RESEARCH PAPER

Evaluation of humoral immunity response to gold nanoparticles carrying *Pseudomonas aeruginosa* type IV pili in BALB/C mouse

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ABSTRACT

Objective(s): *Pseudomonas aeruginosa* is one of the critical multidrug-resistant (MDR) pathogens. Vaccination could offer dual benefits by preventing sepsis caused by antimicrobial-resistant bacteria and curtailing the rise and selection of antimicrobial resistance due to excessive antibiotic use. With this in mind, we designed a vaccine candidate made of gold nanoparticles conjugated to pilin IV protein. Pilin IV protein is an important virulence factor in the pathogenesis of *P. aeruginosa* infections.

Materials and Methods: Gold nanoparticles (AuNPs) were synthesized. The Gold nanoparticles were conjugated to the recombinant protein pilin IV. The recombinant protein pilin IV was emulsified in Montanide ISA 266's adjuvant and was administered to BALB/c mice model via subcutaneous injection. The studied groups included recombinant protein with gold nanoparticles, recombinant protein with Montanide ISA 266's adjuvant, and the control group. After blood sampling at the appropriate time points, ELISA test was performed on the serum of the mice groups.

Resuls: The results showed that the two studied groups could stimulate the mouse's immune system. Both IgG antibody subclasses were stimulated.

*Conclusion: T*his compound can be a new candidate for recombinant vaccine design against *P. aeruginosa* as a dexterous pathogen.

Keywords: *Gold nanoparticles, Nanovaccine, Pseudomonas aeruginosa, Type IV pili*

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INTRODUCTION

Pseudomonas is a type of bacteria found commonly in soil and water. *Pseudomonas aeruginosa* most often causes infections in humans, which can cause irreparable damage to various patients, particularly immunocompromised patients, cystic fibrosis patients, contact lens carriers, and patients suffering from severe burns [1]. According to the WHO, *P. aeruginosa* is a critical and multidrug-resistant (MDR) pathogen requiring urgent research for new treatments [2]. The emergence of multidrug-resistant bacterial species highlights the need to develop alternative antibiotic therapies. Meanwhile, vaccines and immunotherapy have shown promising results as an alternative to antibiotics. Various virulence factors of this bacterium have complicated the design and development of vaccines against infections caused by *P. aeruginosa*. Therefore, it is essential to identify highly immunogenic antigens suitable as vaccine candidates [3]. Since attachment to the host cell is the first step for colonization and infection by this bacterium, vaccines that aim to prevent the attachment of bacteria to the surface of host cells are of great importance [4]. The new generation vaccines, especially the subunit vaccines, are based on recombinant or pure proteins and synthetic peptides that are highly safe but generally appear to have an overall lower immunogenicity and less reactogenic compared to the whole organism

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vaccine; as a result, to increase the safety of this type of vaccine, adjuvants are usually used [5]. An immunological adjuvant is a substance combined with a specific antigen and causes robust immune responses compared to the antigen alone [2]. There are variety of compounds with adjuvant properties and different mechanisms of action. Mineral salts, emulsions, nanoparticles, saponins, cytokines, microbial components/products, and liposomes have all been evaluated as adjuvants [6]. Aluminum salts are the most common and efficient adjuvants, which can be used for human vaccines. Alum adjuvant is relatively weak and rarely stimulates the cellular immune response, but it can activate the humoral immune response; nowadays, evidence shows aluminum salts' direct and indirect neurotoxicity [3]. Therefore, it is essential to find better adjuvant formulations for vaccines.

Meanwhile, Nanoparticles have shown that they are good to choose for biomedical applications. Nanoparticles are solid and small and have a size of 1 to 100 nm. The physical and chemical properties of nanoparticles, including size, shape, surface chemistry, roughness, and surface coatings, are distinctive from bulk materials and have been widely used in biomedical applications, including drug/gene delivery and vaccines [7]. When they carry peptides or proteins, some nanoparticles with adjuvant properties can increase the activity of reticulum endoplasmic system cells and activate macrophages and dendritic cells. Activated macrophages and dendritic cells swallow and process the complex, and immune responses are formed more efficiently. Nanoparticles can increase the immune system's response to the target antigen and effectively direct and guide this system to create a specific type of response. They are using these nanoparticles as antigen carriers to reduce the amount of recombinant protein and antigenic toxicity and the destructive effects of proteases on protein antigens. By attaching to the antigen, nanoparticles increase binding to the immune system and boost the humoral immune response and cellular immunity, thereby increasing the vaccine's effectiveness [2, 7]. Among different nanoparticles already tested and used for the successful development of adjuvants, Gold nanoparticles (GNPs) are advantageous as an adjuvant in designing effective vaccines and preparing high-affinity antibodies to haptens and complete antigens [8]. Some studies have revealed that various immune cells, including macrophages, dendritic cells, and lymphocytes, are stimulated by gold nanoparticles, leading to the production of pro-inflammatory cytokines such as IL-1β and TNF-α and Th1 cytokines like IFN-γ and IL-2; also some reports have shown that AuNPs stimulate a more robust immune response than immunization using complete Freund's adjuvant (CFA) [3, 7]. Therefore, for the first time, this study evaluated the immunogenicity of synthetic Flp pilin protein and gold nanoparticles as an adjuvant in mice models.

MATERIALS AND METHODS

Synthesis of recombinant protein of type IV pili

The recombinant type IV pili protein of *P. aeruginosa* was analyzed by bioinformatics software. The sequence of 49 amino acids was selected, which was able to stimulate T and B immune cells. It was synthesized by the Biomatik Inc. company, Canada. The synthetic type IV pili fragment of bacteria was analyzed by Biomatik Inc and confirmed using Mass spectrometry and high-performance liquid chromatography, too.

Preparation of gold nanoparticles

Citrate-coated AuNPs were prepared by trisodium citrate reduction of tetrachloroauric acid (HAuCl4) [9]with some modifications. 2 ml of HAuCl4 (0.03 mg/ml) was prepared in ultrapure water. The solution was heated to 150 ◦C, and then 2.5 ml of trisodium citrate with 24.5 ml ultrapure water was added to the solution, heated to 70 ◦C, and then stirred for 15 min until the color turned to wine red. Then, the nanoparticle with red color was stirred overnight at room temperature without heating.

Characterization of gold nanoparticle

For evaluation of size and morphology, scanning electron microscopy (SEM) was done. Ultraviolet–visible (UV-Vis) spectrophotometer, from 400 to 700 nm, was used to detect the presence of gold nanoparticles. The maximum peak of UV-Vis spectroscopy for gold nanoparticles typically occurs in the range of 520-550 nanometers (nm). This peak is known as the surface plasmon resonance (SPR) peak and is a characteristic feature of gold nanoparticles.

Preparation of AuNPs-recombinant type IV pili

First, the pH of AuNPs was adjusted to 8 by KOH. AuNPs and recombinant type IV pili conjugated AuNPs were prepared by mixing recombinant protein type IV pili [10].

0.15 ml of 25 mol recombinant type IV pili was added to 1.3 ml of AuNPs (2 nM) with pH:8 and stirred for 10 min. Then, 1 ml BSA (0.1 mg/ml) was added. The mixture was stirred at 300 rpm for 3 hr at room temperature.

After processing, the unbound protein was removed from the solution by centrifugation (15,000×g, 30 min), the supernatant was discarded, and the protein-conjugated AuNPs were resuspended in phosphate buffer pH 7.0. The conjugate stability was assessed by the absence of aggregation (color change from red to blue or gray) after adding 10% aqueous solution of NaCl. The Bradford method determined the concentration of recombinant protein after binding to gold nanoparticles (AuNPs).

Preparation of recombinant type IV pili protein of P. aeruginosa formulated in adjuvant

Due to the enhancement of immunological effects, the synthetic form of the recombinant type IV pili protein has been formulated with two different materials: Montanide as a positive control adjuvant and gold nanoparticle as new adjuvants in this study. Montanide ISA 266 (M-adj) consists of a mixture of mineral oil and 50% metabolizable oil and a highly refined surfactant obtained from purified oleic acid and mannitol [11].

In order to prepare the recombinant adjuvant with protein, the recombinant protein was first prepared by mixing 60 micrograms of type IV synthetic pili with 30 micrograms of Montanide ISA 266 (France). The mixture was prepared by filling and emptying the syringe. Finally, the mixture was dissolved in PBS in a final volume of 400 microliters under sterile conditions.

Animal study

In this study, female BALB/c mice (20-22 gr) (6–8 weeks old) were purchased from the Pasteur Institute of Iran (Tehran, Iran). Mice were retained for 5 days before the experiment and given free access to water and food. The temperature was preserved between 20–25 °C and 12 hr light/dark cycles with the relative humidity set between 45–60%. The use of mice was conducted following the guidelines of the SBU, Tehran, Iran (IR. SBU. ICBS. 98/1018).

Immunological study and immunization of mouse groups

The mice were accidentally divided into test and control groups, each consisting of 5 mice, to evaluate active immunization. Animals were vaccinated thrice on days 0, 14, and 28, inoculating via an intraperitoneal (IP) injection. For immunization, group (I) was inoculated with 40 µg of recombinant type IV pili formulated in M-adj. Group (II) received gold nanoparticles with equal recombinant type IV pili volume. Group (III) received 40 µg gold nanoparticles without antigen. The group (IV) received sterile phosphate-buffered saline (PBS) at equivalent volumes to the control groups. Blood samples were collected from the retro-orbital sinus one week after the last inoculation, and 35 days sera were analyzed for antigen-specific antibody assay by ELISA.

Splenic lymphocytes analysis

One week after the final immunization and after blood sampling, all groups of mice in this study were sacrificed with an overdose of pentobarbital (60 mg/kg) administered intraperitoneally. The spleen was removed, and splenic lymphocytes were dissected from the spleens of immunized mice by density gradient centrifugation using Lymphoprep (specific gravity 1.077) (Sigma-Aldrich) according to the manufacturer's guidelines. Splenocyte suspensions were collected in complete RPMI 1640 containing 1% penicillin-streptomycin L-glutamine and 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at a final count of 4×10^6 cells/ml. The splenocyte cells were incubated at 37 °C in 5% CO_2 for 3 days [12].

Cell proliferation assay

In this experiment, for cell proliferation assay, 100 µL (3 × 105/well) of diluted spleen cells were cultured in 96-well flat-bottom plates and at 37 °C in 5% CO_2 under humidified atmosphere for 72 hours. The splenocyte was stimulated using 10 µg/ml of recombinant protein as an antigen recall. In the next step, 20 µl of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution was added to each well and incubated for 4 h at 37 °C in 5% CO_2. Then, the plates were centrifuged at 1200 g for 15 min, and the supernatant was discarded from the pellet. Finally, 100 µl dimethyl sulfoxide (DMSO) was added to each well of plates test. The absorbance was measured at 570 nm in a microplate reader three times.

Total IgG antibodies ELISA

Flat-bottom 96 well microplates (Sigma, USA) were coated with Recombinant protein (10 µg/ml in carbonate/bicarbonate buffer, pH 9) by incubating at 4 ˚C overnight. Afterward, the plates were washed thrice with PBST (phosphate-buffered saline containing 0.05% Tween-20, pH 7). In the next step, the wells were blocked with 3-5% BSA in PBS buffer at room temperature for 1 hr at 37 ˚C. Then, the wells were washed three times with 250 µL of TBS with 5 mM $CaCl_2$ 0.05% and Tween 20 (wash buffer). As primary antibodies, the collected sera from mice groups at serial dilutions 1:50 to 1:6400 with 1% BSA in PBS1X and Tween 20 were added into each well and incubated at 37 ˚C for 2 hr. The wells of plates were washed five times and incubated for 1 hr at 37 ˚C with 100 µL of 1:2000 dilution anti-mouse antibody of HRP-conjugated for detection. The next level, 100 µl of substrate

containing Tetramethylbenzidine (TMB, Sigma, USA), was added to all wells and incubated in a dark place. After adding 100 µL of H_2SO_4 after 30 min, the ELISA reaction was stopped, and the absorbance was determined at 450 nm by plate reader.

Specific IgG isotypes antibodies ELISA

To determine the types of the immune response, i.e., cellular or humoral, the IgG isotypes (IgG1, IgG2a, and IgG3) were also analyzed using indirect ELISA. Plates were coated with Recombinant protein type (10 µg/ml in carbonate/bicarbonate buffer, pH 9) by incubating and were washed five times with PBS. Then, the wells were blocked at room temperature for 1 hr at 37 ˚C with a blocking buffer. Plates were washed thrice, and then 100 µL of 1:10 diluted sera groups were added to each well. Then, all wells were washed thrice, and 100 µL of 1:1000 dilution of anti-mouse IgG1, IgG2a, and IgG3 subclasses as secondary antibodies were added and incubated. After final washing, 100 µL of HRP-conjugated anti-goat antibody was added to each well. In the next level, the wells of plates were washed, and TMB substrate was added to the wells and incubated for 30 min in a dark place. Finally, the immunoreaction was stopped with H_2SO_4, and the absorbance was read at 450 nm using a plate reader.

Statistical analysis

All statistical analyses were performed using SPSS statistics software version 19 (IBM Corporation, Armonk, NY) and Prism software (GraphPad Software 6.07, Inc., USA). The Post Hoc test was used to compare antibody titers.

Percentages between the non-immune and an indicated immunization group or between two indicated immunization groups. Where applicable, a one-way ANOVA was applied. *P* values of less than 0.05 were considered significant.

RESULTS

Characterization of gold nanoparticle carrying recombinant protein

The diagram in the figure shows that the nanoparticles have the maximum UV-Vis absorption of gold in spectroscopy at 525 nm, indicating the synthesis of gold nanoparticles. The gold nanoparticle's characteristic chemical alterations occurred during the gold nanoparticle's development under different experimental conditions. The size of the gold nanoparticle was determined with scanning electron microscopy about 20-40 nm (Fig. 1 A and B).

Result of proliferation assay

To assess whether recombinant type IV pili antigen as candidate immunization could be able to induce lymphocyte proliferation to provoke cell-mediated immune responses, we examined the reactions of mouse splenocytes after immunization through assessing cell proliferation reaction upon antigen re-stimulation *in vitro* by colorimetric MTT assay. Splenocytes derived from recombinant type IV pili – M-adj as a positive group showed a higher proliferation effect than other groups in this study. In the second proliferation effect level, the Recombinant type IV pili-gold nanoparticle, gold nanoparticle, and control groups after 72 hr (P<0.0001) (Fig. 2), which means immunized T lymphocytes could be stimulated by both types of the antigens recombinant type IV pili –adjuvant and Recombinant type IV pili -gold nanoparticle, after 72 hr.

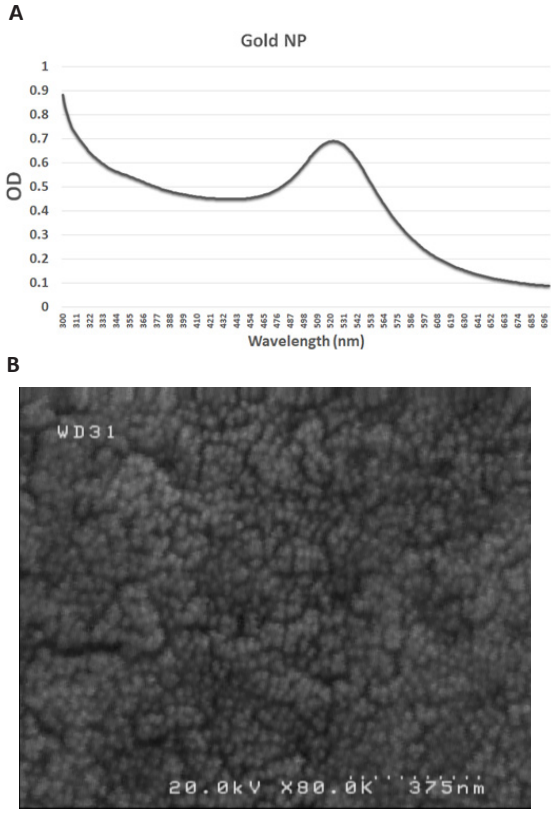

Fig. 1. Characterization of Gold Nanoparticles. (A) the curve of UV–vis spectrophotometry of Gold Nanoparticles with a characteristic peak at 525 nm. (B) scanning electron micrograph revealed spherical AuNPs with a diameter distribution of 30 ± 10 nm

Antibody assay

To evaluate the subtype of antibody response and type of systemic humoral immunity induced by all groups of this study, the levels of total IgG and three subgroups, IgG1, IgG2a, and IgG3, in

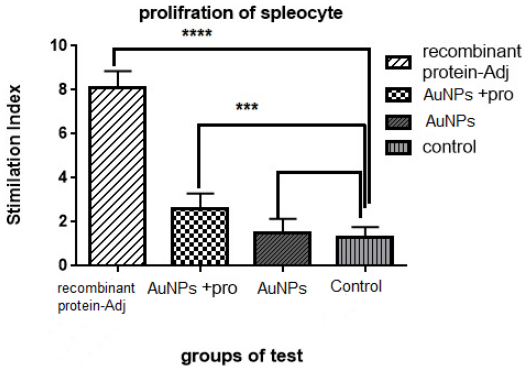

Fig. 2. The proliferation assay (MTT), augmentation of proliferation, and comparison in mice splenocyte with recombinant type IV pili –positive control and recombinant type IV pili -Gold nanoparticle antigens, gold nanoparticle, and control groups (P<0.05)

the immunized mouse sera were also measured. All sera dilutions from the immunized mice (1/50-1/6400) were used to approve the results. After the third inoculation (final booster), Recombinant type IV pili-gold nanoparticle induced antibody response among immunized mice compared to the control group (Fig. 3).

Groups of BALB/c mice (n=4) were immunized with three doses of Recombinant type IV pili - M-adj, Recombinant type IV pili – gold nanoparticle, gold nanoparticle, and PBS (control) on days 0,14 and 28. The animals were bled 7 days after the final immunization. Antisera titers were measured by the ELISA method. To consider the IgG1 (Th2-dependent isotype) and IgG2a and IgG3 (Th1-biased IgG subclasses antibody), in this project, we used the indirect ELISA method to compare the serum Levels of IgG isotypes among the immunized mice which were inoculated with Recombinant type IV pili - M-adj, Recombinant type IV pili -Gold, Gold, and PBS. The IgG isotypes analysis showed that immunization of the groups with Recombinant type IV pili antigen formulated adjuvant and Recombinant type IV pili -Gold nanoparticle potentially induced the specific IgG1, IgG2a, and IgG3 antibodies in comparison to the other groups and PBS control (P<0.05). Remarkably, the level of the specific IgG1 isotype associated with Recombinant type IV pili-adjuvant

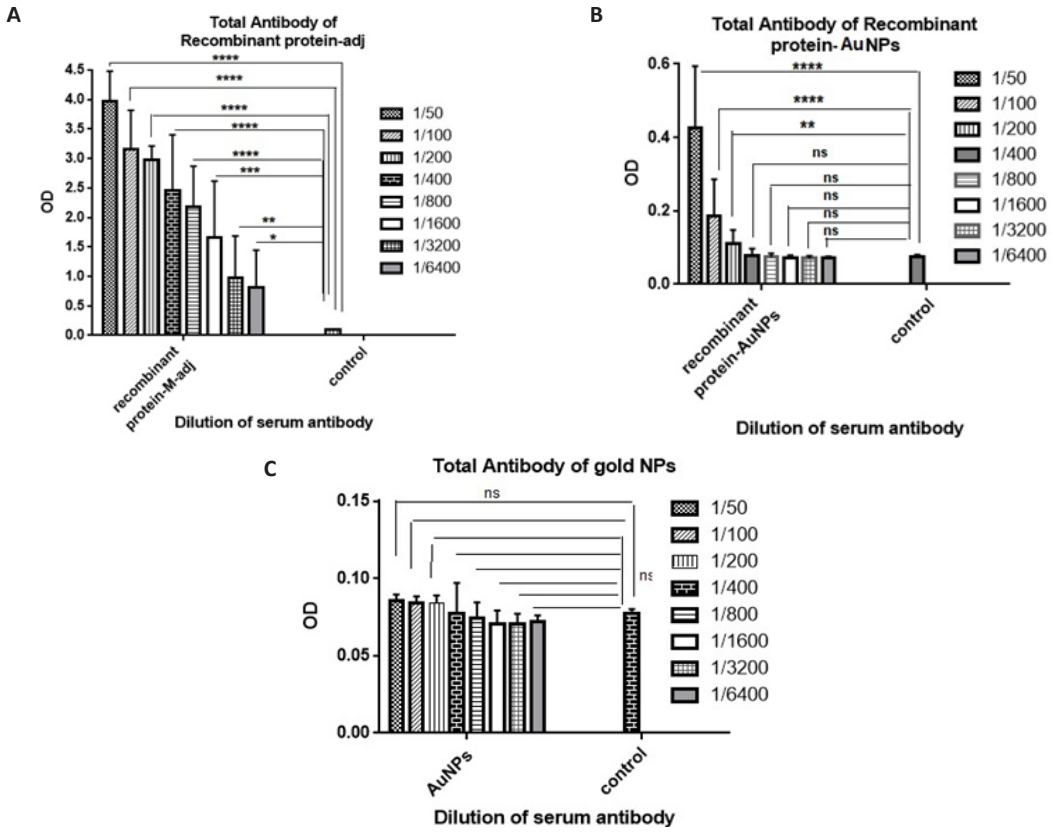

Fig. 3. Analysis of total IgG antibody responses to groups of study. (A) Recombinant type IV pili - M-adj-specific IgG titers. (B) Recombinant type IV pili -Gold specific IgG titers. (C) gold compared to PBS-associated IgG titers. All data are presented as mean ± standard error of the mean P<0.05. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, N.S.)

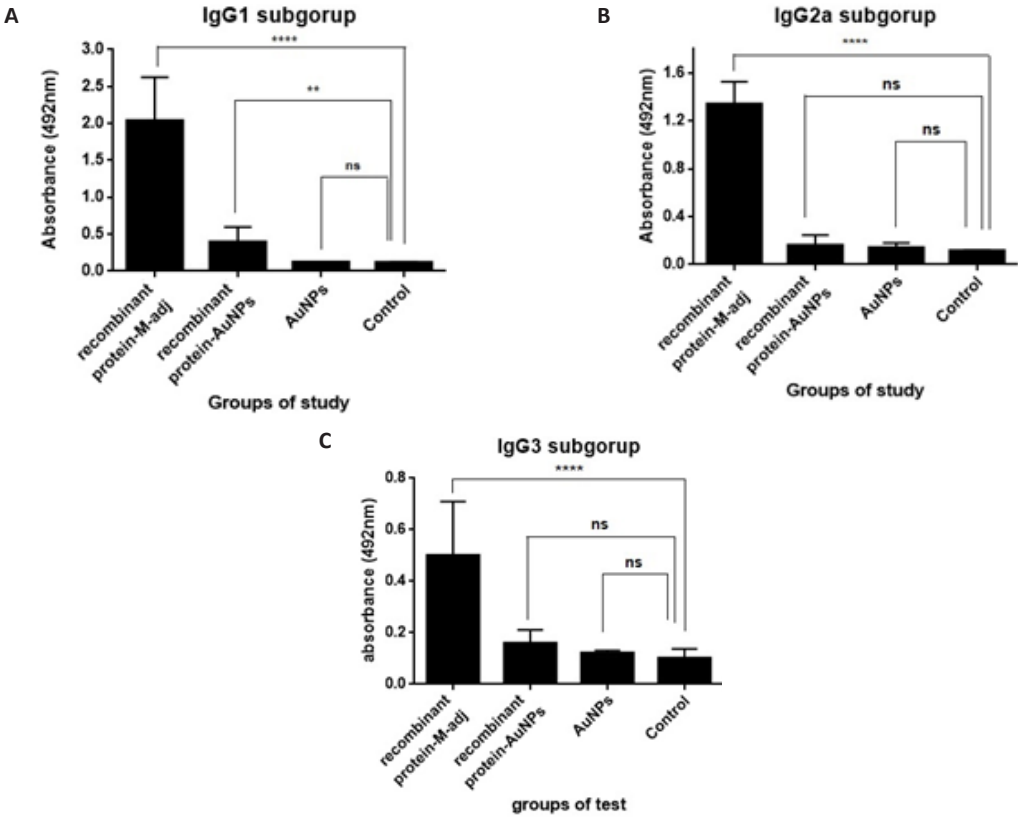

Fig. 4. The effects of recombinant type IV pili on levels of serum IgG isotypes. Recombinant type IV pili-adjuvant and Recombinant type IV pili gold nanoparticles were potent inducers for IgG1 compared to gold nanoparticles, PBS (P<0.001, P<0.01 respectively). The values were represented as mean ± SD based on four mice in each group

and Recombinant type IV pili carrying gold nanoparticles represented a significant difference compared to the control group (P<0.001). It is shown that these two groups could induce the production of IgG1 (Th2-dependent isotype). On the other hand, the comparison of Recombinant type IV pili -gold nanoparticles with the gold group and PBS clarified that Recombinant type IV pili -Gold had enough potential to provoke the IgG subclass, including (P>0.05) (Fig. 4).

DISCUSSION

Pseudomonas aeruginosa was proposed as the organism that caused the most septicemic deaths in the 1960s. *P. aeruginosa* is America's second most common cause of hospital-acquired pneumonia (17%) and the third most common cause of urinary tract infection (7%). The fourth most common agent is the surgical site infection (8%), the seventh most common agent isolated from the bloodstream (2%), and the fifth most common agent isolated from all locations (9%) (13). *Pseudomonas aeruginosa* is a major cause of nosocomial infections and an opportunistic bacterium that causes severe infections in immunocompromised individuals [14]. So, it is necessary to identify suitable highly immunogenic antigens as vaccine candidates. Conserved domains of pathogenic proteins have been among the antigens considered due to their cross-protective effect among different strains for vaccine formulation [15]. Flagellin, pilin, and other *P. aeruginosa* proteins have long been recognized as an inducer of immune system responses [16, 17] and used as a vaccine candidate in the research [18]. For the first time in this study, Flp protein was selected as a highly conserved precursor of the main structural components of Tad pili and an effective immunogen with gold nanoparticles adjuvant. The goal of vaccination is to induce a strong immune response to the antigen in order to provide long-term protection against infection. An adjuvant is needed to achieve this goal. There is an undeniable need for safer and more effective adjuvants suitable for human use. Although many other adjuvants

have been proposed over the years, these adjuvants have not been successful for human use due to toxicity, instability, low bioavailability, and high cost [19]. The use of nanoparticles to increase immune responses is a new science; most of its studies date back to the last two decades. Different types of nanoparticles with different compositions have been used in various studies as conjugated or loaded with various antigens, including microbial antigens, as immune adjuvants [20-22]. In this study, the maximum peak of UV-Vis spectroscopy for gold nanoparticles typically occurs in the range of 520-550 nanometers (nm). This peak is known as the surface plasmon resonance (SPR) peak and is a characteristic feature of gold nanoparticles. The exact value of the peak can vary depending on several factors, including the size, shape, and surface chemistry of the nanoparticles. Larger nanoparticles tend to exhibit a red-shift in the SPR peak, meaning it occurs at longer wavelengths (550 nm or above), while smaller nanoparticles will have a blue-shifted peak (520 nm or below). It is important to note that while the range mentioned is typical for gold nanoparticles, there can be variations based on the specific synthesis method employed and the surrounding environment of the nanoparticles. Additionally, factors such as aggregation, surface modifications, and interactions with other molecules can also influence the position and intensity of the SPR peak. Hence, it is always recommended to measure the UV-Vis spectrum of the gold nanoparticles to determine the specific wavelength of maximum absorption). The most important adjuvant property of nanoparticles is due to their particle size and as a result of increasing their absorption by the cells of the reticuloendothelial system, in which case the possibility of antigen conjugated to nanoparticles entering these cells increases a lot. Gold nanoparticles conjugated with antigens increase respiratory activity and increase mitochondrial activity of macrophages. Also, in various studies that have been conducted on the possible toxic effects of gold nanoparticles in vivo and ex vivo, no specific toxicity of these nanoparticles has been reported. Toxicity is higher at high doses, the presence of some functional groups on the surface of nanoparticles, and their size has been attributed [23, 24]. This research used adjuvants of gold nanoparticles and M-adj as an alternative to Freund's adjuvant in the animal model. Past studies suggested that the binding of immunoglobulin to the flagellar filament causes disturbance in the movement and agglutination of bacteria. Finally, the colony spread in the plate is inhibited [25]. Because movement is an important pathogenic factor [26]. Disruption of this function, with antibodies that inhibit the movement in vivo, may be a suitable measure of the prevention effect against bacteria. The engineered structure of Pili was designed by examining bioinformatics software. The best regions with epitopes stimulating the immune system were identified and selected. The hydrophobic region, which had no antigenic properties, was removed. Also, the antigenic regions were put together. The sequence of 49 amino acids was designed, which was able to stimulate T and B immune cells. The gold nanoparticles facilitate and/or enhance the uptake of adsorbed antigens by antigen-presenting cells (APCs), such as macrophages or dendritic cells, probably the most important function attributed to the adjuvanticity of gold nanoparticles [20, 27]. Although in this study we did not check any serum biochemistry and histopathological or cytokine expression profile of the mice, we did not diagnose any obvious evidence of toxicity after 34 days in the group receiving three doses of protein carrying gold nanoparticles from the point of view of animal behavior, weight, survival, and behavior. In the investigation of antibody production, the adjuvant effect of gold nanoparticles compared to the positive control (M-adj), a significant increase in the total antibody response of the Pili-gold group (average of the last positive titer 1:200) and control positive (average of the last positive titer 1:6400) compared to the gold group and PBS was seen. The result shows the good adjuvant effect of gold nanoparticles like M-adj. Considering the role and importance of type IV pili of *P. aeruginosa* in pathogenesis and in stimulating innate and humoral immunity, this protein has been evaluated as a suitable candidate for preventing *P. aeruginosa* infection. Pili type IV protein -adjuvant and recombined protein carrying gold nanoparticles significantly increase the humoral immune response so that it could effectively clear or prevent bacterial colonization in the early stages.

CONCLUSION

The amount of induction, production, stimulation, and the creation of humoral immune response by adjuvant is higher than gold nanoparticles. Gold nanoparticles carry recombinant protein type IV pili, which directs the immune response to T helper 2 and activates Th2, which can effectively activate macrophages and clean macrophages. According to the studies conducted on the recombinant protein Flp with adjuvant and gold nanoparticles, it can be a good candidate for preventing infection by *Pseudomonas aeruginosa*.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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