helix 12 in C-terminal (251–294 aa.) of PcrV.[42] PcrV protein as a vaccine could increase the titres of the TNF-α, INF-Y, Cluster of Differentiation 3 (CD_3), Cluster of Differentiation 28 (CD_{28}), and Cluster of Differentiation 4 (CD_4).[3] Therefore, all the three proteins selected to design the chimeric antigen have both the ability to activate cellular and humoral immunity. In general, outer membrane proteins can increase the humoral immunity by increasing the (Th_2) CD_4 T Cells (CD_{44} + IL-4) and can cause cellular immunity by increasing the (Th_1) CD_4 T Cells (CD_{44} +IFN-Y).[21,43] Many attempts have also been made to develop multiple vaccines using some outer membrane proteins and some success has been achieved.[3,23,44] Other researchers have turned to development of a multi-antigen vaccine since PA has many factors involved in its pathogenesis. Yang et al have designed a trivalent vaccine ($PcrV_{28-294}$-$OprI_{25-83}$-Hcp_{11-162}) with $Al(OH)_3$ adjuvant and evaluated its protective efficacy in mouse models of pneumonia and burn. Immunization with this trivalent vaccine induced strong immune responses and reduced the bacterial loads, decreased the pathology, inflammatory cytokine expression, and inflammatory cell infiltration.[3] Weimer et al have indicated that intramuscular immunization with a combination of OprF epitope 8 ($OprF_{311-341}$), OprI, and flagellins (types A or B) produced high-affinity IgG antibodies specific to the flagellins, OprI, and OprF that individually promoted extensive deposition of the Complement Component 3 (C3) on PA.[30] Hassan et al have conducted a research on the antigens of trivalent vaccine (OprF, OprI, and flagellin B) and confirmed many results reported in the study by Weimer et al. Both studies were performed to select the same antigens and evaluate their efficacy on non-mucoid pseudomonas infection.[44] Despite significant efforts in raising awareness in producing a vaccine against the PA, to the best of our knowledge, no effective vaccine has been developed yet, suggesting the potential for developing a vaccine to prevent infection.

Conclusion

In summary, our results demonstrated that the chimeric protein could be an effective vaccine candidate for protection against the *P. aeruginosa* infection. Combination of these proteins in the chimer increased the survival rate of the mice compared to any of the antigens applied alone.

Ethical Approval

Acknowledgment

Author Contributions

Prof. Owlia and Mousavi are the supervisor of the PhD thesis of Mohammad Hadi Fakoor. Mohammad Hadi Fakoor as a PhD student performed the experiments and wrote the draft of the article. Ms. Sabokbar participated as a consultant in this thesis project. All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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