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Immunization of mice with chimeric protein-loaded aluminum hydroxide and selenium nanoparticles induces reduction of *Brucella melitensis* infection in mice

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Purpose: Due to the many problems with commercially available vaccines, the production of effective vaccines against brucellosis is a necessity. The aim of this study was to evaluate the immune responses caused by the chimeric protein consisting of trigger factor, Bp26, and Omp31 (TBO) along with aluminum hydroxide (AH/TBO) and selenium (Se/TBO) nanoparticles (NPs) as adjuvants in mouse model.

Materials and Methods: Recombinant antigen expression was induced in *Escherichia coli* BL21 (DE3) bacteria using IPTG (isopropyl-d-1-thiogalactopyranoside). Purification and characterization of recombinant protein was conducted through $NiFe_3O_4$ NPs, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot. NP characteristics, including morphology and particle size, were measured *in vitro*. The recombinant TBO was loaded on to AH and Se NPs and were administered subcutaneously. After mice immunization, measurement of antibody titter and protection assay was performed.

Results: The average sizes of AH and Se NPs were about 60 nm and 150 nm, respectively. The enzyme-linked immunosorbent assay results showed that the serum of mice immunized by subcutaneous injection with both nanovaccines produced significant immunoglobulin G (IgG) responses against the chimeric antigen. The results of TBO-specific IgG isotype (IgG2a/IgG1) analysis showed that both AH and Se NPs induced a type to T-helper immune response. In addition, the results of the challenge with the pathogenic strain of *Brucella melitensis* 16M showed that vaccinated mice with AH/TBO NPs indicated a higher reduction of bacterial culture than immunized mice with Se/TBO NPs and TBO alone.

Conclusion: The results showed that AH NPs carrying chimeric antigen can be a promising vaccine candidate against brucellosis by producing protective immunity.

Keywords: Brucellosis, Aluminum hydroxide, Selenium, Nanoparticles

Introduction

Brucellosis is the causative agent of Malta fever, Mediterranean fever, or feverish fever in humans, which infects more than 500,000 people worldwide each year. *Brucella* spp., which cause the disease, are a group of small gram-negative coccobacilli and facultative intracellular bacteria that affect a wide range of hosts [1,2]. This bacterium has several species; among them, *B. melitensis, B. abortus*, and *B. suis* are the most virulent species in terms of pathogenicity for humans. Due to the presence of pathogenic agents inside macrophages and the intracellular life, treatment of brucellosis is diffi-

cult because Brucella has different strategies to escape the host immune system, which will make the infection chronic. For this reason, only a few antibiotics are used to treat brucellosis in humans [3-7].

Brucellosis is transmitted to humans through contact with infected animals, breaking of the skin, or inhalation of aerosols contaminated with *Brucella*. Therefore, in order to prevent human brucellosis, there is always a need to control the disease in animals. Animal vaccination is one of the most effective strategies against human brucellosis [8].

Vaccines such as *B. abortus* RB51, *B. abortus* S19, and *B. melitensis* Rev1 are currently used to control brucellosis in animals. Despite the effectiveness of these vaccines and disadvantages such as the difficulty in the diagnosis and validation of infection stages in vaccinated animals, the ability to cause disease in humans, residual virulence, and abortion in pregnant animals, there is an immediate need for the production of more effective vaccines [9].

Due to their non-pathogenic properties and the possibility of manipulation to increase the desired properties and reduce the undesirable properties, recombinant vaccines are promising candidates for replacement with live-attenuated vaccines [1,3]. Various antigens in the formulation of subunit vaccines have been studied in recent years, but only a limited number of them can induce protective immune responses against brucellosis in animal models [10]. These include ribosomal L7/L12 protein [11], Dank-Omp31 [12], Bp26 [13], SodC [14], Omp31 [15,16], TF-Bp26, BLS-Omp31 [17,18], TF-Omp31-Bp26, and so forth [1,9].

Omp31 is one of the outer membrane proteins of *Brucella* and its main antigen, which has high immunogenicity. Also, studies have shown that this antigen has a favorable function in inducing humoral and cellular immunity [19]. Furthermore, trigger factor (TF) is one of the cytoplasmic proteins of this bacterium, which has been able to show favorable protection in various studies. Therefore, it is known as an immunogenic antigen [20,21]. The periplasmic protein Bp26, previously known as Omp28, was frequently used in Brucellosis diagnostic tests. Recent studies have shown that this protein can also have an immunogenic property [11].

One of the problems related to recombinant vaccines is their low immunogenicity compared to live-attenuated, or killed vaccines. In order to solve this problem, using an antigen delivery system and adjuvant is essential [6]. Due to their favorable characteristics and effectiveness in improving immunity, it has become common to use nanoparticles (NPs) as vaccine delivery systems and vaccine adjuvants. In previous studies, the effectiveness of aluminum hydroxide (AH) and selenium (Se) NPs as adjuvants was shown, and the results showed that these two NPs can be used in vaccines as adjuvant systems.

In this study, the antigenicity of a chimeric protein composed of three *Brucella* antigens: TF, Bp26, and Omp31 (TBO) along with AH and Se NPs, was assessed in C57 mice as an animal model.

Materials and Methods

Animals and ethics statement

Thirty-two female C57BL/6 mice aged 4 to 6 weeks were obtained from Baqiyatallah University of Medical Sciences (Tehran, Iran). The animals were housed in standard polypropylene cages at 24±2°C and humidity (40%–70%) with weekly floor exchange; they were also kept in customary animal facilities and got water and food ad libitum. Mice were randomly divided into four experimental groups. All experimental procedures on mice were officially agreed upon by the local ethics committee for animal experimentation at Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1397.373).

Bacterial strains and plasmid

Razi Vaccine and Serum Research Institute (Karaj, Iran) provided *B. melitensis* 16M, which was grown on Brucella agar medium for 2–3 days under aerobic conditions at 37°C in a humidified chamber. *E. coli* BL21 (DE3) and the pET28a vector (Novagen, Madison, WI, USA) were used to express TBO recombinant chimeric protein.

Expression and purification of chimeric protein

The TBO recombinant protein was characterized and prepared as previously described [1]. Briefly, *E. coli* BL21 (DE3) containing pET28a-*tbo* was inoculated into 100 mL of Luria Broth medium containing kanamycin (50 μg/mL). The incubation was continued with agitation (180 rpm) to a 0.5 optical density at 600 nm, and then gene expression was induced for 5 hours at 37°C with 1 mM isopropyl-d-1-thiogalactopyranoside (IPTG). The bacteria cells were harvested by centrifugation (5,000 rpm for 10 minutes); after that, the pellet was resuspended in lysis buffer (0.1 M NaH_2PO_4, 8 M urea, and 0.01 M Tris hydrochloride). Bacterial lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the protein expression. After ensuring successful expression of the recombinant protein, it was purified

using nickel magnetic ($NiFe_3O_4$) NPs. For this purpose, 10 mL of TBO-containing supernatant was added to the nickel magnetic NPs and shaken for 30 minutes to attach the TBO chimeric protein to the nickel magnetic NPs via His-tag. The NPs were washed in 20 mL of wash buffer (8 M urea, 50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole) 3 times, each time for 10 minutes with shaking. After that, the recombinant antigen was eluted with an elution buffer (450 mL wash buffer, 500 mM imidazole) for 30 minutes. Finally, the supernatant was separated as the recombinant subunit protein. After all, a Bradford protein assay was used to determine the concentration of recombinant protein.

Protein (antigen) characterization

In order to evaluate the purified proteins, the TBO chimeric protein was analyzed using SDS-PAGE, followed by a western blot using horseradish peroxidase (HRP)-conjugated anti-His tag antibody (Sigma-Aldrich, St. Louis, MO, USA).

Synthesis and characterization of TBO-loading nanoparticles

AH NPs were synthesized based on a previously reported method [22]. Equal volumes of 3.6 mg/mL $AlCl_3.6H_2O$ and 0.04 mol/L NaOH solutions were mixed. After adjusting the pH to 7.0, the mixture was stirred at room temperature for 30 minutes. In the next step, the mixture was sonicated for 10 minutes in an ultrasonic bath. The byproduct NaCl was removed by an AmiconVR Ultra-4 centrifugal filter from Merck Millipore (Darmstadt, Germany). In order to prepare TBO-loaded AH (AH/TBO) NPs, 500 μL of AH NPs were added to 500 μL of purified protein (800 μg/mL).

Arminano (Pardis Technology Park, Commercialization and Technomart Center, Tehran, Iran) provided Se NPs with a particle size of 100–200 nm. To prepare TBO-loaded Se (Se/TBO) NPs, 500 μL of Se NPs were added to 500 μL of purified protein (800 μg/mL).

Dynamic light scattering (Malvern 3000; Malvern Instruments, Malvern, UK) and scanning electron microscopy (SEM) were used to characterize the morphology and size of the NPs. The TBO content of AH/TBO and Se/TBO NPs was determined with a Bradford protein assay, according to the manufacturer's instructions.

Mice immunization

Mice were immunized by the subcutaneous (s.c.) administration route. Four experimental groups of mice either got vaccines or served as control groups: First group was immunized by AH NPs contained 20 μg TBO. The second group was immunized with Se NPs, which contained 20 μg TBO. The third group was immunized with 20 μg of purified protein, and the last group was immunized with 20 μg of phosphate-buffered saline (PBS) phosphate buffer as the negative control group. All experimental groups were vaccinated subcutaneously on days 0, 14, and 28.

Enzyme-linked-immunosorbent assay

Serum samples were collected from all immunized groups of mice on days 0, 10, 25, and 35 after the first vaccination to analyze the antibody production. To detect total immunoglobulin G (IgG), IgG1, and IgG2a, indirect enzyme-linked immunosorbent assay (ELISA) was done with an isotyping ELISA kit (Sigma-Aldrich). ELISA 96-well microplates were coated with 10 μg/mL of TBO chimeric protein and incubated at 37°C for 1 hour. The wells were washed with PBS containing 0.1% Tween 20 thrice after each step. In order to prevent nonspecific binding, the wells were blocked by adding a blocking buffer (5% skim milk in PBS) and incubated at 37°C for 1 hour. After washing, different dilutions of sera (1:500 to 1:160,000) were added to the plates and kept at 37°C for 1 hour. The plates were washed, and antibodies were added. HRP-conjugated anti-mouse antibody (Sigma-Aldrich) was added to the plates to detect whole specific antibodies, and goat anti-mouse subtypes antibody and rabbit anti-goat HRP conjugate antibody (Sigma-Aldrich) were added to detect IgG subclasses (IgG1 and IgG2a). Then, after washing, in the final step, 100 μL of TMB (Sigma-Aldrich) was added to each well and kept at room temperature for 15 minutes. Finally, color development was stopped by the addition of 50 μL of 2N H_2SO_4, and each well was measured for optical density at 450 nm by using a microtiter plate reader (Bio-Rad, Hercules, CA, USA).

Protection assay

To test the protective effects of TBO protein, immunized mice (four mice per group) were challenged intraperitoneally (i.p.) with *B. melitensis* 16M. Four weeks after the challenge, the homogenized spleens of mice were cultured in Brucella agar at 37°C for 3–4 days to determine the number of *Brucella* colonies. The $\log_{10}$ number of colony-forming units (CFUs) per sample was determined.

Statistical analysis

Data were statistically analyzed by Wilcoxon test using IBM SPSS ver. 22.0 software (IBM Corp., Armonk, NY, USA). Dif-

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ferences were considered statistically significant at a p-value ≤0.05. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Kruskal-Wallis test.

Results

Expression of recombinant antigen

Induction of TBO recombinant protein expression was successfully performed on the *E. coli* BL21 (DE3) expression host, which was transformed with pET28a-*tbo* with an N-terminal 6X-His tag. Subsequently, the results of SDS-PAGE analysis indicated the presence of recombinant protein, which was shown in a specific band with a size of 67 kDa (Fig. 1A). Anti-His-tag antibody confirmed the presence of recombinant antigen on Western blot (Fig. 1B). After purifying the chimeric protein, the SDS-PAGE method was used to ensure the correctness of the steps. The findings indicated that the purity was desirable (Fig. 1C).

Characterization of antigen-loaded NPs

A SEM illustrated the morphology and size range of NPs. SEM images indicated that the sizes of AH and Se NPs particles are approximately 60 nm and 150 nm, respectively. Also, the surface of both NPs, as can be seen in the SEM images, is smooth, and their morphology is spherical (Fig. 2).

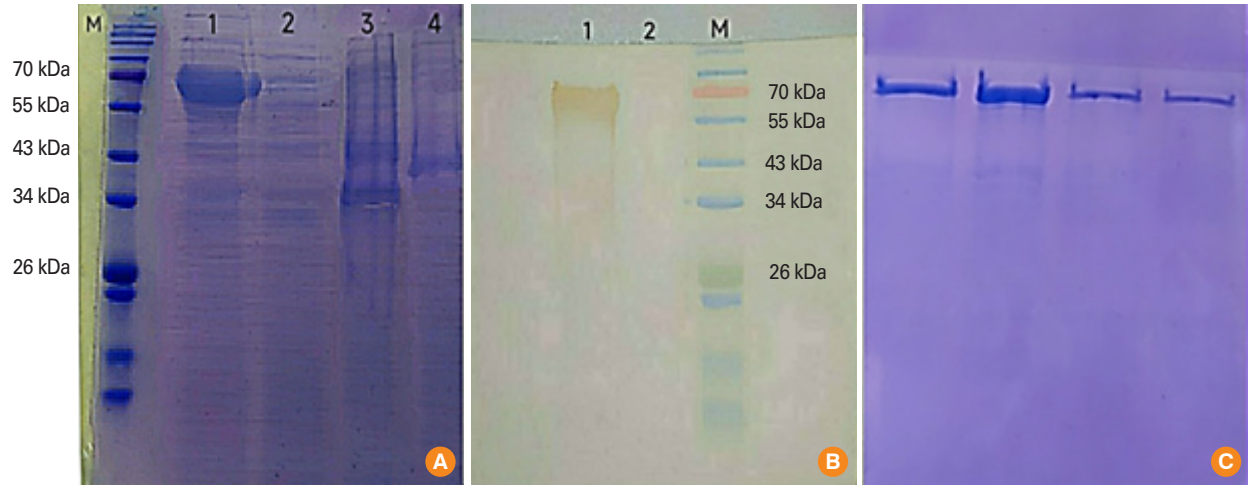

Fig. 1. Characterization of trigger factor/Bp26/Omp31 (TBO) expression. **(A)** The measurement of TBO chimeric protein was performed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. Lane 1 of *Escherichia coli* bacteria containing the recombinant TBO (rTBO) gene after induction of expression with isopropyl-d-1-thiogalactopyranoside (IPTG). Lane 2 cell lysates before induction of expression with IPTG (negative control). Lane 3 and 4 *E. coli* bacteria express the different proteins as a positive control of expression. **(B)** A Western blot using an anti-His antibody showed a single band with the expected size of rTBO; lane 1 illustrates the IPTG-induced *E. coli* that contained pET28a-*tbo*. Lane 2 defines uninduced *E. coli*. **(C)** Analysis of the purified protein with SDS-PAGE ensures the correctness of the protein purification steps. M, protein size marker.

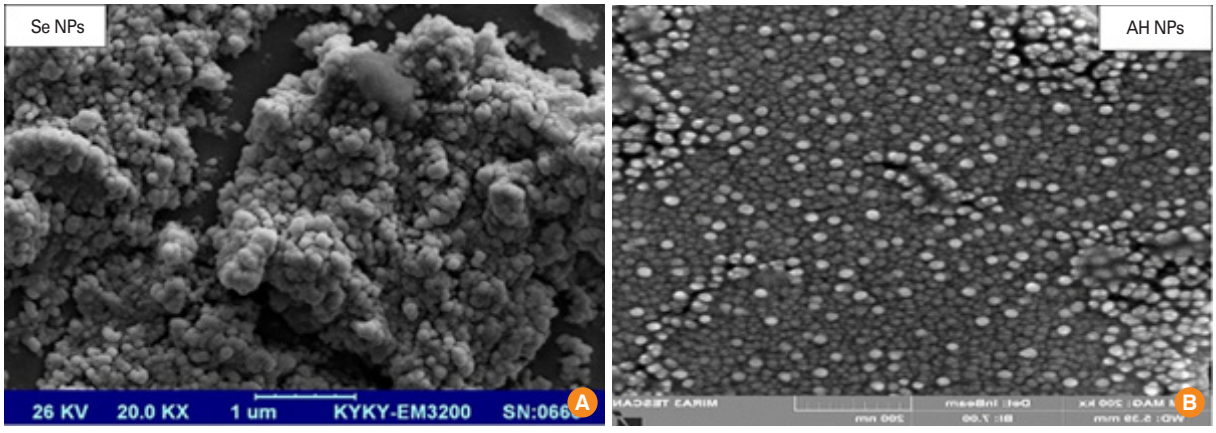

Fig. 2. Scanning electron microscopy images of selenium nanoparticles (Se NPs) **(A)** and aluminum hydroxide nanoparticles (AH NPs) **(B)**.

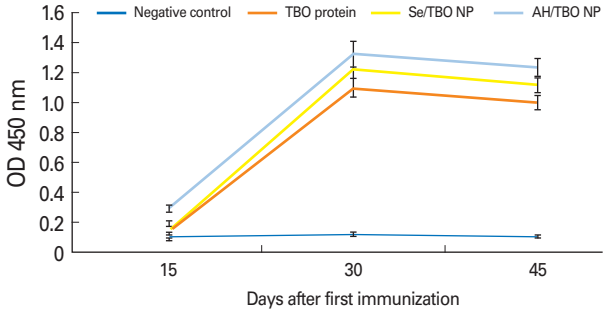

Fig. 3. Anti-trigger factor/Bp26/Omp31 (TBO) antibody levels: an enzyme-linked immunosorbent assay was conducted to analyze the sera in triplicates for aluminum hydroxide (AH)/TBO, selenium (Se)/TBO, and TBO protein without adjuvant-specific immunoglobulin G (IgG) antibodies with comparison to the negative control group. The IgG titration results in the sera of all four groups after each blood sampling on days 15, 30, and 45, respectively. OD, optical density; NP, nanoparticle.

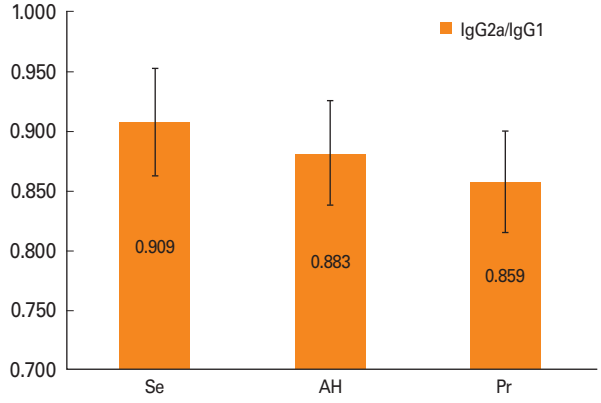

Fig. 4. Evaluation of immunoglobulin G (IgG)2a/IgG1 antibody ratio to specify the type of immune responses. Se, selenium; AH, aluminum hydroxide; Pr, TBO protein alone.

Serum antibody direction

The results showed that total IgG against the chimeric antigen increased significantly in the vaccinated groups with TBO-NPs. The group that received only TBO protein had a lower increase in IgG titer than the vaccinated groups with NPs. But the groups receiving TBO along with AH and Se NPs produced high levels of IgG responses. This indicated the efficiency of NPs used in the formulation of the recombinant vaccine (Fig. 3). In order to assess the kind of immune response, the ratio of IgG2a to IgG1 was measured. In both immunized groups with NPs, this ratio was less than 1, which indicates the induction of humoral immunity against recombinant antigens (Fig. 4).

Table 1. The context of bacteria in spleens

Studied groups	log_{10} CFU of *Brucella melitensis* 16M in spleen	Units of bacterial reduction
Negative control (PBS)	14.50[a]	-
Se/TBO NPs	8.13[b]	6.37
AH/TBO NPs	2.50[c]	12.00
TBO	8.88[b]	5.62

The context of bacteria in spleens is indicated as the mean log CFU±standard deviation per group. The difference between groups was assessed by the Kruskal-Wallis test and comparisons were considered significant at $p \leq 0.05$. Different letters (a, b, and c) represent significant difference between groups. Units of bacterial reduction were determined by deducting the mean log CFU of the vaccinated groups from the mean log CFU of negative control groups.
CFU, colony-forming unit; PBS, phosphate-buffered saline; Se, selenium; TBO, trigger factor/Bp26/Omp31; NPs, nanoparticles; AH, aluminum hydroxide.

Protection experiments

The protective ability of the TBO chimeric vaccine formulation against the virulent strain *B. melitensis* 16M was evaluated. The counting of bacterial colonies in the spleen of vaccinated mice with AH/TBO, Se/TBO NPs, and TBO alone showed a significant increase in immunity levels (Table 1). Compared to the control group, mice immunized with AH/TBO and Se/TBO NPs indicated significant reduction of *B. melitensis* 16M culture, respectively. Therefore, AH NPs had a better function in inducing reduction of bacterial culture than Se NPs and TBO alone.

Discussion

Brucellosis is an unsolved health problem worldwide, affecting more half a million people annually. To counter this disease, the development of safer and more efficient vaccines seems necessary, and since this requires the creation of protective immunity in animals, so far, many researchers have evaluated their ability to induce immunity by using different *Brucella* proteins [1,3,8]. Increasing antigen processing and effective protection of antigens against destruction in the host's body are considered as promising NP features. In addition, they have the ability to stimulate the host's immune system in order to increase immune responses [22].

Se is one of the rare and essential elements with unique antioxidant properties that plays many key roles in various physiological functions, including body metabolism, anticancer and anti-viral effects, and cellular homeostasis. Recently, with the discovery of their effective performance in the field of drug delivery and usability in the targeted drug delivery system, they have been used as vaccine adjuvants [23-26].

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Among the various adjuvants, aluminum-based adjuvants are licensed by the U.S. Food and Drug Administration for use in human vaccines. In 1926, Glenny [27] found that aluminum-deposited diphtheria toxin was more immunogenic than toxoid alone. This study pioneered the use of aluminum compounds in vaccines as adjuvants. Nano-scale AH can be used as a promising material to enhance the antigenicity of vaccines as an effective adjuvant [28,29].

In this study as part of an ongoing project, three *Brucella* antigens as a chimeric protein containing TBO were used in combination with AH and Se NPs as adjuvants against brucellosis.

In general, it has been proven in many studies that multi-antigen (chimeric) vaccines can induce better protection than monovalent vaccines [20]. For this reason, in previous investigations, the potential of the three antigenic components of *Brucella*, both alone and chimeric, has been evaluated in order to induce efficient immunity [1,20,30].

In silico analysis by Ghasemi et al. [31] showed the efficiency of these antigens in inducing immune responses mediated by B cells and T cells, which is an important feature for protective vaccines against brucellosis. Our results also showed that the use of TBO chimeric antigen can induce high levels of IgG antibodies. Thus, the serum of immunized mice contained significant amounts of IgG anti-TBO. These results are in complete agreement with the studies of Karevan et al. [1], which showed that the antigens in the form of fusion were able to induce a high titer of IgG antibodies without any negative effects on each other. In another study, Ghasemi et al. [32] used rabbit models to evaluate the efficacy of TBO recombinant protein. The results of their studies showed that the immunized rabbit pool serum strongly reacted with recombinant TBO. In addition, antibodies against recombinant TBO were detectable in 76.5% of sera obtained from *Brucella*-infected humans. These results indicate the efficiency of TBO protein in different hosts [32].

Although subunit vaccines are a good alternative to current vaccines and solve their limitations, their main problem is poor immunogenicity, and since the goal of vaccination is to achieve strong immune responses and long-term protection, researchers are trying to solve these problems [27,33]. It is necessary to add an adjuvant to most of the subunit vaccine formulations to optimize their induced immunity. Adjuvant-based vaccines have unique advantages such as increasing the speed and intensity of immune responses, inducing longer and broader immune responses, the possibility of reducing the dose of antigen during immunization, and improving vaccine performance in people with a weak immune system. Therefore, the availability of efficient and safe adjuvants has always been considered [18,34].

Currently, the benefits of using NPs as adjuvants have been proven in many studies. They can protect vaccine antigens from degradation (vaccine stability) or take antigens to specific sites and facilitate their uptake and processing by antigen-presenting cells. Appropriate adjuvants must be carefully selected to induce optimal and balanced immune responses [18,35]. One of the most commonly used adjuvants is aluminum salts, which mainly include AH, potassium aluminum sulfate, or aluminum phosphate particles. The widespread use of the adjuvant is due to its low price and high immunogenicity, which has caused these materials to be used in vaccines such as those for diphtheria, tetanus, hepatitis A and B, rabies, etc. for many years. However, they have disadvantages such as allergic responses caused by immunoglobulin E, occasional induction of local reactions and inflammation, and failure to induce cytotoxic T lymphocyte stimulatory responses. Also, aluminum salts mainly elicit humoral immune responses (type 2 T-helper [Th2]/IgG1), although they are able to create cellular responses (type 1 T-helper [Th1]/IgG2a) with less intensity [22,28,33].

The AH/TBO NPs recombinant vaccine clearly enhanced IgG1 responses, according to our findings. These results are consistent with previous studies that showed that AH NPs as adjuvants induce strong humoral immune responses. As a result, in the study by Abkar et al. [22], the use of AH/Omp31 NPs was able to induce IgG1 antibody responses. Also, the results of Pagheh et al. [36] showed the induction of high levels of IgG1 and humoral immunity (Th2) by AH NPs in the pcGRA14+rGRA14-AHPNs vaccine formulation.

In general, the success of subunit vaccines depends on their composition and route of immunization. It has been shown in many studies that both IgG2a and IgG1 titers are sensitive to the route of immunization, and the composition and type of vaccine regime can affect its strategy to induce cellular and humoral immune responses [6,36]. Our results were in opposition to the results of Yousefi et al. [37], who showed that the use of AH NPs together with Omp25 (Brucella outer membrane protein) elicits a greater cellular immune response than humoral immunity from the i.p. injection route. The reason for this difference is probably related to their different immunization routes. This issue has been proven in previous studies, such as that by Abkar et al. [4], who showed that i.p. immunization with TMC/Omp19 NPs stimulated a Th2 immune response, while

oral immunization with TMC/Omp19 NPs induced a mixed Th1/Th17 immune response. Therefore, the route of administration plays an important role in the type of immune response. In fact, the choice of immunization route is an important factor in the success or failure of vaccine development. An incorrect pathway can render an antigen ineffective or reduce its efficacy, even though the same antigen can be highly effective in other immunization pathways [4].

In addition to the route of administration, the type of selected antigen also plays an important role in the immune response, so the use of AH NPs together with EsxV antigen by Amini et al. [29] in the route of s.c. injection was able to increase the secretion of Th1 cytokines and stimulate a strong cellular immune response. Despite the administration route being similar to the present study, the results were contradictory. The possible reason for this is the difference in the antigens used. Abkar et al. [8] found that i.p. administration of TMC/Omp19 NPs induced Th2 immune responses, whereas i.p. administration of TMC/Urease NPs induced Th1/Th2 immune responses. These findings clearly demonstrate the importance of antigen in the type of immune response. Hence, different antigens and delivery systems show different results in inducing immune responses.

Both types of immune response (humoral and cellular) can have important effects on fighting and eliminating intracellular pathogens such as *Brucella*. For this reason, the host's immune responses play a key role in the fate of the infection [36,38]. Due to their ability to absorb and transport antigens to lymph nodes and activate T cells, dendritic cells (DCs) play an important role in the initiation of immune responses. Therefore, effective stimulation of T cells requires efficient signals from DCs. In research conducted by Sokolovska et al. [39], the effects of AH adjuvants on antigens, the expression of important immune molecules by DCs, and their ability to induce the differentiation of T-helper cells were evaluated. Their results showed that AH adjuvants can increase the efficiency and activation ability of T cells through DCs and increase the absorption and efficiency of antigens. This plays an important role in creating Th1 and Th2 responses [39].

Due to their excellent biological properties, low toxicity, high bioavailability, and targeted drug system, Se NPs have attracted a lot of attention in recent years. Se NPs are made in the laboratory both chemically and biologically, and during the many studies that have been done, their effectiveness in stimulating the immune system has been proven by various methods such as innate immunity, T cells, and natural killer cells [25,26].

In this study, our results showed that s.c. administration of Se/TBO NPs led to a significant increase in the titer of IgG and induced Th2 protective immune responses. These data were in agreement with the results of Raahati et al. [25], who showed that the use of Se NPs could induce significant IgG responses and effectively induce humoral immune responses, so that the immunity created by Se NPs was even higher than the commercial vaccine strain Dukoral. Of course, the use of cytokine assay technique in our study could lead to more accurate conclusions about the type of immune response.

Recent studies showed that the use of Se NPs in hepatitis B and breast cancer vaccines administered via the oral route was able to increase the total antibody titer (IgG) and induce strong cellular immunity (Th1), which was not consistent with our results [26,40]. As mentioned earlier, the reason for these differences can be related to different routes of immunization and delivery of NPs and types of Ag. So far, several studies have been conducted with the aim of increasing the effectiveness of vaccines (by changing the composition and delivery routes), all of which have shown the ability of Se NPs as an adjuvant to induce humoral and cellular protective immunity.

Despite the ability of TBO-NPs's chimeric vaccine formulation to increase antibody titers, no significant difference was observed between the groups of mice vaccinated with nanovaccine compared to the group vaccinated with chimeric protein without adjuvant. However, the amount of bacterial reduction created by Se NPs was statistically equal to protein without adjuvant, while the highest reduction of bacterial culture was created by AH NPs, which was more than the other two groups. According to the obtained results, it seems that protein alone has adjuvant properties, which, of course, needs further investigation. Additionally, using the commercial vaccine strain as a positive control could lead to better conclusions about the efficiency of AH NPs. Altogether, the results showed that Se and AH NPs carrying chimeric antigens can be a promising vaccine candidate against brucellosis by producing protective immunity.

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