Original Article

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A comparison between adjuvant and delivering functions of iron oxide and calcium phosphate nanoparticles, using a model protein against *Brucella melitensis*

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Purpose: Calcium phosphate (CaP) and iron oxide (IO) nanoparticles (NPs) are promising adjuvants and delivery systems for vaccination. Furthermore, it has been shown that the chimeric antigen TF/Bp26/Omp31 (TBO) is a good candidate for stimulating protection against virulent *Brucella melitensis*. Our aim in the present study was to compare the roles of CaP and IO NPs for induction of the immune response and protection against *B. melitensis* 16M by using the TBO antigen as a model protein.
Materials and Methods: The *tbo* gene was expressed in the bacterial host and was evaluated by SDS-PAGE and western blot. The recombinant TBO was loaded onto CaP (CaP/TBO) and IO (IO/TBO) NPs. CaP/TBO and IO/TBO NPs were administered subcutaneously.
Results: Antibody levels showed that immunization with both CaP/TBO and IO/TBO NPs stimulated mixed Th1-Th2 immune responses. In addition, immunized mice were challenged with a virulent strain of *B. melitensis* 16M. Immunized mice with CaP/TBO NPs showed a higher degree of protection than vaccinated animals with IO/TBO NPs.
Conclusion: Altogether, our results indicated that the CaP NPs are a potent adjuvant and delivery system for subcutaneously administered *Brucella* antigens.

Keywords: Brucellosis; Calcium phosphate; Iron oxide; Nanoparticles

INTRODUCTION

Brucellosis (also known as Malta fever) is an important worldwide zoonotic disease, and more than 500,000 cases of the disease are reported annually [1,2]. The causative agent of Brucellosis is a Gram-negative, non-spore-forming, non-motile, and facultative intracellular bacteria of the genus *Brucella* that has more than ten species that infect many domestic animals, such as sheep, cattle, and goats. Among them, *B. melitensis*, *B. abortus*, and *B. suis* are the major human pathogen [3-5].

Generally, humans acquire the disease through skin damage, contact with infected animals, consumption of contaminated dairy products, and the transmission of infection from a contaminated environment like inhalation of aerosols contaminated with *Brucella.* Due to complications such as abortion in pregnant animals, infertility, and reduced milk production, the disease causes a lot of economic losses [3,6-8]. Furthermore, the possibility of airborne brucellosis transmission has raised concerns about the use of pathogens in bioterrorism [9,10]. Due to *Brucella*'s intracellular lifestyle, only a few antibiotics are used to treat the disease; in fact, treatment includes rifampin, doxycycline, and an injectable aminoglycoside (gentamicin or streptomycin). Since the prevention of human brucellosis is based on the control of the illness in domestic animals, testing and slaughter programs, along with immunization, are the most important ways to control the disease [5,11-14].

By using live attenuated vaccines (such as *B. abortus* S19, *B. melitensis* Rev. 1, and *B. abortus* RB-51), the disease is now almost completely controlled in many regions. However, some of the disadvantages of these vaccines, like their ability to cause disease in humans, abortion in pregnant animals, and interference with the diagnosis of infected and vaccinated cases, have limited their use. In this regard, researchers have always sought to produce more effective and safer vaccines. The use of subunit vaccines can be a suitable alternative strategy to overcome the disadvantages of live attenuated vaccine strains. Identification of new antigens is essential for the production and development of effective subunit vaccines, but only a small number of these antigens perform well, such as Bp26 (a periplasmic immunogenic protein), Omp31 (outer membrane protein 31), Trigger Factor (TF), Omp19, Urease, Superoxide dismutase, L7/L12, Omp16, and Omp28 [1,6,15].

The main challenge of subunit vaccines is their poor immunogenicity, so various groups of adjuvants have been used to improve the immunogenicity. Nanoparticles (NPs), which have always been used in various fields, are one of the most common adjuvants used in subunit vaccines and are candidates to induce high immunogenicity [16-21]. Calcium phosphate (CaP) NPs are among the most commonly applied inorganic nano-adjuvants that have advantages such as resistance to degradation by lipases and bile salts, low cost, non-toxicity, and excellent biodegradability and biocompatibility. Furthermore, CaP NPs has good potential for inducing immune responses against various infectious diseases [19,22-24]. For many years, CaP has been used as an adjuvant for vaccination against tetanus toxoid. In addition, its use in many research studies has brought promising results in the direction of the desired induction of immune responses [25].

Another adjuvant that stimulates the cellular and humoral immune responses is iron oxide (IO) NPs. Iron is one of the abundant metal elements in the human body that participates in various biological processes, such as helping to transport and store oxygen when combined with haemoglobin and promoting cellular respiration. The US Food and Drug Administration (FDA) has only approved the use of iron-based NPs in mineral nanomedicines such as ferumoxytol, which is used to treat iron deficiency anemia. Therefore, IO NPs have attracted a lot of attention due to their many medical and biomedical applications as well as their unique properties, such as low toxicity and a low production cost. In addition, IO NPs have the ability to transfer antigens to the cells of the immune system, which is why they are used in vaccine candidates as adjuvants and enhancers of antigen properties [19,26,27].

Since CaP and IO NPs have shown promising activities as adjuvants, in the present study, the effects of these two nano-adjuvants on the immunogenicity of TBO (TF, Bp26, and Omp31) antigen (as a model protein) was evaluated.

MATERIAL AND METHODS

Plasmids, bacteria and animals

The 4–6-week-old female C57BL/6 mice were received from laboratory animal research center (Baqiyatallah University of Medical Sciences, Tehran, Iran) and maintained in standard polypropylene cages at 20–22°C, going through 12-hour light/dark cycles. They were acclimatized and randomly divided into 6 experimental groups, so that 6 mice were placed in each group. *B. melitensis* 16 M was purchased. *E. coli* BL21 (DE3) and the pET28a vector (Novagen, Madison, WI, USA) were used for the expression of recombinant protein (TBO).

Expression and purification of recombinant protein

The TBO recombinant protein expression and validation have been performed as previously described [6]. Briefly, *E. coli* BL21 (DE3) incubated into 100 mL of LB medium containing 50 μg/mL kanamycin at 37°C and the incubation was continued with agitation (180 rpm) to 0.5 optical density value at 600 nm and then gene expression was induced with 1mM isopropyl β-d-1-thiogalactopyranoside (IPTG) in 37°C for five hours. 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,

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it was purified using magnetic NPs. For this purpose, the NPs were washed once with the wash buffer (8 M urea, 50 mM NaH2PO4, 500 mM NaCl, and 40 mM imidazole). 10 mL of bacterial lysate was added to magnetic NPs and shaken for 30 minutes to attach the TBO chimeric protein to the magnetic NPs via His-tag. NPs were washed with 20 mL of wash buffer (8 M urea, 50 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole) three times, each time for 10 minutes; then eluted with elution buffer (450 µL wash buffer, 500 mM imidazole) for 30 minutes. The supernatant was separated as the recombinant subunit protein and analyzed with SDS-PAGE. Then the recombinant protein was identified by SDSPAGE and western blot with horseradish peroxidase (HRP)-conjugated anti-His tag antibody (Sigma-Aldrich, St. Louis, MO, USA). The Bradford method determined the concentration of purified protein.

Synthesis and characterization of TBO-loaded nanoparticles

CaP NPs were synthesized according to a previously described method [16]. Briefly, a mixture containing 12.5 mmol calcium chloride from Merck (Darmstadt, Germany), 12.5 mmol disodium hydrogen phosphate from Sigma, and 15.6 mmol sodium citrate from Sigma was prepared, slowly mixed, and stirred for 48 hours to obtain a NP suspension. Then, the mixture was sonicated for 30 minutes in an ultrasonic bath. In order to prepare TBO-loaded CaP (CaP/TBO) NPs, 500 µL of CaP NPs were added to 500 µL of purified protein (800 µg/mL).

IO NPs were synthesize as previously described [28]. To prepare TBO-loaded IO (IO/TBO) NPs, 500 µL of IO NPs were added to 500 µL of purified protein (800 µg/mL).

The zeta potential, size, and morphology of CaP and IO NPs were determined by dynamic light scattering (DLS) (Zetasizer Nano Instrument Malvern 3000, Malvern, UK) and scanning electron microscopy (SEM), respectively.

Mice and vaccination

C57 female mice were vaccinated by the subcutaneous (s.c.) administration route. The mice were classified into 4 groups and immunized 3 times at days 0, 15, and 30. First group was immunized by CaP NPs contained 20 µg TBO. The second group was immunized by IO NPs contained 20 µg TBO. The third group was immunized with 20 µg purified protein, IO and CaP NPs and PBS were used as negative control groups. The positive control group was administered subcutaneously on the 15th day with 1×10^5 CFU of *B. melitensis* Rev.1.

Antibody detection

To analyze the antibody production, serum samples were collected from all immunized groups of mice on days 15, 30, and 45 after the first immunization. Specific indirect enzyme-linked immunosorbent assay (ELISA) was done to detect total immunoglobulin (Ig)G, IgG1, and IgG2a by isotyping the ELISA kit (Sigma-Aldrich). For this purpose, ELISA microplates were coated with 10 µg/mL of purified protein (TBO) and kept at 37°C for 1 hour. Microplates were washed four times after each step with PBST. The plates were blocked with blocking buffer (5% skim milk in PBS) to prevent nonspecific binding and kept for 1 hour at 37°C. Microplate incubation was conducted with different dilutions of sera (1:500 to 1:160,000) at 37°C for 2 hours. To detect specific antibodies against TBO, HRP-conjugated anti-IgG (Sigma-Aldrich) was added to the plates. In order to identify a specific antibody subclass against TBO, a goat anti-mouse subtype antibody and a rabbit anti-goat HRP-conjugated antibody were added to detect IgG subclasses (IgG1 and IgG2a). Finally, 100 µL of TMB (tetramethylbenzidine) was added to each well and incubated at room temperature for 15 minutes; the reaction was stopped after color development. The absorbance of the samples was read at 450 nm with an ELISA reader (Bio-Rad, Hercules, CA, USA).

Protection assay

One month after the final vaccination, five mice from each group were challenged with 2×10^7 CFU (colony forming unit) of *B. melitensis* 16 M through s.c. injection. Four weeks later, the infected mice were sacrificed by cervical dislocation in order to disrupts the spinal cord and brainstem. To determine the number of *Brucella* colonies, their spleens were extracted, homogenized, diluted, and plated on Brucella agar at 37°C for 2–3 days. The results were demonstrated as the mean $\log_{10}$ CFU ± SD per group. Units of protection were calculated by subtracting the mean $\log_{10}$ CFU for the experimental groups from the mean $\log_{10}$ CFU of the negative control group.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS software. All p values ≤0.05 were considered statistically significant. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Dunnett's post hoc test.

Ethics statement

All experiments which were done on animals were conditioned in accordance with the protocol of the National Committee for Ethics in Biomedical Research (IR.BMSU.REC.1397.373). Consent to publish was obtained from the study participants.

RESULTS

Chimeric antigen expression

The bacterium, as an expression host, was transformed with pET28a-*tbo* with an N-terminal 6X-His tag. The results of SDS-PAGE showed a specific protein band with an approximate size of 67 kDa (**Fig. 1A**). Subsequently, western blot analysis of purified protein showed a single band of the same size as the SDS-PAGE results (**Fig. 1B**). The SDS-PAGE analysis showed that the chimeric protein purity was desirable (**Fig. 1C**).

Nanoparticles characterization

The CaP/TBO and IO/TBO particle sizes were determined by DLS and showed an average size of CaP/TBO and IO/TBO NPs about 160 nm and 60 nm, respectively. The images taken by SEM showed a spherical shape and smooth surface of the IO/TBO NPs (**Fig. 2**). The zeta potential (surface charge) of NPs was –20 mV. The stability of size, shape, and zeta potential of NPs was also investigated and showed no significant changes after 21 days of incubation at 4°C or room temperature. Based on the Bradford assay, the adsorption efficiency of antigens to CaP and IO NPs was 50 and 75%, respectively.

Serum antibodies detection

Administration of IO/TBO NPs as well as the administration of CaP/TBO NPs induced high levels of specific IgG. In the group immunized with TBO protein, a moderate increase in immune responses was obtained in antibody level. These results showed the effectiveness of CaP and IO NPs as adjuvants along with TBO protein (**Fig. 3**). Antibody levels showed that immunization with both CaP/TBO and IO/TBO NPs stimulated mixed Th1-Th2 immune responses (**Fig. 4**).

Protection experiments

By s.c. injection of *B. melitensis* 16M to immunized mice, the ability of chimeric immunogen formulation in inducing protection against *B. melitensis* was evaluated. The counting of bacterial colonies in the spleen of groups immunized with both NPs showed an increase in protective immunity compared to the control group (**Table 1**). Immunization with IO/TBO NPs and TBO resulted in 1.16 and 0.87 log protection units, respectively, against *B. melitensis*. When challenged with *B. melitensis*, mice that had been vaccinated with CaP/TBO NPs displayed an equal level of protection compared to the positive control group, with log units of protection measured at 1.75.

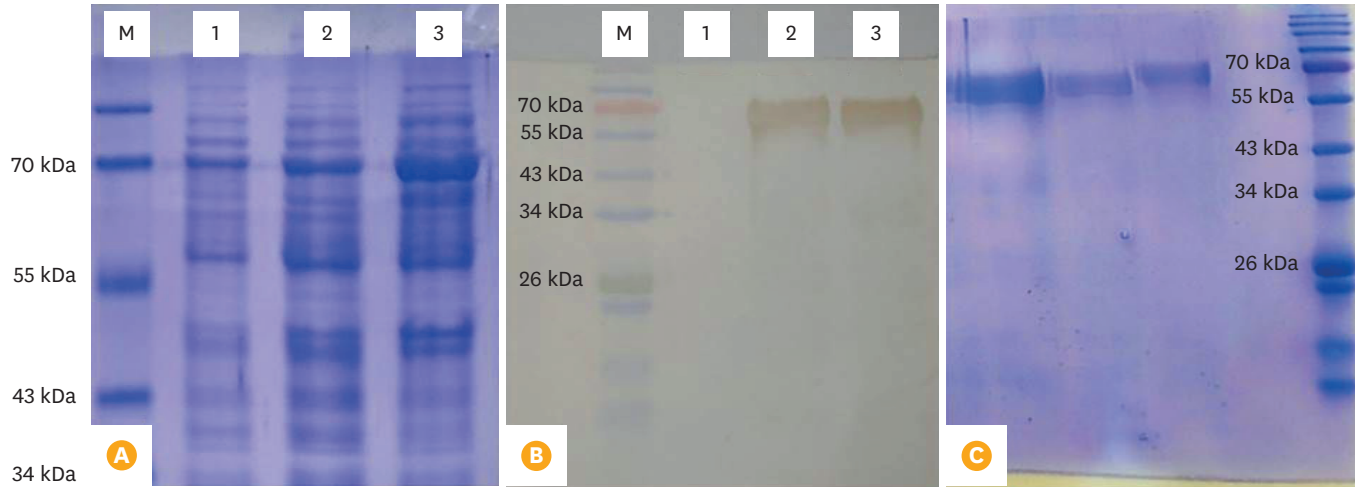

Fig. 1. Characterization of TBO expression. (A) The expression of TBO chimeric protein was analyzed by the SDS-PAGE electrophoresis. After IPTG-induced *E. coli* that contained pET28a-*tbo*, the 67 kDa band indicates the correct expression of the desired protein. Lane 1: *E. coli* BL21(DE3), Lane 2: uninduced transformed *E. coli* BL21 (DE3), Lane 3: Transformed *E. coli* Bl21 (DE3) after induction by IPTG. (B) Western blot using an anti-His antibody shows a single band with the expected size of TBO; Lane 1: uninduced transformed *E. coli* BL21 (DE3), Lane 2 illustrates the IPTG-induced *E. coli* that contained pET28a-*tbo*. Lane 3 indicates purified TBO protein. (C) Analysis of the purified protein with SDS-PAGE to ensure the correctness of the protein purification steps.
M, protein size marker; TBO, trigger factor/Bp26/Omp31; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropyl β-d-1-thiogalactopyranoside.

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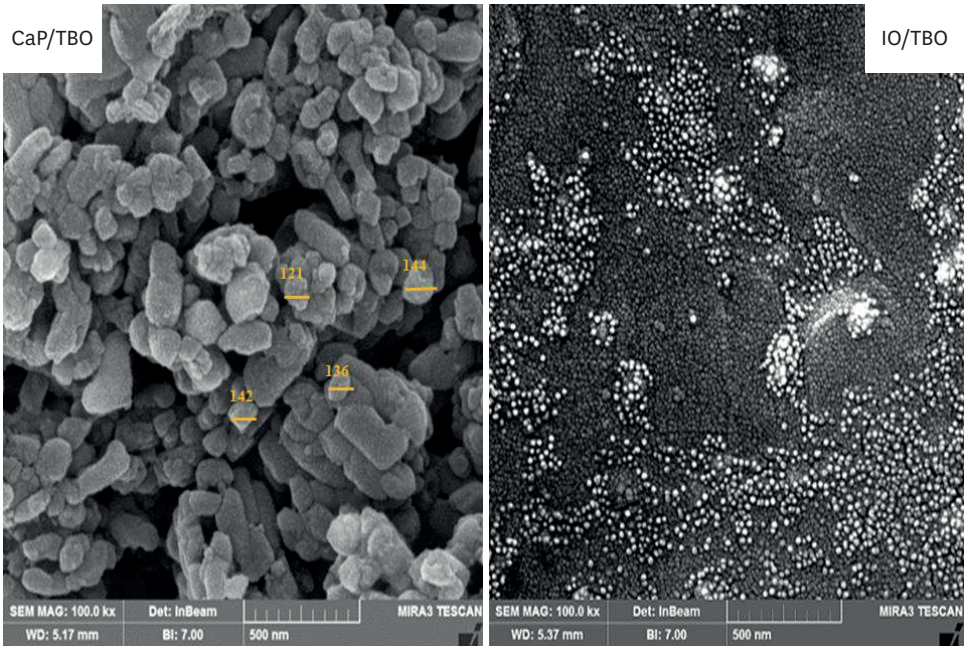

Fig. 2. Scanning electron microscope images of CaP and IO NPs. The figure shows that the CaP/TBO and IO/TBO NPs particles have an average size about 160 nm and 60 nm, respectively. In addition, IO/TBO NPs have a spherical shape and smooth surface.
CaP, calcium phosphate; TBO, trigger factor/Bp26/Omp31; IO, iron oxide; NP, nanoparticle.

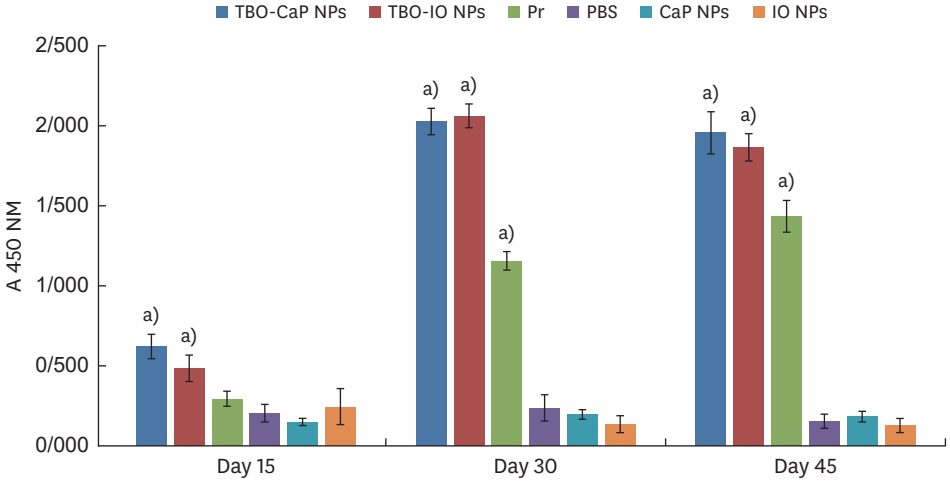

Fig. 3. Anti-TBO antibody level. An enzyme-linked immunosorbent assay was conducted to analyze the sera in triplicates for CaP/TBO, IO/TBO, and TBO proteins without adjuvant-specific IgG antibody in comparison to the negative control group. The IgG level results in the sera (dilution 1:1,000) of all three groups after each blood sampling 15, 30, and 45 days after first immunization.
TBO-CaP NPs, trigger factor/Bp26/Omp31 calcium phosphate nanoparticles; TBO-IO NPs, trigger factor/Bp26/Omp31 iron oxid nanoparticles; CaP NPs, calcium phosphate nanoparticles; IO-NPs, iron oxid nanoparticle; PBS, phosphate-buffered saline; Pr, TBO protein.
[a]Significant difference between groups.

DISCUSSION

Recently, the use of NPs as delivery systems in the formulation of subunit vaccines has been of interest to researchers. NPs have important characteristics that make their use as an adjuvant desirable [25]. In fact, the use of nano-based adjuvants leads to better effectiveness of vaccines and a reduction of booster doses, which subsequently reduces the number of doses consumed and the amount of antigen required. In total, these factors reduce the processes related to vaccine preparation and the final market, which has a significant impact on the cost-effectiveness of vaccine

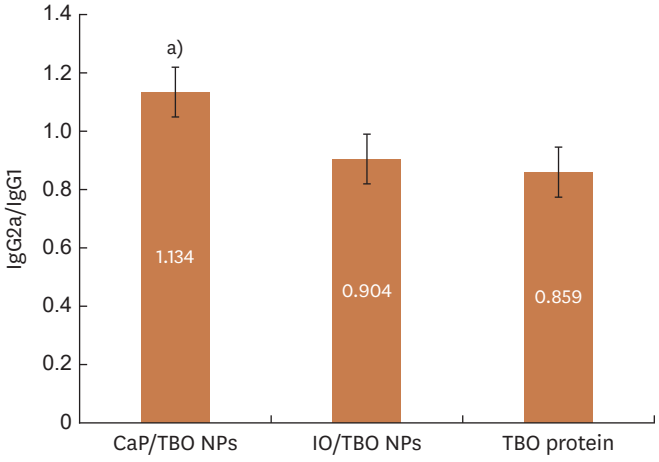

Fig. 4. Evaluation of the IgG2a/IgG1 antibody ratio to specify the type of immune response. The serum dilution used was 1:1,000.
IgG, immunoglobulin G; CaP, calcium phosphate; TBO, trigger factor/Bp26/Omp31; NP, nanoparticle; IO, iron oxide.
[a]Significant difference between groups. Error bar indicates standard deviation.

Table 1. The context of bacteria in spleens is indicated as the mean log CFU ± standard deviation per group

Groups (N=5)	Log_{10} CFU of *B. melitensis* 16M in the spleen	Protection units
PBS	6.07±0.37[a]	0
TBO	5.20±0.23[b]	0.87
B. melitensis Rev.1	4.06±0.24[d]	2.01
CaP NPs	5.98±0.18[a]	0
CaP/TBO	4.23±0.19[d]	1.75
IO NPs	5.90±0.13[a]	0
IO/TBO	4.74±0.14[c]	1.16

Units of protection were determined by deducting the mean log CFU of the vaccinated groups from the mean log CFU of negative control groups. The difference between groups was assessed by the t-test and comparisons were considered significant at $p \leq 0.05$. Different letters (a, b, c and d) represent significant difference between groups. PBS is considered as a negative control for *B.melitensis* Rev.1 and TBO, whereas CaP NPs and IO NPs are considered as negative control for CaP/TBO and IO/TBO, respectively.
TBO, trigger factor/Bp26/Omp31; CaP, calcium phosphate; NP, nanoparticle; IO, iron oxide.

production [18]. The use of nanotechnology has made it possible to make different nanoadjuvants in desired dimensions and surface charges, which has led to their wide use in various fields. In such a way, the application of synthetic and biological NPs has been confirmed in human use through clinical and preclinical investigations [19,25].

The main challenge of available commercial vaccines is their weak immunogenicity; the use of a suitable adjuvant can solve the concern in this field [29]. The present study examines the effects of CaP and IO NPs in increasing the immunogenicity of TBO chimeric protein.

One of the natural compounds of living organisms is calcium, which has high absorption and is safe. For this reason, the use of calcium as an adjuvant in human vaccination has long been common [25,30]. CaP NPs have always been considered due to properties such as low production cost, a simple manufacturing process, acceptable safety without adverse effects, high stability and effective immune induction [16]. This compound not only has high biodegradability but also, due to its biocompatibility, can be used as an antigen delivery system for the immune system [18].

In the present study, CaP NPs were used as an adjuvant. Our results showed a significant increase in IgG antibody responses against recombinant TBO protein (IgG anti-TBO). These results are consistent with Pagheh et al.'s study [31], which achieved high levels of total IgG responses using CaP NPs in their bivalent immunogen formulation (rGRA14+pcGRA14-CaPNPs). Pagheh et al. [31] evaluated the protective immunity of BALB/c mice using a prime/boost vaccination strategy using GRA14 antigen. The study found that the prime-boost strategy, involving plasmid DNA and recombinant protein CaP and Aluminum hydroxide (AH) NPs, significantly stimulated the production of specific IgG antibodies and cytokines against *Toxoplasma gondii* infection. The CaPNs-based prime-boost vaccine demonstrated the longest survival time and lowest parasitic load in brain tissues, indicating its potential for future vaccine development [31]. Also, the results of Dodangeh et al. [32] showed that the use of CaP NPs as an adjuvant in the formulation of the multi-epitope vaccine candidate, including MIC3, ROP8, and SAG1 (MRS), can increase the efficacy of protective immunity. Another study showed that CaP NPs can induce strong IgG responses against herpes simplex virus type 2 (HSV-2) antigen [30].

Efficient protective immunity against intracellular microorganisms is often cellular immunity, which plays an important role in eliminating infections. *Brucella* species require cellular immunity due to their intracellular lifestyle (mostly residing in macrophages). Th1, cytotoxic T lymphocyte, and IgG2a responses, which facilitate phagocytosis with the opsonization process of the pathogen, are important protective components against *Brucella* and play a key role in immunity to the microorganism [1,23,33]. In fact, cellular immune responses are able to activate the functions of bactericidal and antigen-presenting cells (APCs), and subsequently, the effective activation of these cells will cause effective elimination of the infection [34]. Our results showed the ability of CaP NPs to effectively increase IgG2a responses and induce cellular immunity (Th1-Th2), which is consistent with previous studies. Abkar et al. the functions of CaP, aluminum hydroxide (AH) and chitosan (CS) NPs

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compared in terms of their ability to stimulate the immune response and provide protection against *B. melitensis* using omp31 as a representative protein. Vaccination with CaP/Omp31 and AH/Omp31 NPs resulted in the induction of a Th1-Th2 immune response, as evidenced by the cytokine profile and subclasses of the antibody. On the other hand, immunization with CS/Omp31 NPs only induced a Th1 immune response. CaP/Omp31 NPs demonstrated a level of protection against *B. melitensis* challenge that was equivalent to the vaccine strain *B. melitensis* Rev.1. However, compared to CS/Omp31 NPs, CaP/Omp31 NPs only exhibited a modest increase in the level of protection against *B. melitensis* 16 M. Taking into account the fact that calcium phosphate nanoparticles (CaP NPs) possess a commendable level of defense against *B. melitensis*, it is evident that our findings are in line with the conclusions drawn in the study conducted by Abkar et al. [25]. In another study, in a mouse model, the capacity of a CaP NPs to develop immunity to HSV-2 and Epstein-Barr virus infections was compared to that of regularly used aluminium (alum) adjuvants. CaP was found to be more effective as an adjuvant than alum, to cause little or no inflammation at the site of administration, to create high levels of IgG2a antibody and neutralizing antibody, and to provide a high percentage of protection against HSV-2 infection [30].

In another study, Sadeghi et al. [16] showed that the use of CaP NPs in the formulation of multi-antigen vaccines induced Th1 responses. Three *Brucella* antigens (FliC, 7-HSDH, and BhuA) and two multi-epitopes (poly B and poly T) absorbed by CaP NPs were employed. Mice immunized with several vaccine candidate formulations were protected against *B. melitensis* 16M and *B. abortus* 544, and demonstrated the same levels of protection as commercial vaccines (*B. melitensis* Rev.1 and *B. abortus* RB51). In a study conducted by Rahimi et al. [23], it was shown that CaP NPs induce Th1 immune responses and high protection against microorganisms such as *T. gondii*. Overall, our results show stimulation of a Th1-2 response following vaccination with CaP NPs, whereas the results of Sadeghi and Rahimi show a Th1 response. This discrepancy may be due to the type of antigen used in vaccination, as different types of antigens can induce different types of immune responses.

IO NPs have many applications. Due to their excellent safety profile, low production cost, affordability, application in the fields of drug delivery, and use in vaccines as an adjuvant, they have attracted the attention of researchers [35].

We showed that the use of IO NPs in combination with TBO results in high level of IgG and mixed Th1-Th2 immune responses. Our results were in agreement with the studies of Zhao et al. [19], who showed that the use of IO NPs to a large extent activates immune cells and causes cellular and humoral immune responses. Furthermore, these NPs in the formulation of the rMSP1 (merozoite surface protein 1) vaccine candidate against malaria was able to induce humoral immune responses and produce high levels of parasite-inhibiting antibodies [35].

A recent investigation conducted an evaluation on the immune responses initiated by the chimeric protein, composed of TBO combined with aluminum hydroxide (AH/TBO) and selenium (Se/TBO) nanoparticles (NPs), when used as adjuvants in a mouse model. The ELISA findings exhibited that the serum of mice, which were immunized via subcutaneous injection with both nanovaccines, generated notable IgG responses against the chimeric antigen. Furthermore, the outcomes of the IgG isotype analysis specific to TBO (IgG2a/IgG1) demonstrated that both AH and Se NPs triggered a T-helper immune response. Additionally, the results of the challenge involving the pathogenic strain of *B. melitensis* 16M revealed that mice vaccinated with AH/TBO NPs displayed a greater reduction in bacterial culture compared to mice immunized with Se/TBO NPs and TBO alone. Consequently, their findings demonstrated that AH NPs, carrying the chimeric antigen, hold promise as a potential vaccine candidate against brucellosis by inducing protective immunity [36]. In present investigation, the protective examinations demonstrated that the immunization of mice with CaP/TBO NPs elicits significant protection against *B. melitensis* 16M contamination (equivalent to *B. melitensis* Rev.1). This suggests that CaP NPs present a superior alternative to IO NPs owing to their enhanced level of protection. Neto et al. [27] demonstrated that vaccination using IO-based NPs as an adjuvant elicit cellular immune responses (Th1, Th17, and TCD8), demonstrating good adjuvant properties. Furthermore, the immunological response elicited by the vaccine's subcutaneous administration reduced the bacterial load of *Mycobacterium tuberculosis* (Mtb) challenged animals, indicating the vaccine's potential for further development as a tuberculosis vaccine [27].

Previous studies showed that IO NPs are absorbed by the reticuloendothelial system and lead to the exposure of immune cells to NPs. They can also affect the function of APCs and stimulate cellular and humoral immune responses [19]. Investigations by Powles et al. [37] showed that IO NPs coated with pullulan (pIONPs) produced cellular immune responses (Th1) against malaria antigens (MSP4/5). Also, IO NPs coated with citrate as an adjuvant in combination with CMX fusion protein induced the titer of cellular immune responses (Th1) [27]. Both of these studies disagreed with our results. It is possible that the coating of IO NPs with different groups can affect the type of induced

immune response; however, this conclusion requires more extensive and detailed investigations.

The characteristics of NPs, such as size, shape, and surface charge, are one of the important factors in absorbing them and provoking the host's immune responses. In particular, the size of NPs is an important factor that affects the type of distribution and absorption of them by APCs [3]. In a study conducted by Kanchan and Panda [38], NPs with sizes of 200–600 nm are absorbed with high efficiency by macrophages. NPs with a size range of 20 to 100 nm are also able to directly enter the lymphatic vessels and become available to dendritic cells by transferring to the lymph nodes [38]. In our study, DLS and SEM results showed that CaP and IO NPs have an approximate size of 160 nm and 60 nm, respectively. In previous studies, many efforts were made to understand the relationship between size and its effect on the type of induced immune response. Some authors believe that larger particles work better than smaller ones, while others disagree. Nevertheless, our results were consistent with the studies of Abkar et al. [25], who showed that NPs with a larger size perform better than smaller NPs. In the present study, 160 nm CaP NPs showed better performance in inducing cellular immune responses than 60 nm IO NPs. Most likely, one of the reasons for the better response of CaP NPs is due to their larger size compared to IO NPs. Maybe if the size of IO NPs was larger, it could induce a better immune response. However, there is still debate as to whether larger or smaller NPs favour Th1 versus Th2, or cellular immunity versus antibody responses [25].

The injection of antigen into the skin provides good conditions for the absorption and transfer of the antigen to the lymph nodes in order to induce immune responses. Skin has many immunological properties. As a result, it has always been regarded as an ideal route for vaccine injection. Our results showed that the use of subcutaneous (s.c.) injection routes to immunize mouse models was able to produce high immune responses. According to the findings of Abkar et al. [39], s.c. Urease injection induced stronger protective responses than intraperitoneal (i.p). immunization. Also, in the studies of Mao et al. [24], it was found that the levels of antibodies produced in the s.c. route are ten times higher than those produced in the intramuscular route, which indicates the s.c. injection is a proper vaccination route.

In some groups of mice immunized with CaP NPs, slight local inflammation was observed in the subcutaneous tissue. Previous research showed that minor inflammations without clinical symptoms or systemic adverse effects can be an advantage for the effectiveness of the vaccine. This inflammation is probably due to the local presence of CaP NPs remaining at the injection site, which causes more effective absorption of macrophages and immune cells. As a result, the slow absorption of CaP NPs from the injection site facilitates the slow release of antigen, its absorption, and its presentation to macrophages [40].

Both NPs along with the chimeric antigen were able to create favorable levels of protection against the pathogenic strain of *B. melitensis* 16M, which is consistent with other studies. However, the group immunized with CaP/TBO NPs performed remarkably better and was more successful in challenge tests compared to the IO/TBO NPs group. According to the protection results, it appears that the TBO protein alone has adjuvant properties, though more research is needed in this area. In conclusion, the protection experiments showed that immunization of mice with CaP/TBO NPs induces high protection against *B. melitensis* 16M infection, indicating that CaP NPs are a better option than IO NPs due to their higher level of protection.

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No potential conflict of interest relevant to this article was reported.

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